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JOURNAL OF CHROMATOGRAPHY

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PRACTICAL USE OF AN OPTIMIZATION STRATEGY IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE SEPARATION OF A LIMITED SUBSET OF COMPONENTS IN A REACTION MIXTURE

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(First received September 15th, 1988; revised manuscript received November 14th, 1988)

SUMMARY

A step-by-step development of a high-performance liquid chromatographic method for the optimized separation of a largely unknown sample is presented. A limited subset of two main components is separated from a ten-component reaction mixture of pyrroloquinoline quinone and cyclopropanol, using tetrahydrofuran–water binary eluents buffered to pH 2.5 with triethylamine phosphate on a reversed-phase column packed with 5-μm ODS-Hypersil. After the definition of the separation problem, the mobile phase parameters are selected rationally on the basis of a systematic gradient scouting procedure. The optimization search area is defined in accordance to the complexity of the sample mixture (two out of ten components are of interest) by utilizing a statistical approach, while the selectivity optimization is carried out using binary eluents of variable eluotropic strength.

INTRODUCTION

The separation of a few key components in sample mixtures containing a larger number of solutes has not received much attention. Although many samples are believed to fall into this category, most schemes described in the literature on experimental optimization address the separation of all components.

In a previous paper[1], we evaluated the merits of separating a limited subset of components by systematic optimization of the ternary mobile phase composition in reversed-phase high-performance liquid chromatography (HPLC). It was shown[1] that the analysis time can be reduced substantially by assigning more rigorous starting conditions for the optimization, when only few solutes are of analytical interest.

In this paper the other advantage of such "limited optimizations" is evaluated, *i.e.*, addressing the separation of only a limited subset of components (NI) will generally allow the analysis of sample mixtures containing large numbers of solutes, *M*.

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Schoenmakers and Mulholland[2] have pointed out the importance of the definition of the problem and the goal of the analysis as the first step of the chromatographic method development. We will also examine how these requirements can be translated into a meaningful design of the chromatographic experiments for the special case of limited optimization.

A reaction mixture of pyrroloquinoline quinone (PQQ) and cyclopropanol was selected as a typical example for limited optimization. The extent of conversion and mechanism of this reaction are of interest for studies in the inhibition of quinoprotein, *i.e.*, PQQ-containing methanol dehydrogenase (MDH)[3]. In solution the reaction of PQQ with cyclopropanol can occur only in the presence of a suitable catalyst. For studies of the mechanism of this reaction, the effect of different catalysts on the conversion of PQQ into the main product PQQ-M[3] (see Fig. 1) was also investigated. In order to monitor the extent of conversion, PQQ and PQQ-M must be separated from each other and from all the other (co)products in the sample mixture.

This reaction mixture represents an interesting example from another point of view. One of the most important steps in HPLC method development is the system selection, *e.g.*, reversed *versus* normal phase and the definition of the parameter space, *i.e.*, the combination and limits of chromatographic parameters which influence the separation selectivity of the relevant sample components. After the selection of the chromatographic system, initial scouting experiments should be carried out to limit the search for the optimum experimental conditions to a reduced parameter space (constrained from the relevant parameters). This parameter space was shown[4] to depend on the nature and complexity of the sample mixture.

In the case of our reaction mixture, little information was available on the

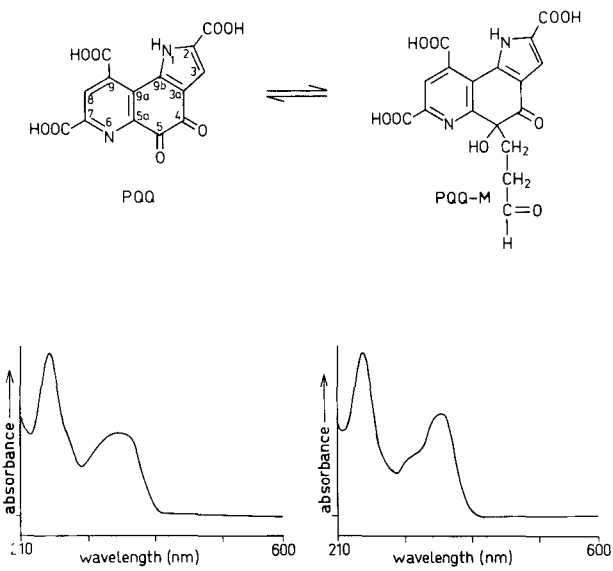

Fig. 1. The chemical structure and UV absorption spectra (taken in 50% methanol–triethylamine phosphate buffer at pH 2.5) of pyrroloquinoline quinone (PQQ) and its derivative pyrroloquinoline quinone (C_5)-3-propanal adduct (PQQ-M), formed in the reaction with cyclopropanol.

number and nature of the components in the sample. Pure standards were available only for the two compounds of interest, their UV absorption spectra are also shown in Fig. 1. The reaction products may also be ionizable solutes with (three or less) carboxyl groups like PQQ and PQQ-M and therefore different separation modes, *e.g.*, ion suppression and ion pairing might be applicable.

Recently, a systematic gradient scouting procedure has been developed by Low *et al.*[5] for reversed-phase HPLC, to track the nature of the solutes in such (relatively) unknown sample mixtures. The results of these scouting experiments can be used for the rational selection of the optimization parameters[4]. The application of this procedure is clearly required in the case of the present sample.

Once the mobile phase parameters have been selected, the statistical approach developed by Herman *et al.*[6] can be consulted to define retention limits between which the sample should be eluted in order to establish reasonably high probabilities (peak capacities) of separation success in the reversed-phase mode. Eluent compositions which provide the required retention limits will finally determine the optimization parameter space, where the systematic eluent optimization is carried out.

In this work, the step-by-step method development of an optimized separation of a relatively unknown reaction mixture is discussed in detail. We intend to demonstrate; (i) the importance of the correct definition of the separation problem and the goal of the analysis; (ii) the rational selection of the separation mode and the optimization parameters on the basis of systematic scouting experiments; (iii) the utilization of the statistical approach to determine starting eluent conditions (optimization search area) which are adapted to the complexity of the sample and (iv) the practical use of the limited optimization procedure using binary eluents of variable eluotropic strength, to locate the optimum separation of two (main) components of interest in a complex sample mixture.

EXPERIMENTAL

Chemicals and instrumentation

HPLC grade organic solvents were obtained from Rathburn (Walkerburn, U.K.). Distilled, deionized water was prepared in-house using a Milli-Q water purification system (Millipore, Molsheim, France). Buffer solutions contained 15 m*M* triethylamine (Gold Mark quality; Aldrich Chemie, Steinheim, F.R.G.) adjusted to pH 2.5 and 7.0 by the addition of phosporic acid (H_3PO_4). Tetrabutylammonium bromide (TBA) and sodium octanesulphonate ($OctSO_3$) were obtained from Janssen Chim. (Beerse, Belgium) and used as 1 *M* concentrated solutions in the pulse injection experiments. Pyrroloquinoline quinone (PQQ) was obtained from Sigma (St. Louis, MO, U.S.A.) and used as received.

ODS-Hypersil, 5 μm, (Shandon Southern Products, U.K.), was used as the stationary phase and slurry-packed into a 20 cm × 4.6 mm I.D. HPLC Valco column (Chrompack, Middelburg, The Netherlands).

The chromatographic system consisted of a Model 1090 chromatograph autoinjector and a Model 1040A linear photodiode array UV–VIS spectrometer (Hewlett-Packard, Waldbronn, F.R.G.).

The gradient scouting method used in this study was as described in ref. 5. All measurements were made at room temperature (25°C).

The computer program for limited optimization using binary eluent mixtures has been developed in PRO/BASIC and run on a Waters 840 data management system, equipped with 512 Kbyte of memory, a dual diskette drive (2 × 400 Kbyte), integral 10-Mbyte Winchester disk drive, extended bit map graphics with colour monitor and a letterprinter LA-100 (all from Digital Equipment Corporation, Maynard, MA, U.S.A.).

Sample preparation

The reaction mixtures of PQQ and cyclopropanol were prepared as described[3], using ZnO as a catalyst. Prior to HPLC analysis the samples were diluted in 0.02 *M* HNO_3 and adsorbed on a Sep-Pak C_{18} cartridge (Millipore). After washing with 10 ml 0.002 *M* HNO_3, the components were eluted with 1 ml methanol and the solutions were stored in a refrigerator.

RESULTS AND DISCUSSION

Defining the problem

In the definition of this limited optimization problem we will follow some of the guidelines given by Schoenmakers and Mulholland[2]. All information collected about the sample is of importance later during the course of the optimization and method development.

The separation of the reaction mixture of PQQ and cyclopropanol had been performed earlier by a linear gradient of 28.5–53% (v/v) methanol and 0.4% H_3PO_4 on Novapak-C_{18} RCM cartridges (Millipore-Waters Assoc., Milford, MA, U.S.A.), as described in ref. 3. We were looking for an isocratic method to perform the quantitative separation of the two main components of this reaction mixture. The isocratic method was to be performed occasionally, not on a routine basis. It was intended to favour reduction of analysis time by changing from gradient conditions to isocratic, setting the highest acceptable limit to 30 min.

The samples were available in methanol after the sample preparation step (see Experimental for details). Standards were available only for the two components of interest (PQQ and PQQ-M), the total number of components in these samples being expected to vary between 9 and 16 (depending also on the reaction conditions applied and/or catalysts) according to the earlier gradient experiments.

The concentration range for the main components was expected to be 1–20 mg/ml, and UV absorption detection at 320 nm was applied to record the chromatograms.

Based on the chromatographic information above (reversed-phase gradient at acidic pH) the use of reversed-phase HPLC along with pH adjustment is a possible solution. Alternatively, ion-pairing reagents might also be used at neutral pH, *e.g.*, 7.0 for retention and selectivity control, where the weak carboxylic acid groups of PQQ will be dissociated. An additional problem is that the nature and number of the other (co)products in the reaction mixture are not known; such knowledge can help to reduce the possible range and number of mobile phase parameters. Therefore, the application of a systematic scouting procedure is in order here for the selection of the proper reversed-phase mode and set of mobile phase parameters.

Selection of the optimization parameters by systematic gradient scanning

One of the first steps in the development of an HPLC method is to decide which phase system would be used. The selection of the chromatographic method will depend heavily on the nature and complexity of the sample. When reversed phase seems appropriate, first usually a water to methanol gradient scan is performed in order to determine whether there is sufficient retention of the solutes. However, when the solutes are ionizable, variation of the eluent pH and/or the addition of an ion pairing agent might cause significant shifts in the retention of the solutes.

Recently a systematic gradient scanning strategy has been developed in our laboratory[5], to search for the relative hydrophobicity and charge type of the solutes in unknown sample mixtures. It consists of four linear gradients from 0 to 90% methanol: two at pH 2.5, one of which is performed with a pulse injection of an anionic ion pairing agent (sodium octanesulphonate), and the other two at pH 7.5, one of which is also performed with a pulse injection of a cationic ion pairing agent (tetrabutylammonium bromide) (see Fig. 2).

From the unique retention movement pattern of the (groups of) compounds, information can be deduced as to the nature of the solutes in the sample mixture. The nature and complexity of the sample has primary importance in the selection of the separation mode (ion suppression, ion pairing, etc.) and the optimization vector space. When the two gradients at pH 2.5 without (Fig. 2a) and with the pulse injection of octanesulphonate (Fig. 2b) are compared, no significant shifts in retention are seen, indicating that all solutes are in a uncharged form at this eluent pH. When the gradient elution is performed at pH 7.5, the retention times of all peaks are found to be shorter

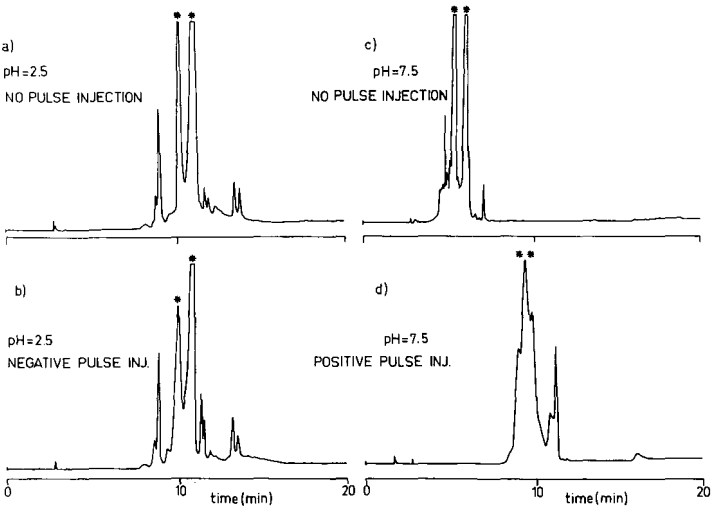

Fig. 2. Gradient elution chromatograms of the PQQ reaction mixture. Linear gradient of 0–90% methanol–triethylamine phosphate buffer in 15 min, at (a) pH 2.5 without pulse injection, (b) pH 2.5 with pulse injection of negatively charged octanesulphonate (as sodium salt), (c) pH 7.5 without pulse injection, (d) pH 7.5 with pulse injection of positively charged tetrabutylammonium (as bromide). Column: 20 cm × 4.6 mm I.D., packed with 5-μm ODS-Hypersil. Flow-rate: 1.0 ml/min ($t_0 = 2.05$ min). UV detection at 320 nm. Asterisks indicate positions of PQQ and PQQ-M.

(Fig. 2c) by about 50%, which is the result of the lower hydrophobic retention of the ionized (dissociated) weakly acidic groups.

At this point we know that all the solutes are weak acids. However, we do not know whether all contain the same number of charged groups, as one or two negative charges may cause an equally early elution in the gradient at pH 7.5. If the number of charged (carboxyl) groups is different for given solutes, the separation selectivity may be increased by the addition of a positively charged ion pairing agent. The fourth gradient, however, shows the entire collection of peaks moving in similar fashion (see Fig. 2d), indicating that all (co)products of the reaction have equal negative charge(s).

On the basis of the results of the scouting experiments we can select the eluent parameters (reversed-phase mode) to be optimized from the following three: the organic modifier concentration, the eluent pH and the concentration of a positively charged pairing ion.

From Fig. 2c we conclude that at pH 7.5 the isocratic retention of the sample is estimated to fall below $k' = 4$ even at 0% methanol, resulting in a very early elution of the (at least ten-component) sample mixture. Also, the UV spectrum of the weak acids may vary substantially with the eluent pH, which may cause problems in recognizing the (relevant) chromatographic peaks in the subsequently measured chromatograms. Thirdly, the larger the optimization vector space (range and number of parameters), the more experimental effort is needed to locate the optimum.

As a "procedural rule", we suggested that the most direct and simple approach should be taken in such cases[4]. Therefore, when it is possible to avoid the simultaneous variation of two or several parameters, the less complex mobile phase system is selected first. As a consequence, for our sample we select the reversed-phase ion-suppression mode at pH 2.5, where the hydrophobic retention of the sample allows the addition of organic modifiers. The optimization in the reversed-phase mode can advantageously be carried out using different organic modifier–water buffer combinations, while less variation is expected in the UV spectrum and in the solute retention.

Definition of the optimization search area

Once the separation parameters have been selected, the optimization search area, *i.e.*, the initial value of the parameters, must be defined. In reversed-phase chromatography (performed in this case at a constant pH of 2.5, *i.e.*, in ion-suppression mode for weak acids) binary, ternary or quaternary eluent mixtures (the last two with fixed or variable eluotropic strength) can be used for selectivity optimization.

Interest has only recently been focused on the selection of the optimization search area, and different approaches have been formulated by representative research groups[6–11]. Unfortunately, no simple rules are readily available for the analyst to decide on the scheme to be preferred.

The statistical approach suggested by Herman *et al.*[6] gives some guidelines on the choice of the optimization parameters in the reversed-phase chromatographic mode. For moderately complex mixtures where the (equivalent) number of components to be separated is less than seven, both binary and ternary solvent mixtures can be used to optimize the separation, with a reasonably high ($> 50\%$) probability of success. The lower limit of the maximum number of components in our sample mixture is estimated to be 10 on the basis of the gradient experiments shown in Fig. 2, while the

polarity range index is found to be 5 (see ref. 6 for details). The 2-out-of-10 limited optimization problem is calculated to be equivalent to a 6-out-of-6 full optimization problem, using the empirical relationships described in ref. 6.

Optionally, either isoeluotropic ternary or variable eluotropic strength binary (or ternary) eluent optimization can be applied. Optimized ternary eluents generally offer a better use of the separation space and shorter analysis time compared to binary systems[6], and this possibility is examined first.

Before starting the isoeluotropic ternary eluent optimization, chromatograms of the sample must be recorded in initial binary eluents which provide closely identical solute retention limits (peak capacities). Usually the results of the linear water–methanol gradient experiment and empirical transfer rules can be applied for the rapid determination of such mobile phase compositions[1,2]. Samples which deviate from the "average" behaviour may need further retention adjustment.

Our reaction mixture contained several homologues of PQQ-M, and predictions based on the average solute behaviour had to be corrected. Efficient experimental procedures described by Herman *et al.*[7], as well as by Sekulic and Haddad[8], can be applied to predict eluent compositions on the basis of isocratically measured retention data. Examples of the operation of such a correction procedure can be found in refs. 1, 7 and 8. In the present example, two or three isocratic measurements were needed to locate the methanol–, acetonitrile–, tetrahydrofuran (THF)–water buffer binaries where the retention of the PQQ reaction mixture was similar (see Fig. 3).

Before starting the optimization procedure it is always advisable to check the possible gain in selectivity by mixing these eluents. Comparison of the initial chromatograms reveals that the two main components (PQQ and PQQ-M) are eluted early and in the same order in these experiments. Retentions are especially short in the acetonitrile binary. The retention times and retention order of the two components of interest are closely identical for the binaries containing methanol and THF. Clearly, not much selectivity improvement is expected, when these binaries are mixed to obtain ternary eluents (likely behaviour is to be expected in a ternary methanol–THF–water mobile phase).

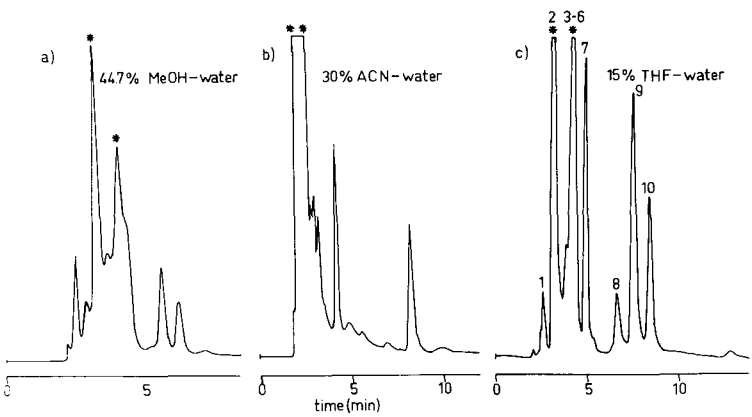

Fig. 3. Isocratic chromatograms of the reaction mixture of PQQ in binary eluents of organic modifier–water buffer (pH 2.5): (a) 0.447 methanol (MeOH); (b) 0.3 acetonitrile (ACN); (c) 0.15 THF. Asterisks indicate positions of PQQ and PQQ-M.

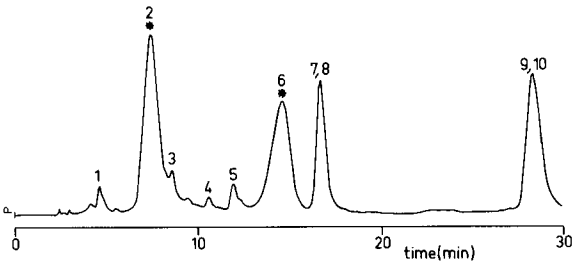

Fig. 4. Chromatogram of the PQQ reaction mixture in triethylamine phosphate buffer (pH 2.5) containing 7.5% THF.

Before (re)considering a more complex optimization vector space, *e.g.*, quaternary mixtures or pH variation it is worthwile to examine the behaviour of the sample in the THF–water binary eluent system because the chromatogram shows more profiles then in methanol–water (see Fig. 3c). This system shows the best distribution of all peaks and the two main components of interest are separated from each other and from interfering coproducts, which is not the case in the other two systems. The variation of the concentration of the organic modifier in this binary eluent will significantly change the retention, but may alter the selectivity, too. The concentration of THF (0.15) in this eluent can be considered as an upper limit, as its further increase will result in even shorter retention. The lower limit of its concentration is determined by the maximum retention allowed for this sample, *i.e.*, the predetermined highest acceptable analysis time of 30 min.

Two additional isocratic experiments were needed to identify an eluent composition of 7.5% THF for eluting the last component at 28.2 min, $k' = 12.5$ (see Fig. 4). In the resulting chromatogram a better spread of all peaks is obtained, but the separation of PQQ from one of the reaction product is still not satisfactory. The retention limits for the first and last peaks eluted at 7.5% and 15% THF ($X = 0$ at 7.5% THF and $X = 1$ at 15% THF; X is a parameter running from 0 to 1, indicating the composition of the variable constituent of the mobile phase) define peak capacities of 9.5 and 14.6 for these chromatograms. A plate count of 3500 and a minimum required resolution of 2 (set to an high value to account for the unequal height of the neighbouring peaks) were used in these calculations. The corresponding probabilities of separation success are found to be 0.06 and 0.56. Therefore, the probability of optimizing the separation by mixing these two eluents containing 7.5 and 15% THF, respectively, is estimated to be about 0.50 (50%).

These starting conditions seemed to be acceptable to start the eluent optimization in the THF–water binary eluent system.

Limited optimization using binary eluent mixtures

The application of solvent optimization procedures to specific problem such as the separation of a limited subset of components requires the recognition of the two solutes of interest in subsequently measured chromatograms, special optimization criteria —which reflect only the separation of the peaks of interest— and a check on peak purity during the method validation procedure.

The "predictive" optimization procedures such as the iterative regression

TABLE I

SOLUTE RETENTION DATA FROM THE SEQUENTIALLY MEASURED CHROMATOGRAMS OF THE TEN-COMPONENT REACTION MIXTURE DURING THE LIMITED OPTIMIZATION PROCEDURE

Tetrahydrofuran–triethylamine phosphate buffer (pH 2.5) as binary eluent mixtures; 5-μm ODS-Hypersil as the stationary phase (column plate count 3500). The value of the weighting factor, w_i, is 1 for the peaks of interest and 0 for the unimportant peaks.

Solute	W_i	*Retention times (min) in chromatograms*				
		1	*2*	*3*	*4*	*5*
1	0	4.5	2.83	3.40	3.74	3.98
2 PQQ	1	7.27	3.45	4.69	5.38	5.92
3	0	8.42	4.21	5.78	6.57	7.15
4	0	10.42	4.30	6.64	7.77	8.65
5	0	11.75	4.40	7.00	8.71	9.74
6 PQQ-M	1	14.47	4.61	7.65	9.55	11.04
7	0	16.53	5.35	9.04	11.28	13.01
8	0	16.53	7.05	10.29	11.90	13.01
9	0	28.20	7.97	14.51	18.48	21.70
10	0	28.20	8.88	15.52	19.20	21.70
Parameter X		0	1	0.386	0.20	0.933
Fraction of THF		0.075	0.15	0.104	0.09	0.082
Fraction of buffer		0.925	0.85	0.896	0.91	0.918
Number of figure		4	3c	6a	6b	7

method[12,13] require the recognition of all peaks. Peak tracking in our case was mainly done on the basis of the UV spectra of the components. First the spectra of the PQQ, PQQ-M standards and the apparently pure peaks in the initial chromatograms (see Figs. 3c and 4) were collected. Coelution of peaks 9 and 10 in chromatogram 1 (see Table I) was found according to the peak areas (from chromatogram 2). Peaks 7 and 8 have very different spectra (taken from chromatogram 2) and their coelution in chromatogram 1 was easily recognizable. A number of small peaks (3–5) were recognized to be due to homologues of PQQ-M, and their retention was estimated to fall between those of PQQ and PQQ-M in chromatogram 2. All the assumptions on the identity and order of the peaks were justified by further measurements. Without giving all the details of peak tracking, the main steps of the limited optimization are discussed below.

Recently, we have adapted some resolution-based criteria in order to express the quality of separation in limited optimization[14]. Weighting factors, w_i, with values of 1 (important) and 0 (unimportant) are assigned to each component of the sample (see Table I). The resolution between two consecutive peaks is taken as relevant when (w_i OR w_{i+1}) = 1, $i = 0 \ldots (M-1)$, where OR means the logical OR function, $i = 0$ for the solvent peak and M is the total number of peaks. A sequential approach was suggested[14] and tested[1] for the selection of different optimization criteria, adjusted to the goal of the analyst. Our primary goal here is to reach a satisfactory (required $R_{s,req} = 2.0$) resolution for the two peaks of interest, within the time constraints set by the two initial chromatograms. For this purpose the minimum resolution criterion,

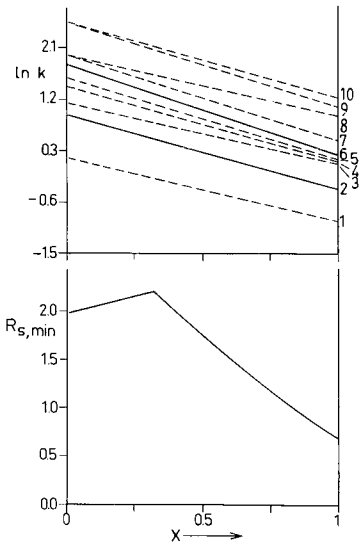

Fig. 5. Phase selection diagram in the THF–water buffer binary eluent system (0 = 7.5% THF and 1 = 15% THF), constructed from the chromatograms shown in Figs. 3c and 4. (top) Plots of ln k; solid lines refer to PQQ and PQQ-M; (bottom) response surface for the minimum resolution criterion, $R_{s,min}$.

$R_{s,min}$, can be used. The position of the two important solutes (PQQ and PQQ-M) is indicated by the symbol (★) in the chromatograms recorded in the THF–water buffer binary eluents.

Using the procedure applied for the optimization of ternary mobile phases, the logarithm of the solute capacity factors is assumed to be a linear function of the binary eluent composition (shown in Fig. 5a). These retention plots are used to calculate the minimum resolution criterion, over the concentration range of 0.075 to 0.15 THF in water (Fig. 5b). A maximum value of $R_{s,min} = 2.2$ is predicted for the eluent containing 9.9% THF.

After two additional measurements at "shifted" compositions (see Fig. 6) predicted by the iterative optimization method[12,13], a final optimum is found at 8.2% THF.

The chromatogram verifying this optimum is shown in Fig. 7, with an analysis time of 22 min. The two peaks of interest are separated from their (small) neighbouring peaks, while some pairs of peaks, which were assigned to be not of interest, are coeluted, *e.g.*, 7–8, 9–10. The $R_{s,min}$ is calculated to be greater than 2 (using retention data and a constant plate count of 3500), a value representative of the extent of the separation for the large peaks (which are of interest in this case) as a result of different peak heights[15]. The resolution obtained between peaks 2 and 3 and 5 and 6 may be less than 2 due to tailing bands, but quantitation of the relevant peaks 2 and 6 should be no problem.

The optimum was considered to be adequate for the requirements formulated at the beginning of this discussion, and no attempt was made to optimize for higher R_s or shorter analysis time. If the analysis were performed on a routine basis, both R_s and the analysis time may need further adjustments. Nevertheless, a "better" optimum would

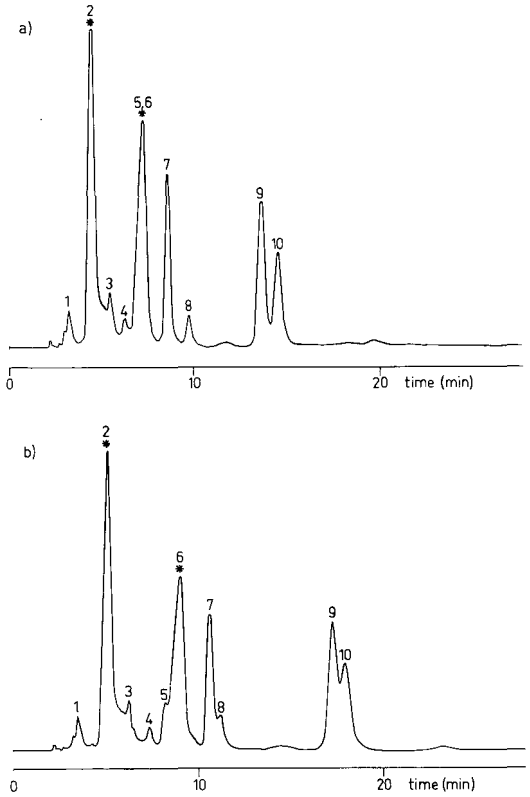

Fig. 6. Chromatograms measured during the iterative regression optimization procedure in the THF–water buffer (pH 2.5) binary eluent system. (a) 0.104 THF; (b) 0.091 THF.

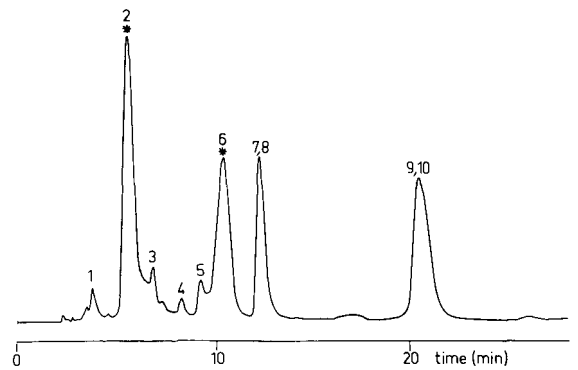

Fig. 7. Separation of the two solutes of interest (PQQ and PQQ-M) in the reaction mixture of PQQ and cyclopropanol under optimum conditions, in triethylamine phosphate buffer (pH 2.5) containing 0.082 THF.

need more effort, possibly reconsideration of the optimization search area, *e.g.*, variable eluotropic strength ternary eluent optimization or even the separation mode, *e.g.*, pH variation or ion pairing. Obviously, in that case one must perform some "backtracking" to an earlier stage in the method development scheme, as suggested by Schoenmakers and Mulholland[2].

When the optimum is accepted, a final step is the check on the purity of the peaks of interest. This is undoubtedly needed, as the peak of PQQ-M is much broader than the other peaks of the chromatogram. However, when the UV spectrum and chromatographic characteristics of the respective peaks are compared for the standards and the sample, no differences are found. Additional information on PQQ-M indicated[3], that a ring closure to a tetrahydrofuran structure between the C(5)–CH_2–CH_2–CHO and the C(5)–OH groups (see Fig. 1) can result in another possible configuration of this compound. The simultaneous existence of these two configurations both for the "standard" and the reaction mixture may be responsible for the broader sample peak with very similar spectral characteristics.

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FACTOR ANALYSIS AND EXPERIMENT DESIGN IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

VIII[a]. CHARGE-TRANSFER *VERSUS* STANDARD STATIONARY PHASES IN NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation mechanism and selectivity of the separation of a group of the 4,4′-substituted *E-s-cis* and *Z-s-cis* isomeric chalcones on charge-transfer stationary phases were compared with those on diol, CN, NO_2, etc., stationary phases. Correspondence factor analysis and the hierarchical ascending classification method were used. The results obtained provide evidence of the efficiency of charge-transfer stationary phases in the separation of configurational isomers.

INTRODUCTION

In recent years the effects of charge-transfer interactions have played an important role in high-performance liquid chromatographic (HPLC) separations. Chemically bonded charge-transfer phases are often applied to separate coal liquids and other aromatic mixtures. In order to broaden the range of applicability of charge-transfer stationary phase and to define their specific properties more closely, we have compared the retention mechanisms of a wide range of electron-rich

[a] For Part VII, see *J. Chromatogr.*, 395 (1987) 183.

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compounds on charge-transfer stationary phases and on standard chemically bonded stationary phases of the diol, CN, NO_2, etc., type, which are commonly used in normal-phase HPLC.

EXPERIMENTAL

Samples

A series of 4- and 4′-substituted chalcones (*E-s-cis* and *Z-s-cis*), $XC_6H_4CH=CHCOC_6H_4Y$, were chromatographed using eleven normal-phase HPLC systems; the substituents X and Y are given in Table II. *E-s-cis* chalcones were synthesized[1] and the corresponding *Z-s-cis* isomers formed spontaneously in dichloromethane solution. Throughout this paper *E-s-cis* chalcones are referred to as X–Y and *Z-s-cis* chalcones as X–Y*.

Chromatographic systems

The mobile phase was HPLC-grade heptane–tetrahydrofuran (97:3, v/v), purchased from Merck (Darmstadt, F.R.G.). The stationary phases are listed in Table I.

Instruments

The HPLC equipment consisted of a Bruker LC-31 pump, a Rheodyne Model 7125 injection valve, a Schoeffel Model SF 770 spectrophotometer as detector, set at 280 nm, and a Shimadzu C-R1B recorder.

Chromatographic procedure

Prior to measurements, the columns were washed with the mobile phase until a constant value was obtained for the retention of chalcones. Sample solutions (2 mg

TABLE I

COLUMN PACKINGS

Stationary phases 7–11 were synthesized in the Laboratoire de Chimie Organique du Silicium et de l'Etain of the Université de Bordeaux, France, and were kindly donated by Dr. G. Felix.

No.	*Column packing*[a]	*Dimensions (mm × mm)*	*Supplier*	*Ref.*	*Abbreviation*
1	Zorbax NH_2	250 × 4.6	DuPont		NH_2
2	LiChrospher 100 diol	250 × 4.6	Merck		diol
3	MicroPak CN	300 × 4	Varian		CN
4	Zorbax ODS	150 × 4.6	DuPont		ODS
5	Zorbax C_8	90 × 4	DuPont		C_8
6	RSIL NO_2	250 × 4.6	Alltech		NO_2
7	Spherisorb AP	90 × 4		2	AP
8	Spherisorb DNAP	90 × 4		3	DNAP
9	Spherisorb DNB	90 × 4		4	DNB
10	Spherisorb TCP	90 × 4		5	TCP
11	Spherisorb TB	90 × 4		6	TB

[a] AP = *n*-propyl picryl ether; DNAP = 3-(2,4-dinitroanilino)propyl; DNB = 3,5-dinitrobenzamidopropyl; TCP = tetrachlorophthalimidopropyl; TB = caffeine.

per 25 ml) were prepared in dichloromethane. All data points were collected as averages from two or three reproducible separations. The dead time, t_0, of the column was determined using benzene and cyclohexane markers. The capacity factor, k', was calculated from the retention time of the solute, t_R, according to the equation $k' = (t_R - t_0)/t_0$.

Data processing

A set of "abstract" factors affecting the selectivity in the normal-phase HPLC systems were extracted by correspondence factor analysis (CFA)[7,8], which is a method for the analysis of a table of positive data arranged as matrix elements k_{ij} in n rows ($i = 1,2,\ldots, n$) and p columns ($j = 1,2,\ldots, p$). CFA can be used to analyse how a studied data matrix K differs from a hypothetical matrix K^*, its elements defined by

$$k_{ij}^* = (k_{i\cdot}\, k_{\cdot j})/k$$

where

$$k_{i\cdot} = \sum_{j=1}^{p} k_{ij};\ k_{\cdot j} = \sum_{i=1}^{n} k_{ij};\ k = \sum_{i=1}^{n} \sum_{j=1}^{p} k_{ij}$$

The characteristic property of a hypothetical K^* matrix is that its rows are proportional to each other, and so are its columns.

The principle of CFA is to associate with every row of the K matrix a point in p-dimensional space so that the distance between two rows i and i' clearly shows the deviation from proportionality between them. Simultaneously, a second cluster of j points associated with every column of the matrix K is built in n-dimensional space, so that the distance between two columns j and j' shows their deviation from proportionality.

To establish the relative locations of the $(n + p)$ points (as the rows and columns play a symmetrical role in CFA), a small-dimensional subspace is constructed, in which the cluster of projected points is most extended. The factors of CFA that define this subspace are the eigenvectors of a correspondence matrix obtained as the covariance matrix of the original data divided by the square root of the row and column sums.

The results of the analysis can be demonstrated as a projection of the $(n+p)$ points on the planes defined by the successive main axes of cluster inertia (the CFA factors), *e.g.*, on the plane defined by the two main axes (axes 1 and 2), then on the plane defined by axes 2 and 3, etc.

In order to determine the number of factors (*i.e.*, the dimension of the constructed subspace) we use principal component analysis (PCA)[9]. The results of PCA indicated that when comparing the retentions of chalcones using the eleven chromatographic systems as many as seven factors ought to be taken into consideration. The error of the data reproduction is then 5.05%. It is known[8] that the number of significant CFA factors is equal to the number of significant PCA factors minus 1, which means that in the CFA six factors need to be taken into consideration.

To establish the similarity of the chromatographic behaviours of solutes and/or chromatographic systems, the hierarchical ascending classification (HAC)[10] was used,

the basis of which can be presented in the following way. Let n denote the number of objects, N the number of observations on these objects and x_{ij} (where $i=1,2,\ldots,n$ and $j=1,2,\ldots,N$) the numerical value related to the jth observation performed on the ith object. The arranged series of numerals $\{x_{i1}, x_{i2},\ldots, x_{iN}\}$ can be regarded the observation vector of the ith object, and the individual numerals x_{ij} are components of this vector. These components determine the position of the ith object in the N-dimensional observation space. Hence, from the geometrical standpoint the considered objects can be viewed as a set of n points in the N-dimensional observation space, in which the inter-object distances can furnish a criterion of their similarity. These distances can be defined in a number of different ways[10]; in this paper inter-object distance is expressed by the Euclidean distance measure.

The first step in the HAC procedure depends on calculating the distances between all pairs of object, and from the n objects one chooses those two which are the closest to one another (*i.e*, in this case, in which the inter-object distance is the smallest). These two objects are fused to form a group, and then replaced by the coordinates of their centroid. In the next step, one calculates distances between the pairs of $(n-1)$ objects, and again the closest two are joined. The procedure is repeated until all the objects are eventually agglomerated. The subsequent agglomerations are presented in the form of a classification tree (*i.e.*, dendrogram), and each agglomeration level corresponds to a numerical value representing the distance of the objects. Because we consider those objects for wich the distance is zero to be identical (similarity = 100%), and the objects for which the relatively greatest distance is observed to be dissimilar (similarity = 0%), the relative scale of object similarity varies from 100 to 0%.

RESULTS AND DISCUSSION

Polarity of phases

The experimental results obtained for the chromatographic systems with stationary phases 1–5 (see Table I) have been published[11]. The capacity factors, k', for chalcones chromatographed using the remaining six chromatographic systems are presented in Table II.

With respect to the mean capacity factors ($\bar{k}'_j = \sum_i^{38} k'_{ij}/38$; see Table III), the investigated chromatographic systems can be arranged in the order DNAP > DNB > NH_2 > TCP > CN > NO_2 > TB > AP > diol > ODS > C_8. Hence the polarities of the charge-transfer stationary phases cover a fairly wide range (the mean capacity factor for DNAP is almost five times higher than that for AP; Table III).

Retention mechanism

Examination of the log k'_{ij} *vs.* log $k'_{ij'}$ relationship (where j and j' denote the jth and j'th stationary phases, respectively) can help in comparing the retention mechanisms of the studied group of chalcones in the individual chromatographic systems. Let us assume the following classification rules according to Melander *et al.*[12]: with a correlation coefficient $r \geqslant 0.95$ for the log k'_{ij} *vs.* log $k'_{ij'}$ relationship, the retention mechanisms can be regarded as homo- or homeoenergetic; and with $r < 0.95$, the retention mechanisms on the jth and j'th stationary phases can be regarded as heteroenergetic.

TABLE II

CAPACITY FACTORS, k', FOR 38 *E-s-cis* AND *Z-s-cis* CHALCONES SEPARATED ON DIFFERENT COLUMNS

No.	*Chalcone X–Y*	*Stationary phase*					
		NO_2	*AP*	*DNAP*	*DNB*	*TCP*	*TB*
1	H–CF_3	1.74	0.92	2.84	2.40	2.52	1.78
2	H–*t*-C_4H_9	2.69	1.33	7.01	5.77	3.25	1.85
3	H–*i*-C_3H_7	2.77	1.37	7.07	5.79	3.70	1.91
4	H–H	2.92	1.61	6.14	5.38	5.12	2.41
5	F–H	1.93	1.66	6.18	5.10	5.17	2.61
6	H–F	2.61	1.35	4.70	3.99	4.32	2.30
7	H–C_2H_5	3.06	1.55	7.77	6.42	4.97	2.14
8	H–CH_3	3.42	1.79	8.56	7.07	6.32	2.57
9	F–CH_3	3.89	1.81	8.22	6.51	6.14	2.70
10	F–F	3.13	1.50	4.99	3.97	4.41	2.57
11	CH_3O–CH_3	10.42	4.90	29.57	21.78	21.02	6.05
12	CH_3–CH_3O	5.82	4.70	31.88	21.89	20.33	6.12
13	F–CH_3O	5.49	4.67	22.57	15.57	17.91	6.45
14	H–NO_2	6.05	2.77	12.01	9.38	10.10	6.83
15	NO_2–CH_3	10.35	4.43	23.96	17.06	16.80	8.97
16	NO_2–H	9.78	4.33	18.91	13.81	14.59	2.21
17	CH_3O–CH_3O	12.23	10.84	82.46	49.44	47.92	13.69
18	NO_2–F	12.44	4.29	19.34	12.74	13.59	9.88
19	CH_3O–C_6H_5	14.67	6.72	43.26	29.38	36.92	10.63
20	CH_3–C_6H_5	5.40	2.82	17.00	12.36	13.19	4.61
21	H–CF_3*	1.39	0.67	1.63	1.63	1.25	1.16
22	H–*t*-C_4H_9*	1.89	0.89	3.65	4.18	1.51	1.31
23	H–*i*-C_3H_7*	1.96	0.94	3.69	4.22	1.72	1.37
24	H–H*	2.02	1.09	3.12	3.60	2.45	1.67
25	F–H*	1.59	1.02	2.71	2.81	2.32	1.70
26	H–F*	1.93	0.96	2.48	2.75	2.10	1.48
27	H–C_2H_5*	2.11	1.02	3.99	4.55	2.30	1.51
28	H–CH_3*	2.32	1.22	4.20	4.90	2.97	1.83
29	F–CH_3*	2.12	1.09	3.37	3.60	2.65	1.76
30	F–F*	2.03	1.02	2.37	2.36	2.08	1.67
31	CH_3O–CH_3*	4.70	2.48	9.09	11.61	8.40	3.54
32	CH_3–CH_3O*	3.39	2.90	10.94	12.77	8.40	4.01
33	F–CH_3O*	4.72	2.63	8.05	8.36	6.74	4.01
34	H–NO_2*	4.61	1.91	6.22	5.64	5.20	3.99
35	NO_2–CH_3*	7.53	3.50	15.18	13.10	9.01	7.28
36	NO_2–H*	7.39	3.65	13.23	11.20	8.43	1.56
37	CH_3O–CH_3O*	10.78	5.82	23.19	27.41	19.84	7.99
38	NO_2–F*	8.78	3.84	13.67	10.65	8.30	7.88

The results in Table IV indicate that the separation mechanism on most pairs of stationary phases is heteroenergetic; $r > 0.95$ only for 15 of the 55 pairs of systems.

A detailed analysis of the chromatographic data and the specific properties of the individual stationary phases was performed with the help of CFA. The data matrix was constructed of the capacity factors for 38 chalcones developed with the eleven chromatographic systems. Fig. 1 gives an example of chalcones and chromatographic systems projected on the plane defined by the two main CFA axes.

The factor space obtained is six-dimensional, the percentage contributions of the

TABLE III

MEAN CAPACITY FACTORS, $\bar{k}'$, AND MEAN SELECTIVITY PARAMETERS, $\bar{\alpha}_{X-Y/X-Y^*}$, FOR THE INVESTIGATED CHROMATOGRAPHIC SYSTEMS

No.	*System*	$\bar{k}'$	$\bar{\alpha}_{X-Y/X-Y^*}$
1	NH_2	9.76	1.10
2	diol	2.50	1.40
3	CN	7.28	1.26
4	ODS	1.34	1.21
5	C_8	0.76	1.02
6	NO_2	5.05	1.45
7	AP	2.68	1.52
8	DNAP	13.03	2.18
9	DNB	10.29	1.56
10	TCP	9.31	2.15
11	TB	4.05	1.50

individual factors to the total cluster inertia being 70.66, 8.90, 6.93, 5.89, 3.24 and 2.78%. The mutual distance of the chromatographic systems in the six-dimensional factor space is χ^2, defined by

$$\chi^2(j,j') = k \sum_{i=1}^{n} (k'_{ij}/k_{\cdot j} - k'_{ij'}/k_{\cdot j'})/k_{i\cdot}$$

where

$$k_{i\cdot} = \sum_{j=1}^{p} k'_{ij}$$

$$k_{\cdot j} = \sum_{i=1}^{n} k'_{ij}$$

$$k = \sum_{i=1}^{n} \sum_{j=1}^{p} k'_{ij}$$

TABLE IV

NUMERICAL VALUES OF THE CORRELATION COEFFICIENT, r, OF THE LOG k'_{ij} *vs.* LOG $k'_{ij'}$, RELATIONSHIPS FOR 55 PAIRS OF THE CHROMATOGRAPHIC SYSTEMS

	NH_2	*diol*	*CN*	*ODS*	*C_8*	*NO_2*	*AP*	*DNAP*	*DNB*	*TCP*	*TB*
NH_2	1.000										
diol	0.975	1.000									
CN	0.990	0.993	1.000								
ODS	0.938	0.919	0.925	1.000							
C_8	0.945	0.900	0.920	0.960	1.000						
NO_2	0.931	0.956	0.952	0.867	0.853	1.000					
AP	0.933	0.963	0.952	0.918	0.910	0.952	1.000				
DNAP	0.849	0.906	0.884	0.882	0.823	0.923	0.973	1.000			
DNB	0.856	0.882	0.877	0.831	0.851	0.911	0.968	0.982	1.000		
TCP	0.857	0.920	0.893	0.862	0.822	0.922	0.978	0.975	0.957	1.000	
TB	0.857	0.886	0.868	0.834	0.793	0.867	0.892	0.869	0.853	0.890	1.000

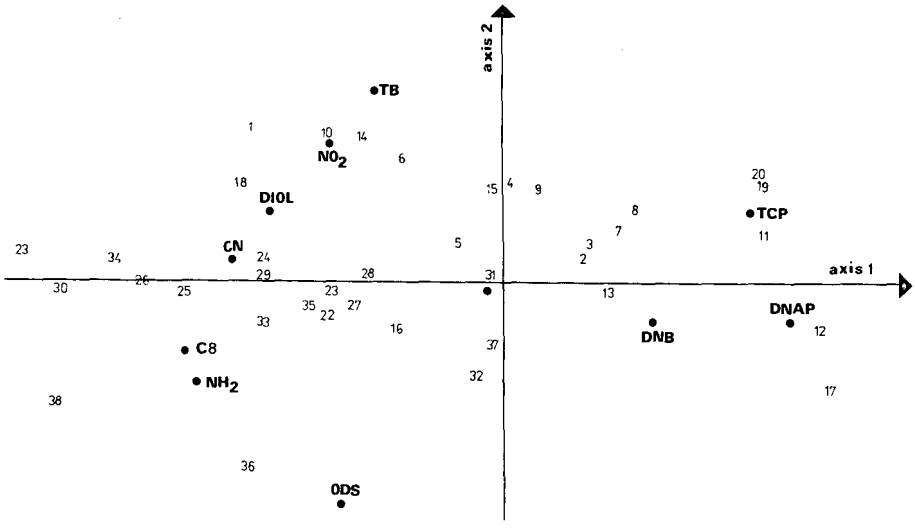

Fig. 1. Projection of chalcones (compounds 1–38; see Table II) and the chromatographic systems on the plane defined by the two main CFA axes.

p and n are the number of stationary phases and compounds, respectively, and k'_{ij} is the capacity factor of the ith solute in the jth chromatographic system.

The χ^2 (j,j') distance can be used as a criterion of similarity of the separation mechanisms on the jth and j'th stationary phases[13], because χ^2 (j,j') describes the deviation from proportionality between the capacity factors measured on the column pairs (j and j'); for the chromatographic systems showing a homoenergetic separation mechanism, χ^2 (j,j') = 0. Analogously, the χ^2 (i,i') distance can serve as a measure of similarity of the chromatographic behaviours of the ith and i'th compound, and χ^2 (i,j) permits a rapid evaluation of the specifity of the jth chromatographic system toward the ith compound.

Assuming the χ^2 distance to be a measure of similarity or differentiation of the investigated objects, we classified chalcones and the chromatographic systems by means of the HAC. The results obtained presented in form of a classification tree are shown in Fig. 2.

The data in Fig. 2 indicate that only at an aggregation level of 21% do the investigated chromatographic systems form three distinct sub-groups: (A) NH_2, diol, CN, NO_2, ODS and C_8; (B) TB; and (C) DNB, AP, TCP and DNAP. The stationary phase TB seems slightly closer to those in the sub-group A than to those in sub-group C. Among the phases in sub-group A the greatest similarity is observed for diol and CN. In sub-group C the pairs TCP–DNAP and DNB–AP as the most similar.

At an aggregation level of 50% we can distinguish the following sub-groups of the investigated chalcones: (a) H–H*, F–CH_3*, F–CH_3O*, NO_2–H*, NO_2–CH_3*, H–F, F–F, H–NO_2, H–CF_3 and NO_2–F; (b) NO_2–F*, H–CF_3*, F–F* and H–NO_2*; (c) NO_2–H; (d) NO_2–H*; (e) H–*t*-C_4H_9, H–*i*-C_3H_7, H–C_2H_5 and H–CH_3; (f) H–*t*-C_4H_9, H–*i*-C_3H_7, H–C_2H_5, H–CH_3, H–H, F–CH_3, NO_2–CH_3, F–H, F–CH_3O, CH_3O–CH_3*, CH_3O–CH_3O* and CH_3–CH_3O*; and (g) CH_3O–C_6H_5, CH_3–C_6H_5,

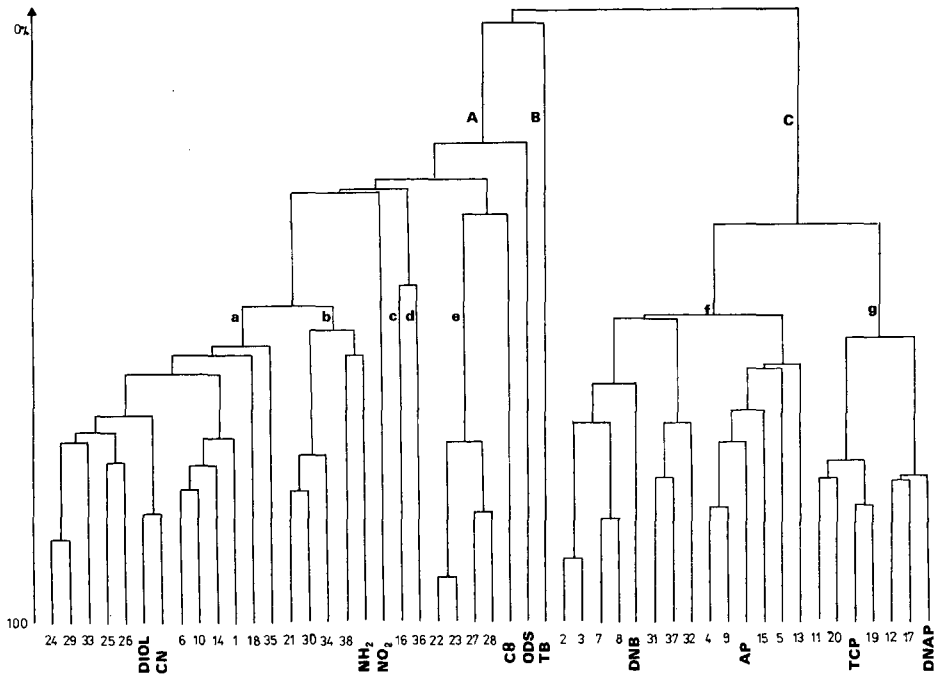

Fig. 2. Classification tree from the HCA for 38 chalcones and 11 normal-phase HPLC systems. The classification criterion is the euclidian distance in the six-dimensional factor space built by means of CFA.

CH_3O–CH_3, CH_3–CH_3O and CH_3O–CH_3O. These sub-groups of solutes are based on their specific behaviour toward the individual stationary phases. Thus the solutes in sub-group a show a relatively higher retention on stationary phase C_8, those in sub-group b on NH_2, those in sub-group c on diol and CN, etc. The specific properties of the charge-transfer stationary phases in sub-group C depend on their relatively more effective interactions (higher k' values) with chalcones in sub-group f and g.

Separation of substitutional isomers

Let us consider the reltive retentions of the pairs of *E-s-cis* substitutional isomers NO_2–H, H–NO_2; F–H, H–F; and CH_3–CH_3O, CH_3O–CH_3 (see Fig. 3a). It can be stated that the relatively best separation is observed for the isomer pair NO_2–H and H–NO_2. With stationary phases 1–10 $\alpha_{NO_2-H/H-NO_2}$ changes from 1.42 to 1.59. On stationary phase TB the elution order for this pair of isomers reverses, and consequently $\alpha_{NO_2-H/H-NO_2} = 0.32$. The selectivity of the separation of the isomer pair F–H and H–F is lower than in the previous instance, although it is satisfactory on each stationary phase ($\alpha_{F-H/H-F}$ varies in the range 1.13–1.31, and the elution order reverses only on the NO_2 phase, while the respective $\alpha_{F-H/H-F} = 0.74$). In contrast to the aforementioned examples, separation of the substitutional isomers CH_3–CH_3O and CH_3O–CH_3 is much poorer ($\alpha_{CH_3-CH_3O/CH_3O-CH_3}$ range from 0.96 to 1.08). A satisfactory separation of these isomers is achieved exclusively on the NO_2 phase ($\alpha = 0.56$).

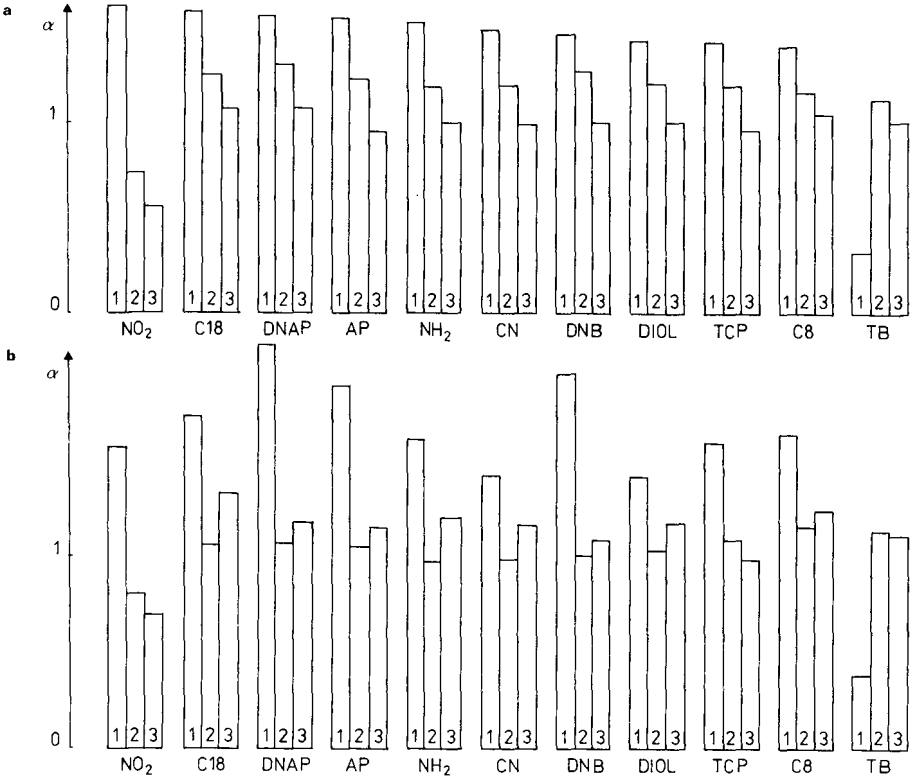

Fig. 3. Comparison of the selectivity parameter, α, for the pairs of substitutional isomers (1) NO_2–H, H–NO_2, (2) F–H, H–F and (3) CH_3–CH_3O, CH_3O–CH_3, with configurations (a) *E-s-cis* and (b) *Z-s-cis*.

The selectivity of separation of the analogous *Z-s-cis* substitutional isomers is considerably influenced by the stationary phase (see Fig. 3b). $\alpha_{NO_2-H^*/H-NO_2^*}$ varies in the range 1.43–2.13 for stationary phases 1–10 ($\alpha_{NO_2-H^*/H-NO_2^*} = 0.39$ for phase 11). The reverse situation occurs with the isomers F–H* and H–F*. $\alpha_{F-H/H-F^*}$ is lower than $\alpha_{F-H/H-F}$, and the only exception in this instance is furnished by phase 11. A relatively improved selectivity of separation is observed with isomers CH_3–CH_3O* and CH_3O–CH_3*. Only with phase 10 is $\alpha_{CH_3-CH_3O^*/CH_3O-CH_3^*} = 1$, wereas for phases 1–8 and 11 $\alpha_{CH_3-CH_3O^*/CH_3O-CH_3^*}$ varies in the range 1.10–1.36. For the NO_2 phase (9) the retention order of the discussed isomers reverses, and consequently $\alpha_{CH_3-CH_3O^*/CH_3O-CH_3^*} = 0.72$.

Separation of configurational isomers (E-s-cis and Z-s-cis)

The selectivity of separation of the isomeric *E-s-cis* and *Z-s-cis* chalcones varies over a very wide range on shifting from one chromatographic system to another. The mean selectivity parameters $\bar{\alpha}_{X-Y/X-Y^*}$ determined for eighteen pairs of isomers are given in Table III and indicate that the charge-transfer stationary phases separate the *E-s-cis* and *Z-s-cis* isomers far better than the standard phases. Particularly high efficiency of separation is observed with DNAP and TCP, for which the mean selectivity parameters $\bar{\alpha}_{X-Y/X-Y^*}$ are 2.18 and 2.15, respectively. Fig. 4 shows an

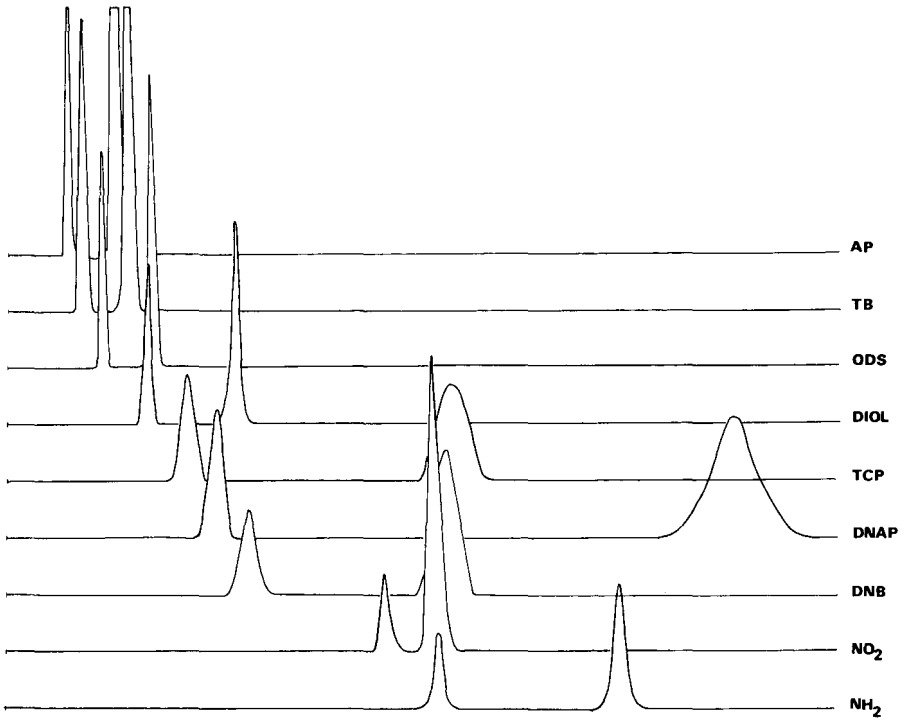

Fig. 4. Separation of the isomers CH_3O–CH_3O (*E-s-cis*) and CH_3O–CH_3O* (*Z-s-cis*) on the selected stationary phases. Mobile phase: heptane–THF (97:3, v/v). Detection: UV, 280 nm.

example of the separation of the isomeric CH_3O–CH_3O and CH_3O–CH_3O* chalcones on the selected stationary phases.

The selectivity of separation of the *E-s-cis* and *Z-s-cis* isomers depends, however, on the chemical nature of the substituents present in the chalcone molecule. The degree of separation similarity for the *E-s-cis* and *Z-s-cis* isomeric pairs on the eleven stationary phases can be established from the results of HAC, given in Fig. 5. This classification indicates that the isomeric monosubstituted chalcones (H–Y and H–Y*) appear in sub-groups A and C. The isomers with an NO_2 substituent in position 4 are all in sub-group B. Sub-groups D, E and F contain the isomers with a CH_3O substituent in position 4 and/or 4′ and an F substituent in position 4.

The analogous classification of the stationary phases allows their similarity to be described with respect to the separation selectivity for the eighteen pairs of the *E-s-cis* and *Z-s-cis* isomeric chalcones (see Fig. 6). The classification tree obtained indicates that the stationary phases can be divided into three sub-groups: (A) AP, DNB, TB, diol, CN and NO_2; (B) NH_2, ODS and C_8; and (C) DNAP and TCP. It should be emphasized that the stationary phases DNAP and TCP differ to the greatest extent from the others.

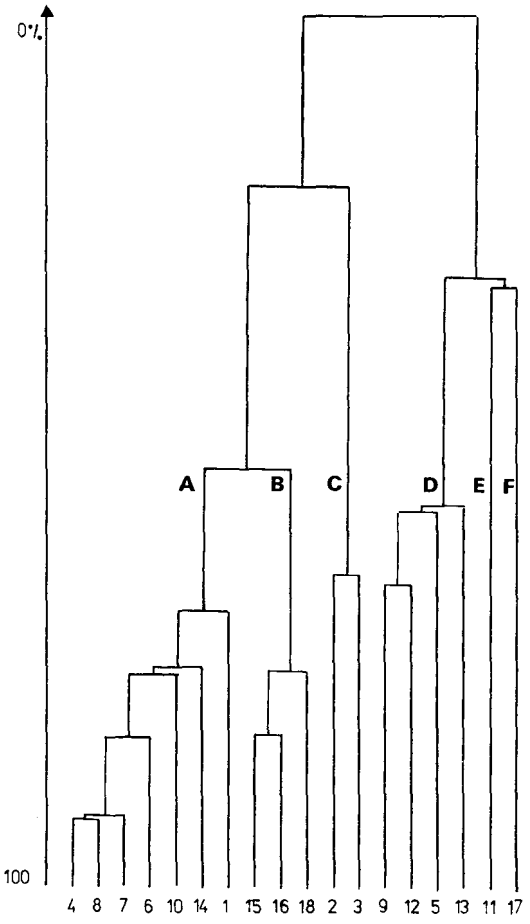

Fig. 5. Classification tree from the HAC for the selectivity parameters $\alpha_{X-Y/X-Y^*}$ for 18 pairs of *E-s-cis* (X–Y) and *Z-s-cis* (X–Y*) isomeric chalcones. The numbering of the parameters $\alpha_{X-Y/X-Y^*}$ conforms with the numbering of chalcones X–Y in Table II.

CONCLUSIONS

The polarity of the investigated charge-transfer stationary phases changes to a relatively great extent in the order DNAP > DNB > TCP > TB > AP. The retention mechanism on the charge-transfer stationary phases compared with that on standard phases (diol, CN, NH_2, etc.) is heteroenergetic (see Table IV) (the only exception is phase AP). The separation of the substitutional isomers on the charge-transfer phases does not differ substantially from that on the standard phases (see Fig. 3). The selectivity of separation of the *E-s-cis* and *Z-s-cis* chalcone isomers on the charge-transfer stationary phases is far better than that on the standard phases. Special attention in this respect should be paid to the phases DNAP and TCP (see Table III and Fig. 4).

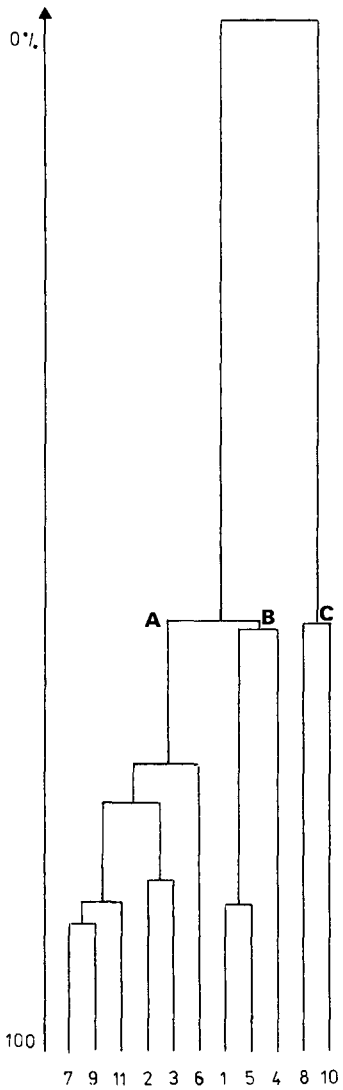

Fig. 6. Classification tree from the HAC for eleven normal-phase HPLC systems. The classification criterion is the euclidian distance in the eighteen-dimensional space of the parameters $\alpha_{X-Y/X-Y}$*. The numbering of the systems is given in Table I.

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PROPERTIES OF TOPOLOGICAL INDICES AS STRUCTURAL PARAMETERS FOR COMPOUNDS CONTAINING OLIGOOXYETHYLENE CHAIN(S)

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SUMMARY

The application of different topological indices as structural descriptors is discussed for several groups of compounds exhibiting surface activity and potential extractants of metals. The influence of different structural fragments, such as alkyl groups, oligooxyethylene and/or thioethylene chains and heteroatoms, on the topological indices is discussed.

INTRODUCTION

Attempts to describe the structure of a compound by quantitative parameters led to the definition of several topological indices. Randič[1–4] and Kier and Hall[5,6] developed a system of molecular and valence molecular connectivity indices. These were extensively examined in quantitative structure–property (QSPR) or structure–activity (QSAR) relationships[7–14]. The application of molecular connectivity indices in correlation analysis was recently reviewed by Ekiert *et al.*[15].

Other topological indices are based on the concept of the molecular distance matrix. The Rouvray index[16], Wiener number[17] and Balaban index[18] have been considered as descriptors of structure. These parameters were first calculated for saturated hydrocarbons but the work of Balaban[18] allowed the range of compounds to be extended to unsaturated and aromatic hydrocarbons. Barysz *et al.*[19] reported on the construction of the distance matrix for graphs representing any molecular system, including systems containing heteroatoms.

Some studies on retention–structure[20–28] and polarity–structure relationships[29–35] have been published. The structures of examined compounds, such as liquid stationary phases in gas chromatographic columns, were described by using the numbers of characteristic fragments in their molecules. Some effect of neighbouring groups was also considered[36–39]. In many instances the description of the structure of a compound was rather qualitative[40,41].

The application of different topological indices to describe the structures of alkane and alkene molecules has been examined[42–44]. Kaliszan[45] stated that the connectivity index does not adequately reflect the retention index differences caused by

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varying unsaturation. This conclusion was confirmed in part, by Voelkel[44], when the use of dipole moment as a parameter of electric interactions was necessary in order to obtain satisfactory retention–structure correlations. Kaliszan[45] discussed the connectivity index as a useful descriptor of non-specific dispersive interaction increments of retention. If dispersion forces predominate (*i.e.*, on non-polar liquid stationary phases), the chromatographic behaviour of solutes could be explained by means of connectivity parameters. With polar stationary phases and/or solutes of varying polarity, the connectivity indices become of less importance for retention as the specific polar interactions predominate. However, as was shown for alkenes[44], the discriminating power of topological indices [I_B, $W(G)$, $R(G)$] is higher and they could probably much better act as a useful tool for compounds containing heteroatoms.

The aim of this paper is to discuss the usefulness of the examined parameters in describing the structures of a selected group of compounds with different structural elements. Such compounds exhibit surface-active properties and some are used as extractants of metals[29–35,45–47]. The application of the considered topological indices as structural parameters in the relationship between the polarity and structure of the examined compounds is considered below.

EXPERIMENTAL

Topological indices were calculated for four groups of compounds containing oligooxyethylene chain(s) in their molecules:

(a) oligooxyethylene glycol dialkyl ethers, $RO(CH_2CH_2O)_nR$ (**1**), where R = C_4H_9, C_8H_{17} or $C_{12}H_{25}$ and n = 3–9, and some of their sulphur analogues, $C_4H_9(OCH_2CH_2)_n(SCH_2CH_2)_mS(CH_2CH_2S)_m(CH_2CH_2O)_nC_4H_9$ (**2**), where m = 0–2 and n = 1–4;

(b) amino ether alcohols, $[R(OCH_2CH_2)_n]_2NCH_2CH_2OH$ (**3**), and their ethers, $[R(OCH_2CH_2)_n]_2N(CH_2CH_2O)_{n+1}H$ (**4**), where R = C_4H_9, C_6H_{13} or C_8H_{17} and n = 1–3;

(c) 1,3-bis[ω-alkoxyoligo(oxyethylene)]propan-2-ols, $RO(CH_2CH_2O)_nCH_2CH(OH)CH_2(OCH_2CH_2)_mR^1$ (**5**), where $R = R^1$ or $R \neq R^1$ = C_4H_9, C_6H_{13} or C_8H_{17} and $n = m$ or $n \neq m$ = 0–4;

(d) oligooxyethylene derivatives of aliphatic alcohols, thio alcohols and alkylamines, $RX(CH_2CH_2O)_nR'$ (**6**), where R = C_4H_9, C_6H_{13}, C_8H_{17}, $C_{10}H_{21}$, $C_{12}H_{25}$ or $C_{14}H_{29}$, R′ = H or CH_3, X = –O–, –S–, –NH–, =NCH_3 or =NC_4H_9 and n = 1–5.

For comparison, topological indices were also calculated for some linear and branched alkanes and some primary and secondary alcohols.

The molecular connectivity indices of the first ($^1\kappa$), second ($^2\kappa$) and third order ($^3\kappa$) and the appropriate valence molecular connectivity indices ($^1\kappa^v$, $^2\kappa^v$, $^3\kappa^v$) were calculated according to the procedures of Randič[1–4] and Kier and Hall[5,6].

The Rouvray index, $R(G)$, the Wiener number, $W(G)$, and the Balaban index, I_B, were calculated according to the procedures given by these authors, using the values of diagonal and off-diagonal elements of the distance matrix as given by Barysz *et al.*[19].

RESULTS AND DISCUSSION

A useful and sensitive structural parameter should describe any change in the structure of a molecule. The main structural elements present in the compounds discussed are alkyl groups of different length, oligooxyethylene and thioethylene chains(s) containing different numbers of oxy- or thioethylene units and heteroatoms.

The first step was to examine the properties of topological indices for alkanes. An increase in the alkane chain length increases all the topological indices considered, but the characteristics of the relationships between each topological index and the chain length differ. The connectivity indices of the first, second and third order increase linearly (Fig. 1) with increase in the alkane chain length and the slopes decrease with increase of the index order. The Balaban index increases asymptotically with increase in the alkane chain length whereas the relationship between the Wiener number and the alkane chain length is parabolic (Fig. 1). Appropriate equations are given in Table I. This observation is consistent with those of Balaban[18], who stated that for an increasing number of carbon atoms from n to ∞ the Balaban index increases to π. The relationship between the Rouvray index and the alkane chain length is similar to that for the Wiener number.

Exemplary values of topological and molecular connectivity indices calculated for polyoxyethylene glycol dialkyl ethers (**1**) and their sulphur analogues (**2**) are presented in Tables II and III, respectively.

An increase in the length of the alkyl chain in polyoxyethylene glycol dialkyl ethers influences the topological indices in a different way (Fig. 2). An increase in the alkyl chain length results in a decrease in the Balaban index. This parameter calculated for alkanes having a number of carbon atoms equal to the total number of atoms in compounds **1** increases with increase in the carbon number. The values of the Balaban index for the hydrocarbon analogues are much lower than those for compounds **1**. The

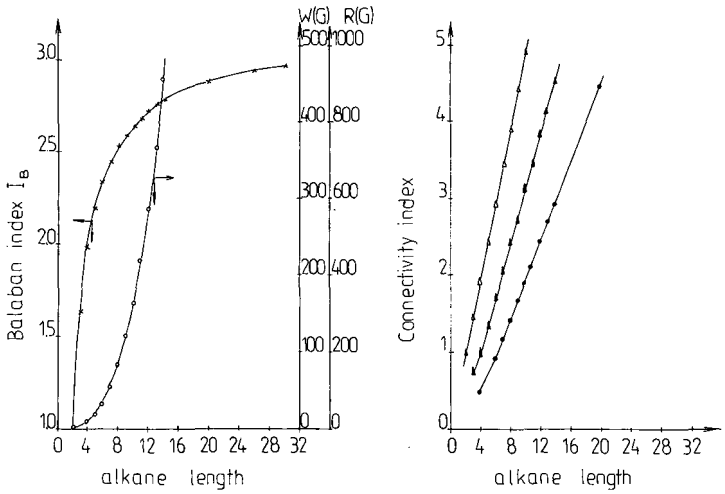

Fig. 1. Relationships between the topological indices and the length of the carbon chain in n-alkanes (n). ×, I_B *vs.* n; ○, $W(G)$ *vs.* n and $R(G)$ *vs.* n; △, $^1\kappa$ *vs.* n; ▲, $^2\kappa$ *vs.* n; ●, $^3\kappa$ *vs.* n.

TABLE I

RELATIONSHIPS BETWEEN THE TOPOLOGICAL INDICES AND THE NUMBER OF CARBON ATOMS FOR *n*-ALKANES ($N = 27$)

Topological index	*Equation*	*r*
I_B	$I_B = \pi(-1.3656/n + 0.9808)$	0.9348
$W(G)$	$W(G) = 1290.06 - 336.24n + 20.47n^2$	0.9965
$R(G)$	$R(G) = 645.03 - 168.12n + 10.23n^2$	0.9965
$^1\kappa$	$^1\kappa = -0.0742 + 0.5000n$	1.0000
$^2\kappa$	$^2\kappa = -0.4142 + 0.3535n$	1.0000
$^3\kappa$	$^3\kappa = -0.5429 + 0.25n$	1.0000

TABLE II

TOPOLOGICAL INDICES FOR POLYOXYETHYLENE GLYCOL DIALKYL ETHERS **1** AND THEIR SULPHUR ANALOGUES **2**

Group of compounds	*R*	*n*	*m*	*Balaban index, I_B*	*Wiener number, $W(G)$*	*Rouvray index, $R(G)$*
1	$C_{12}H_{25}$	3	–	3.2934	5990.5	11981
	$C_{12}H_{25}$	4	–	3.3480	7626.625	15325.25
	$C_{12}H_{25}$	5	–	3.3930	9540.25	19080.5
	$C_{12}H_{25}$	6	–	3.4314	11753.875	23507.75
	$C_{12}H_{25}$	7	–	3.4650	14290	28580
	$C_{12}H_{25}$	8	–	3.4896	17171.125	34342.25
1	C_8H_{17}	4	–	3.3880	3580.625	7161.25
	C_8H_{17}	7	–	3.5039	7886	15772
1	C_4H_9	4	–	3.4240	1310.625	2621.25
	C_4H_9	5	–	3.4730	1948.25	3896.5
	C_4H_9	6	–	3.5100	2765.875	5531.75
	C_4H_9	7	–	3.5380	3786	7572
	C_4H_9	8	–	3.5616	5031.125	10062.25
	C_4H_9	9	–	3.5805	6523.75	13047.5
2	C_4H_9	1	0	3.6092	443.5625	887.125
	C_4H_9	2	0	3.6800	1228.3125	2456.625
	C_4H_9	3	0	3.7128	2629.5625	5259.125
	C_4H_9	4	0	3.7280	4827.3125	9654.625
2	C_4H_9	1	1	4.2380	1077.1875	2154.375
	C_4H_9	2	1	4.1548	2370.4375	4740.875
	C_4H_9	3	1	4.0928	4433.1875	8866.375
	C_4H_9	4	1	4.0432	7445.4375	14890.875
2	C_4H_9	1	2	4.5812	2151.8125	4303.625
	C_4H_9	2	2	4.4610	4079.5625	8159.125
	C_4H_9	3	2	4.3662	6929.8125	13859.625
	C_4H_9	4	2	4.2900	10882.562	21765.125

TABLE III

MOLECULAR CONNECTIVITY INDICES FOR POLYOXYETHYLENE GLYCOL DIALKYL ETHERS 1 AND THEIR SULPHUR ANALOGUES 2

Group of compounds	*R*	*n*	*m*	*Molecular connectivity indices*			*Valence molecular connectivity index*		
				$^1\kappa$	$^2\kappa$	$^3\kappa$	$^1\kappa$	$^2\kappa$	$^3\kappa$
1	$C_{12}H_{25}$	3	–	16.9142	15.2236	11.6066	11.2234	15.2333	12.2222
	$C_{12}H_{25}$	4	–	18.4142	12.6673	8.7071	16.3010	10.4528	6.7725
	$C_{12}H_{25}$	5	–	19.9142	13.7279	9.4571	17.3783	11.0392	7.1445
	$C_{12}H_{25}$	6	–	21.4142	14.7886	10.2070	18.4557	11.6506	7.5165
	$C_{12}H_{25}$	7	–	22.9142	15.8492	10.9570	19.5330	12.2629	7.8885
	$C_{12}H_{25}$	8	–	24.4142	16.9099	11.7070	20.6104	12.8753	8.2605
1	C_8H_{17}	4	–	14.4142	9.8388	6.7071	12.3010	7.5974	4.7725
	C_8H_{17}	7	–	18.9142	13.0208	8.9571	15.5330	9.4345	5.8885
1	C_4H_9	4	–	10.4142	7.0104	4.7071	8.3010	4.7690	2.7725
	C_4H_9	5	–	11.9142	8.0711	5.4571	9.3783	5.3813	3.1445
	C_4H_9	6	–	13.4142	9.1317	6.2071	10.4557	5.9937	3.5165
	C_4H_9	7	–	14.9142	10.1924	6.9571	11.5330	6.6091	3.8885
	C_4H_9	8	–	16.4142	11.2530	7.7071	12.6104	7.2185	4.2605
	C_4H_9	9	–	17.9142	12.3137	8.4571	13.6877	7.8308	4.6325
2	C_4H_9	1	0	7.4142	4.8891	3.2071	7.9267	4.7644	2.9358
	C_4H_9	2	0	10.4142	7.0104	4.7071	9.4514	5.9891	3.6798
	C_4H_9	3	0	13.4142	9.1317	6.2071	11.6061	7.2139	4.4238
	C_4H_9	4	0	16.4142	11.2530	7.7071	13.7608	8.4386	5.1678
2	C_4H_9	1	1	10.4142	7.0104	4.7071	11.7521	8.4295	6.1560
	C_4H_9	2	1	13.4142	9.1317	6.2071	13.9068	9.6542	6.9000
	C_4H_9	3	1	16.4142	11.2530	7.7071	16.0615	10.8790	7.6441
	C_4H_9	4	1	19.4142	13.3744	9.2071	18.2162	12.1037	8.3881
2	C_4H_9	1	2	13.4142	9.1317	6.2071	16.2076	12.0946	9.3763
	C_4H_9	2	2	16.4142	11.2530	7.7071	18.3623	13.3193	10.1203
	C_4H_9	3	2	19.4142	13.3743	9.2071	20.5170	14.5440	10.8643
	C_4H_9	4	2	22.4142	15.4957	10.7071	22.6717	15.7688	11.6084

Wiener number, the Rouvray index and the connectivity indices increase with increase in the length of the alkyl chain, as was observed for saturated hydrocarbons. The values of $W(G)$ and $R(G)$ are lower than those obtained for corresponding hydrocarbon analogues. The connectivity indices do not distinguish between alkanes and compounds **1** having several oxygen atoms in the polyoxyethylene chain; the values of $^1\kappa$, $^2\kappa$ and $^3\kappa$ are the same for both groups of compounds. The use of valence molecular connectivity indices allows one to discriminate between these compounds; lower values were obtained for compounds **1**, although the trend of the connectivity index *vs.* alkyl chain length relationship remains unchanged.

The same relationships were observed between the topological indices and the number of carbon atoms in alkyl groups for compounds **3–6** (Fig. 3).

The change in the character of the relationships considered may be attributed to the presence of the oligooxyethylene chain. The Balaban index calculated for corresponding secondary and primary alcohols changes in a similar way as for

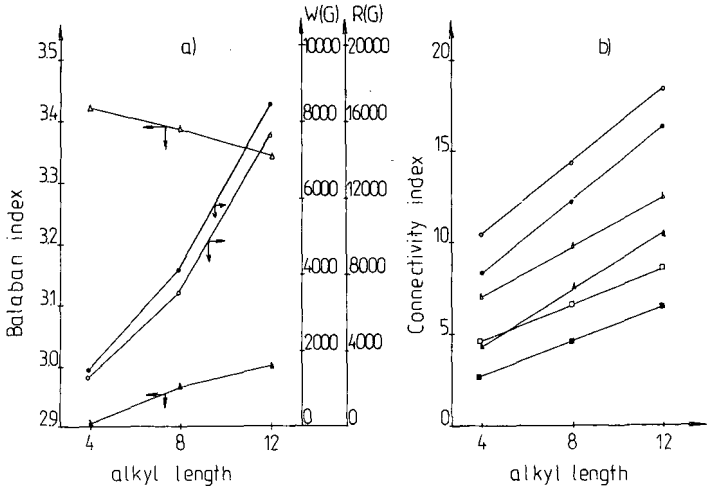

Fig. 2. Influence of the alkyl chain length (n) on the topological indices of polyoxyethylene glycol dialkyl ethers and their hydrocarbon analogues. (a) △,▲, I_B *vs.* n; ○,●, $W(G)$ *vs.* n and $R(G)$ *vs.* n; △,○, for compounds **1**: ▲,●, for alkane analogues of compounds **1**. (b) ○, $^1\kappa$ *vs.* n; ●, $^1\kappa^v$ *vs.* n; △, $^2\kappa$ *vs.* n; ▲, $^2\kappa^v$ *vs.* n; □, $^3\kappa$ *vs.* n; ■, $^3\kappa^v$ *vs.* n.

saturated hydrocarbons having the same number of atoms (Fig. 4). The term "alcohol analogue" denotes a primary or secondary alcohol corresponding to the examined compound in which all the ether oxygen atoms are replaced with methylene group, *e.g.*, an "alcohol analogue" for $ROCH_2CH(OH)CH_2(OCH_2CH_2)_2OR$ has the structure $RCH_2CH_2CH(OH)CH_2(CH_2CH_2CH_2)_2CH_2R$. However, higher values of the Balaban index were obtained for alcohols than alkanes.

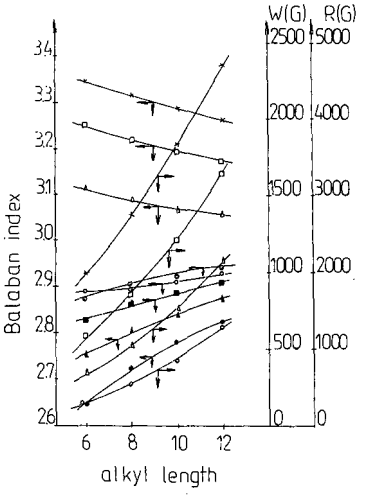

Fig. 3. Influence of alkyl chain length on the topological indices of oligooxyethylene derivatives of alcohols, $RO(CH_2CH_2O)_nR$. ○,●, For $n = 1$; △,▲, for $n = 2$; □,■, for $n = 3$; ×,⊗, for $n = 4$. ○,△,□,×, For oligooxyethylene alcohols; ●,▲,■,⊗, for their hydrocarbon analogues.

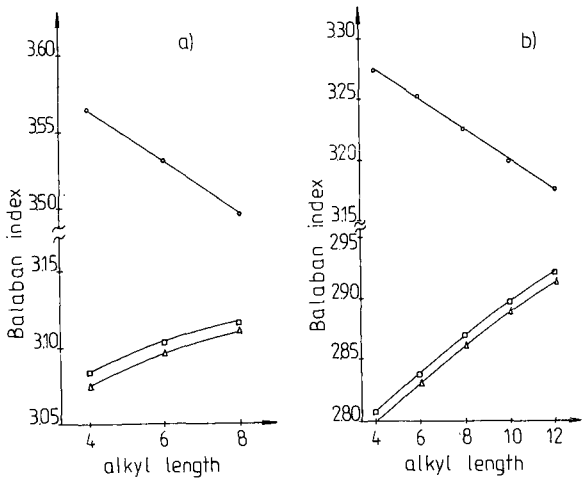

Fig. 4. Influence of alkyl chain length on the Balaban index of (a) $ROCH_2CH(OH)CH_2(OCH_2CH_2)_2R$ and (b) $RO(CH_2CH_2O)_3H$ and their (△) hydrocarbon and (□) "alcohol" analogues.

An increasing length of the oligooxyethylene chain in derivatives of aliphatic alcohols also significantly influences the relationship between the Balaban index and the alkyl chain length (Fig. 3). Other relationships such as those between the topological index and the alkyl chain length remain the same as above.

The important structural fragment in the molecules of the compounds examined is the oligooxyethylene chain. An increase in the oligooxyethylene chain length in compounds **1–6** increases all the topological indices discussed (Fig. 5). Only the relationships involving the connectivity indices have a linear character and $^1\kappa$, $^2\kappa$, $^3\kappa$ increase by 1.5, 1.0606 and 0.75 i.u., respectively, for each additional oxyethylene group.

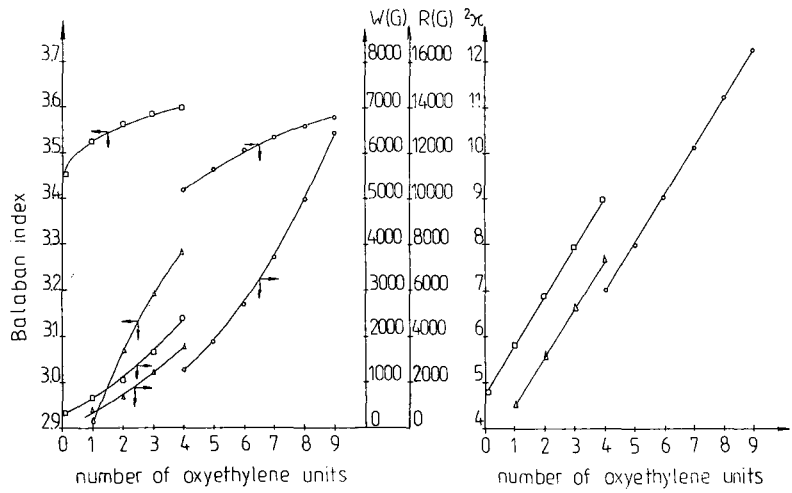

Fig. 5. Influence of the oligooxyethylene chain length on the topological indices for (○) $C_4H_9O(CH_2CH_2O)_nC_4H_9$, (△) $C_{10}H_{21}O(CH_2CH_2O)_nH$ and (□) $C_4H_9OCH_2CH(OH)CH_2O(CH_2CH_2O)_nC_4H_9$.

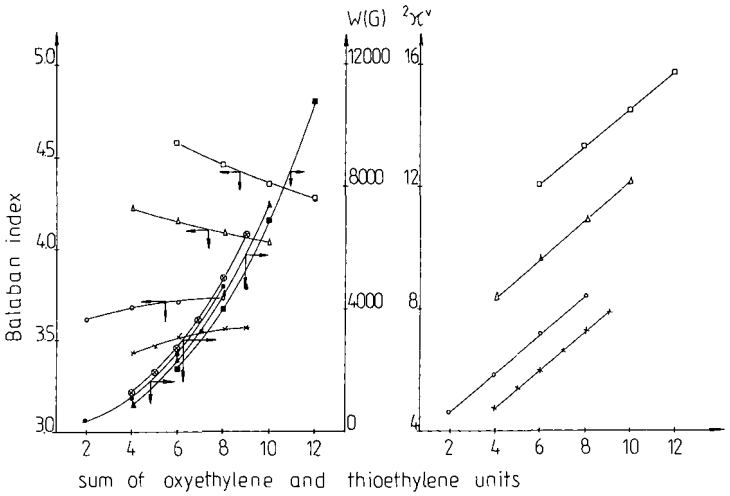

Fig. 6. Influence of the length of the hydrophilic chain on the topological indices for compounds **1** and **2**. ×,⊗, **1** (R=C_4H_9); ○,●, **2**, m = 0; △,▲, **2**, m = 1; □,■, **2**, m = 2. ×,○,△,□, For I_B and $^2\kappa^v$; ⊗,●,▲,■, for $W(G)$ and $R(G)$.

The replacement of oxygen with sulphur atoms (in compounds **2**) causes a decrease in polarity[29,30]. The more sulphur atoms in the molecule, the lower is the polarity exhibited by compounds **2** in comparison with their oxygen analogues **1**. As shown in Fig. 6, the Balaban index changes with increase in the hydrophilic chain length but the character of this relationship depends on the number of sulphur atoms present in the molecule. The same conclusion is valid for the molecular connectivity indices. The Wiener number and Rouvray index increase with increase in the number of sulphur atoms in compounds **2** (Fig. 7) when the length of the hydrophilic chain (the

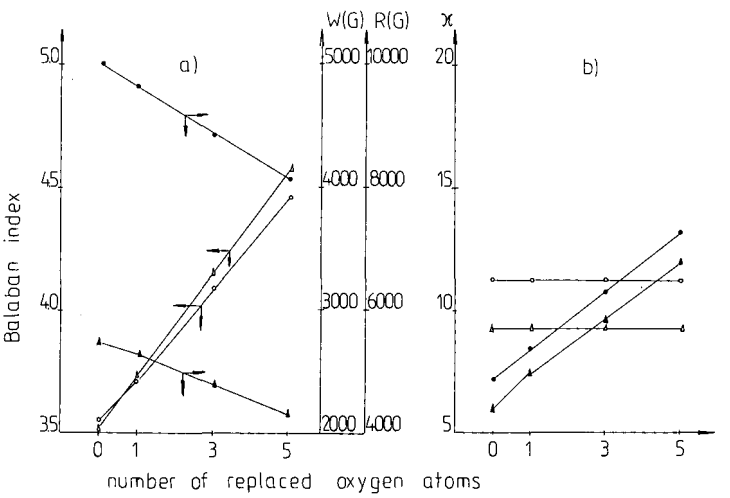

Fig. 7. Influence of the number of replaced oxygen atoms on the topological indices. △,▲, $n_{EO} + n_{ES}$ = 7; ○,●, $n_{EO} + n_{ES}$ = 9. △,○, For (a) I_B and (b) $^2\kappa$; ▲,●, for (a) $W(G)$, $R(G)$ and (b) $^2\kappa^v$.

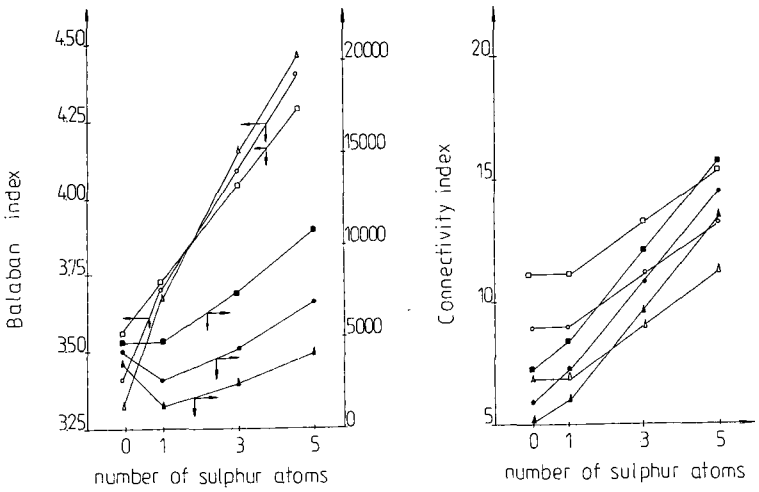

Fig. 8. Influence of the number of sulphur atoms on the topological indices. △,▲, $n_{EO} = 4$; ○,●, $n_{EO} = 6$; □,■, $n_{EO} = 8$. △,○,□, For I_B and $^2\kappa$; ▲,●,■, for $W(B)$ and $^2\kappa^v$.

sum of oxyethylene and thioethylene units) remains constant. The molecular connectivity indices do not change whereas the valence molecular connectivity indices increase with increase in the number of replaced oxygen atoms. An increase in the thioethylene chain length increases slightly the polarity of compounds **2**[29,30] when the number of oxyethylene units is constant (Figs. 7–9 in ref. 20). The replacement of the first oxygen with a sulphur atom causes a decrease in $W(G)$, whereas the introduction of each additional thioethylene unit increases $W(G)$ (Fig. 8). An analogues relationship is observed for the Rouvray index.

The location of a single sulphur atom in the molecule significantly influences the topological indices. The Balaban index decreases whereas the Wiener number and Rouvray index increase with increasing asymmetry of the molecule. The Wiener number and Rouvray index decrease in the order $Bu(OE)_2O(EO)_2Bu$ > $BuS(EO)_4Bu$ > $Bu(OE)S(EO)_3Bu$ > $Bu(OE)_2S(EO)_2Bu$ where Bu = C_4H_9, OE = OCH_2CH_2 and EO = CH_2CH_2O (Table IV).

TABLE IV

TOPOLOGICAL AND POLARITY PARAMETERS FOR ASYMMETRIC SULPHUR ANALOGUES

Compound[a]	*Topological indices*		*Polarity parameters*[29]	
	$W(G)$	I_B	$I_R^{C_2H_5OH}$	$PI^{C_2H_5OH}$ [b]
$Bu(OE)_2O(EO)_2Bu$	1310.625	3.4240	666.0	88.4
$BuS(EO)Bu$	1255.3125	3.5660	659.6	87.2
$Bu(OE)S(EO)_3Bu$	1235.0625	3.6480	653.7	85.7
$Bu(OE)_2S(EO)_2Bu$	1228.3125	3.6800	645.2	83.4

[a] Bu = C_4H_9; EO = $-OCH_2CH_2-$; EO = $-CH_2CH_2O-$.

[b] $PI^{C_2H_5OH}$ = Polarity index of ethanol as polar solute[29].

TABLE V

INFLUENCE OF THE DISTRIBUTION OF OLIGOOXYETHYLENE UNITS ON THE TOPOLOGICAL AND CONNECTIVITY INDICES FOR $C_4H_9O(CH_2CH_2O)_nCH_2CH(OH)CH_2(OCH_2CH_2)_m$-$C_4H_9$

n	m	*Topological indices*			*Molecular connectivity indices*					
		I_B	$W(G)$	$R(G)$	^1K	^2K	^3K	$^1K^v$	$^2K^v$	$^3K^v$
0	3	3.6025	2402.125	4804.25	12.8081	9.0465	6.3896	9.953	5.9602	3.5221
1	3	3.6425	2384.125	4768.25	12.8081	9.0465	6.3896	9.953	5.9602	3.5221
2	2	3.6550	2378.125	4756.25	12.8081	9.0465	6.3896	9.953	5.9602	3.5221

Compounds **3** also contain homologues with different lengths of both oligooxyethylene chains and different terminal alkyl groups. The influence of the asymmetry of the molecules on their polarity has been discussed previously[32]. The comparison of the values of the topological indices, when the influence of the distribution of the oxyethylene units is taken into account, is limited to compounds having the same total length of the oligooxyethylene chains and the same length and distribution of alkyl groups. The increasing asymmetry of the distribution of the oligooxyethylene units in the molecule decreases the Balaban index, whereas the Wiener number and Rouvray index increase (Table V).

The asymmetry of the hydrocarbon groups could be discussed for compounds having the same length and distribution of oligooxyethylene chains and the same number of carbon atoms in both alkyl groups. Appropriate data are presented in Table VI. The values of the Balaban index are lower for asymmetric compounds, whereas the Wiener number and Rouvray index increase slightly.

The molecular connectivity indices and valence molecular connectivity indices do not change with the increasing asymmetry of either the polar or apolar part of the molecule.

A change in the heteroatoms present in compounds **6** also influences their structural parameters (Fig. 9). The Balaban index, the Wiener number and the Rouvray index distinguish between derivatives of alcohols, alkylamines and thio alcohols. The highest I_B values were obtained for derivatives of thio alcohols and the

TABLE VI

INFLUENCE OF THE DISTRIBUTION OF CARBON ATOMS IN ALKYL GROUPS ON THE TOPOLOGICAL INDICES AND CONNECTIVITY INDICES FOR $RO(CH_2CH_2O)_nCH_2CH(OH)$-$CH_2(OCH_2CH_2)_mR^1$

n	m	R,R^1	I_B	$W(G)$	$R(G)$	^1K	^2K	$^1K^v$	$^2K^v$
0	0	$R = R^1 =$	3.4102	826.625	1653.25	2.8081	9.0465	9.953	5.9602
0	1	C_6H_{13}	3.4880	1299	2598	2.8081	9.0465	9.953	5.9602
1	1		3.5512	1922.875	3845.75	2.8081	9.0465	9.953	5.9602
0	0	$R = C_8H_{17}$,	3.3643	834.625	1669.25	2.8081	9.0465	9.953	5.9602
0	1	$R^1 = C_4H_9$	3.4680	1305	2610	2.8081	9.9465	9.953	5.9602
1	1		3.5236	1934.875	3869.25	2.8081	9.0465	9.953	5.9602

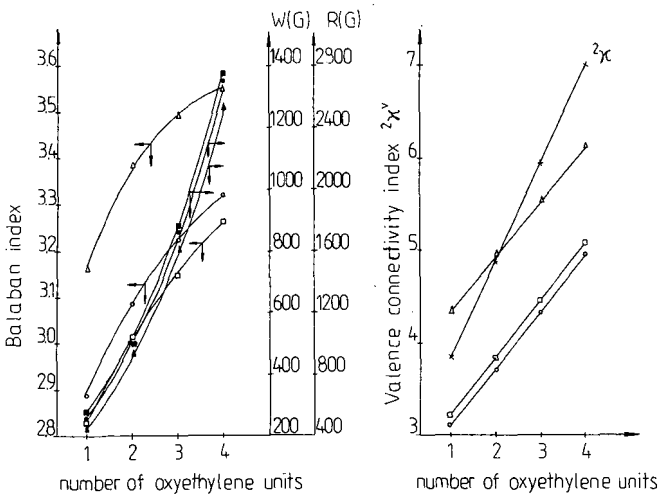

Fig. 9. Influence of the oligooxyethylene chain length on the topological indices for $C_8H_{17}X(CH_2CH_2O)_nH$. ○,●, X = –O–; △,▲, X = –S–; □,■, X = –NH–.

lowest for derivatives of alkylamines. The same values of the molecular connectivity indices $^m\kappa$ were obtained for all compounds **6**. The use of the valence molecular connectivity indices $^m\kappa^v$ allows one to distinguish between these derivatives. The values of $^2\kappa^v$ decrease in the order thio alcohols > alkylamines > alcohols.

CONCLUSIONS

The Balaban index, the Wiener number and Rouvray index seem to be the suitable parameters to describe the structure of a compound and could be used, for example, in the relationships between the structure and activity of the compounds examined. However, the topological parameters considered describe the structure of the compounds examined in different ways. These differences arise from the definitions of the topological indices. The Balaban index may be treated, from its definition, as the average distance between two vertices of a molecular graph. Other parameters are calculated as the sums of distances between vertices in the molecular graph, and therefore they increase with increasing number of adjacent atoms in the molecule. The possible variations of the Wiener number caused, for example, by the presence of an oligooxyethylene chain may be suppressed by the influence of the increasing molecular mass.

The Balaban index, increases with decrease in the alkyl length and increase in the oligooxyethylene chain length. The influence of a given structural element depends significantly on the presence and number of other structural fragments. Topological indices are sensitive to the asymmetry in the polar and hydrocarbon parts of the molecule. The replacement of one oxygen atom with sulphur or an NH group changes the Balaban index in the order derivatives of thio alcohols > alcohols > alkylamines. Of the group of connectivity indices, the valence molecular connectivity index is more suitable for such use than the molecular connectivity index, because the latter does not distinguish between different heteroatoms.

The question of which topological index differentiates best the individual structures of organic compounds remains open. One should take into account that graph theoretical descriptors have been designed for closely congeneric sets of compounds (linear and branched alkanes). Kier and Hall's and Barysz *et al.*'s modifications of topological indices lie outside the close graph theory and they include an electronic (the number of valency electrons) and not only a structural component. The discriminating power of a given topological index may be limited to an examined group of compounds. Therefore, the chromatographer looking for an appropriate structural parameter should select the one from the group of topological indices that best discriminates between the examined set of compounds.

The application of the considered topological indices as structural descriptors in retention–structure or polarity–structure relationships will be discussed in detail in subsequent papers. The retention–structure relationships for oligooxyethylene derivatives of alcohols, thio alcohols and alkylamines on standard stationary phases of different polarity will be discussed. The compounds considered in this paper and also others were used as liquid stationary phases and their polarity–structure relationships will be evaluated. As the compounds are of medium and relatively high polarity[29–35,48–50], it is expected that the future relationships will include not only topological indices but also parameters describing electrical and/or chemical properties of compounds.

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SELECTION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS IN PHARMACEUTICAL ANALYSIS

III[a]. METHOD VALIDATION

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(First received November 13th, 1987; revised manuscript received November 7th, 1988)

SUMMARY

The most important steps in the validation of high-performance liquid chromatographic (HPLC) methods are discussed. The establishment of system suitability data and the assessment of peak purity are demonstrated on the example of bisquaternary amino steroids. For the recognition of incomplete resolution of adjacent peak pairs, the absorbance-ratio method in which the ratio of absorbances at two preselected wavelengths are plotted as a function of time in combination with the separation of sample components subjected to various chemical and physico-chemical treatments (stress conditions) is applied. The separation power and performance of the HPLC systems are characterized by the system resolution (SR) and system selectivity (SS). The special demands of stability-indicating methods are summarized.

INTRODUCTION

The main aim of pharmaceutical analysis is to obtain necessary qualitative and quantitative information about the sample to be tested. Since the quantitative analysis includes the total analytical procedure from the sample treatment to the evaluation of the analytical results, each step of the procedure can be separately evaluated to determine the weakest step that may influence the analytical results. The overall validation of a high-performance liquid chromatographic (HPLC) method can be considered as the sum of different validation steps to be included in the analytical process (both chromatographic and pre-chromatographic investigations).

In general, method validation involves the performance and interpretation of a series of experiments designed to reveal the most important characteristic of an HPLC method. Previous parts of our paper[1,2] dealt with the optimization of the mobile phase in reversed-phase and normal-phase systems. In this part, different aspects of the validation of previously optimized HPLC methods will be discussed. The principles used in the authors' laboratory will be demonstrated on the example of the

[a] For Part II, see ref. 2.

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TABLE I
COMPOUNDS INVESTIGATED AND PEAK NUMBERS
Structures are shown in Fig. 6.

Compound	*Peak No.*
2β,16β-Bis(4'-methyl-1'-piperazino)-3α,17β-diacetoxy-5α-androstane	1
2β-(4'-Methyl-1'-piperazino)-16β-(4'-dimethyl-1'-piperazino)-3α,17β-diacetoxy-5α-androstane bromide	2
2β-(4'-Dimethyl-1'-piperazino)-16β-(4'-methyl-1'-piperazino)-3α,17β-diacetoxy-5α-androstane bromide	3
2β-(4'-Dimethyl-1'-piperazino)-16β-(4'-dimethyl-1'-piperazin-2',3'-ene)-3α,17β-diacetoxy-5α-androstane dibromide	4
2β-(4'-Dimethyl-1'-piperazin-2',3'-ene)-16β-(4'-dimethyl-1'-piperazino)-3α,17β-diacetoxy-5α-androstane dibromide	4
2β,16β-Bis-(4'-dimethyl-1'-piperazino)-3α,17β-diacetoxy-5α-androstane dibromide (Pipecuronium bromide)	5
2β,16β-Bis-(4'-dimethyl-1'-piperazino)-3α-hydroxy-17β-acetoxy-5α-androstane dibromide	6
2β,16β-Bis-(4'-dimethyl-1'-piperazino)-3α-acetoxy-17β-hydroxy-4α-androstane dibromide	7
2β,16β-Bis-(4'-dimethyl-1'-piperazino)-3α,17β-dihydroxy-5α-androstane dibromide	8

separation of pipecuronium bromide and related substances. Optimization of the separation system has been published[3,4].

EXPERIMENTAL

The same instrumentation (HP 1090A) as described in Part I[1] was used. Separations were performed on a LiChrosorb Si 60 (5 μm) column (250 × 4.6 mm I.D.) (Chrompack, Middelburg, The Netherlands). The eluent was methanol–acetonitrile–concentrated ammonia solution (43:43:14) containing 100 m*M* each of ammonium carbonate and ammonium chloride. The flow-rate was 1 ml/min and the steroids were detected at 213 and 225 nm.

The compounds to be tested were prepared at the Chemical Works of Gedeon Richter (Budapest, Hungary) and their quality was checked by HPLC prior to use. The compounds are listed in Table I and their structures can be seen in Fig. 6.

RESULTS AND DISCUSSION

The validation of every HPLC procedure involves at least four distinct steps: validation of sample pre-treatment and derivatization; chromatographic separation; elaboration of system suitability data; and peak purity determination. The analytical method itself using HPLC can be separated into the following five distinct parts: (i) sample pretreatment and preparation, including pre-column derivatization if necessary; (ii) introduction of the sample into the chromatographic system; (iii) chromatographic separation; (iv) detection and amplification of detector signals; and (v) transformation of detector signals into numerical data. The last four parts are termed instrumental components, because the scale of errors depends on the degree of instrumentation, and therefore validation of sample pre-treatment and preparation can be distinguished and discussed.

Validation for sample pretreatment and derivatization

Several books and reviews have dealt with the problems of sample preparation and derivatization and their contribution to the errors made during the analysis[5–9]. The errors made during sample preparation can be classified into five groups: sampling error; extraction of sample from the matrices; sample clean-up and enrichment; stability of the sample during the preparation of the test solution and during its storage; and errors caused by derivatization. Error contributions in sample pretreatment have been discussed by Snyder and Van der Wal[5], who developed a comprehensive theory of the various contributions to assay imprecision, providing specific conclusions and recommendations for significant improvements in precision.

Here, only three aspects closely connected with the practical approach of method validation are considered: recovery; stability of sample components during sample preparation and storage; and determination of the accuracy and precision of the sample pretreatment procedure.

Recovery of sample components. Recovery is a measure of the efficiency of the extraction of the analyte from the sample matrix. With respect to the sample type being analysed, two different types of samples can be distinguished. First, a mixture of a known number of essentially known compounds (*e.g.*, formulated pharmaceuticals) is analysed. Recovery can be determined by analysing a spiked placebo containing all ingredients except the active substance. Known concentrations of standard prepared from the active substance in increasing amounts (50, 75, 100, 125 and 150% of the labelled amount for dosage forms) are added to the placebo. The procedure is carried out in a manner identical (the sample is pulverized, milled, dried, homogenized, extracted and analysed) with that for the real sample. With a knowledge of the added and measured amounts , the recovery of the sample preparation can be calculated. In the second type, a mixture of a known number of components with a number of unknown (background) compounds is analysed. Here, a standard recovery graph is produced by adding increasing amounts of standard to the untreated sample.

Stability of sample components during sample preparation and storage. Many solutes readily decompose prior to chromatographic investigations during the preparation of sample solutions (extraction, clean-up, phase transfer, etc.) and also in ready-made sample solutions. To avoid this problem, several possibilities exist if we consider the possible reason(s) for the undesirable decomposition of sample components.

To determine the stability of the samples being analysed in a sample solution, the term of "system stability" (St_s) is defined. It is a measure of bias in the assay results within a preselected time interval (*e.g.*, every hour up to 4–6 h) using single solution. System stability should be determined by replicate analyses of the same sample solution and the results are evaluated for major and/or minor components.

System stablity is considered to be appropriate if the relative standard deviation calculated on the assay results obtained in different time intervals does not exceed more than 20% of the corresponding value of the system precision (discussed later). If the value is higher on plotting the assay results as a function of time, the maximum duration of the usability of the sample solution can be calculated.

Determination of precision of sample preparation. Because the method validation data for the overall analytical procedure are calculated from the detector responses after chromatographic separation, the precision data of sample pretreatment can be

separated from those of chromatographic separation. In the authors' laboratory the following procedure is used for this purpose. From seven independent weighings, seven sample solutions are prepared using an identical sample treatment in each instance. From each individual sample solution a single injection is performed. From the data, the precision characteristic of the overall analytical procedure is obtained (method precision, σ_m). From a single solution, seven determinations are made; the precision characteristic of the chromatographic procedure can be determined (system precision, σ_s). Finally the precision of sample preparation (σ_p) can be calculated from the first two precision data:

$$\sigma_p = \sqrt{\sigma_m^2 - \sigma_s^2} \quad (1)$$

Validation of chromatographic separation

Current concepts for an HPLC method validation procedure have been reported[10] and discussed in detail recently[11,12]. According to these guidelines,

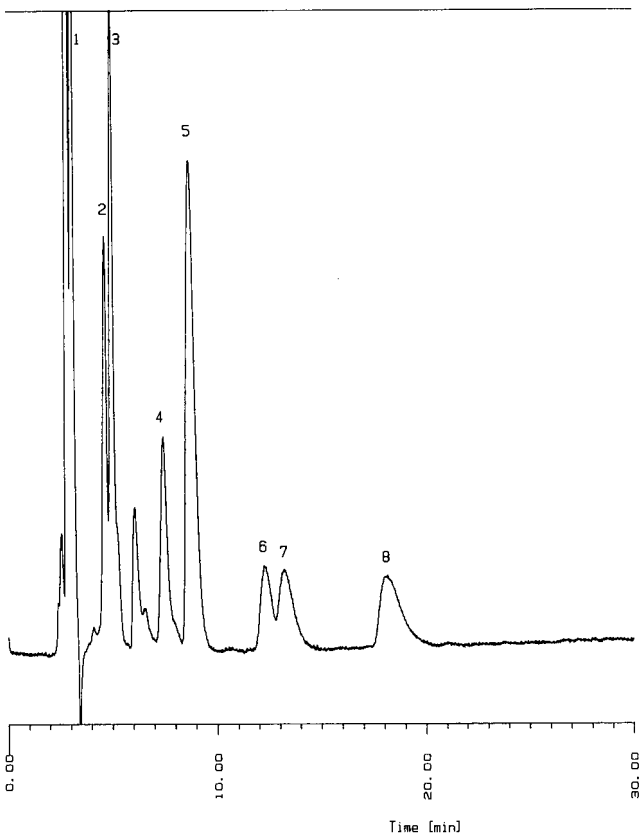

Fig. 1. Separation of pipecuronium bromide and its possible impurities. Conditions: column, LiChrosorb Si 60 (5 μm) (250 × 4.6 mm I.D.); eluent, methanol–acetonitrile–concentrated ammonia solution (43:43:14) containing 100 m*M* each of ammonium chloride and ammonium carbonate; flow-rate, 1 ml/min; detection at 213 nm. For peak numbers see Table I.

demonstration of the following data elements are required for HPLC method validation: accuracy, precision, limit of quantitation, selectivity, range, linearity and ruggedness. According to our classification, the validation data can be divided into four major groups:

(a) Data elements which can be statistically evaluated (their definitions and determinations can be found elsewhere[10–12]), such as accuracy, precision, reproducibility (repeatability), day-to-day reproducibility, inter-laboratory reproducibility (ruggedness), detector linearity and range (these data elements are not discussed here).

(b) System suitability data containing measures of the resolving power of the HPLC system and comprising criteria established for acceptance or rejection of any analytical results including additional data to characterize the performance of the separation system, such as column loadability, depending on the size of column hardware and type of stationary phase filled into the column.

(c) Peak purity test to verify the homogeneity of a chromatographic peak.

(d) Additional data such as system resolution (SR) and system selectivity (SS), which directly express the quality of the separation, proportional to the performance (selectivity of the separation) and power (efficiency of the separation) of an HPLC

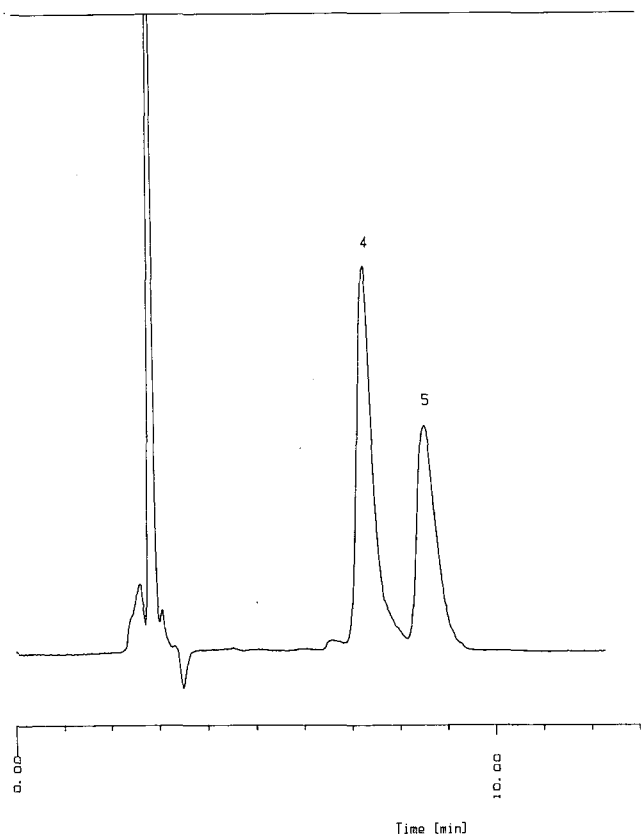

Fig. 2. Chromatogram used for the determination of system suitability. Conditions as in Fig. 1. For peak numbers see Table I.

system, and characterize the applicability of an HPLC method for solving a particular analytical problem.

System suitability data. The data elements belonging to the term system suitability can be controlled each day for a preselected sample (mainly for standard solutions used for quantitation) prior to the use of method for routine analysis to ensure that the system is performing up to specified standards. These are illustrated on the example of the separation and determination of pipecuronium bromide and its impurities listed in Table I. The standard chromatogram and the chromatogram used for the system suitability test are shown in Figs. 1 and 2. System suitability data are collected in Table II. The following points can be considered.

(a) Minimum required resolution measured between two adjacent peak pairs: as can be seen in Table II, the poorest separated pair of peaks are 2 and 3 (two monoquaternary derivatives), with an $R_{s,min}$ value of 1.13. However, these are impurities present at low concentrations. The separation between peaks 4 and 5 (characterized by R_{sb}; see Part I[1]) is more important, as compound 4 is the main degradation product. Therefore, the minimum value of the required resolution (R_s) is determined for these two components (4 and 5).

(b) The approximate capacity ratio is determined for pipecuronium bromide to estimate the possible retentions of the other components.

(c) The maximum allowable value of the peak asymmetry is determined for the peak of pipecuronium bromide and serves to control the conditions of the separation column.

(d) The approximate value of the peak height is also determined for the peak of pipecuronium bromide using fixed detection and amplification parameters to control the detection conditions.

(e) The maximum value of the column loadability can be characterized by the amount of sample resulting in not more than a 20% decrease in the theoretical plate number[13]. It is determined for the main component (pipecuronium bromide) and can be considered when larger amounts of sample are introduced in order to improve an unsatisfactory detectability of trace components.

(f) The limit of quantitation (lowest detectable quantity, LDQ) is a parameter of quantitative assays for low levels of compounds in sample matrices, such as impurities in bulk drug substances and degradation products in pharmaceuticals. It can be expressed as the lowest concentration (quantity) of an analyte in the sample which can be determined under the prescribed experimental conditions with acceptable accuracy and precision. It should be determined for the major component and for minor compounds differing significantly in structure (*e.g.*, for peaks 4 and 5).

(g) The maximum acceptable value for the relative standard deviation is determined for pipecuronium bromide by multiple injections of a standard solution.

(h) Linearity is usually expressed in terms of the variance around the slope of the regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with various concentrations of analyte. The slope of the regression line and its variance provide a mathematical measure of linearity; the intercept on the ordinate is a measure of the potential assay bias.

Peak purity. One of the most important parts of the method validation procedure is to confirm the purity of a chromatographic peak. Several published techniques[14–23] are available for examining the purity of a peak profile. One of the

TABLE II
METHOD VALIDATION DATA FOR THE DETERMINATION OF PIPECURONIUM BROMIDE
Conditions as in Fig. 1.

Data	*Parameter*	*Value*	
System suitability data	Retention times:		
	For peak 4 (decomposition product)	7.3 min	
	For peak 5 (pipecuronium bromide)	8.5 min	
	Resolution for peaks 4 and 5	Min. 1.5	
	Relative standard deviation for peak 5	Max. 1.0%	
	Asymmetry factor (for peak 5)	Max. 2.0	
	Column loadability (for peak 5)	100 μg	
Method validation data	R_{sb} (for peaks 4 and 5)	1.74	
	R_{sa} (for peaks 5 and 6)	3.81	
	$R_{s,min}$ (for peaks 2 and 3)	1.13	
	D_{min} (for peaks 2 and 3)	0.072	
	SS	−0.013	
	SR	1.85	
	Asymmetry factor for peak 5	1.55	
	Lowest detectable quantity:		
	for peak 5 (pipecuronium bromide)	25 ng	
	for peak 4	4 ng	
	Relative standard deviation (for peak 5)	0.81%	
	Day-to-day reproducibility (for peak 5)	1.97%	
	r^2	0.999	
	F	999.9	
		Response ratio measured at 213 and 225 nm	
		Treated sample	*Spiked sample*
Peak purity	Stress conditions:		
	Standard	3.927	
	Reflected light, 14 days	3.999	3.999
	40°C, 80% relative humidity, 14 days	3.922	3.919
	105°C, 48 h	3.987	3.975
	UV light (254 nm), 24 h	3.935	3.891
	pH 2, RT[a], 24 h, 2% solution	3.866	3.850
	pH 12, RT[a], 30 min, 2% solution	3.699	3.712
	Relative standard deviation ($n=13$)	3.899 ± 2.51%	
	$D = (k_2 - k_1)/(k_2 + 1)$		

[a] Room temperature.

simplest methods is to plot the ratio of the absorbances at two (or more) preselected wavelengths as a function of time[20]; peak inhomogeneity is indicated by a discontinuity in the plot. The techniques, however, have a common problem, namely that peak inhomogeneity can be mostly recognized when the spectral properties of the overlapping compounds are sufficiently different and total overlap of two peaks does not occur.

To improve further the assessment of peak purity investigations, a combination of the method based on the measurement of absorbance ratios with time[20] with the

separation of samples subjected to appropriate stress conditions producing decomposition products may be applied in order to recognize peak overlapping. The length of time a substance is subjected to stress conditions depends on the rate of degradation. A degradation of 10–15% is considered to be adequate. (Stress conditions used for the peak purity investigation of pipecuronium bromide are listed in Table II.

The experiments start with the determination of the absorbance ratio(s) for a chromatographically pure (standard) compound at two (or more) preselected wavelengths with respect to time from the standard chromatograms. The chromatograms of treated samples without and with spiking with known concentrations of a standard are recorded at the selected wavelengths. The absorbance ratios (for the peak of pipecuronium bromide) are determined as a function of time.

The following possibilities may exist:

(a) When two peaks do not totally overlap and the difference in spectral properties is sufficient, peak inhomogeneity can be recognized from the discontinuity in the absorbance ratio(s) with time.

(b) When two peaks do not totally overlap, but the compounds possess similar spectral characteristics, existing differences in their decomposition rates can cause changes in the peak shape, resulting in different absorbance ratios for the treated, spiked and standard samples.

(c) When two peaks totally overlap, but the spectral properties of the two compounds are sufficiently different, peak inhomogeneity can be recogized from the apparent difference in the degree of degradation observed for real and reference pure samples owing to the different decomposition rates. The degree of degradation seems to be higher when the main component has a significantly lower absorbance at the selected wavelengths and a slower decomposition rate than the co-eluting compound. The reverse situation exists when the main component has a higher absorbance and a faster decomposition rate.

(d) The most problematic cases of possible peak inhomogenicity are as follows:

(i) peak inhomogeneity belongs to group (b), but no significant difference in decomposition rates exists between the co-eluting compounds;

(ii) peak inhomogeneity belongs to group (c), but the main component has a higher absorbance and slower decomposition rate than the co-eluting compound and, conversely, when the co-eluting compound has a higher absorbance and a slower decomposition rate;

(iii) two peaks are totally overlapped and the spectral properties of the co-eluting compounds are similar.

In this instance the peak inhomogeneity can be recognized only from the chromatograms obtained for the treated sample, assuming different decomposition products are formed from the co-eluting compounds under stress conditions resulting in alteration in the elution patterns for the treated standard and sample solutions.

To increase the possibility of the formation of different degradation products, stress conditions include heat, light and humidity treatments and decomposition due to the pH in aqueous solutions (or for solubility reasons in a mixture of water and organic solvents).

In our practical experience, when the peak absorbance ratios at two (or more) wavelengths determined for treated, spiked and non-treated samples are within acceptable limits ($\pm 5\%$), the chromatographic peak can be considered to be pure. A typical example (pipecuronium bromide) is shown in Fig. 3.

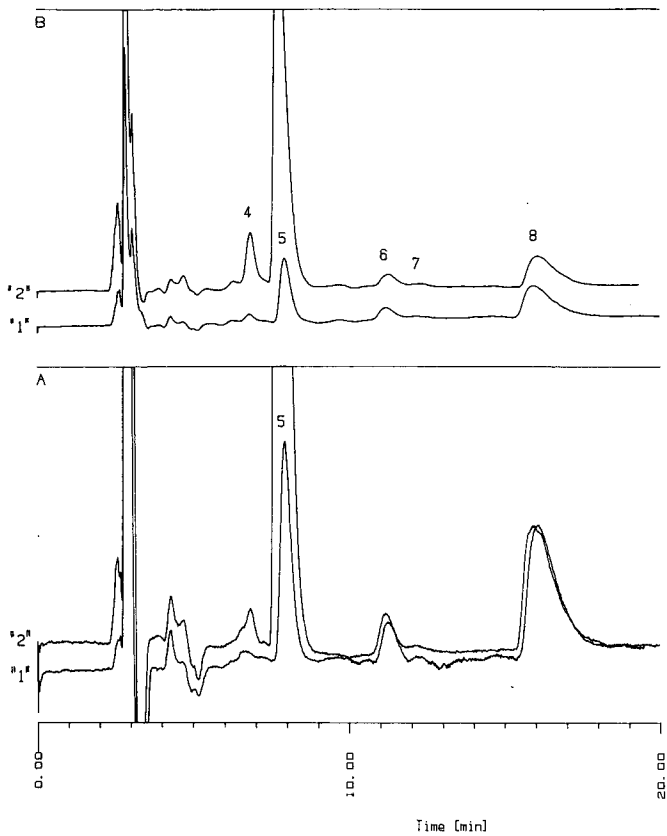

Fig. 3. Assessment of peak purity. (A) Detection at 213 nm; (B) detection at 225 nm. (1) Chromatogram of treated sample (2% aqueous solution at pH 12; sample allowed to stand at room temperature for 30 min). (2) chromatogram of treated sample spiked with standard pipecuronium bromide. Peak ratios (measured for pipecuronium bromide): 3.699 for treated sample and 3.712 for spiked sample. Conditions as in Fig. 1. For peak numbers see Table I.

It should be noted that the application of direct thermospray mass spectrometry to liquid chromatographic eluents is an extremely powerful method of validating peak purity in every instance considered above.

Method validation data for the HPLC determination of pipecuronium bromide are collected in Table II.

System selectivity (SS) and system resolution (SR). When further information may be necessary about the quality of the separations from the point of view of the difficulties created by the analytical problems to be solved, it may be obtainable from the data from previous experiments carried out to formulate criteria that directly express the quality of the separation and are proportional to the performance (system selectivity) and power (system resolution) of an HPLC system.

The separation power of any HPLC system depends mainly on three parameters: the resolutions achieved between the peak of the main component and preceding (R_{sb}) and following (R_{sa}) peaks, and the lowest value of the resolution ($R_{s,min}$) obtained for

any pair of peaks in the chromatogram. Values required for R_{sb} and R_{sa} are the functions of the relative concentrations of compounds in the sample (peak ratios of the adjacent peak pairs), and these are also dependent on the analytical problems to be solved (this will be discussed in Part IV). Here their recommended values are indicated by b (for R_{sb}) and a (for R_{sa}). If an HPLC system possesses these recommended resolution data, that system can be considered to be applicable for solving the analytical problem. The most advantageous value for both R_{sb}/b and R_{sa}/a is 1, as a lower value is not sufficient for a perfect separation and a higher value will increase the analysis time.

When all the peaks on the chromatograms are treated as being of equal importance, the minimal resolution measured for the worst separated peak pairs should also be considered. Based on the above-mentioned considerations, the system resolution (SR) can be expressed by the following equation:

$$\mathrm{SR} = \frac{R_{sb}}{b} \cdot \frac{R_{sa}}{a} (1 + R_{s,min}) \tag{1}$$

The first part of the SR equation is important when the compounds are present at different concentrations. The second part is important when compounds at similar concentrations can be identified. The maximum values for R_{sb}/b and R_{sa}/a are limited to unity to avoid false results being obtained by multiplication of a high value of one with a low value of another (in this instance SR is a function of $R_{s,min}$). When no peak elutes before or after the peak of the main component, the ratio is also unity.

System selectivity (SS) refers to the selectivity of the total separation system and can be expressed by the equation

$$\mathrm{SS} = \frac{D_a^z}{(1 - D_{min}^{z+1})^{1-z}} - \frac{D_a^v}{(1 - D_{min}^{v+1})^{1-v}} = Z - V \tag{2}$$

where z is the number of peak eluting before the peak of the main component; v is the number of peaks eluting after the peak of the main component;

$$D_a^z = \frac{\Sigma D^{z+1}}{z} = \text{average } D \text{ value calculated for peaks "}z\text{";}$$

$$D_a^v = \frac{\Sigma D^{v+1}}{v} = \text{average } D \text{ value calculated for peaks "}v\text{";}$$

$$D_{min}^{z+1} = \text{lowest value of } D \text{ for peaks "}z\text{";}$$

and

$$D_{min}^{v+1} = \text{lowest value of } D \text{ for peaks "}v\text{".}$$

The system selectivity depends on the values of Z and V and directly correlates with the separation for the peaks eluting before (z) and after (v) the main component. When the

value of Z is higher than V, it provides better separation conditions. Values of Z are higher (a) when more peaks elute before the peak of the main component than after it (z is higher than v); (b) the average normalized resolution (D_a) and/or the minimum value of the normalized resolution (D_{min}) calculated for the peaks eluting before the main component is higher than the corresponding value for the peaks eluting after the main component.

When no peak elutes before the main component ($z=0$), the system selectivity is equivalent to the value of V, and similarly SS has a positive value when no peak elutes after the main components ($v=0$, $SS=Z$). When only one peak elutes before or after the main components, the values of Z and V are equivalent to the corresponding values of the minimum normalized resolutions.

With respect to the system selectivity, it can be generally concluded that its value is dependent mainly on the elution order provided by the performance of the given separation system and has great importance when trace compounds are determined. A prefered separation system can be selected on the basis of the numerical value of SS (see Part IV).

Stability-indicating methods

The purpose of stability tests is to obtain adequate information that enables proposals to be made for the shelf-life of pharmaceutical products and to recommend storage conditions. Appropriate stability tests require the use of appropriate stability-indicating methods. Special demands placed on stability-indicating methods can be summarized as follows:

(a) The peak of the main component (drug substance) should not co-elute with any other peaks originating from its production (by-products) or formed by decomposition (degradation products); the method should be able to follow the decrease in active content during the period of the stability investigations (stability-indicating assay method).

(b) Desired resolutions between the peak of the main component and adjacent peak pairs (R_{sb} and R_{sa}) can be selected to be higher than in other instances (this will be discussed in more detail in Part IV), in order to identify possible degradation products similar in structure and chromatographic characteristics, and formed during various storage conditions at low concentrations (stability-indicating purity testing method).

(c) The optimum k' value for the main component is between 5 and 8 to achieve the necessary band spacing for the possible decomposition products with different chemical natures.

(d) The desired value of the precision of stability-indicating assay methods should not be more than $\pm 1.0\%$, in order that small decreases in active content can be accurately measured.

(e) The peak(s) of decomposition product(s) with different chemical natures should be separated from those of impurities present in the sample at the start of the investigations, as the results of assay and purity tests are evaluated together and can be corrected using the original impurity content.

(f) Peaks of secondary decomposition products (formed by degradation of by-products and/or decomposition products) can also be separated from other peaks.

To satisfy these requirements, good chromatographic resolution (suitable values of SR and SS) and well defined stress conditions (see Table II) can be established.

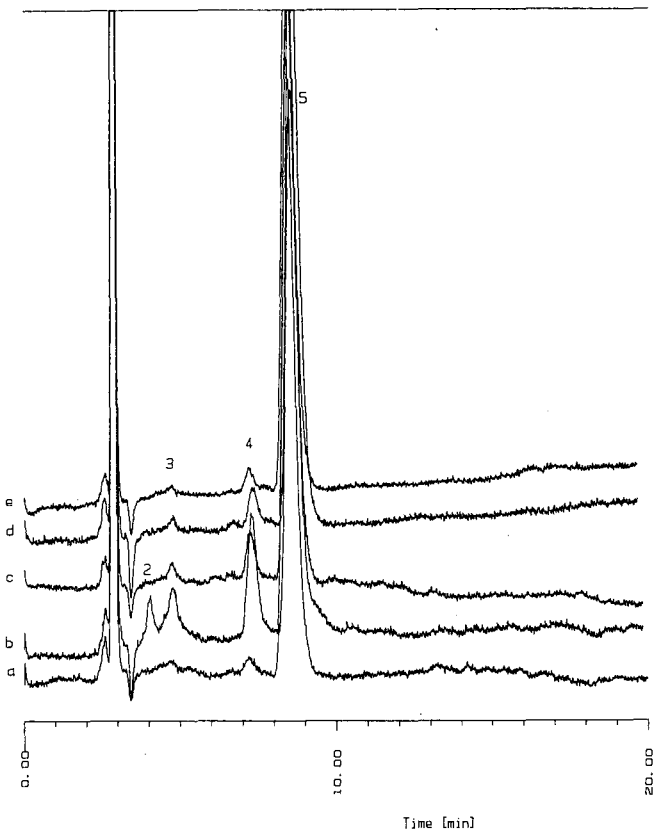

Fig. 4. Chromatograms of pipecuronium bromide subjected to various treatments in solid form. (a) untreated sample; (b) heated at 105°C for 48 h; (c) irradiated with reflected light for 14 days; (d) heated at 40°C and 80% relative humidity for 14 days; (e) irradiated with UV light (254 nm) for 24 h. Conditions as in Fig. 1. For peak numbers see Table I.

Chromatograms obtained for a pipecuronium bromide sample subjected to different stress conditions are shown in Fig. 4 (treatments made in solid form) and in Fig. 5 (treatments made in solution).

CONCLUSIONS

Based on the chromatograms obtained for pipecuronium bromide subjected to various treatments, the degradation pathway shown in Fig. 6 can be proposed.

Considering the most important characteristics of the validation of HPLC methods, it can be generally concluded that in addition to the well known criteria (accuracy, precision, linearity, range, sensitivity, ruggedness) published as guidelines[10], proof of the selectivity (specificity) of an HPLC method is the main interest. This may be accomplished by adding known compounds (impurities, degradation products) in small amounts to known amounts of drug substances or by subjecting samples to appropriate stress conditions such as those mentioned above in order to

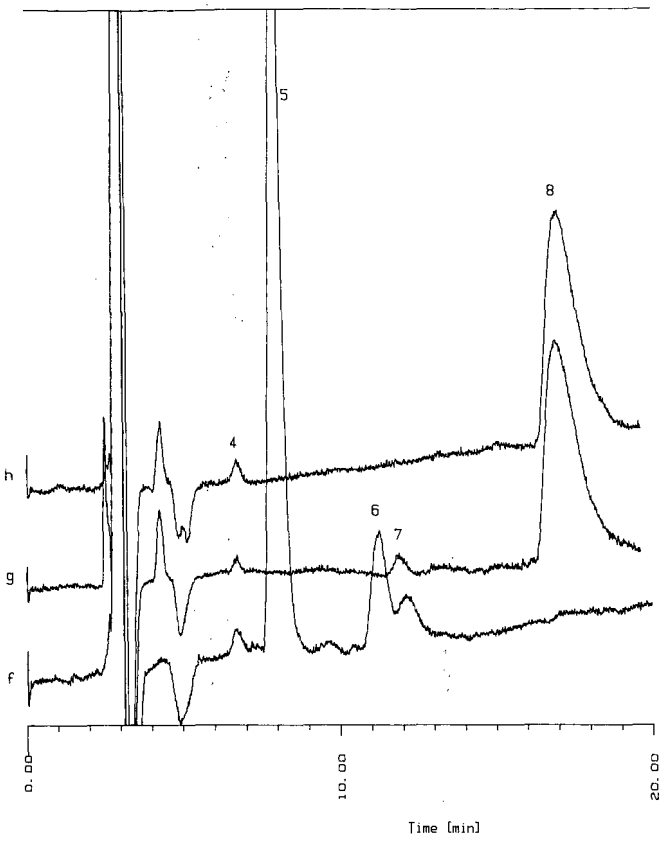

Fig. 5. Chromatograms of pipecuronium bromide subjected to various treatments in solution. (f) 2% aqueous solution at pH 2 and room temperature for 24 h; (g) same as (f) but at 100°C for 8 h; (h) 2% aqueous solution at pH 12 and 100°C for 8 h. Conditions as in Fig. 1. For peak numbers see Table I.

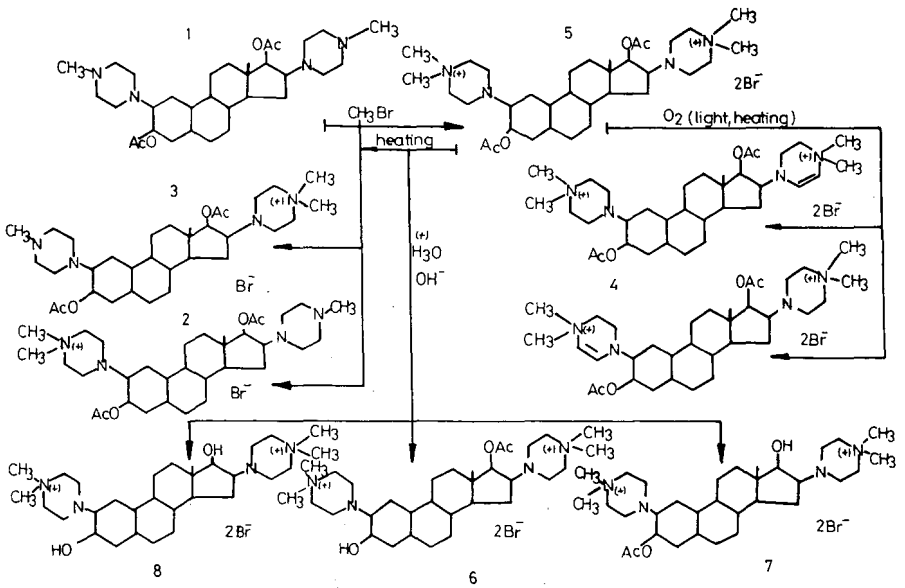

Fig. 6. Degradation pathway for pipecuronium bromide. Ac = acetyl.

generate "real" degradation products. Subsequently the purity of the peak of the main component should be determined adequately by absorbance ratio comparisons. Finally, the usability of the HPLC method for solving various analytical problems can be expressed in terms of SR and SS. The use of SS and SR as validation criteria can be recommended in pharmaceutical analysis.

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SELECTION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS IN PHARMACEUTICAL ANALYSIS

IV[a]. SELECTION OF MOST APPLICABLE SEPARATION SYSTEM

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SUMMARY

Different analytical tasks in the pharmaceutical analysis can be classified according to the separation problems into three main groups: trace analysis, assay methods and separation of closely related compounds including isomers. The most important requirements of high-performance liquid chromatographic (HPLC) methods with respect of the separation problems are summarized. Considerations and recommendations for the selection of the most applicable HPLC system to solve particular analytical problems are discussed. HPLC methods can be compared on the basis of the system resolution (SR) and system selectivity (SS). Criteria developed for the characterization of HPLC methods considering the difficulties created by the different analytical problems are established. The principles of the selection of the most applicable separation systems are demonstrated through some practical examples in pharmaceutical analysis.

INTRODUCTION

As discussed in Parts I and II[1,2], two HPLC systems (normal- and reversed-phase) were developed and optimized in order to minimize the possibility of band overlap and failure to recognize the presence of some unknown species. Based on validation data, the systems can be compared[3]. To establish the suitability of any high-performance liquid chromatographic (HPLC) method in pharmaceutical analysis, further information about the quality of the separations considering the difficulties created by the analytical problems to be solved may be necessary. Method validation data provide important information about the separation systems and criteria can be formulated to express directly the quality of the separation. These criteria are the system resolution (SR) and system selectivity (SS) and relate to the applicability of a separation system. As a continuation of our previous work[1–3], the

[a] For Part III, see ref. 3.

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basic principles used in our laboratory to compare different separation systems considering the most important features of the individual analytical tasks are discussed in this paper.

EXPERIMENTAL

The following experimental conditions were used.

Steroid separation

The same instrumentation (HP 1090A) and conditions were used as in Parts I and II[1,2].

Stability test on sulfinpyrazone tablets

A Varian 8500 liquid chromatograph equipped with a loop-type injector, variable-wavelength UV detector (both from Labor MIM) and a Hewlett-Packard HP 3392A electronic integrator was used. Separations were performed on the same types of column (Nucleosil C_{18}, 10 μm; LiChrosorb Si 60, 5 μm) as used in steroid investigations[1,2].

Purity test on ergotamine tartrate

A Liquochrom 2010 liquid chromatograph equipped with a loop-type injector, variable-wavelength UV detector (all from Labor MIM) and HP 3392A electronic integrator was used. Separations were performed on the same types of column as used in steroid separations[1,2].

RESULTS AND DISCUSSION

Most important analytical tasks in pharmaceutical analysis (connection between method used and analytical problem to be solved)

The most important analytical tasks in pharmaceutical analysis and their characteristics are collected in Table I. Considering the chromatographic separation problems, the analytical tasks can be further classified as follows.

Trace analysis. Several of the analytical tasks indicated in Table I may occur which necessitate the separation of compounds present at low concentrations in the sample in the presence of large amounts of unknown (A-1, A-2, A-3 and E) or known (B-1, C-1, C-4, D-2 and D-5) components. Three main types of trace analytical problems can be distinguished:

(a) A limited number of components are of interest and can be separated from each other and from the unknown background materials (A-1–A-3 and E), which occur at high concentrations. The most important considerations can be summarized as follows:

(i) A high selectivity and resolution of the HPLC method are required in order to achieve adequate separation of the compounds from many unidentified background materials.

(ii) Detectability of the trace components requires their early elution. However, in most instances the unknown materials also elute with short retention times, resulting in several unidentified peaks on the chromatograms. Late retention of trace

TABLE I

CHARACTERIZATION OF THE MOST IMPORTANT ANALYTICAL TASKS IN PHARMACEUTICAL ANALYSIS

Analytical task	*Symbol*	*Characteristics*[a]				
		Recommended resolution			*Peak of interest*	*Aim of analysis*
		b	*a*	$R_{s,min}$		
Investigation of starting raw materials:	A					
Plant extracts	A-1	1.5	1.8	1.0	L	T
Extracts of animal organs	A-2	1.5	1.8	1.0	L	T
Fermentation mixture	A-3	1.2	1.5	1.0	L	T
Investigation of intermediates and crude products:	B					
Intermediates and crude products	B-1	1.3	1.5	0.8	E	T
Reaction mixture	B-2	1.2	1.5	0.8	E	A
Mother liquors and secondary products	B-3	1.3	1.8	0.8	L	T
Investigation of active ingredients:	C					
Purity test	C-1	1.8	2.0	1.2	E	T
Assay	C-2	1.2	1.2	0.7	L	A
Separation of closely related compounds	C-3	1.5	1.8	1.0	E	T
Stability test	C-4	2.0	2.5	1.2	E	A+T
Investigation of formulated products:	D					
Assay	D-1	1.2	1.2	0.8	L	A
Purity test	D-2	1.7	2.0	1.0	E	T
Content uniformity test	D-3	1.2	1.2	1.0	L	A
Dissolution test	D-4	1.2	1.2	1.0	L	A
Stability test	D-5	2.5	3.0	1.2	E	A+T
Pharmacokinetic and metabolic studies	E	1.5	1.8	1.0	L	T

[a] Symbols: *b* and *a*, recommended values of R_{sb} and R_{sa}; L, limited number of peaks of interest; E, equal importance of peaks; T, trace analysis; A, assay.

compounds may result in their easier separation from the matrix materials, but their detectability may be more difficult. A good compromise can be found between chromatographic resolution and detectability.

(iii) Precise and accurate sample preparation involving optimization of extraction, clean-up and sample concentration procedures are necessary.

(iv) The use of selective and sensitive detection is an important factor including pre- or post-column derivatizations, if necessary.

(b) Separation and quantitative determination of a known number of essentially known impurities at low concentrations in the sample (B-1, C-1 and D-2). Depending on the aim of the analysis, the impurities may be present in the concentration range 0.01–1% in the sample. A sufficiently high selectivity of the separation and suitable sensitivity of the detection are the most relevant factors. One of the essential considerations is the order of elution of the separated peaks. It can be expressed by the system selectivity, (SS), as discussed in Part III[3]. The minimum resolution measured between the worst separated pair of peaks at any place in the chromatogram ($R_{s,min}$)

and the resolution between the peak of the main component and the preceding (R_{sb}) and following (R_{sa}) peaks can characterize the separation power of the HPLC system. The effect of these three parameters on the separation of trace components is expressed by the system resolution term[3] and it is recommended that it be considered when an appropriate HPLC system is selected.

(c) Stability-indicating methods (C-4 and D-5). A stability test is a specific case of trace analysis, and the method development requires suitable skill and expertise. As discussed in Part III[3], special demands arise with regard to the HPLC system as impurities with similar chromatographic properties can be separated and identified. Investigation of the peak purity of the main component to recognize incomplete resolution (discussed in detail in Part III[3]) by the absorbance-ratio method, plotting the ratio of the absorbances at two (or more) preselected wavelengths as a function of time using samples subjected to various stress conditions in order to produce real degradation products has been recommended[3].

The accuracy and precision of the methods are not critical factors. A relative standard deviation of 5% is adequate.

Assay methods (B-1, B-3, C-2, D-1, D-3 and partly C-4 and D-5). When the analytical task is to determine the active content of bulk drug substances, the selectivity and efficiency of the separation are not as critical as in other instances. The aim is to separate the main components from the impurities, but the separation of impurities from each other is not required. The accuracy and precision of the method are of more importance.The relative standard deviation should not exceed 2%.

Separation of closely related compounds and isomers. Almost every group of analytical problems requires the separation of closely related compounds and isomers. This task involves different degrees of difficulty during the method development. In the separation of related compounds, greater structural differences are mostly sufficient for their easy separation by reversed-phase or normal-phase chromatography. Method development for the separation of isomers, except the separation of optical isomers, requires more time and expertise, but in most instances the separations can be solved without using special HPLC techniques.

The most difficult problem is the separation of enantiomeric forms of pharmaceutically important compounds. At present no universal method is available for solving this analytical problem. According to literature data the methods suitable for enantiomeric separations can be divided into four groups: (a) separation in the form of diastereomeric derivatives using a chiral reagent for pre-column derivatization; (b) separation on dynamically coated or chemically bonded chiral stationary phases; (c) separation on conventional stationary phases (bare silica or chemically bonded phases) using a chiral eluent; and (d) separation by inclusion complex formation. The different separation possibilities have been excellently reviewed by Souter[4], Lindner and Petterson[5], Armstrong[6] and Wainer[7].

To establish finally the suitability of any HPLC method in pharmaceutical analysis, further information about the quality of separations considering the difficulties created by the analytical problems to be solved may be necessary. Table I contains data for the different characteristics of various analytical tasks (recommended resolutions, peak of interest, aim of analysis). Method validation data provide important information about the separation systems[3] and criteria (SR and SS) can be formulated in order to express the quality of the separation. The system resolution

TABLE II

DATA ELEMENTS RECOMMENDED FOR METHOD VALIDATION

Category I = trace analysis: LN, limited number of peaks of interest; a, in the presence of unknown materials (A-1, A-2, A-3, E); b, known number of essentially known components (B-3, D-2); EN, all peaks are of equal importance (B-1, B-2, C-1). Category II = assay methods (B-2, C-2, D-1, D-3, D-4). Category III = separation of closely related compounds (C-3, C-4, D-5). Symbols: +, recommended (importance is expressed by the number of + signs); (+), depending on the analytical tasks; −, determination is not necessary.

Analytical performance parameter	*Category I*			*Category II*	*Category III*
	LN		*EN*		
	a	*b*			
Accuracy	++	+	+	+++	++
Precision	+	++	+	+++	++
Linearity	+	+	+	++	++
Range	+	+	+	+	++
Lowest detectable quantity (LDQ)	+++	++	++	(+)	+++
Recovery	+++	(+)	(+)	(+)	(+)
Ruggedness	++	++	++	++	++
$R_{s,\min}$	+++	++	++	−	+++
R_{sb}	−	−	++	−	+++
R_{sa}	−	−	++	−	+++
SR	−	−	++	−	+++
SS	−	−	++	−	+++

TABLE III

CRITERIA FOR HPLC METHODS

Symbol	*SR*	*SS*[a]	*Sample preparation*	*R.S.D.*[b] *(%)*	*Desired number of peaks*	*Recovery (%)*	*LDQ*[a]
A-1	2.0	n.d.	Problematic	≤5	2–3	Min. 95	1–5 ng
A-2	2.0	n.d.	Problematic	≤5	2–3	Min. 95	1–5 ng
A-3	2.0	n.i.	Problematic	≤5	2–3	Min. 95	1–5 ng
B-1	1.8	+	Easy	≤5	2–5	100	0.1%
B-2	1.8	n.i.	Easy	≤2	2–3	100	n.i.
B-3	1.8	+	Easy	≤5	2–5	100	0.1%
C-1	2.2	+	Easy	≤2	2–10	100	0.01%
C-2	1.7	n.i.	Easy	≤1.5	2–3	100	n.d.
C-3	2.0	+	Easy	≤2	2–5	100	0.01%
C-4	2.2	+	Easy	≤2	2–10	100	0.01%
D-1	1.8	n.i.	Should be problematic	≤2	1–2	Min. 99	n.d.
D-2	2.0	+	No problem	≤5	2–5	Min. 98	0.01%
D-3	1.8	n.i.	Problematic	≤2	2–3	Min. 98	n.d.
D-4	1.8	n.i.	n.d.	≤5	2–3	n.d.	1–5 ng
D-5	2.2	+	No problem	≤2	5–15	Min. 99	0.1%
E	2.0	n.d.	Problematic	≤5	2–10	Min. 90	100 pg

[a] n.d., not defined; n.i., not important; +, elution order is important, should be as positive as possible.

[b] Relative standard deviation.

(SR) and system selectivity (SS) correlate closely with the applicability of a separation system.

The variety of analytical tasks require the application of different validation schemes. Recommended validation data elements for various analytical tasks are given in Table II.

Considering the difficulties created by the various analytical problems, criteria to be formulated for HPLC methods can be established (Table III).

Selection of the most applicable separation system

Based on the data in Table III, the most applicable separation system for solving the particular analytical problem is selected. The following scheme is used in the authors' laboratory, assuming that two HPLC methods are available to solve the separation problems. The first decision is based on the SR values obtained for the two systems. Higher SR values provide better separation conditions.

When the systems possess similar SR values (both HPLC methods can solve the analytical tasks) the SS values are compared. The recommended separation system can be selected on the basis of the numerical value of SS; a higher value means a more advantageous elution order and enhanced selectivity. Finally, the validation data are taken into consideration in method selection.

The principles used for the selection of a recommended separation system are now demonstrated with some practical examples. The first example (which is theoretical) is based on the experiments described in Parts I and II[1,2]. Six different steroids were used as model compounds (for the names and symbols, see Table IV) for the experiments. The chromatograms obtained with the optimized systems were presented in Parts I and II[1,2]. Norgestrel (NG) is assumed to be the main component and the others are impurities. The criteria for both normal- and reversed-phase systems were calculated and are given in Table IV.

The data in Table IV indicate the more advantageous properties of reversed-phase chromatographic separation owing to its higher separation power (SR). In a normal-phase system the minimum recommended value of SR cannot be achieved.

Another example is when various steroids at different concentrations can be

TABLE IV

CALCULATED DATA FOR STEROIDS IN REVERSED-PHASE (SYSTEM A) AND NORMAL-PHASE (SYSTEM B) SYSTEMS (PURITY TEST)

System A: column, Nucleosil C_{18} (10 μm) (250 × 4.6 mm I.D.); eluent, acetonitrile–tetrahydrofuran–water (12.9:22.4:64.7). System B: column, LiChrosorb Si 60 (5 μm) (250 × 4.6 mm I.D.); eluent, hexane–dioxane–isopropanol (95:3:2). Compounds: main component, norgestrel (NG); others, traces.

Parameter	*System A*	*System B*
Desired number of peaks (n)	6	6
Number of peaks before the main component (z)	2	3
Number of peaks after the main component (v)	4	3
System resolution (SR)	3.05	2.17
System selectivity (SS)	−0.094	+0.077
Running time (min)	40	25

TABLE V

CALCULATED DATA FOR STEROIDS IN REVERSED-PHASE (SYSTEM A) AND NORMAL-PHASE (SYSTEM B) SYSTEMS (STEROID ASSAY)

Systems A and B: as in Table IV. System C: (ref. 8) column, silica; eluent, cyclohexane-isopropanol (97:39). System D: (ref. 9) column, octadecylsilica; eluent, methanol–10 m*M* buffer (4:1). Compounds: northehindrone (N), ethinylestradiol (E), norgestrel (NG), mestranol (M).

Parameter	*Composition*							
	NG–E		*N–E*		*N–M*			
	A	*B*	*A*	*B*	*A*	*B*	*C*	*D*
Desired number of peaks (*n*)	2	2	2	2	2	2	2	2
System resolution (SR)	3.27	3.66	10.52	2.31	18.8	9.26	6.85	6.28
System selectivity (SS)	−0.099	−0.229	−0.418	+0.138	−0.781	+0.712	+0.216	−0.233
Running time (min)	18	15	18	25	40	25	10	10

determined in oral contraceptives. Calculated data for the criteria are shown in Table V. For the N + E combination, the use of a reversed-phase system (system A) can be recommended owing to its higher SR value. For NG + E tablets, both systems provide the same elution order. The values of SR are close to each other but a higher SS value is obtained with system A. This result supports the use of system A for steroid assay. With the N + M combination, high SR values were obtained. Based on the significantly better SS value obtained with the normal-phase system (system B), it can be recommended for steroid assay. Similar conclusions can be drawn from examples taken from the literature[8,9], where the higher SS value obtained with the normal-phase system (system C) provides better detection for small amounts of M.

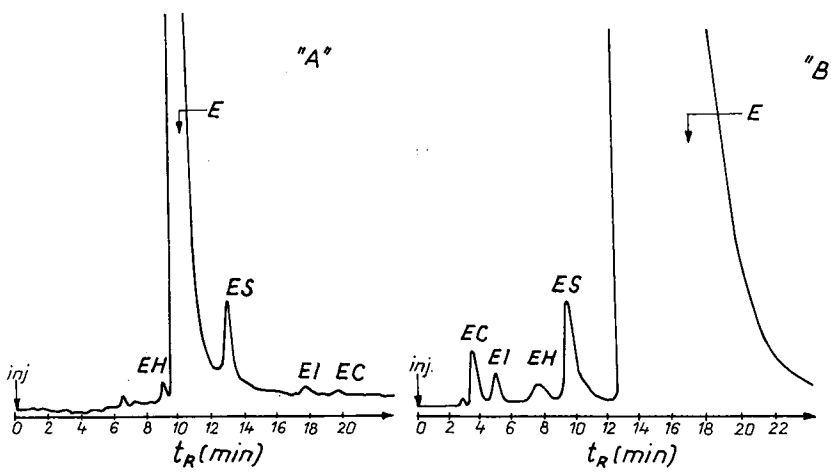

Fig. 1. Separation of ergotamine tartrate with (A) reversed-phase and (B) normal-phase systems. Conditions as in Table VI.

TABLE VI

CALCULATED DATA FOR ERGOTAMINE TARTRATE IN REVERSED-PHASE (SYSTEM A) AND NORMAL-PHASE (SYSTEM B) SYSTEMS (PURITY TEST)

System A: column, Nucleosil C_{18} (10 μm) (250 × 4.6 mm I.D.); eluent, acetonitrile–10 m*M* ammonium carbonate solution (1:1). System B: column, Micropack SI-10 (10 μm) (250 × 2 mm I.D.); eluent, chloroform–methanol (95:5). Compounds: ergotamine (E), ergocristine (EC), ergosine (ES), 8-hydroxyergotamine (EH).

Parameter	*System A*	*System B*
Desired number of peaks (n)	5	5
Number of peaks before the main component (z)	2	5
Number of peaks after the main component (v)	3	–
System resolution (SR)	2.30	3.01
System selectivity (SS)	+0.031	+0.363
Running time (min)	25	30

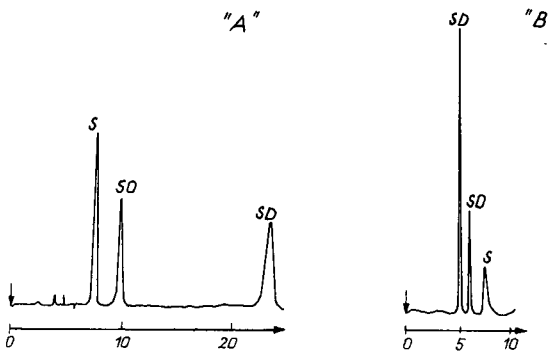

Fig. 2. Separation of sulfinpyrazone and its impurities. (A) Reversed-phase system; (B) normal-phase system. Conditions as in Table VII.

TABLE VII

CALCULATED DATA FOR SULFINPYRAZONE IN REVERSED-PHASE (SYSTEM A) AND NORMAL-PHASE (SYSTEM B) SYSTEMS (STABILITY TEST, PRELIMINARY INVESTIGATIONS)

System A: column, Nucleosil C_{18} (10 μm) (250 × 4.6 mm I.D.); eluent, acetonitrile–water (1:1), pH 3. System B: LiChrosorb Si 60 (5 μm) (250 × 4.6 mm I.D.); eluent, hexane–tetrahydrofuran–methanol–glacial acetic acid (40:50:4:6). Compounds: sulfinpyrazone (S), 1,2-diphenyl-4-(2-phenylsulphonylethyl)pyrazolidine-3,5-dione (SO); 1,2-diphenyl-4-(2-phenylthioethyl)pyrazolidine-3,5-dione (SD); others unknown.

Parameter	*System A*	*System B*
Desired number of peaks (n)	3	3
Number of peaks before the main component (z)	–	2
Number of peaks after the main component (v)	2	–
System resolution (SR)	4.53	3.28
System selectivity (SS)	−0.529	+0.152
Running time (min)	25	10

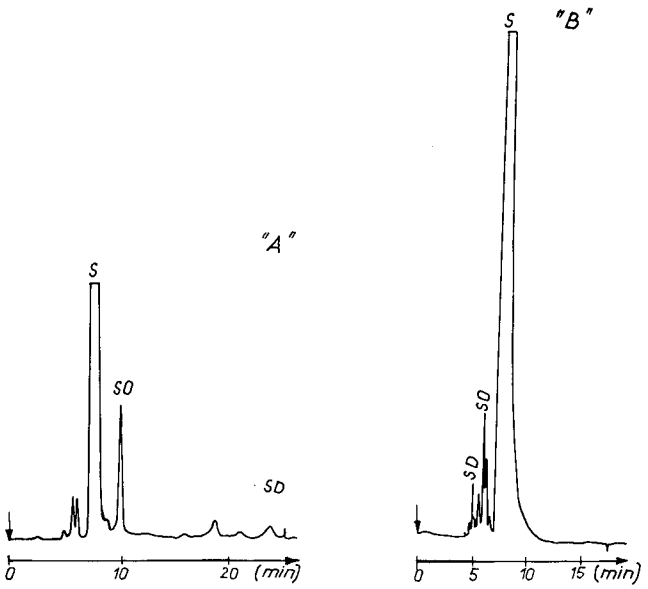

Fig. 3. Chromatograms of tablets subjected to heat treatment. Conditions as in Table VII.

The next example relates to the selection of an HPLC system suitable for purity testing of ergotamine tartrate[10,11].

Chromatograms obtained with reversed- and normal-phase systems are shown in Fig. 1 and the data are given in Table VI. High SR values are obtained by using both HPLC methods, but owing to the significantly higher SS value obtained with the normal-phase system (system B), it can be recommended for routine analysis.

The last example is connected with the stability testing of sulfinpyrazone tablets (a reversed-phase HPLC method has recently been published[12]). Chromatograms obtained with reversed- and normal-phase systems for model compounds are shown in Fig. 2 and the data are given in Table VII. Based on these data, system B was selected

TABLE VIII

CALCULATED DATA FOR SULFINPYRAZONE IN REVERSED-PHASE (SYSTEM A) AND NORMAL-PHASE (SYSTEM B) SYSTEMS (STABILITY TEST, CORRECTED DATA ON THE BASIS OF STABILITY INVESTIGATIONS)

Conditions as in Table VII.

Parameter	*System A*	*System B*
Desired number of peaks (n)	10	7
Number of peaks before the main component (z)	3	6
Number of peaks after the main component (v)	6	–
System resolution (SR)	1.84	1.45
System selectivity (SS)	−0.136	+0.079
Running time (min)	30	15

for stability testing owing to its significantly higher SS value (the SR values are similar to each other).

When the first experimental runs with the tablets subjected to various stress conditions were performed and the criteria were re-calculated (chromatograms are shown in Fig. 3 and the calculated data are given in Table VIII), the application of system A (reversed-phase system) for stability testing can be recommended, as it provides higher SR and $R_{s,min}$ values than system B.

CONCLUSIONS

As the analytical aims in pharmaceutical analyses may vary widely, the effects of several factors on the method development have to be taken into consideration. The examples presented here indicate the advantageous characterization of HPLC systems with SR and SS criteria for the selection of the most applicable separation systems.

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CHIRAL RECOGNITION BY BIOLOGICAL MACROMOLECULES

PARTIAL RESOLUTION OF RACEMIC ENONES BY ALBUMIN

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SUMMARY

A novel approach to the optical resolution of racemic enones has been introduced by using the binding properties of the transport protein albumin, which chemically binds preferentially one antipode of some α,β-unsaturated cyclic enones in a reversible manner. Simple separation of the macromolecules by ultrafiltration leads to partial resolution of racemates.

INTRODUCTION

Serum albumin is the most familiar plasma protein. It is the principal agent responsible for the transport of fatty acids and for the sequestration of physiological molecules, *viz.*, bilirubins, hormones, amino acids, etc., and also many drugs, from aspirin to antibiotics[1]. Its binding action appears to be connected to a variety of sites, a unique characteristic of this non-enzymatic, non-structural protein among the plasma proteins. Chiral interactions of bovine serum albumin (BSA) with smaller molecules were recognized some time ago; interesting examples are optical activation of the achiral bilirubin chromophore[2,3] and asymmetric induction in reactions involving prochiral substrates[4]. Recently, a commercial chiral high-performance liquid chromatographic (HPLC) column using BSA covalently bound to silica gel was introduced for the resolution of acidic and basic low-molecular-weight compounds[5–9]. We have now investigated whether novel enantiomeric enrichment approaches can be found for other substrates, based on the evident binding tendency of this transport protein.

We report here that BSA preferentially binds one antipode of some racemic α,β-unsaturated cyclic enones, and that binding is due to the formation of a reversible chemical bond. Here the BSA–enone interactions basically differ from those usually found between BSA and chromatographic analytes. As a consequence of this chemical bonding, separation of the macromolecular adducts from the low-molecular-weight species, accomplished by simple ultrafiltration, led ultimately to optically enriched samples of both (+)- and (−)-antipodes.

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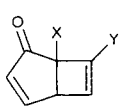
EXPERIMENTAL

Apparatus

UV measurements were performed with a Pye Unicam SP150 spectrophotometer. A Jasco J 500 C spectropolarimeter was used to measure circular dichroism (CD) spectra, from which the optical purity (OP) of the samples was obtained, OP = $|\delta\varepsilon/\Delta\varepsilon|$, $\delta\varepsilon$ and $\Delta\varepsilon$ being the CD, at some suitable maximum, of the sample and of the pure enantiomer, respectively. HPLC determinations were made with a Jasco BIP1 chromatograph equipped with a spectrophotometric detector (Uvidec 100V) and a laboratory-made data processor. A Merck RP-8 (7 μm) (25 cm $\times$ 4.6 mm I.D.) column was used with acetonitrile–water (25:75) as the mobile phase at a flow-rate of 1 ml/min and with UV detection at 225 nm. Ultrafiltrations were carried out with a Spectra/por stirred cell (S25-10) pressurized by nitrogen at about 3 atm and equipped with Spectrum C20K membranes, 20 000 daltons cutoff (Spectrum Medical Industries, Los Angeles, CA, U.S.A.).

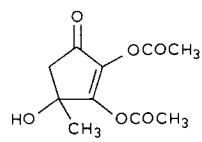
Materials

Racemic 1-methoxybicyclo[3.2.0]hepta-3,6-dien-2-one, (±)-**1**[10–12], and 7-acetylaminobicyclo[3.2.0]hepta-3,6-dien-2-one, (±)-**2**[13,14], were prepared according to the literature and 2,3-diacetyl-4-hydroxy-4-methylcyclopent-2-en-1-one, (±)-**3**, was kindly provided by Prof. M. Scotton (University of Siena). BSA, crystallized and essentially free from fatty acids, was purchased from Sigma (St. Louis, MO, U.S.A.).



1 X = OCH_3, Y = H

2 X = H, Y = $NHCOCH_3$



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RESULTS AND DISCUSSION

When the racemic bicyclic α,β-unsaturated enone (±)-**1** is dissolved in an aqueous solution of BSA, the mixture shows increasing circular dichroism with time in the range 320–430 nm. This is a spectral region where BSA is almost transparent and devoid of Cotton effects (Fig. 1a). Simultaneously, a decrease in absorbance in the same spectral region, the n $\rightarrow$ π^* absorption band of the enone and an increase in absorbance below 320 nm are observed (Fig. 1b). With molar ratios of **1**:BSA ranging from 1.8 to 30, at a constant BSA concentration of $1 \cdot 10^{-4}$ *M*, time-dependent changes of the UV and CD spectra were observed in the BSA absorption spectral zone (<320 nm) up to the peptide zone (<240 nm), where variations in the ellipticity are not measurable. In particular we noted a reduction in CD in the 280–315 nm region and an increase in CD in the range 240–275 nm with respect to the CD spectrum of pure BSA solutions. In conclusion, we have a first zone, the enone (>320 nm) with no BSA chromophore, a second zone (320–240 nm), in which relatively few BSA chromo-

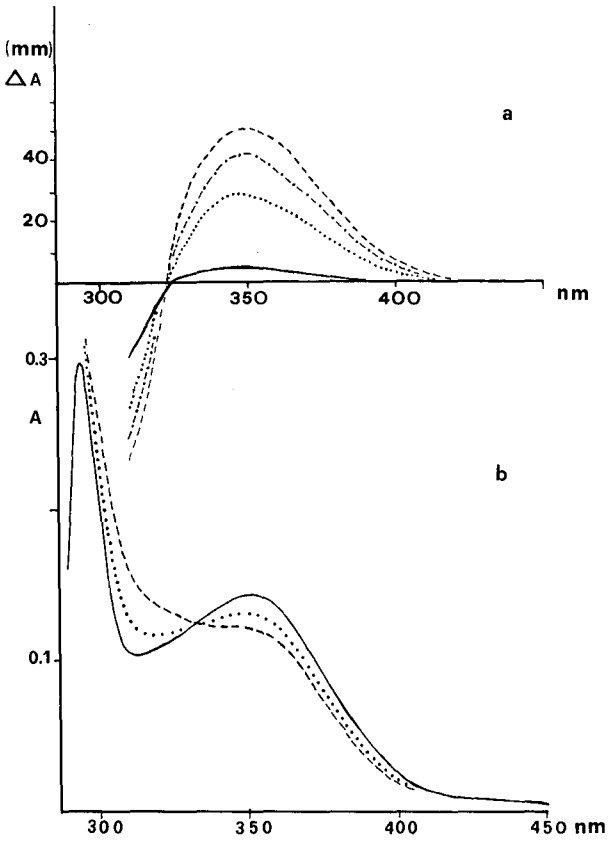

Fig. 1. CD and UV spectra after mixing **1** and BSA. (a) CD elongation 5, 60, 100 and 1100 min after dissolving 1.9 mg ($1.4 \cdot 10^{-2}$ mmol) of (±)-**1** in 9 ml of a $6.2 \cdot 10^{-5}$ *M* aqueous solution of BSA (pH 6.6). Differential circular absorbance increases with the time. (b) UV absorbance 15, 100 and 500 min after mixing. The reference cell contained a solution with the same concentration of BSA as in the sample cell. The absorbance decreases with time above 330 nm.

phores are present (48 aromatic and 17 disulphided), and a third zone (<240 nm), where the 580 peptide chromophores together with the aromatic chromophores override both in chiral and in achiral light absorption the effects due to the few enone chromophores. Note that the constancy of the CD in the spectral zone 190–240 nm implies no relevant modification of the content of α-helix and β-structure of the protein due to the binding of the enone[1]. From the variations in both the CD and UV spectra with time in the n → π* spectral region we estimate a half-life of about 1 h for the binding process at room temperature. Similar indications resulted from HPLC determinations at various times of the unbound **1** in the **1**–BSA mixtures.

Free **1** can be separated from high-molecular-weight molecules by ultrafiltration of the aqueous **1**–BSA mixture using membranes with a cut-off of 20 000 daltons. With these high-performance membranes, ultrafiltration of 10–20 ml of feed solution, with collection of 2–3 ml, is carried out rapidly (3–8 min) with respect to the reaction time.

The composition of the permeate (*i.e.*, the solution of low-molecular-weight compounds passed through the membrane) essentially reflects the composition of the feed solution, as far as free **1** is concerned, at the moment of the ultrafiltration, especially with values of the volume reduction factor (*i.e.*, the ratio of the volume of the permeate to the volume of the residual feed solution, the retentate) such as 0.2–0.4. In fact, the permeate showed a strong positive Cotton effect in the enone chromophore spectral zone (320–430 nm), owing to the enantiomeric excess of (+)-**1** present: in the same spectral region a positive CD signal was also observed in the retentate.

The enone bound to BSA can be recovered by dialysing the retentate or by dichloromethane extraction of the retentate: (−)-**1** of optical purity definitely higher than that of (+)-**1** in the permeate can thus be obtained. Hence it is clear that optical activation of **1** derives from the preferential binding of the (−)-**1** antipode to the BSA molecule. As the CD of the retentate in the enone n → π^* region is positive and in the same region there was a decrease in the absorbance after mixing **1** and BSA, we conclude that **1** bound to BSA no longer absorbs any near-UV light and therefore the observed positive CD is essentially due to the free **1** present in the retentate.

These observations indicate that binding to BSA involves a transformation of the enone chromophore. The variations in the spectroscopic properties of **1** bound to BSA and the reversible nature of the binding can be explained by the formation of a Schiff base between the carbonyl group of **1** and the ε-amino group of some lysine in the binding site of BSA. We observe that ketoimine adducts are common intermediates in the transformation of carbonyl-containing substrates catalysed by enzymes of the lysine class[15]. Alternative explanations could be the formation of a reversible Michael adduct between the enone moiety and BSA or formation of an aminoketal adduct. In any case, a shift at least of 40–50 nm of the long-wavelength absorption band towards shorter wavelengths is expected[16]. On the basis of the present data, none of these possibilities can be ruled out. Anyway, the interactions responsible for the above reaction are basically different from the weak interactions usually found under chromatographic conditions, as expected.

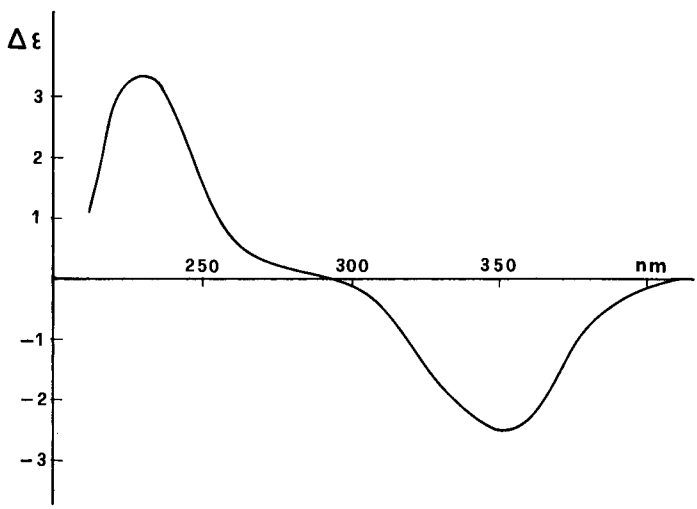

Fig. 2. CD spectrum of (−)-**1** from a sample with an optical purity of 17.1% in water ($\Delta\varepsilon$ in l mol^{-1} cm^{-1}).

The enantiomeric excess of (−)-**1** bound to BSA, ee^b, is obviously an important parameter. This ee, which is greater than the measured OP of (−)-**1** extracted from the overall retentate because of the free (+)-**1** present in it, can be evaluated from the measured optical purities of **1** in the permeate and in the retentate and from the mass of **1** in the permeate and the mass of free **1** in the retentate (see Appendix 1). In a typical experiment, with an initial [**1**]:[BSA] ratio of 17, [BSA] = $1.3 \cdot 10^{-4}$ and a volume reduction factor of 8.9, (−)-**1** with an optical purity of 17.1% (Fig. 2) was obtained from the retentate, whereas (+)-**1** with an optical purity of 5.1% was found in the permeate. Insertion of the experimental data in eqn. 3 (Appendix 1) gives 16% of total **1** bound to BSA. From the masses of "free" **1** in the permeate and in the retentate, the amount of bound **1** can also be obtained as the difference from total **1**; hence about 14% of **1** was bound. The above values correspond to less than three molecules of **1** bound to each molecule of BSA. From eqn. 4 (Appendix I), the enantiomeric excess of this bound **1** is about 24%. Therefore, with a [**1**]:[BSA] ratio of 17 and under the above-defined experimental conditions, the maximum OP that can be obtained for **1** is of the order of 25%. This value was effectively approached by a three-fold iteration of the process, each time dissolving fresh BSA in the permeate previously obtained.

In order to clarify as far as possible the dependence of the OP of **1** (permeate) on the ratio of [**1**] to [BSA], a set of experiments was performed under strictly controlled conditions. With a fixed BSA concentration of 7.5 mg/ml and variable amounts of **1** the data represented in Fig. 3 were obtained. With the hypothesis of n binding sites in each BSA molecule with a very high association constant we expect that at [**1**]:[BSA] ratios higher than n the optical purity of **1** in the permeate would be "diluted" because of saturation of the enantioselective sites. The experimental curve in Fig. 3, at its steepest part, gives some support to this simplified model when n is of the order of unity. In fact, experimental data, including the HPLC determinations of free **1**, are

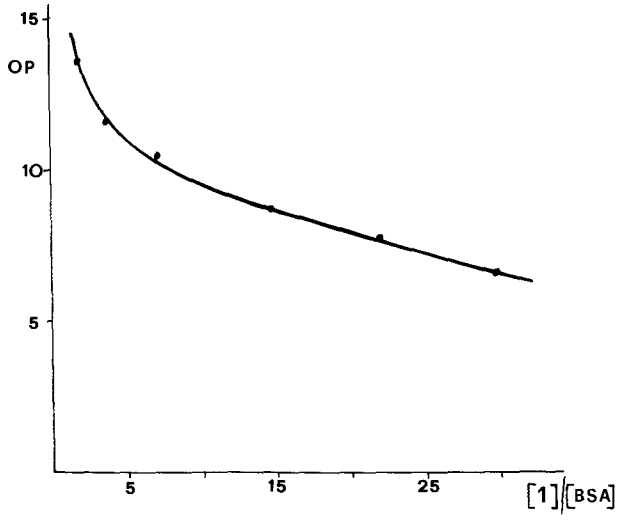

Fig. 3. Optical purity of **1** in the permeate and initial [**1**]:[BSA] molar ratio. [BSA] = $1.1 \cdot 10^{-4}$ M; ageing for 100 min at 25°C and, subsequently, 22 h at 0°C; volume reduction factor, 0.43; initial volume, 10 ml; volume of the permeate, 3 ml, obtained with 3 min of ultrafiltration.

unable to give a precise value for the number and the binding constants for each type of binding site of the protein.

More precise data, especially for small [**1**]:[BSA] ratios (0–1) would require the use of BSA purified from bound impurities and, probably the separation of monomeric from oligomeric BSA[1].

The dependence of the OP of **1** in the permeate on other variables was observed. These variables include temperature, ageing of **1**–BSA mixtures, concentration of BSA and speed of ultrafiltration. In particular, we noted a progressive degradation of **1** with ageing of **1**-BSA mixtures, especially on increasing the BSA concentration. Some reduction in the OP of **1** (permeate) was also found.

Other substrates show similar chiral interactions with BSA, as observed with the bicyclic ketone **2**, an anti-inflammatory product[13,14], and with the cyclic enone **3**. With [**2**]:[BSA] = 35 and [**2**] = $2 \cdot 10^{-3}$ *M* we observed a negative dichroism in the ketone $n \rightarrow \pi^*$ spectral band after 10 min of ageing of the proteic solution. The measured maximum of the CD at 345 nm compared with that of the pure antipode[13,14] gave an OP of 0.6%. Interestingly, in this instance the bound antipode is the positive one. For **3**, under similar experimental conditions, we observed a negative CD with a maximum at 330 nm ($\delta\varepsilon = -0.08$ l mol^{-1} cm^{-1}). In the absence of chiroptical data referring to the pure antipode, the OP of free **3** was not determined.

CONCLUSIONS

The OP of **1** ranged from 5 to 17%. Although these values are usually considered of minor interest, it should be emphasized that they are sufficient for many spectroscopic studies (*e.g.*, chiroptical determinations, chiral NMR experiments) or stereochemical correlations, the minimum OP level required being determined by the sensitivity of the instrumentation. Hence efforts to obtain higher OP are not really justified in most instances. In fact, we could measure the CD spectrum of (−)-**1** (Fig. 2) up to 200 nm, which was not possible for **1** with OP = 1%, previously obtained by partial photolysis of (±)-**1** with circularly polarized UV light[10]. However, OP for **1** greater than the above values would certainly be achieved with a substrate: BSA molar ratio of less than 1.8, as suggested by the data in Fig. 3. These data were obtained by deliberately using commercially available "pure" BSA batches. As already noted, with such low ratios the use of highly purified BSA becomes essential in order to obtain meaningful and reproducible results.

We have shown that the BSA molecule contains at least one reaction site able to discriminate the chirality of the reacting ketone **1**; probably this site would react with other ketones as, in fact, was observed with **2** and **3**. Hence the above examples partially define the possibilities of using the transport protein BSA as a chiral template for reactions involving ketones.

APPENDIX 1

Assuming no reaction other than the binding of **1** to BSA, the enantiomeric excess of **1** bound to the protein, ee^b, and that of free **1** must balance each other, and the following equation holds:

$$(m^{rf} + m^p)OP^p = m^{rb}ee^b \qquad (A1)$$

where m^{rf}, m^{p} and m^{rb} are the masses of **1** free in the retentate, of **1** in the permeate and of **1** bound to protein, respectively, and OP^{p} is the optical purity of **1** in the permeate.

Another conservation equation is obtained by imposing optical compensation between **1** in the retentate and **1** in the permeate:

$$(m^{rf} + m^{rb})OP^{r} = m^{p}OP^{p} \tag{A2}$$

where OP^{r} is the optical purity of **1** extracted from the retentate. Then, using eqns. A1 and A2, the quantities m^{rb} and ee^{b}, not directly measured, can be expressed as functions of the experimental quantities m^{p}, m^{rf} (from HPLC), OP^{p} and OP^{r} (from CD measurements):

$$m^{rb} = \frac{m^{p}OP^{p}}{OP^{r}} - m^{rf} \tag{A3}$$

$$ee^{b} = OP^{r}OP^{p} \cdot \frac{m^{p} + m^{rf}}{m^{p}OP^{p} - m^{rf}OP^{r}} \tag{A4}$$

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COMPARISON OF THE SELECTIVITY OF CYANO-BONDED SILICA STATIONARY PHASES IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

The selectivities of three brands of cyano-bonded silica stationary phase (CPS-Hypersil, Spherisorb-CN and Ultrasphere cyano) have been compared for a series of test compounds using a range of methanol–phosphate buffer (pH 7.0) and acetonitrile–phosphate buffer (pH 7.0) eluents. The retentions were expressed as retention indices using the alkyl aryl ketone scale. Considerable differences were found on elution with the different eluents and between the different brands of stationary phases. The retentions were also compared to separations on ODS-Hypersil.

INTRODUCTION

The majority of separations carried out by reversed-phase high-performance liquid chromatography (HPLC) use an octadecylsilyl-(ODS), or other alkyl-bonded silica stationary phase. However, all these materials have relatively similar selectivity properties although there are differences in the overall retentive capacity between phases with different alkyl chain lengths. There are also often small differences in relative retentions on different brands of nominally equivalent packing materials. As a consequence, because changing brands or the length of the carbon chain will have relatively little effect on selectivity, almost all optimisation strategies in liquid chromatography concentrate on the influence of changing the pH or organic modifier in the mobile phase. The only exceptions are with analytes, such as basic drugs, which are partially retained by specific interactions with residual silanol groups on the surface of the silica. These interactions can vary considerably between brands and chain length, because of differences in the chemistry of the bonding reaction and of the protection afforded to the column surface.

However, other column materials are available for reversed-phase chromatography, which have different selectivity properties so that analytes containing different functional groups will show different relative retentions and thus potentially could improve or alter the separation of the components of a mixture. These stationary

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phases are starting to attract considerable interest and include cyano- and amino-bonded silicas[1] and polymeric materials, such as polystyrene–divinylbenzene[2] and the very specific chiral phases. The cyano-bonded columns are of particular interest as it is reported that they can be used for both normal- and reversed-phase separations[3]. Much work has been carried out by De Smet *et al.*[4] who have suggested that a single cyano-bonded column used with eluents prepared from a limited group of mobile phases, dichloromethane, tetrahydrofuran (THF), acetonitrile, *n*-hexane, and water, can separate a wide range of drug compounds. They have subsequently examined in detail the separations using methanol–buffer eluents[5]. The effects of eluent pH, ionic strength and proportion of organic modifiers on the separation of a series of acidic, neutral, and basic drugs were examined using a LiChrosorb CN column. In most cases the behaviour of the drugs to these parameters closely resembled effects observed on ODS-bonded columns although the selectivities differed. Other workers have suggested that in reversed-phase chromatography the primary retention of cyanopropyl-bonded columns is similar to a short chain alkyl-bonded silica[1]. In their studies Massart and his co-workers initially used a Micropak-CN column[3] but subsequently changed to a LiChrosorb CN column[4], which was reported to have better selectivity.

The retention properties in reversed-phase chromatography of individual cyano-bonded columns have been examined previously and compared with other types of stationary phases. Cooper and Lin[6] studied the retention of the simple test compounds phenol, aniline and nitrobenzene relative to toluene. They demonstrated an increase in stationary phase polarity in the order octyl-, phenyl-, to cyano-bonded silica. In a similar study Moats and Leskinen[7] compared cyano-bonded silica, polymeric and silica columns over a wide composition range of aqueous acetonitrile eluents for the separation of penicillins. Pietrogrande *et al.*[8] have compared the separation of benzodiazepines on octadecyl-, phenyl- and cyano-bonded silica and have related the capacity factors to octanol–water partition coefficients. Zorbax-CN has been compared with alkyl- and phenyl-bonded Zorbax phases for the separation of phenylthiohydantoin amino acid derivatives[9] and a range of retention test compounds[10].

However, each of these studies has only examined a single brand of cyano-bonded column. As noted earlier differences can be found between different brands of ODS-bonded silica and in previous studies from these laboratories it has been possible to make direct comparisons between phases by using a set of column test compounds[11,12]. The retentions of the test compounds were determined relative to the alkyl aryl ketone retention index scale[13,14] in a similar manner to the methods of McReynolds and Rohrschneider for the comparison of stationary phases in gas–liquid chromatography. This approach has the advantage that because the retentions are calculated by interpolation between the retention index standards, the values are insensitive to differences in the overall retention capacity of the column or to small changes in the experimental conditions.

In the present study the retentions of the standard test compounds have been used to compare three different brands of cyano-bonded silica using methanol–buffer and acetonitrile–buffer as eluents. The results have also been compared with the selectivity on ODS-Hypersil in the same eluents.

EXPERIMENTAL

Chemicals and reagents

Standard alkyl aryl ketones (acetophenone, propiophenone, butyrophenone, and valerophenone) and column test compounds, toluene, nitrobenzene, N-methylaniline, methyl benzoate, 2-phenylethanol, and *p*-cresol were laboratory grade from a number of suppliers. Methanol and acetonitrile were HPLC grade and disodium hydrogenphosphate, and sodium dihydrogenphosphate for buffers were analytical reagent grade from FSA Laboratory Suppliers (Loughborough, U.K.).

High-performance liquid chromatography

HPLC separations were carried out using a Kontron LC 410 pump and a Pye-Unicam PU 4020 variable-wavelength detector set at 254 nm. The samples (10 μl) were injected, using a Rheodyne 7125 valve with a 20-μl loop, onto the column which was surrounded by jacket through which was circulated water at 30°C from a thermostated bath. The peaks were recorded using a Spectra-Physics 4270 integrator.

The cyano-bonded columns were CPS-Hypersil 5 μm (Batch No. 12/1423, Shandon Southern, Runcorn, U.K.) (100 × 5 mm I.D.), Spherisorb-CN 3 μm (Batch 19/205, Phase-Separations, Queensferry, U.K.) (150 × 5 mm I.D.) and Ultrasphere cyano 5 μm (Batch 3UEC122N, Beckman) (250 × 4.6 mm I.D.).

Methods

The analytes as dilute solutions in methanol–water were separated using methanol–pH 7 buffer or acetonitrile–pH 7 buffer in different proportions as the mobile phase. The pH 7.0 buffer was prepared using disodium hydrogenphosphate (0.6850 g) and sodium dihydrogenphosphate (0.7882 g) in water (500 ml). A sample of 10% aqueous sodium nitrate (5 μl) was used to determine the column void volume.

The capacity factors were calculated from the mean retention time of triplicate injections. The retention indices were calculated from log k' (capacity factor) of the analytes as reported earlier[13] by comparison with the linear least squares relationship between log k' of the alkyl aryl ketones and their carbon number × 100.

RESULTS AND DISCUSSION

The retention times of the alkyl aryl ketones and column test compounds, (nitrobenzene, toluene, 2-phenylethanol, *p*-cresol, and N-methylaniline, and methyl benzoate) were measured on each of three cyano-bonded columns (CPS-Hypersil, Ultrasphere cyano, and Spherisorb-CN) using 10–40% methanol–pH 7.0 buffer and 10–30% acetonitrile–pH 7.0 buffer as eluents. The first four column test compounds had been selected previously to be representative of the different interactions that can occur on a column[8]. N-Methylaniline was included to test for interactions with acidic uncapped silanols on the silica surface. Methyl benzoate has previously been found to have a retention index value on ODS-silica columns that was virtually independent of the brand of stationary phase and of the eluent composition over a wide range and can be used as a secondary standard. Buffered eluents were used to ensure that the effects of the underlying silanols on the silica surface and the degree of ionisation of *p*-cresol and N-methylaniline would be constant throughout the study. The peak shapes on all

TABLE I

CAPACITY FACTORS OF ALKYL ARYL KETONES AND TEST COMPOUNDS ON CPS-HYPERSIL COLUMN

Methanol–phosphate buffer (pH 7.0) and acetonitrile–phosphate buffer (pH 7.0) are used as mobile phases.

Compound	*Capacity factors*						
	% Methanol				*% Acetonitrile*		
	10	*20*	*30*	*40*	*10*	*20*	*30*
Retention index standards							
Acetophenone	2.57	1.80	1.19	0.81	2.28	1.74	1.31
Propiophenone	4.56	2.95	1.80	1.19	3.99	2.90	1.69
Butyrophenone	8.34	5.00	2.85	1.74	7.27	4.86	2.47
Valerophenone	17.7	9.54	4.87	2.72	14.74	8.84	3.74
Test compounds							
2-Phenylethanol	1.59	1.19	0.95	0.66	1.62	1.28	0.79
N-Methylaniline	1.74	1.37	1.06	0.80	2.03	1.77	1.27
p-Cresol	2.11	1.70	1.20	0.86	2.20	1.74	1.08
Nitrobenzene	2.55	2.05	1.61	1.20	2.86	2.53	1.66
Toluene	3.51	2.74	2.04	1.46	4.04	3.68	2.14
Methyl benzoate	4.05	2.77	1.83	1.22	3.75	2.76	1.54
Void volume (min)	2.03	2.07	2.10	2.12	1.94	1.92	1.98

TABLE II

CAPACITY FACTORS OF ALKYL ARYL KETONES AND TEST COMPOUNDS ON SPHERISORB-CN COLUMN

Methanol–phosphate buffer (pH 7.0) and acetonitrile–phosphate buffer (pH 7.0) are used as mobile phases.

Compound	*Capacity factors*						
	% Methanol				*% Acetonitrile*		
	10	*20*	*30*	*40*	*10*	*20*	*30*
Retention index standards							
Acetophenone	2.20	1.32	0.87	0.66	1.33	0.92	0.70
Propiophenone	3.11	1.87	1.13	0.77	1.89	1.21	0.89
Butyrophenone	4.98	2.69	1.49	0.91	2.68	1.60	1.12
Valerophenone	8.10	3.98	2.02	1.10	4.02	2.17	1.44
Test compounds							
2-Phenylethanol	0.85	0.43	0.45	0.45	0.76	0.60	0.49
N-Methylaniline	1.21	0.88	0.64	0.64	1.08	0.87	0.71
p-Cresol	0.80	0.61	0.45	0.44	0.77	0.62	0.51
Nitrobenzene	1.83	1.29	0.90	0.73	1.32	0.99	0.77
Toluene	1.39	1.15	0.84	0.70	1.39	1.14	0.94
Methyl benzoate	2.01	1.39	0.93	0.71	1.50	1.07	0.78
Void volume (min)	2.37	2.44	2.42	2.33	2.40	2.30	2.31

TABLE III

CAPACITY FACTORS OF ALKYL ARYL KETONES AND TEST COMPOUNDS ON ULTRASPHERE CYANO COLUMN

Methanol–phosphate buffer (pH 7.0) and acetonitrile–phosphate buffer (pH 7.0) are used as mobile phases.

Compound	*Capacity factors*						
	% Methanol				*% Acetonitrile*		
	10	*20*	*30*	*40*	*10*	*20*	*30*
Retention index standard							
Acetophenone	2.99	1.65	1.03	0.76	1.50	1.10	0.82
Propiophenone	5.09	2.47	1.35	0.95	2.23	1.55	1.07
Butyrophenone	8.66	3.71	1.84	1.18	3.33	2.24	1.40
Valerophenone	–	5.75	2.58	1.53	5.26	3.18	1.85
Test compounds							
2-Phenylethanol	1.24	0.84	0.63	0.51	0.87	0.76	0.60
N-Methylaniline	1.55	1.00	0.76	0.62	1.10	1.02	0.84
p-Cresol	1.33	0.92	0.62	0.52	0.94	0.83	0.67
Nitrobenzene	2.48	1.53	1.03	0.81	1.42	1.26	0.99
Toluene	2.17	1.50	1.02	0.82	1.58	1.53	1.23
Methyl benzoate	3.57	2.06	1.24	0.86	1.90	1.23	1.00
Void volume (min)	2.69	2.72	2.66	2.71	2.61	2.60	2.59

three columns were symmetrical and suggested that silanol interactions were minimal. This would be expected as at pH 7.0, N-methylaniline should not be protonated and thus would behave as a neutral analyte. In most cases the column efficiencies were significantly higher in the acetonitrile–buffer eluents than the methanol–buffer eluents [*i.e.*, nitrobenzene on CPS-Hypersil $N = 1950$ with methanol–buffer (10:90) and $N = 3400$ with acetonitrile–buffer (10:90)].

Using the retention times and the column void volumes measured with an aqueous sodium nitrate solution the capacity factors on the three columns were calculated (Tables I–III). The capacity factors varied considerably, for example valerophenone on elution with methanol–buffer (20:80) had capacity factors of 3.98, 5.75, and 9.54 on the different columns. These differences confirmed the difficulty of making direct comparisons between columns using just capacity factors. Overall, the k' values are much shorter than those for the same analytes on ODS columns and are even shorter than those measured previously on the short alkyl-bonded SAS-Hypersil column [with methanol–water (30:70) as the eluent: butyrophenone, $k' = 20.6$ and nitrobenzene, $k' = 4.93$)[13]].

In each case there was a linear relationship between the logarithm of the capacity factors of the alkyl aryl ketones and their carbon numbers (log k' = carbon number ketones × slope + constant) (Table IV) with correlations comparable to the results from studies on ODS columns[13,15]. In their study, De Smet and Massart[5] found that over all their drug compounds, there was a reasonable relationship between size, expressed as carbon number, and retention on the LiChrosorb CN column, although in methanol–buffer (30:70) all the drug compounds with fewer than 10 carbon atoms were eluted with k' values less than 1.

TABLE IV
CORRELATION BETWEEN CARBON NUMBER OF ALKYL ARYL KETONES AND LOG k' DIFFERENT CYANO-BONDED SILICA COLUMNS

Compound	*% Methanol*				*% Acetonitrile*		
	10	*20*	*30*	*40*	*10*	*20*	*30*
CPS-Hypersil							
Correlation	0.998	0.998	0.998	0.999	0.999	0.999	0.999
Slope × 10^3	2.8	2.4	2.0	1.7	2.7	2.3	1.7
Spherisorb-CN							
Correlation	0.999	0.999	0.999	0.998	0.999	0.999	0.999
Slope × 10^3	2.1	1.6	1.2	0.74	1.6	1.2	1.0
Ultrasphere cyano							
Correlation	0.999	0.999	0.998	0.998	0.999	0.999	0.999
Slope × 10^3	2.3	1.8	1.3	1.0	1.8	1.5	1.2

Using the linear relationships for the ketones on the present columns the retention indices of the column test compounds were calculated (Table V). In each case the change in retention indices with composition was much smaller than the differences in capacity factors. However, particularly with the eluents containing the higher proportions of organic modifier all the retention times were very short ($k' < 1.5$) and the slopes of the curves for the ketones standards were very flat [methanol–water (30:70) eluent: CPS-Hypersil, slope $= 2.00 \cdot 10^{-3}$; Spherisorb-CN, slope $1.2 \cdot 10^{-3}$ and Ultrasphere cyano, slope $1.3 \cdot 10^{-3}$]. These contrast with the much higher methylene selectivity in the same eluent on the SAS-Hypersil column (slope $3.35 \cdot 10^{-3}$)[13]. On the cyano-bonded columns the capacity factors of the test compounds in these eluents were also low so that even very small differences in retention times would cause significant variations in retention indices (I). This was reflected in a poor repeatability of retention indices based on individual separations, for example successive individual injections of nitrobenzene [in methanol–water (40:60), average $k' = 0.81$] gave I values of 802, 840 and 854 on the Ultrasphere cyano column. Differences in the I values of up to 30 units are therefore probably not significant for analytes with these low retentions, although normally retention indices can be determined with a precision of better than 5 units (standard deviation) even over a prolonged period[16]. There are noticeable differences in the selectivities of the separations with the two organic modifiers and in particular N-methylaniline and toluene were relatively more highly retained with the acetonitrile containing eluents.

The separations on two of the columns, Spherisorb-CN and Ultrasphere cyano, were relatively similar with differences in the retention indices in most cases of less than 30 units. However, the CPS-Hypersil column was markedly different and the retention indices with this column, particularily for *p*-cresol and 2-phenylethanol, differed by over 100 units from the other two brands. However, according to the manufacturers all three columns have similar carbon loadings (3.5, 3.9 and 4.4%) and have been prepared from monofunctional cyanopropylsilane reagents. The differences between brands were larger than observed earlier for the same test compounds on different

TABLE V

RETENTION INDICES OF TEST COMPOUNDS ON THREE CYANO-BONDED STATIONARY PHASES AND COMPARISON WITH ALKYL-BONDED HYPERSIL COLUMNS

Methanol–phosphate buffer (pH 7.0) and acetonitrile–phosphate buffer (pH 7.0) are used as mobile phases.

Compound	*Retention indices*						
	% Methanol				*% Acetonitrile*		
	10	*20*	*30*	*40*	*10*	*20*	*30*
CPS-Hypersil							
2-Phenylethanol	730	732	746	752	750	746	709
N-Methylaniline	744	757	782	800	787	807	830
p-Cresol	774	795	808	817	800	804	789
Nitrobenzene	804	830	871	900	842	873	898
Toluene	854	882	921	950	898	942	961
Methyl benzoate	877	884	899	905	886	889	877
Spherisorb-CN							
2-Phenylethanol	616	499	574	579	651	653	651
N-Methylaniline	692	693	698	782	748	781	807
p-Cresol	604	596	574	562	654	663	662
Nitrobenzene	782	797	817	864	801	829	841
Toluene	723	765	793	843	815	877	921
Methyl benzoate	802	817	826	850	836	857	846
Ultrasphere cyano							
2-Phenylethanol	635	640	647	632	672	699	688
N-Methylaniline	678	683	706	714	729	779	809
p-Cresol	649	661	642	644	692	722	726
Nitrobenzene	766	785	807	833	790	839	869
Toluene	741	779	804	835	816	895	953
Methyl benzoate	834	855	869	856	860	834	873
ODS-Hypersil[15,a]							
2-Phenylethanol			773	778	752	735	713
N-Methylaniline			776	787	783	799	828
p-Cresol			797	800	790	784	776
Nitrobenzene			813	829	820	842	869
Toluene			957	985			996
Methyl benzoate			901	916	885	882	886
SAS-Hypersil[13]							
2-Phenylethanol			747				
p-Cresol			779				
Nitrobenzene			816				
Toluene			892				
Methyl benzoate			895				

[a] Values are for methanol–phosphate buffer (pH 8.5)[17] except for methyl benzoate, methanol–water[13].

brands of ODS-silicas. Particularly noticeable in the present study was the variation in the retention index value of methyl benzoate, which ranged from $I = 846$ to 877 with acetonitrile–buffer (30:70). In contrast, on six different brands of ODS-bonded silicas it had only varied between $I = 881$ to 891 with acetonitrile–water (50:50)[8].

The changes in the retention indices of the column test compounds with eluent composition also differed on the different brands (Table V). With increasing proportions of methanol the retention indices of all the column test compounds increased on the CPS-Hypersil column, whereas on the Spherisorb-CN column the indices of *p*-cresol and 2-phenylethanol decreased but those of the other test compounds increased. On the Ultrasphere cyano column the retention indices of compounds containing hydroxyl groups were relatively constant (Fig. 1).

With acetonitrile containing eluents, *p*-cresol and 2-phenylethanol decreased markedly with increasing modifier on the CPS-Hypersil columns but showed only small changes on the other two columns. The other test compounds, toluene, nitrobenzene, N-methylaniline and methyl benzoate all increased on all three columns.

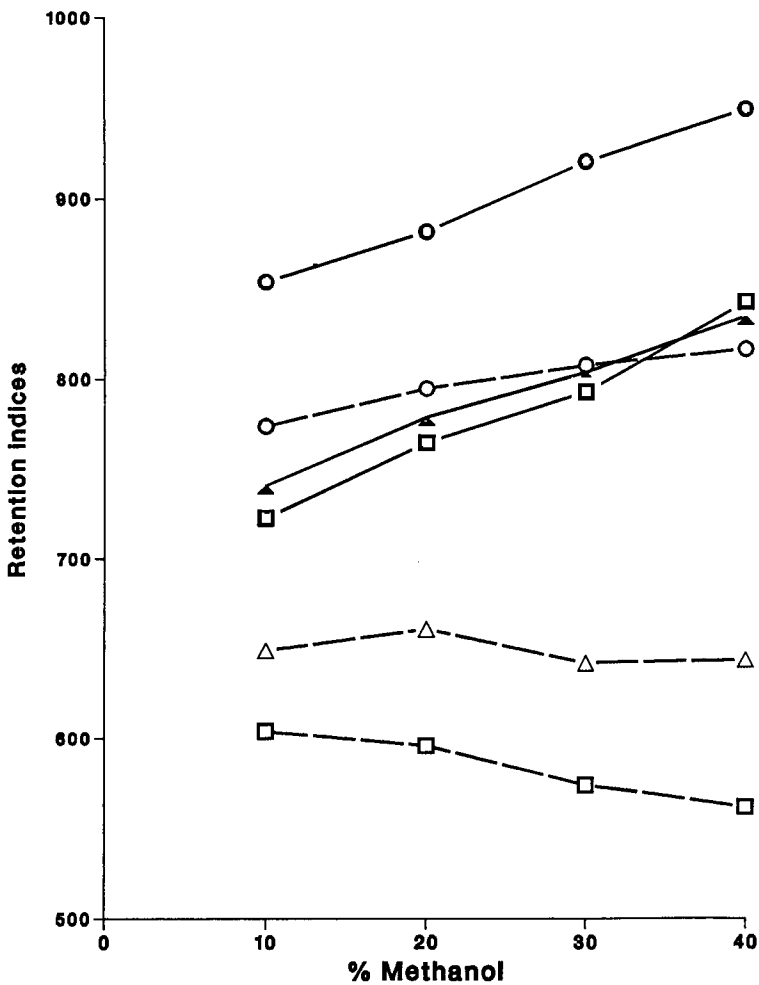

Fig. 1. Comparison of the changes in the retention index with methanol percentage on three cyano-bonded silica columns for toluene (solid symbols) and *p*-cresol (open symbols). Columns; ● and ○, CPS-Hypersil; ■ and □, Spherisorb-CN; ▲ and △, Ultrasphere cyano.

Again there is a marked difference in the I values on the CPS-Hypersil column, which were higher than the other two columns.

The retention indices can also be compared with the retention indices of these test compounds on ODS-Hypersil and SAS-Hypersil measured in earlier studies in these laboratories using the same or closely similar mobile phase[13,15,17] (Table V). As expected there were often differences between the selectivity of the bonded phases suggesting that changing columns could offer considerable optimisation potential. However, in methanol–water (30:70) the separation on the CPS-Hypersil column was quite similar to that on the SAS-Hypersil column (for example on CPS- and ODS-Hypersil: 2-phenylethanol, I = 746 and 747, respectively; *p*-cresol, I = 808 and 779; and toluene, I = 921 and 892) whereas the retention indices on the other two cyano columns were very different (Spherisorb-CN and Ultrasphere cyano: 2-phenylethanol, I = 574 and 647, respectively; *p*-cresol, I = 574 and 642, toluene, I = 793 and 804). This suggested that a specific cyano interaction with the analytes might not be significant on the CPS-Hypersil column. This lack of an interaction agrees with the comments that cyano-bonded columns in reversed-phase can effectively act as short alkyl-silanes[1] and the finding that in normal phase separations a cyano-bonded column usually behaves as a weakly retentive silica column with few specific interactions[18].

However, the results from the Spherisorb-CN and Ultrasphere cyano columns suggest that the differences between columns may reflect marked variations in interactions and possibly specific cyano effects. This would agree with other workers who have suggested that more specific interactions can take place if the length of the alkyl chain carrying the cyano group enables it to screen residual silanols on the surface of the silica[19].

CONCLUSION

The selectivity of different brands of cyano-bonded silica varies considerably, which may make the transfer of methods between brands difficult. The effects of changing the organic modifier or its proportion in the mobile phase also causes different effects on the relative retention with the different brands.

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ION-PAIR, ANION-EXCHANGE AND LIGAND-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF TENUAZONIC ACID AND 3-ACETYL 5-SUBSTITUTED PYRROLIDINE-2,4-DIONES

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SUMMARY

The ion-pair, ligand-exchange and anion-exchange chromatography of the fungal metabolic tenuazonic acid (TA) and its related 3-acetyl 5-substituted pyrrolidine-2,4-diones were studied. Ion-pair chromatography was performed on a C_{18} column with a mobile phase composed of cetrimide, phosphate buffer in water–methanol and a metal complexant (ethylenediamine) to improve the peak sharpness. Addition of the same metal complexant to the mobile phase of the anion-exchange chromatographic system also improved its efficiency. TA and its 5-substituted analogues derived from valine and leucine were separated with the ion-pair and anion-exchange chromatographic systems. With ligand-exchange chromatography, TA could only be separated from its valine analogue. These chromatographic systems were used for the detection of TA in the culture filtrates of the fungus *Pyricularia oryzae* and in infected rice leaves. Deproteinated culture filtrates could be rapidly analysed for their TA content by anion-exchange chromatography. However, this system was not suitable for the detection of TA in the infected rice leaf as interfering compounds were coeluted with TA. Ion-pair and ligand-exchange chromatographic systems allowed the efficient quantification of TA in infected leaves.

INTRODUCTION

Tenuazonic acid (3-acetyl 5-*sec.*-butylpyrrolidine-2,4-dione; TA; Fig. 1) is a toxic metabolite derived from isoleucine[1,2] which is produced by several fungi: *Alternaria tenuis, alternata, longipes, mali, citri, oryzae and kikuchiana*[3–7], *Phoma sorghina*[8] and *Pyricularia oryzae*[9–11]. TA exhibits toxicity towards plants[5,9] and animals[6,12,13] and is weakly bactericidal[14] and viricidal[15].

Chromatographic methods have been developed for the detection of TA in *Alternaria*-contaminated food and feeds for animals. First, Harvan and Pero[16] described the gas chromatography of TA trimethylsilyl derivatives. This technique was

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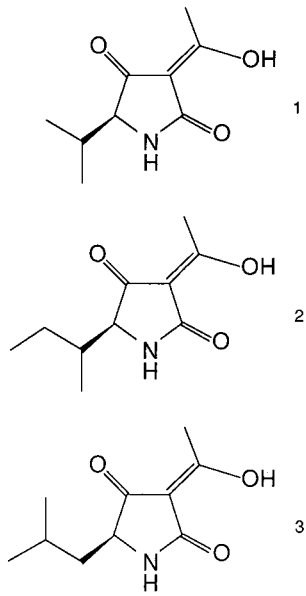

Fig. 1. Structures of 3-acetyl-5-isopropylpyrrolidine-2,4-dione (1), 3-acetyl-5-*sec.*-butylpyrrolidine-2,4-dione (2) and 3-acetyl-5-isobutylpyrrolidine-2,4-dione (3).

applied to the detection of TA in contaminated tomatoes[17]. Then, reversed-phase chromatography with UV detection at 280 nm was employed by Heisler *et al.*[18] using a 10-μm C_{18} column with methanol–water (90:10) as the mobile phase. TA was more retained with increasing proportion of methanol, suggesting that residual silanol groups were responsible for its retention. This technique has been used in the detection of TA in contaminated fruits[19,20]. As TA has metal-chelating properties[3,21], Scott and Kanhere[22] developed a ligand-exchange chromatographic system with dodecyldiethylenetriamine and zinc sulphate in water–acetonitrile (50:50) with a 10-μm C_{18} column. This method allowed the detection of TA in tomato paste[22] and was applied with slight modifications to the chromatography of cyclopiazonic acid[23,24], which has the same metal-complexing structure as TA. A simplification of this method was described by Stack *et al.*[25], who used zinc sulphate in methanol–water solutions with a C_{18} packing. Scott and Kanhere[22] also used a silica-based weak anion exchanger to detect TA. Finally, a gradient of acetonitrile buffered with trifluoroacetic acid and a C_{18} packing were described by Frisvad[26].

In addition to these chromatographic improvements, Joshi *et al.*[27] detected, together with TA, a small amount of a TA analogue derived from valine (3-acetyl 5-isopropylpyrrolidine-2,4-dione) by mass spectrometric analysis of their copper salts. This compound was also detected by Stack *et al.*[25] in *Alternaria*-contaminated tomatoes, without derivation, by capillary gas chromatography coupled with mass spectrometry. This metabolite might interfere with the determination of TA if they are coeluted. Therefore, we have compared different high-performance liquid chromatographic (HPLC) systems for their ability to separate TA from other 3-acetyl 5-substituted pyrrolidine-2,4-diones. We used isocratic systems, which allow the

analysis of a large number of samples in a short time (less than 15 min per sample). Three distinct chromatographic systems were studied. First, ion-pair chromatography with alkylammonium ion was adapted to the chromatography of TA and its 5-substituted analogues with 5-μm C_{18} columns. Then, we studied the behaviour of TA and its 5-substituted analogues during anion-exchange and ligand-exchange chromatography. We have applied these methods to the detection of TA produced by the fungus *Pyricularia oryzae* in culture media and in infected rice leaves.

EXPERIMENTAL

Extraction of metabolites from culture filtrates

Isolates of *Pyricularia oryzae* were obtained from the CIRAD-IRAT culture collection and stored as dried paper disks at $-20°C$. The strains were grown on rice medium agar (using paddy rice 20 g, Difco yeast extract 2 g and agar 20 g per litre). Liquid cultures (Fries medium[28]) in Roux flasks (100 ml) were inoculated with mycelial implants. The flasks were incubated for 15–30 days at 26°C in the dark. At the end of the culture, the mycelium was separated from the culture medium by filtration through a filter-paper (No. 111, Durieux). The culture filtrate was either extracted with an organic solvent or treated with acetonitrile to precipitate the proteins. In the first instance, 2 ml of the culture filtrate were acidified to pH 2 with 5 *M* hydrochloric acid and extracted with 2 ml of ethyl acetate. After agitation of the tube on a Vortex mixer and decantation for 30 min, the organic phase was taken up with a Pasteur pipette and transferred to another tube. This ethyl acetate phase was then evaporated in a Speed-Vac evaporator and treated with 2 ml of 5% sodium hydrogencarbonate solution and 2 ml of methylene chloride. The aqueous phase was removed and centrifuged at 1500 *g* for 10 min to remove particles in suspension. For direct injection of the culture filtrates, proteins were removed as described by Jehl *et al.*[29], by precipitation with acetonitrile followed by a centrifugation at 1500 *g* for 10 min.

Inoculation of rice with Pyricularia oryzae spores and extraction of infected leaves

Pyricularia oryzae spores were produced on rice agar, suspended in water (10^5 spores ml^{-1}) containing 0.8% gelatine (bovine type II, Sigma) and sprayed on the rice leaves (5 leaf stage) with a hand atomizer. Seven days after the inoculation, the infected leaves were collected and crushed in ethanol with an Ultra-Turrax (100 mg fresh weight in 1 ml). After filtration on a glass-fibre filter (Whatman GF/C), the ethanol was evaporated in a Speed-Vac apparatus. The residue was dissolved in acidified water and extracted with ethyl acetate. The organic phase was then extracted with 5% sodium hydrogencarbonate solution. The aqueous phase was centrifuged at 1500 *g* for 10 min and injected.

Tenuazonic acid and 3-acetyl 5-substituted pyrrolidine2,4-diones

Physical data for TA and its analogues synthesized from different amino acids have been presented elsewhere[30]. D-*allo*-TA was synthesized independently from D-*allo*-isoleucine (Bachem, Switzerland). Its characteristics are as follows: $[\alpha]_D^{22}$, $+150°$ (H_2O; *c* 1); ^1H NMR (250 MHz, sodium salt, $C^2H_3O^2H$), δ 0.78 (3H, d, C-9), 1.0 (3H, t, C-8), 1.4 (2H, m, C-7), 1.9 (H, m, C-6), 2.45 (3H, s, C-11), 3.72 ppm (H, d, C-5).

Reagents

HPLC-grade acetonitrile was obtained from Carlo Erba and methanol (Rectapur) from Prolabo. Water was obtained daily from a Milli-Ro-Milli-Q system (Millipore). Tetrabutylammonium hydroxide (TBAOH, Aldrich), hexadecyltrimethylammonium bromide ($C_{16}N$, Merck), octadecyltrimethylammonium bromide ($C_{18}N$, Fluka, 4-dodecyldiethylenetriamine (C_{12}-dien, Kodak), ethylenediamine (Merck), Na_2EDTA (Merck), zinc sulphate (Merck), ammonium acetate (Merck), potassium monophosphate (Merck), phosphoric acid (Merck) and potassium chloride (Merck) were of analytical-reagent grade. For stock solutions, tetrabutylammonium hydroxide and ethylenediamine were dissolved in water (1 *M*) and the solution was adjusted to pH 6 with 80% phosphoric acid.

Chromatographic equipment

The isocratic liquid chromatograph consisted of a Model 110A pump (Beckman), a Model 210 injector (Altex) with a 20-μl loop and a Model 330 UV detector (Beckman) with a 8-μl cell and filters for detection at 254 and 280 nm. Chromatograms were recorded on an HP-3380 A calculator (Hewlett-Packard). Reversed-phase C_{18} columns were obtained from Beckman (Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D., V_0 = 1.4 ml, stainless steel) and anion-exchange columns from Chrompack (Ionospher-A, 10 μm, 250 × 4.6 mm I.D., V_0 = 3.2 ml or 100 × 4.6 mm I.D., V_0 = 1.25 ml, stainless steel; 5 μm, 100 × 3 mm I.D., V_0 = 0.7 ml, glass). The support for the 5-μm Ionospher-A cartridge was obtained from Chrompack (Chromsep, 135 mm). Dead volumes of each column were measured by weighing the column filled with two different solvents[31].

Composition of mobile phases

Ion-pair chromatography. The ion-pairing reagents (tetrabutylammonium phosphate, hexadecyltrimethylammonium bromide or octadecyltrimethylammonium bromide) were added at a concentration of 5 m*M* to the water–methanol (45:55) mobile phase buffered with potassium phosphate (10 m*M*, pH 6). Ethylenediamine phosphate or Na_2EDTA were added to this mobile phase at a concentration of 1 m*M*, if necessary. The flow-rate was fixed at 1 ml/min.

Anion-exchange chromatography. Acetonitrile (5–20%) was mixed with water buffered with potassium phosphate (10 m*M*, pH 6). Ethylenediamine phosphate (1 m*M*) or Na_2EDTA (0.3 m*M*) were added if necessary. The flow-rate was fixed at 2 ml/min for the 10-μm Ionispher-A packing and at 1 ml/min for the 5-μm Ionospher-A packing.

Ligand-exchange chromatography. 4-Dodecyldiethylenetriamine (5 m*M*) and zinc sulphate (5 m*M*) were added to the water–methanol (25:75) mobile phase buffered with ammonium acetate (30 m*M*, pH 6). The flow-rate was fixed at 1 ml/min.

Determination of pK values in water

p*K* values were obtained from measurements of the UV absorption of solutions of defined pH. Sodium salts of 3-acetyl 5-substituted pyrrolidine-2,4-diones have two UV absorption maxima, at 240 and 280 nm. Their acidic forms have a low UV absorption at 240 nm and maxima at 220 and 275 nm. Sodium salts of TA or its analogues were dissolved in 0.01 *M* potassium chloride solution at a concentration of

0.1 m*M*. UV absorptions were recorded at 240 nm from pH 6 to 2. These values (obtained from three independent experiments) were used to construct a graph with the equation $y = pH_i = a + bx$, with $x = \log[D_i - D_{min})/(D_{max} - D_i)]$, where D_i is the absorption at 240 nm of the solution at pH_i, D_{max} the absorption of the sodium salt (pH $\geqslant$ 7) at 240 nm and D_{min} the absorption of the acid at 240 nm. p*K* values were calculated from the linear regression curve for $x = 0$ ($y = pK$).

RESULTS

Tenuazonic acid has a strong metal complexation capacity[3,21,32] and maximum UV absorbance at 280 nm[1,2]. Isocratic reversed-phase chromatography of TA on a fully capped C_{18} column with different water–methanol mixtures at neutral pH led to broad peaks or a very low retention. The addition of a metal complexing reagent (EDTA) or the acidification of the mobile phase with phosphoric acid (pH 2) did not give better results. Therefore, we chromatographed TA on a C_{18} column by ion pairing or ligand exchange and with a silica-based anion-exchange column.

Ion-pair chromatography of tenuazonic acid

Scott and Kanhere[22] used the ion-pairing reagent tetrabutylammonium phosphate (TBA, 5 m*M*) for the chromatography of TA[22] with water–methanol (40:60) as the mobile phase and a 10-μm C_{18} column (μBondapack, Waters Assoc.). Adaptation of this method to a 5-μm C_{18} column (Ultrasphere-ODS, Beckman) did not give good results. TA was rapidly eluted in water–methanol (50:50), even with a high TBA concentration ($k' = 1.8$ with 10 m*M* TBA, Fig. 2). Further, the efficiency of this chromatographic system was low ($N \leqslant 300$). Another ion-pairing reagent, cetrimide ($C_{16}N$), has been widely used for the ion-pair chromatography of organic acids[33], and also for the bitter iso-α-acids from hops[34], which have an enolic structure comparable

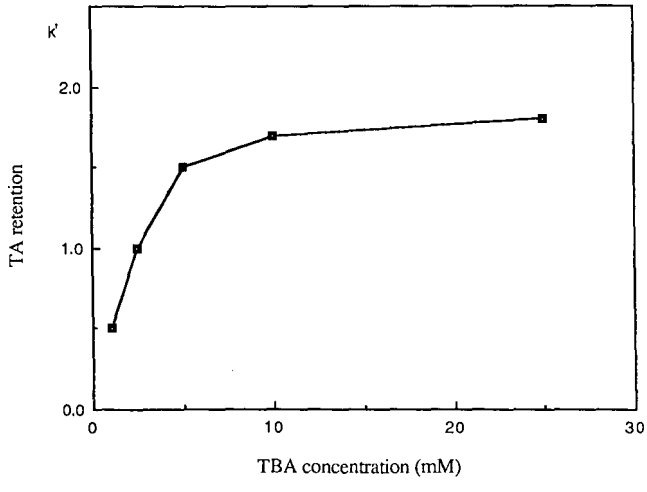

Fig. 2. Effect of the concentration of the ion-pairing reagent TBA on TA retention. TBA = tetrabutylammonium phosphate. Mobile phase, methanol–water (1:1) buffered with phosphate (10 m*M*, pH 6), column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D.; flow-rate, 1 ml/min.

TABLE I

EFFECT OF ION-PAIRING REAGENTS AND METAL COMPLEXANTS ON THE ION-PAIR CHROMATOGRAPHY OF TA

Mobile phase: 55% methanol in phosphate buffer (10 m*M*, pH 6) at 1 ml/min. Column: C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D., V_0 = 1.4 ml.

Compound	*TA retention (k')*	*Selectivity (α)*		*Efficiency (N)*
		TA–VT	*LT–TA*	
$C_{18}N$ (5 m*M*)	5.4	1.63	1.23	2000
$C_{16}N$ (5 m*M*)	4.5	1.60	1.18	2000
$C_{16}N$ (5 m*M*) + EDTA (1 m*M*)	3.3	1.70	1.20	1700
$C_{16}N$ (5 m*M*) + ethylenediamine (1 m*M*)	4.8	1.60	1.20	2500

to that of TA. Addition of 5 m*M* $C_{16}N$ to a water–methanol (45:55) mobile phase buffered with phosphate (10 m*M*, pH 6) allowed a sufficient retention of TA on a 5-μm C_{18} column, (k' = 4.5, N = 2000, Table I). When an ion-pairing reagent with a longer apolar chain (5 m*M*, $C_{18}N$) is added to the water–methanol (45:55) mobile phase buffered with phosphate, TA is slightly more retained (k' = 5.4, Table I). However,

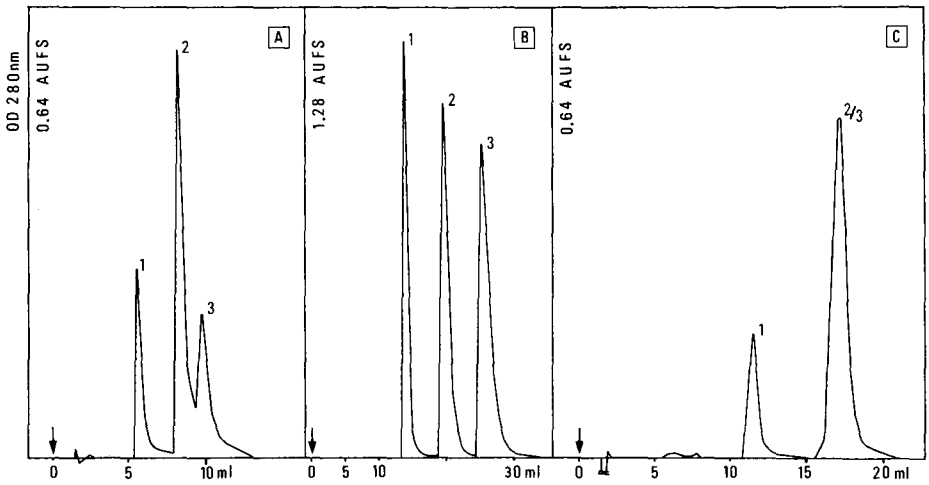

Fig. 3. Separation of 3-acetyl 5-substituted pyrrolidine-2,4-diones by ion-pair, anion-exchange and ligand-exchange HPLC. Injection of 20 μl of VT, TA and LT (0.1, 0.3 and 0.1 m*M* in A and C; 1 m*M* each in B). Peaks: 1 = VT (3-acetyl-5-isopropylpyrrolidine-2,4-dione); 2 = TA (3-acetyl-5-*sec.*-butylpyrrolidine-2,4-dione); 3 = LT (3-acetyl 5-isobutylpyrrolidine-2,4-dione). (A) Ion-pair chromatography. Mobile phase, cetrimide (5 m*M*), ethylenediamine (1 m*M*) and phosphate buffer (10 mM) (pH 6) with 55% methanol at 1 ml/min; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D., V_0 = 1.4 ml; detection at 280 nm, 0.64 a.u.f.s. (B) Anion-exchange chromatography. Mobile phase, phosphate buffer (10 m*M*) (pH 7) and KCl (0.1 *M*) with 2.5% acetonitrile at 2 ml/min; column, Ionospher-A, 10 μm, 250 × 4.6 mm I.D., V_0 = 3.2 ml; detection at 280 nm, 1.28 a.u.f.s. (C) Ligand-exchange chromatography. Mobile phase, ammonium acetate buffer (30 m*M*) (pH 6), C_{12}-dien (5 m*M*), $ZnSO_4$ (5 m*M*) with 70% methanol at 1 ml/min; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D.; detection at 280 nm, 0.64 a.u.f.s.

the low solubility of $C_{18}N$ in water–methanol mixtures makes it less suitable than $C_{16}N$. Changing the buffer from phosphate to acetate increased the retention time of TA but decreased the efficiency and gave broad peaks. The separation of TA from its analogues derived from valine (VT) and leucine (LT) is shown in Fig. 3a. With water–methanol (45:55) and 5 m*M* $C_{16}N$, TA is clearly separated from VT ($\alpha = 1.6$) but less satisfactorily from LT ($\alpha = 1.18$). The TA–LT separation was slightly improved with increase in the water content of the mobile phase (60% water, $\alpha = 1.3$).

The efficiency of this chromatographic system decreased with time, as a result of two major alterations to the column. First, ion-pairing reagents slowly dissolved the silica particles at the head of the column[35]. Repacking of the column with small amounts of the original solid phase restored the column efficiency[36]. This column deterioration can be prevented if a silica saturation column is placed before the injector[35]. Second, the accumulation of metal ions or complexes on the solid phase might interfere with the retention of TA. Such problems have been reported during the chromatography of iso-α-acids of hops[37], leukotrienes[38] and fusaric acid[39]. For the last two, the addition of EDTA to the mobile phase was necessary in order to achieve good peak resolution[38,39]. Therefore, we cleaned up the column with a mobile phase containing a metal-complexing reagent (10 m*M*, EDTA). This treatment restored its original efficiency. Further, the addition to the mobile phase of a metal-complexing agent (1 m*M* EDTA or ethylenediamine phosphate) prevented this column alteration. The addition of ethylenediamine phosphate to the mobile phase did not change the retention time of TA. In contrast, EDTA slightly decreased the retention time of TA (Table I). The reproducibility of the retention time of TA was satisfactory with ethylenediamine in the mobile phase ($k' = 4.8 \pm 0.2$, $n = 5$).

Anion-exchange chromatography of tenuazonic acid

Scott and Kanhere[22] used a 10-μm weak anion-exchange column and a mobile phase composed of acetonitrile (2.5%) buffered with phosphate (50 m*M*) and potassium chloride (0.1 *M*) for the chromatography of TA. We tested different anion-exchange columns (Chrompack, Ionosher-A, 5 and 10 μm) for their chromatographic behaviour towards TA. The retention of TA was dependent on the proportion of acetonitrile in the mobile phase (Table II, $k' = 4.6$ with 5% acetonitrile and 0.1 *M* potassium chloride), but also on the type and the concentration of the buffer. For example, a 30 m*M* sodium acetate buffer gave the same retention time as a 10 m*M* phosphate buffer, but an ammonium acetate buffer increased the retention time of TA (1.5-fold). The separation of TA from its 5-substituted analogues is complete with the 10-μm Ionospher-A packing and the mobile phase described by Scott and Kanhere[22] (Fig. 3b). An increase in the proportion of water in the mobile phase improved the selectivity of this separation (Table II). However, the selectivity of the separation with a 5-μm Iomospher-A packing was not improved.

The efficiency of the anion-exchange column decreased with time. This column deterioration was experimentally reproduced by the injection (ten times) of concentrated metal salt solutions which are used for the growth of the fungus. Washing the column with EDTA (10 m*M*) overnight restored its original efficiency. This column deterioration could be prevented by the addition of a metal-complexing reagent to the mobile phase (Table III). EDTA (1 m*M*) decreased the retention time of TA, particularly with the 5-μm Ionospher packing, for which a 0.3 m*M* concentration

TABLE II

EFFECT OF THE PERCENTAGE OF ACETONITRILE IN THE MOBILE PHASE ON THE SEPARATION OF TA AND ITS 5-SUBSTITUTED ANALOGUES BY ANION-EXCHANGE CHROMATOGRAPHY

Mobile phase: phosphate buffer (10 m*M*, pH 7)–KCl (0.1 *M*)–acetonitrile (2.5–10%) at 2 ml/min. Column: Ionospher-A, 10 μm, 250 × 4.6 mm I.D., V_0 = 3.2 ml.

Acetonitrile (%)	*TA retention (k')*	*Selectivity (α)*	
		VT–TA	*TA–LT*
2.5	5.2	1.55	1.35
5	4.6	1.50	1.30
10	3.6	1.45	1.25

should be used to allow a sufficient retention of TA. In contrast, ethylenediamine phosphate only slightly decreased the retention time of TA. Neither reagent modified the selectivity of the separation of TA from its 5-substituted analogues. Therefore, we used ethylenediamine for the ion-exchange chromatography of TA.

When crude culture filtrates samples were injected, the retention time of TA irreversibly decreased with time, even with the addition of a metal-complexing reagent to the mobile phase. Deproteinization of the culture filtrates by precipitation with acetonitrile overcame this problem. The reproducibility of the retention time of TA between experiments was then more satisfactory ($k' = 6.2 \pm 0.34$, $n = 4$) with a mobile phase consisting of 5% acetonitrile, 10 m*M* phosphate buffer (pH 6) and 1 m*M* ethylenediamine phosphate.

Ligand-exchange and metal complex chromatography

We adapted the ligand-exchange chromatographic system described by Scott and Kanhere[22] to short 5-μm C_{18} columns. We used a water–methanol (25:75) mobile

TABLE III

EFFECT OF ADDITION OF METAL-COMPLEXING REAGENTS ON THE ION-EXCHANGE CHROMATOGRAPHY OF TA

Mobile phase for Ionospher-A (5 μm) column: phosphate buffer (10 m*M*, pH 6)–acetonitrile(15%) at 1 ml/min. Mobile phase for Ionospher-A (10 μm) column: phosphate buffer (10 m*M*, pH 6)–acetonitrile (5%) at 2 ml/min. Columns: Ionospher-A (5 μm), 100 × 3 mm I.D., V_0 = 0.7 ml; Ionospher-A (10 μm), 250 × 10 min I.D., V_0 = 3.2 ml.

Eluent	*TA retention (k')*	
	Ionospher-A (5 μm)	*Ionospher-A (10 μm)*
Mobile phase	7	6.8
Mobile phase + 1 m*M* EDTA	2.7	5.1
Mobile phase + 1 m*M* ethylenediamine phosphate	5.6	6.2

phase with an ammonium acetate buffer (pH 6, 30 m*M*), zinc sulfate (5 m*M*) and C_{12}-dien (5 m*M*). This mobile phase gave a good retention of TA ($k' = 5.8$). The separation of the 5-substituted TA analogues was only partially achieved with this system as LT was coeluted with TA (Fig. 3c, Table V). Further, all the 5-substituted TA analogues with side-chains of the same size as TA (*n*-butyl) or longer (5-benzyl) were not separated from TA. Hence this chromatographic system is not selective for these TA analogues.

With this chromatographic system, the reproducibility of the retention time of TA was not satisfactory ($k' = 5.8 \pm 1.2$, $n = 5$). We studied the effect of different parameters that could be responsible for this low reproducibility of the retention time of TA. First, we compared the effect of temperature with a thermostated column. An increase in temperature from 22 to 28°C decreased the retention time of TA from 6 to 5.2 (with 75% methanol), but increased the column efficiency (from $N = 1500$ to 2000). These temperatures may occur as the lower and upper limits of room temperature. Therefore, the column must be thermostated in order to reduce the variability in retention time. Second, the addition of zinc in a slight excess (1.3 fold) increased the retention time of TA 1.05-fold but decreased the efficiency 0.75-fold. In contrast, an excess of C_{12}-dien (2-fold) decreased the retention time of TA (0.85-fold). Hence these slight modifications to the mobile phase could lead to differences in the retention time of TA from one batch to another.

Withdrawal of the C_{12}-dien from the mobile phase as described by Stack *et al.*[25] led to a poor retention of TA, even with 60% water ($k' = 1.8$), which is different from their results. This difference might be due to the C_{18} columns employed, as Stack *et al.* used a Waters C_{18} column (μBondapack), which might be more hydrophobic than the column we used (Ultrasphere-ODS). Further, this last chromatographic system did not show any selectivity for the TA analogues as they were all coeluted.

Separation of tenuazonic acid diastereoisomers

Synthetic or natural TA (L-*iso* diastereoisomer) can be contaminated by various amounts of its D-*allo* diastereoisomer[30]. To make a quantitative measurement of TA, we have to take into account this contaminating isomer. For this reason we compared different chromatographic systems (ion-pair, anion-exchange, ligand-exchange) for their ability to separate the four TA diastereoisomers. The reference compounds were obtained individually by synthesis from the corresponding amino acids[30]. Ion-pair chromatography did not allow their separation. L-*allo*-TA and D-*allo*-TA were slightly separated from L-*iso*-TA by anion-exchange and ligand-exchange chromatography ($\alpha = 1.06$–1.10, Table IV). This is in agreement with the report of Scott and Kanhere[22], who showed that ligand-exchange chromatography on a C_{18} column with zinc and C_{12}-dien was able to separate L-*iso*-TA from D-*allo*-TA. D-TA and L-TA were not separated with any of the chromatographic systems tested. Hence, ligand- and anion-exchange chromatography can be used to determine the amount of D-*allo*-TA in synthetic or natural samples of L-TA.

Separation of 3-acetyl 5-substituted pyrrolidine-2,4-diones

Synthetic analogues of TA with apolar substituents of increasing size at position 5 were submitted to ion-pair, ligand-exchange and anion-exchange chromatography. The analogues with the longer substituents were the more retained in all chromato-

TABLE IV

SEPARATION OF TA DIASTEREOISOMERS BY LIGAND-EXCHANGE AND ANION-EXCHANGE CHROMATOGRAPHY

Values given are retentions (k') with selectivity factors (k'_1/k'_2) in parentheses. Ligand-exchange chromatography: mobile phase, 70% methanol in ammonium acetate buffer (30 m*M*, pH 6), C_{12}-dien (5 m*M*) and zinc sulphate (5 m*M*), at 1 ml/min; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D., V_0 = 1.4 ml. Anion-exchange chromatography: mobile phase, 5% acetonitrile in phosphate buffer (10 m*M*, pH 7) and KCl (0.1 *M*) at 2 ml/min; column, Ionospher-A, 10 μm, 250 × 4.6 mm I.D., V_0 = 3.2 ml.

Mode	L-*TA*	L-*allo-TA*	D-*TA*	D-*allo-TA*
Ligand-exchange	11[a]	10.3[a] (1.07)	11 (1.00)	10.3 (1.07)
Anion-exchange	4.6	4.1 (1.10)	4.6 (1.00)	4.1 (1.10)

[a] Retention of the compound alone.

graphic systems (Table V). However, the separation of TA from its isomers (*iso*- and *n*-butyl) or from the analogue with a longer substituent at position 5 (benzyl) was achieved only with ion-pair and anion-exchange chromatography. The order of elution of these compounds cannot be related to their p*K* values as they are all similar (Table V). Hence the hydrophobic side-chains of these compounds might participate in their retention on the column.

Determination of tenuazonic acid produced by Pyricularia oryzae

Pyricularia oryzae is able to produce TA when grown on synthetic media[9,10] or in infected rice leaves[11] at concentrations between 0.01 and 1 m*M*. Its quantification requires a rapid and sensitive chromatographic assay with no interferences from the culture media or plants from which it is isolated. Quantification of TA was performed with external TA standard solutions with concentrations ranging from 10 μ*M* to 1 m*M* (20-μl loop) with detection at 280 nm. For all the chromatographic systems tested, a linear relationship between the logarithm of the amount of TA injected and the

TABLE V

SEPARATION OF 3-ACETYL 5-SUBSTITUTED PYRROLIDINE-2,4-DIONES BY ION-PAIR, ANION-EXCHANGE AND LIGAND-EXCHANGE CHROMATOGRAPHY

Values given are retentions (k'). Ion-pair chromatography: mobile phase, 55% methanol in phosphate buffer (10 m*M*, pH 6) and cetrimide (5 m*M*) at 1 ml/min; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D., V_0 = 1.4 ml. Anion-exchange chromatography: mobile phase, 5% acetonitrile in phosphate buffer (10 m*M*, pH 7) and KCl (0.1 *M*) at 2 ml/min; column, Ionospher-A, 10 μm, 250 × 4.6 mm I.D., V_0 = 3.2 ml. Ligand-exchange chromatography: mobile phase, 75% methanol in ammonium acetate buffer (10 m*M*, pH 6) and $ZnSO_4$–C_{12}-dien (5 m*M*) at 1 ml/min; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D., V_0 = 1.4 ml.

Mode	*5-Methyl*	*5-Ethyl*	*5-Isopropyl (VT)*	*5-sec.-Butyl (TA)*	*5-Isobutyl (LT)*	*5-n-Butyl*	*5-Benzyl*
Ion-pair	1.65	2	2.85	4.5	5.6	5.8	7.1
Anion-exchange	2.4	2.75	3.05	4.6	6	6.5	11.5
Ligand-exchange	2.8	3.4	4.35	5.8	5.8	5.8	5.8
p*K*	3.00	3.00	3.05	3.10	3.05	3.00	2.95

logarithm of the peak area was obtained over this range of concentrations. This linear relationship was extended to 3 μM TA with the ion-pair and ligand-exchange chromatographic systems. We were not able to detect TA in amounts lower than 3 μM (12 ng) by anion-exchange chromatography, as it has a low efficiency at low TA concentrations and a variable baseline at low attenuation. In contrast, we were able to detect as little as 1 μM (4 ng) TA by ion-pair and ligand-exchange chromatography (Table VI).

For the analysis of culture filtrates, different preparations of the samples to be analysed were performed, depending on the chromatographic system used. For ion-pair or ligand-exchanged chromatography, removal of the apolar compounds from the culture filtrates was necessary in order to avoid contamination of the C_{18} packing of the column. For this purpose, the culture filtrates were acifified to pH 2 and extracted with ethyl acetate. The organic phase was then extracted with sodium hydrogencarbonate. With this procedure, TA was recovered in 75% yield ($\pm$6%, with 0.2 mM TA, $n = 3$). Anion-exchange chromatography required the removal of the proteins from the culture filtrates to avoid deterioration of the column. A rapid protein precipitation was performed with the addition of acetonitrile (50% final concentra-

TABLE VI

COMPARISON OF ION-PAIR, LIGAND-EXCHANGE AND ANION-EXCHANGE CHROMATOGRAPHY OF TA AND 3-ACETYL 5-SUBSTITUTED PYRROLIDINE-2,4-DIONES

Ion-pair chromatography: mobile phase, 55% methanol in phosphate buffer (10 mM, pH 6), ethylenediamine (1 mM), cetrimide (5 mM) at 1 ml/min; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D. Ligand-exchange chromatography: mobile phase, 75% methanol in ammonium acetate buffer (30 mM, pH 6), C_{12}-dien (5 mM) and zinc sulphate (5 mM) at 1 ml/min and 22°C; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D., V_0 = 1.4 ml. Anion-exchange chromatography: mobile phase, 5% acetonitrile in phosphate buffer (10 mM, pH 6), ethylenediamine (1 mM) at 2 ml/min; column, Ionospher-A, 10 μm, 250 × 4.6 mm I.D., V_0 = 3.2 ml.

Parameter	*Ion-pair chromatography*	*Ligand-exchange chromatography*	*Anion-exchange chromatography*
Retention [k'(TA)]	4.8	5.8	6.2
Efficiency (N)	2500	2000	1000
Selectivity (VT–TA)	1.60	1.30	1.45
Selectivity (TA–LT)	1.2	1	1.25
Selectivity (L-*allo*-TA–LTA)	1	1.05	1.10
Advantages	Reproducibility sensitivity (4 ng[a]), selectivity (TA–LT–VT), no interference (leaf)	Sensitivity (4 ng[a]), strong retention of TA, no interference (leaf)	Reproducibility, selectivity (TA–LT–VT), rapid sample preparation
Disadvantages	Prepurification of sample	Prepurification of sample, low selectivity (TA–LT), low reproductibility	Interference (leaf), low sensitivity (12 ng[a])

[a] Minimum amount detected, injection of 20 μl and detection at 280 nm.

tion) followed by the removal of acetonitrile by extraction with methylene chloride[29]. With this treatment, TA was recovered in 90% yield from culture filtrates ($\pm 4\%$, with 0.03–0.3 m*M* TA, $n = 4$). As shown in Fig. 4, TA was the major UV-absorbing metabolite of *Pyricularia oryzae* culture filtrate. The extraction and the purification by sodium hydrogencarbonate partition of the culture filtrate removed most of the UV-absorbing metabolites, but not all. We detected trace amounts of a metabolite that could correspond to the valine analogue of TA (arrow 1 in Fig. 4). The production of TA varied from 10 μM to 1 m*M*, depending on the strain of *Pyricularia oryzae*, but most of the strains produced between 0.3 and 0.6 m*M* TA.

The analysis of the infected plants required purification of the ethanolic leaf extract. After evaporation of the ethanol, the residue was partitioned between acidified water and ethyl acetate and the organic phase was extracted with sodium hydrogencarbonate. Extracts from non-infected plants were analysed with the three chromatographic systems. We found compounds that were eluted almost at the same retention time as TA in anion-exchange chromatography (Fig. 5, B_1 and B_2). The presence of these compounds did not prevent the detection of TA in the infected leaves, but

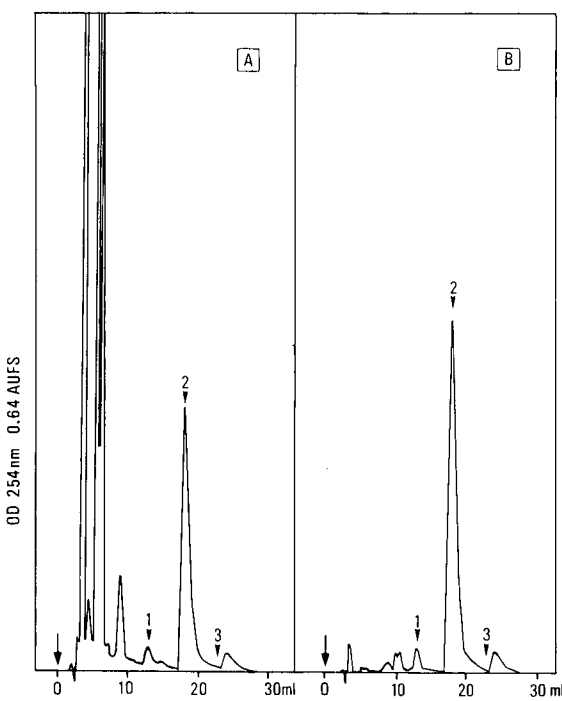

Fig. 4. Anion-exchange chromatography of *Pyricularia oryzae* culture filtrates. (A) Injection of the deproteinated culture filtrate containing 1 m*M* TA. (B) Injection of the culture filtrate extract purified by partition with sodium hydrogencarbonate (× 1.5 compared with A). Retention times of VT (1), TA (2) and LT (3) are indicated by arrows and were determined by co-injection of a standard solution with the sample. Culture filtrates were obtained from the *Pyricularia oryzae* strain MAD4 (Madagascar) after 30 days of growth on Fries medium. Mobile phase, phosphate buffer (10 m*M*) (pH 7), KCl (0.1 *M*) and 5% acetonitrile at 2 ml/min; column, Ionospher-A, 10 μm, 250 × 4.6 mm I.D.. Detection at 254 nm to detect most of the UV-absorbing metabolites, 0.64 a.u.f.s., 20 μl injected.

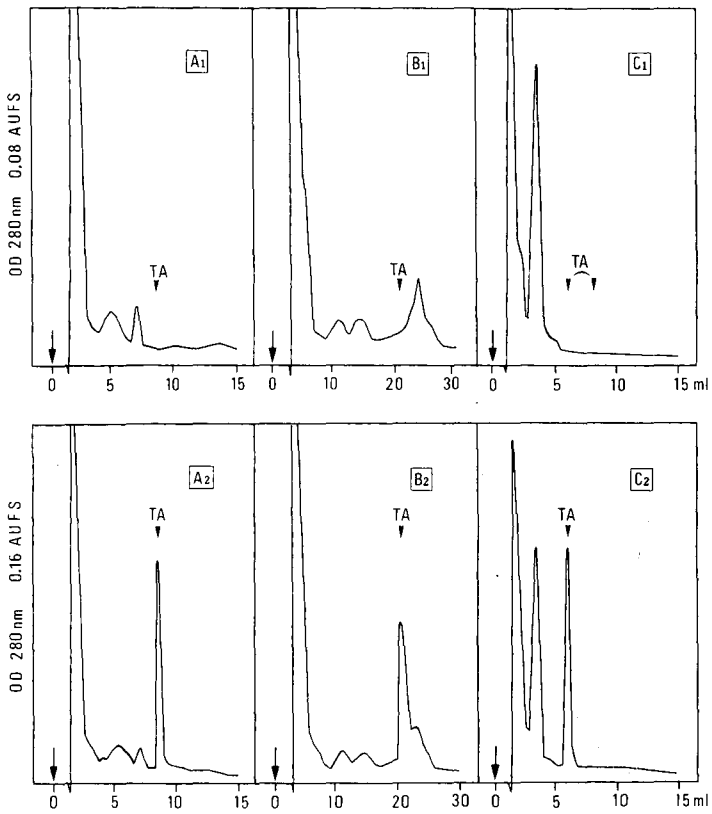

Fig. 5. Anion-exchange, ion-pair and ligand-exchange HPLC of rice leaf extracts. The TA retention time, determined by co-injection of TA and the sample, is indicated by an arrow. Samples injected (20 μl): (1) extract of non-infected rice leaves, dilution 0.25; (2) extract of leaves infected by *Pyricularia oryzae* containing 0.16 m*M* TA (7 days after inoculation), dilution 0.25. (A) Ion-pair chromatography. Mobile phase, cetrimide (5 m*M*) and phosphate buffer (10 m*M*) (pH 6), ethylenediamine (1 m*M*) with 55% methanol at 1 ml/min; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D.; detection 280 nm. (B) Anion-exchange chromatography. Mobile phase, phosphate buffer (10 m*M*) (pH 6), ethylenediamine (1 m*M*) with 5% acetonitrile at 2 ml/min; column, Ionospher-A, 10 μm, 250 × 4.6 mm I.D.; detection at 280 nm. (C) Ligand-exchange chromatography. Mobile phase, C_{12}-dien (5 m*M*) and zinc sulphate (5 m*M*) with ammonium acetate buffer (30 m*M*) (pH 6) with 80% methanol at 1 ml/min; column, C_{18} Ultrasphere-ODS, 5 μm, 150 × 4.6 mm I.D.; detection at 280 nm.

interfered with its quantification. Further, we were not able to detect small amounts of TA (10 μM; 40 ng). With ion-pair and ligand-exchange chromatography, TA was eluted after the plant components (Fig. 5, A_1, A_2, C_1 and C_2). These two last chromatographic systems were used for the determination of TA in infected leaves. The TA concentration that we detected in these leaves was around 0.1 m*M* 7 days after the beginning of the infection. We did not detect any TA analogues in the infected leaf extracts.

DISCUSSION

The three isocratic liquid chromatographic systems studied were applied to the determination of TA in fungal culture filtrates and infected leaves (Table VI). Anion-exchange chromatography was the best suited for the rapid monitoring of TA production by *Pyricularia oryzae* strains. The main advantages of this system are its rapidity and its selectivity, as it allowed the use of culture filtrates without extraction and a good separation of 5-substituted TA analogues. However, we were not able to detect trace amounts of TA ($< 3\ \mu M$) because of its lower efficiency ($N = 1000$). In contrast, ion-pair or ligand-exchange chromatography could be used to detect TA in this range (1–10 μM). Ion-pair and ligand-exchange chromatography were best suited for the determination of TA in infected rice leaves. We were not able to use anion-exchange chromatography as TA was not separated from compounds present in the non-infected leaves. TA was more retained with ligand-exchange than ion-pair chromatography as it was necessary to use a higher proportion of methanol to obtain the same retention time (80% compared with 55% for $k' = 5$). This is related to the strong metal-complexation capacity of TA. Therefore, TA was more clearly eluted after plant components than with ion-pair chromatography. Hence ligand-exchange chromatography was used to determine TA in leaf extracts, despite the low reproducibility of the retention time of TA and the low selectivity of the separation of the 5-substituted analogues of TA. Because of this last characteristic, some samples of infected leaf extracts were also analysed by ion-pair chromatography to detect whether or not some TA analogues were produced. No TA analogues were detected in any of the leaf samples analysed. This result allowed us to use ligand-exchange chromatography for the determination of TA in leaf extracts.

CONCLUSIONS

We have studied the behaviour of tenuazonic acid in three different HPLC systems. Ion-pair chromatography was performed with the best results with the ion-pairing reagent cetrimide and a phosphate buffer with water–methanol mixtures. With this chromatographic system, we were able to separate TA analogues substituted at position 5 with apolar substituents of different size. Anion-exchange chromatography with silica-based packings allowed the separation of all the 5-substituted TA analogues and also some of its diastereoisomers (D-*allo*-TA and L-*allo*-TA). The selectivity factors of these separations were higher than with ion-pair chromatography. With time, the efficiency of ion-pair and anion-exchange chromatography might decrease. Washing with EDTA solution restored the initial performances of the columns. Addition of a metal-complexing reagent (ethylenediamine) to the mobile phase prevented this column deterioration. These chromatographic systems were used to detect and determine TA in *Pyricularia oryzae* culture filtrates and infected rice leaves. We chose anion-exchange chromatography for the analysis of the culture filtrates for its rapidity, simplicity and selectivity. Extracts of infected leaves could only be analysed by ion-pair and ligand-exchange chromatography as interfering compounds were coeluted with TA during anion-exchange chromatography.

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SIZE FRACTIONATION OF OLIGOSACCHARIDES BY LIQUID CHROMATOGRAPHY ON A CATION-EXCHANGE COLUMN

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SUMMARY

Oligosaccharides, labelled with 2-aminopyridine at their reducing ends, were satisfactorily fractionated according to the sugar sizes on Shodex RSpak DC-613, a cation-exchange resin column (Na^+ form), with water–acetonitrile as the eluent in the presence of sodium acetate or triethylammonium acetate buffer. For the fractionation of sugar samples, dextran hydrolyzates, chitin oligomers and oligosaccharide moieties of ovomucoid were used. The oligosaccharides were strongly adsorbed to the resin column with solvents containing less water and at lower temperature, and were eluted in order of increasing molecular size above the critical concentration of acetonitrile. Baseline separation of a dextran hydrolyzate up to oligomers having 20 glucose units was observed by gradient elution. The separation efficiency and elution pattern were investigated by changing the buffer concentration, mobile phase pH and temperature.

INTRODUCTION

For the structural elucidation of sugar chains of glycoproteins it may be essential to obtain the component oligosaccharides from the macromolecules, and inevitable to purify each oligosaccharide. Size fractionation of oligosaccharides may be useful for this purpose in the first step. Recently, this has been performed by a preparative gel filtration on a Bio-Gel P-4 column[1]. On the other hand, high-performance liquid chromatography (HPLC) is convenient, quick, accurate and quantitative for size fractionation of oligosaccharides. The following three separation modes have been used: (1) gel permeation using an ion-exchange resin[2–4], which shows poor separation for high-molecular-weight oligosaccharides compared to the following two modes; (2) reversed-phase partition[5–8], relating to hydrophobicity, not strictly to sugar size, and (3) normal-phase partition (amine adsorption)[9] using amine-bonded silica gel. This column basically with the solvent system of acetonitrile and water shows satisfactory separation for neutral oligosaccharides[10–15]. Especially, gradient elution using increasing water contents with the addition of buffer has enabled a good separation even of anionic oligosaccharides[16–18], the mechanism being called "ion suppression

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amine adsorption". However, this silica-based stationary phase is subject to deterioration and fouling, and not durable upon repeated analysis.

As a detection method for sugars in HPLC, refractivity has been widely monitored, but this shows low sensitivity and is susceptible to changes in solvent composition. Tritium labelling at the reducing end of the sugar chain shows high sensitivity[19], but one cannot utilize this specific facility at any time. Sensitive and selective post-column labelling methods have been devised[20], but it is difficult to recover the fractionated samples. Recently, Hase *et al.*[21] devised a highly sensitive detection method by pyridylamination at the reducing end of sugars. This method has been used for the structural analysis of oligosaccharides[22–24], and Hase *et al.*[25] reported that size fractionation of pyridylaminated (PA-) sugars was achieved by the ion suppression amine adsorption mode.

Samuelson and co-workers[26–29] have systematically investigated size fractionation of oligosaccharides or oligomeric sugar alcohols by open-column partition chromatography on ion-exchange resins in aqueous ethanol. They have reported that the sugars were eluted in order of increasing molecular size, and the distribution coefficients were influenced by the type of glycosidic linkage, and decreased with non-polar groups in the sugar molecule.

In this study, we investigate the usefulness of a cation-exchange polymer column according to the normal-phase partition mode for the purpose of size fractionation of PA derivatives of oligosaccharides prepared from dextran, chitin and ovomucoid.

EXPERIMENTAL

Chemicals

2-Aminopyridine was obtained from Tokyo Chemical Industry (Tokyo, Japan), and recrystallized from 1-hexane. Triethylamine was from Wako Pure Chemical (Osaka, Japan), and distilled once by flash evaporation. Anhydrous hydrazine and sodium cyanoborohydride were obtained from Aldrich (Milwaukee, WI, U.S.A.) and used without any pretreatment. Ovomucoid (trypsin inhibitor, Type III-0) was obtained from Sigma Chemical (St. Louis, MO, U.S.A.). Water was deionized and distilled before use. All other chemicals were of analytical reagent grade.

Preparation of oligosaccharides

Glucose (Glc) oligomers were prepared from dextran by partial acid hydrolysis[1] (0.1 *M* HCl, 4 h, 100°C), and excess of acid was removed on a Dowex 1-X8 (OH^- form) resin column. The hydrolyzate was applied on a Sephadex G-15 column (61 cm × 1 cm), eluted with distilled water at a flow-rate of 8–10 ml/h at room temperature, and high-molecular-weight Glc oligomers were pooled in the void volume fraction. Chitobiose, chitotriose, chitotetraose and chitopentaose were provided by Dr. E. F. Walborg (The University of Texas System Cancer Center, Science Park Research Division, Smithville, TX, U.S.A.). Oligosaccharides from ovomucoid were prepared by hydrazinolysis and sequential N-reacetylation for free amino groups according to the method of Takasaki *et al.*[30].

Pyridylamination of sugars

Aldehyde groups of the reducing ends of oligosaccharides were coupled with

2-aminopyridine by reductive amination with sodium cyanoborohydride according to the method of Hase *et al.*[21]. The following four samples were derivatized: (1) Glc oligomers from an hydrolyzate of 9 mg dextran; (2) high-molecular-weight Glc oligomers from an hydrolyzate of 64 mg dextran; (3) N-acetylglucosamine (GlcNAc) oligomers (mixture of 140, 99, 104, 102 and 118 nmol of GlcNAc, chitobiose, chitotriose, chitotetraose and chitopentaose); (4) oligosaccharide fraction of an hydrazinolyzate of 3.1 mg ovomucoid. Each sample was dissolved in 0.5 ml of a 2-aminopyridine solution (prepared by dissolving 1 g of 2-aminopyridine in 0.76 ml of concentrated hydrochloric acid) in a hydrolyzing tube (12 cm × 1.6 cm) with a PTFE screw cap, and warmed at 100°C for 13 min. After cooling, 25 μl of the reducing reagent (prepared by mixing 10 mg sodium cyanoborohydride, 20 μl of the 2-aminopyridine solution and 30 μl of water) were added to the tube, and warmed at 90°C overnight. The PA-sugars were separated from the reaction mixture by gel filtration on Sephadex G-15 column (61 cm × 1 cm) eluted with 10 m*M* ammonium acetate (pH 6.0) at a flow-rate of 8–10 ml/h at room temperature, and 2.5-ml fractions being collected. Fractions 6–16 were pooled, concentrated by evaporation and applied on a Dowex 50W-X2 (H^+ form) resin column (4.7 cm × 2 cm). After washing with 160 ml of water, PA-sugars were eluted with 100 ml of 3% aqueous ammonium solution, and concentrated by evaporation. Excess of 2-aminopyridine was removed by evaporation with repeated additions of triethylamine. The residue was dissolved in 250 μl of water, and used as a sample solution.

High-performance liquid chromatography

The following HPLC 800-series instruments (Japan Spectroscopic, Tokyo, Japan) were used for a high-pressure mixing gradient elution. From the two reservoirs containing different ratios of acetonitrile and water with an appropriate buffer, the solvents were delivered by two 880-PU pumps and passed through a 880-50 degasser. The two solvents were mixed in the programmed ratio at the solvent mixing module, and sent to the column at a flow-rate of 1 ml/min. The column temperature was regulated by a 860-CO column oven. After the sample injection by a Reodyne (Cotati, CA, U.S.A.) 7125 sample injector, a linear gradient elution was performed. The elution solvent and the sample solution were passed through a 0.45-μm membrane filter before use. A RSpak DC-613 column (15 cm × 0.6 cm), a highly cross-linked, sulphonated polystyrene resin in the sodium form (Showa Denko, Tokyo, Japan), was used. The samples were detected by a 820-FP spectrofluorometer, the excitation and emission wavelengths being 320 and 400 nm, respectively. The amount of PA-sugars was calculated from the peak area on the elution chromatogram by an 805-GI graphic integrator.

RESULTS AND DISCUSSION

Elution conditions for pyridylaminated sugars

PA derivatives of oligosaccharides were not eluted from the RSpak DC-613 column in acetonitrile–water solvents (from 9:1 to 4:6, v/v), indicating the strong adsorption of the PA-sugars to the resin. It is likely that PA-sugars protonated in this solvent displaced sodium ion from the cation exchanger. In an attempt to elute the PA-sugars, sodium acetate and triethylammonium acetate buffers were used. The

following two sections indicate elution conditions with respect to the column temperature (23, 40, 60°C), buffer concentration (25, 175 m*M*) and mobile phase pH (7.5, 8.5, 9.5).

Elution in the presence of sodium acetate

Sodium ion, the counter ion of this resin, was added to the elution solvent, at 25 m*M*, the concentration at its maximum solubility. The pH of the mobile phase was an important factor in the elution of PA-sugars. Below pH 6.7, PA-sugars were eluted considerably later or not eluted in acetonitrile–water (from 8:2 to 4:6, v/v). Therefore, the pH was adjusted to above 7.5 in order to suppress the protonation of the aminopyridyl group possessing pK_a 6.7. The samples were adsorbed to the column more strongly with solvents of lower water contents. The temperature had a considerable influence on the separation of PA-sugars, *i.e.*, at 23°C satisfactory elution was not obtained for the PA-Glc oligomers. Raising the column temperature led to a decrease in their retention times, and gave a higher separation efficiency. The oligosaccharides were eluted in order of increasing molecular size. When the water content was between 56 and 60% (v/v), PA-Glc oligomers were eluted as a single peak. This acetonitrile concentration is denoted the "critical concentration" as described by Havlicek and Samuelson[27–29]. When the water content was above 60% (v/v), the elution order was reversed, being in the order of decreasing molecular size.

Fig. 1 shows the elution pattern of PA-oligosaccharides where a linear gradient

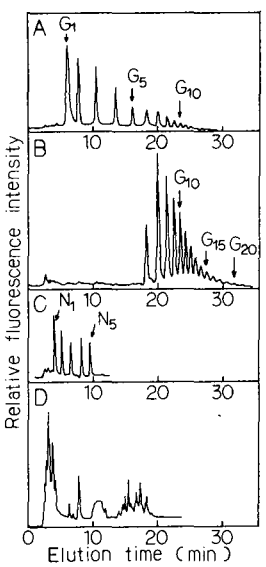

Fig. 1. Separation of pyridylaminated derivatives of oligosaccharides on an RSpak DC-613 column in the presence of sodium acetate. (A) PA derivatives of dextran hydrolyzate (0.1 μl); (B) PA derivatives of the high-molecular-weight fraction of a dextran hydrolyzate (PA-HMWF, 0.15 μl); (C) PA-GlcNAc oligomers (0.2 μl); (D) PA derivatives of ovomucoid oligosaccharides (PA-OM, 1 μl). The linear gradient elution from 24 to 54% water was performed in 20 min in the acetonitrile mobile phase containing 25 m*M* sodium hydroxide, adjusted to pH 7.5 with acetic acid. The column temperature was 60°C. G_1 is PA-Glc, G_5, G_{10}, G_{15} and G_{20} are PA-Glc oligomers of DP 5, 10, 15 and 20, respectively, and N_1 and N_5 are PA-GlcNAc and PA-chitopentaose.

elution was performed from 24 to 54% water in 20 min at 60°C, the pH of the mobile phase being 7.5. The gradient system was chosen so that all species might be well resolved and in an appropriate time. The boundary of the acetonitrile content was from the concentration at which PA-Glc and PA-isomaltose were well separated to the critical concentration. Baseline resolution of PA derivatives of dextran hydrolyzates containing 1–13 Glc oligomers was obtained (Fig. 1A). As the degree of polymerization (DP) of Glc increased, their peak areas decreased exponentially. Separation and detection of Glc oligomers up to DP 20 was observed from the high-molecular-weight fraction of dextran hydrolyzate (Fig. 1B). Moreover, the baseline resolution of GlcNAc oligomers, DP 1–5 was obtained (Fig. 1C). PA-GlcNAc oligomers possessed retention times about two-thirds those of PA-Glc oligomers of like DPs. Raising the pH of the mobile phase in the same elution system led to a decrease in the maximum detectable DP of PA-Glc oligomers. However, it did not affect the resolution of low-molecular-weight PA-Glc oligomers (Table I).

Ovomucoid is known to have highly heterogeneous oligosaccharides, its sugar size being 5–14 monosaccharide units[31–33]. In the gel filtration (Fig. 2), PA derivatives of oligosaccharide fractions of ovomucoids were separated into two main peaks. One was eluted at the void volume and the other at the retention time between PA-di- and trisaccharides. In HPLC (Fig. 1D), the elution profile of PA-ovomucoid oligosaccharides was constituted of several peaks, and their retention times were all shorter than that of PA-isomaltohexaose.

Elution in the presence of triethylammonium acetate

In the presence of buffered triethylamine (TEA), the elution pattern of PA-sugars was much influenced by its concentration. The results are as follows at lower (25 m*M*) and higher (175 m*M*) TEA concentrations.

TABLE I

COMPARISON OF RESOLUTION WITHIN THE DIFFERENT FOUR ELUTION CONDITIONS

Column: Shodex RSpak DC-613 (15 mm × 6 mm I.D.); elution solvent, water in acetonitrile-containing buffer; flow-rate, 1.0 ml/min.

Buffer	*pH*	*Temperature* (°C)	*Gradient* (water)	*Resolution*[a]		*Maximum DP*[b]	$\frac{t_R(N_5)^c}{t_R(G_5)}$
				$G_3 \leftrightarrow G_4$	$G_5 \leftrightarrow G_6$		
25 m*M* Na^+	7.5	60	24 → 54%/20 min	6.0	3.7	20	0.61
25 m*M* Na^+	8.5	60	24 → 54%/20 min	5.9	3.7	16	0.69
25 m*M* Na^+	10.0	60	24 → 54%/20 min	5.9	4.0	12	0.69
25 m*M* TEA	7.5	60	10 → 34%/24 min	3.3	1.5	11	0.73
175 m*M* TEA	7.5	60	10 → 24%/24 min	2.8	1.8	12	0.81
175 m*M* TEA	7.5	23	10 → 24%/24 min	2.6	2.2	16	0.89
175 m*M* TEA	8.5	60	10 → 26%/24 min	3.4	2.1	11	0.79
175 m*M* TEA	8.5	23	10 → 26%/24 min	3.7	2.5	15	0.98
175 m*M* TEA	10.0	60	14 → 32%/24 min	3.9	2.2	10	0.85
175 m*M* TEA	10.0	23	14 → 32%/24 min	4.5	2.3	14	0.94

[a] Calculated according to ref. 39. G_n represents the PA-Glc oligomer of DP = n.

[b] Maximum detectable DP of PA-Glc oligomer.

[c] Ratio of retention time for PA-chitopentaose to that for PA-isomaltopentaose.

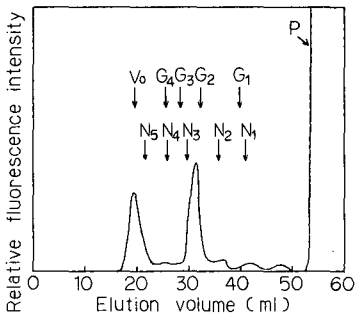

Fig. 2. Gel filtration of pyridylaminated oligosaccharides from ovomucoid. The pyridylaminated sample was applied to a Sephadex G-15 column (61 cm × 1 cm) equilibrated with 10 m*M* ammonium acetate, pH 6.0, and the column was eluted with the same buffer. The eluate was passed through the spectrofluorometer (820-FP), and PA-sugars were detected, the excitation and emission wavelengths being 320 and 400 nm, respectively. Arrows indicate the elution position of PA-sugars. G_1 is PA-Glc, G_2–G_4 are PA-Glc oligomers of DP 2–4, N_1 is PA-GlcNAc and N_2–N_5 are PA-GlcNAc oligomers of DP 2–5. V_0 = Void volume; P = 2-aminopyridine.

25 mM TEA concentration (pH 7.5). Raising the temperature led to a decrease of the retention time, and significant increase in the column efficiency. However, even with the lowest water content (10%), the baseline separation of PA-Glc and PA-isomaltose was not observed. PA-oligosaccharides were eluted in order of increasing molecular size above the critical concentration of acetonitrile (64–70%). Below it, the elution order was reversed. The gradient elution pattern is shown in Fig. 3. The detectable limit of PA-Glc oligomers was obtained only up to DP 12. PA-GlcNAc oligomers possessed retention times about two-thirds those of PA-Glc oligomers of like DPs. The elution profile of PA-ovomucoid oligosaccharides was constituted of several peaks, and their retention times were all shorter than that of PA-isomaltononaose.

175 mM TEA concentration. Raising the temperature led to a decrease in the retention time. In the isocratic elution at a water content of 10%, the number of theoretical plates was slightly increased. However, the resolution of PA-isomaltotriose and PA-isomaltotetraose was almost the same at 23, 40 and 60°C. PA-oligosaccharides were eluted in order of increasing molecular size above the critical concentration of acetonitrile (71–75%). In the gradient elution (water content from 10 to 24% in 24 min, pH 7.5), raising the temperature led to a decrease in both the resolution of PA-isomaltopentaose and PA-isomaltohexaose (2.2 at 23, 1.9 at 40 and 1.8 at 60°C) and the maximum detectable DP of PA-Glc oligomers (DP 12 at 23, 10 at 40 and 8 at 60°C). Fig. 4 shows the gradient elution pattern at 23°C. PA-Glc oligomers were detected up to DP 16. PA-GlcNAc oligomers possessed retention times about four-fifths those of PA-Glc oligomers of like DPs. The elution profile of PA-ovomucoid oligosaccharides was constituted of several peaks, and their retention times were all shorter than that of the PA-Glc oligomer of DP 13. Raising the pH of the mobile phase led to an alteration of the adequate gradient boundary and an increase in resolution, both at 23 and 60°C. However, it led to decrease in the maximum detectable DP of PA-Glc oligomers.

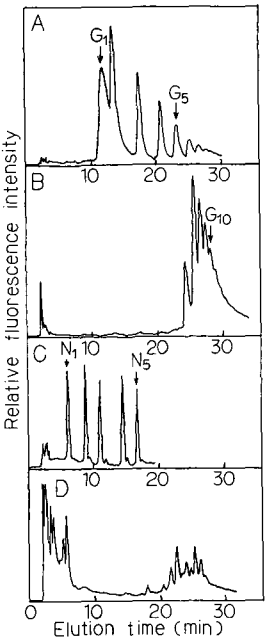

Fig. 3. Separation of pyridylaminated oligosaccharides on an RSpak DC-613 column in the presence of triethylammonium acetate. (A) PA-Glc oligomers (0.4 μl); (B) PA-HMWF (0.4 μl); (C) PA-GlcNAc oligomers (0.8 μl); (D) PA-ovomucoid oligosaccharides (4 μl). Conditions as in Fig. 1 except for the gradient (10 → 34%/24 min) and the mobile phase anion (25 mM).

A wide range of linearity was observed between the sample content and the peak area on the chromatogram, from the detection limit (about 2 pmol; signal-to-noise ratio S/N = 2) to about 25 nmol for PA-Glc (upper limit of fluorescence linearity of the detector used), and about 1 nmol for each PA-GlcNAc oligomer. At greater than 1 nmol, the PA-GlcNAc oligomer did not show linearity, suggesting its adsorption on this column at higher concentrations.

Sodium ion binding to the sulphonyl group of the resin was gradually replaced by protonated TEA with a decrease in retention time. After equilibration with the elution solvent (200 ml) in the column, the retention time for PA-Glc oligomers did not change significantly during the analysis in a day. The percentage deviation (n = 5) was calculated as 0.66 and 1.5% for the retention times of PA-Glc and PA-isomaltoheptaose in the elution system shown in Fig. 4. After elution of 500 ml solvent, the retention time was decreased by 6.6% for PA-Glc and 7.9% for PA-isomaltoheptaose, respectively, as often appears to be the case on amine-bonded phases[34]. However, this column was renewed by washing with diluted aqueous sodium hydroxide followed by water, and the column capacity was restored reversibly. Therefore, this column is superior to an amine-bonded silica column in stability and long-term durability.

Comparison of the elution systems

A comparison of the elution systems in size fractionation of PA-sugars with respect to the column efficiencies (resolution and maximum detectable DP) and

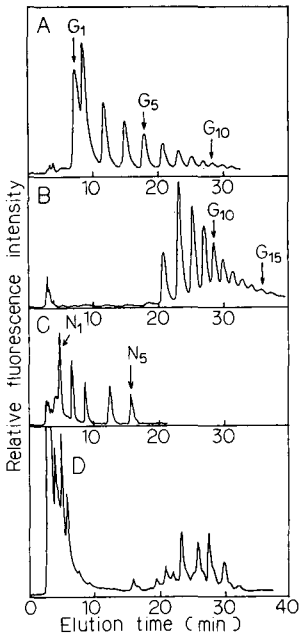

Fig. 4. Separation of pyridylaminated oligosaccharides on an RSpak DC-613 column in the presence of triethylammonium acetate. (A) PA-Glc oligomers (0.6 μl); (B) PA-HMWF (0.4 μl); (C) PA-GlcNAc oligomers (0.8 μl); (D) PA-ovomucoid oligosaccharides (4 μl). Conditions in Fig. 1 except for the gradient (10 → 24%/24 min), the mobile phase anion (175 m*M* TEA) and temperature (23°C).

separation characteristics between PA-Glc oligomers and PA-GlcNAc oligomers is shown in Table I. A good separation efficiency was obtained in the presence of sodium ions rather than in the presence of TEA. However, because of its non-volatility and low solubility in aqueous acetonitrile, this sodium acetate buffer is not suitable for preparative HPLC. The higher separation efficiency was obtained at higher temperature using the diluted TEA or sodium acetate buffer, as reported on an ion-exchange resin[35] and on an amine-bonded silica column[15]. Raising the pH of the mobile phase did not increase the separation efficiency. GlcNAc oligomers and ovomucoid oligosaccharides were eluted significantly earlier than Glc oligomers, similar to the amine adsorption mode where GlcNAc and fucose behaved like a 0.5 Glc unit[24]. On the other hand, at the high concentration of TEA, an higher separation efficiency was obtained at lower temperature. Raising the pH led to higher separation efficiency for low-molecular-weight PA-Glc oligomers, but lower efficiency for high-molecular-weight ones. Moreover, GlcNAc oligomers were eluted almost at the retention times corresponding to the Glc oligomers of the same DPs. Also, elutions of ovomucoid oligosaccharides were widely distributed, comparable to Glc oligomers.

The sulphonated polystyrene resin column used in this experiment (RSpak DC-613) has been used for the separation of monosaccharides, especially for anomers (in its sodium or calcium form)[36], and for monosaccharides present in glycoproteins (in its proton form)[37]. The separation mechanism on this column is regarded as a ligand-exchange interaction between water molecules in aquated metal ions and

hydroxyl groups in sugar molecules[38]. Alternatively, the partition of sugars between the mobile and stationary liquid phases can be considered[26–29]. In the oligosaccharide separation, the partition mechanism may be predominant on consideration of the reversal of the elution order below the critical concentration of acetonitrile.

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DETERMINATION OF SOME POLYNUCLEAR AROMATIC HYDROCARBONS IN NEW, USED AND REGENERATED OILS

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SUMMARY

The levels of polynuclear aromatic hydrocarbons (PAHs) in oils were determined using a three-step process: extraction and concentration by charge-transfer liquid chromatography on an improved tetrachlorophthalimidopropyl-bonded silica; oxidation of the thiophenes, which are extracted and concentrated at the same time, and separation of the resulting sulphones from the PAHs by adsorption chromatography on bare silica; and separation of the PAH fraction of improved purity on tetrachlorophthalimidopropyl-bonded silica. The recoveries for six PAHs were greater than 70%, except for indeno[1,2,3-*cd*]pyrene (28%), and they can be determined in new and used lubricating and regenerated oils.

INTRODUCTION

Charge-transfer liquid chromatography is mainly used for the separation of polynuclear aromatic hydrocarbons (PAHs)[1–3] and has been the subject of a number of reviews[4–6]. The retention of a solute in charge-transfer liquid chromatography is based on π–π interactions between the solutes and the stationary phase[7–9] and the main parameter governing PAH retention is the density of their electronic cloud constituted by delocalized π-electrons. Recently, we defined a series of structural parameters which leads to a rapid determination of the retention order of unsubstituted and substituted PAHs[10].

One of the interesting aspects of charge-transfer liquid chromatography is the high capacity factors for PAHs achieved using a π-electron acceptor-bonded silica with an apolar mobile phase. We improved the synthesis of tetrachlorophthalimidopropyl-bonded silica[11], a support described by Holstein in 1981[12], and on this improved

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support, with isooctane as mobile phase, the capacity factors were higher than 75 for four-ring PAHs, 700 for five-ring PAHs and 2000 for six-ring PAHs[11].

An obvious application of charge-transfer liquid chromatography on tetrachlorophthalimidopropyl-bonded silica is the extraction and concentration of PAHs in apolar media such as oils. These are mainly composed of saturated hydrocarbons which have no delocalized π-electrons, and are not retained by charge-transfer liquid chromatography (olefinic hydrocarbons are very weakly retained).

In comparison with the usual methods of determination, this approach partially resolved the following problems: the low recovery obtained with liquid–liquid extraction[13–15] or with adsorption chromatography[1,16]; the low solubility of the samples in aqueous mobile phases used for reversed-phase liquid chromatography[17,18]; the low volatility of real samples when they are analysed by gas chromatography; and the fact that fluorescence detection is much more selective than flame ionization detection[19,20] and more sensitive than mass spectrometry[21].

However, the determination of the concentration of PAHs in oils on tetrachlorophthalimidopropyl-bonded silica requires an intermediate step, *viz.*, the separation of PAHs from polynuclear derivatives with a heterocycle, especially thiophenes, which are also strongly retained by charge-transfer liquid chromatography.

This approach has been applied to the measurement of the concentations of PAHs in a series of new, used and regenerated lubricating oils and the results are reported in this paper.

EXPERIMENTAL

Apparatus

A Model 5020 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) was used in conjunction with an LDC Spectromonitor III detector (Milton Roy, LDC Division, Riviera Beach, FL, U.S.A.) or with an LS4 spectrofluorometric detector (Perkin-Elmer, Norwalk, CT, U.S.A.). The integrator was a Chromatopac C-R3A (Shimadzu, Kyoto, Japan). The temperature was set at 40 $\pm$ 0.1°C with a constant-temperature water-bath.

Stationary phases

Two chromatographic columns were packed with tetrachlorophthalimidopropyl-bonded silica, which was prepared as described previously[11]. The ligand density was 2.7 μmol m^{-2}. The dimensions of the column used for the concentration step were 15 cm $\times$ 6.9 mm I.D. The dimensions of the analytical column were 25 cm $\times$ 4.6 mm I.D.

The columns were polished stainless-steel tubes fitted with a 2-μm porous stainless-steel frit. They were packed by using the classical slurry packing technique: the bonded silica was suspended in carbon tetrachloride–methyl iodide (67:33, v/v) and the slurry was pushed with ethanol into the chromatographic column for 30 min under a 500-bar pressure.

C_{18} columns (5 cm $\times$ 4.6 mm I.D.; particle size 3 μm) were provided by Chrompack (Middelburg, The Netherlands).

Chemicals

The porous silica used to synthesize the bonded electron acceptor phase was 10-μm Lichrosorb Si 100 (pore diameter 100 Å; specific surface area 350 $m^2\ g^{-1}$) (Merck, Darmstadt, F.R.G.). Adsorption chromatography was carried out on 63–200-μm silica gel 100 (Merck).

PAHs were provided by the Community Bureau of Reference (Brussels, Belgium), Fluka (Buchs, Switzerland) and Janssen Chimica (Beerse, Belgium), the thiophenes were from Aldrich (Milwaukee, WI, U.S.A.) and the oils and the additives were obtained from Régie Renault (Rueil-Malmaison, France).

Isooctane and methylene choride were purchased from Rathburn (Walkerburn, U.K.), toluene, acetonitrile and hydrogen peroxide from Merck and acetic acid from Prolabo (Paris, France). Water was doubly distilled.

RESULTS

Procedure

The determination of PAHs in apolar media was accomplished in three steps, as follows.

Extraction and concentration. The column was initially equilibrated with isooctane. Then, an apolar matrix, $V = 300$ ml, was percolated into the column, where $V = V_1 + V_2$, V_1 being the volume of oil and V_2 the volume of isooctane added. The addition of isooctane was necessary to reduce the viscosity of the percolated medium, *i.e.*, to reduce the pressure drop. The strongly fixed PAHs and eventually other molecules (*e.g.*, thiophenes, see later) were eluted from the column by back-flushing with methylene chloride. This solvent has two interesting characteristics: (1) from the fixation isotherms, its affinity with respect to the tetrachlorophthalimidopropyl groups, and consequently its eluting power, are very high[10], so the PAHs are eluted very rapidly; and (2) it is very volatile and the concentration of the PAH-containing fraction (to V_3) is rapid. In this work, the column was completely eluted with 50 ml of methylene chloride, the eluate being evaporated to 0.5 ml: the enrichment factor was equal to $V_1/V_3 = 300$.

Separation of PAHs and thiophenes. In the previous step, the PAHs were

TABLE I

RETENTION OF PAHs AND THIOPHENES ON TETRACHLOROPHTHALIMIDOPROPYL-BONDED SILICA

Column, 25 cm × 4.6 mm I.D.; stationary phase, tetrachlorophthalimidopropyl-bonded LiChrosorb Si 100 (10 μm), ligand density 2.7 μmol m^{-2}; mobile phase, isooctane–methylene chloride; flow-rate, 1.2 ml min^{-1}; detection, UV absorbance at 254 nm. Solutes: Fl = fluorene; DBT = dibenzothiophene; Ant = anthracene; Ft = fluoranthene; BNT = benzo[*b*]naphtho[2,1-*d*]thiophene; B[a]A = benz[*a*]anthracene.

Isooctane–methylene chloride	*Fl*	*DBT*	*Ant*	*Ft*	*BNT*	*B[a]A*
70:30 (v/v)	–	3.0	3.2	6.5	7.4	7.6
80:20 (v/v)	–	4.9	5.3	11.2	15.0	15.7
90:10 (v/v)	–	11.0	11.6	24.6	39	41
100:0 (v/v)	20	44	50[a]	80[a]	–	–

[a] Extrapolated values.

concentrated, but also were polynuclear derivatives that contain a heterocycle, especially thiophenes (Table I). This is due to both the electron pairs on the sulphur atom and π-electron delocalization. In a ring containing four carbon atoms plus one sulphur atom, the π-electron delocalization is the same as that for a benzene ring and, for example, a four-benzene-ring PAH and a four-ring thiophene have almost the same retention on this stationary phase. Hence the separation of PAHs and thiophenes was necessary and required the oxidation of the thiophenes to sulphones (after an intermediate oxidation to sulphoxides). Sulphones are very polar whereas the PAHs are apolar, so the separation of PAHs from sulphones can be achieved by adsorption chromatography on bare silica. The oxidation procedure was as follows[22]: the concentrated PAH mixture was added to 100 ml of toluene–acetic acid (50:50, v/v), the solution was brought to reflux, 50 ml of hydrogen peroxide were added during 1 h and reflux was continued for 16 h. The aqueous and organic phases were separated and the toluene phase washed with 250 ml of distilled water, the resulting aqueous phase then being washed with 50 ml of toluene. The 100 ml of toluene (50 ml from the initial mixture + 50 ml from the washing of water) were evaporated to 10 ml, which were percolated on the top of a bare silica column (40 g of silica). The PAHs were eluted with 100 ml of water-saturated toluene, and the eluate was evaporated to less than 0.5 ml.

Separation of PAHs. This step was effected by charge-transfer liquid chromatography on tetrachlorophthalimidopropyl-bonded silica. The mobile phase was isooctane–methylene chloride with gradient elution. The temperature was maintained at 40 $\pm$ 0.1°C to improve the resolution and keep the duration of analysis short. A spectrofluorimetric detector was used, which was time-programmable with respect to the excitation and emission wavelengths to improve the selectivity and the sensitivity. Of course, this detection method needed a very reproducible separation. For the determination of PAH concentration, the PAH extract was diluted to a determined volume, *e.g.*, 0.5, 1 or 2 ml.

TABLE II

ADDED AND MEASURED AMOUNTS OF FLUORANTHENE, BENZO[*k*]FLUORANTHENE AND BENZO[*a*]PYRENE AND RECOVERIES OF THE EXTRACTION STEP

Column: 15 cm × 6.9 mm I.D. Abbreviations as in Table I.

Percolated solution	*PAH*	*Amount (mg)*		*Recovery (%)*
		Added	*Measured*	
300 ml isooctane				
+ 13.2 mg l^{-1} Ft	Ft	3.96	3.63	91.7
1.45 mg l^{-1} B[k]F	B[k]F	0.43	0.47	>100
5.10 mg l^{-1} B[a]P	B[a]P	1.53	1.48	96.7
300 ml standard	Ft	3.96	3.86	97.5
solution	B[k]F	0.43	0.50	>100
+ 50 ml Neutral 350	B[a]P	1.53	1.47	96.1
300 ml standard	Ft	3.96	3.86	97.5
solution	B[k]F	0.43	0.52	>100
+ 50 ml Neutral 100	B[a]P	1.53	1.44	94.1

Comments

Extraction and concentration. The volume percolated was established from the dead volume of the column (4.2 ml) and the retention of the least retained PAH, fluoranthene. Its capacity factor on tetrachlorophthalimidopropyl-bonded silica, with pure isooctane as the mobile phase, is 75; consequently, if an almost 100% recovery is required, the volume percolated has to be lower than $4.2 \times 75 = 315$ ml (the column will never be overloaded, owing to the low concentrations of PAH in oils).

We chose 300 ml, and we verified the quantitativeness of the process with three solutions: a "standard" solution composed of 4.4 mg of fluoranthene, 0.48 mg of benzo[*k*]fluoranthene and 1.7 mg of benzo[*a*]pyrene in 100 ml of pure isooctane; 300 ml of the "standard" solution + 50 ml of new Neutral 350; and 300 ml of the "standard" solution + 50 ml of new Neutral 100. Neutral 350 and Neutral 100 are base oils containing aromatic compounds and without any additives. Table II presents the results and, within the experimenal error, the extraction and concentration step can be considered to be quantitative. The worst recovery was 92% and the precision was 5%.

For lubricating oils we had to determine the influence of the additives that they contain. None of them is polyaromatic and they were not strongly retained on tetrachlorophthalimidopropyl-bonded silica (Table III). The highest capacity factor was lower than 3 with isooctane as the mobile phase. Consequently, the additives were not retained during the concentration step.

All PAHs are eluted from the column with 50 ml of methylene chloride and the support is then perfectly clean. In addition, the evaporation of methylene chloride does not add any interfering peaks. Under these conditions, the tetrachlorophthalimidopropyl-bonded silica was stable for more than 1 year.

Separation of PAHs and thiophenes. Sulphur is the third most abundant element in mineral oils, after carbon and hydrogen, and thiophenes are always present in oils.

TABLE III

FUNCTION AND RETENTION OF OIL ADDITIVES ON TETRACHLOROPHTHALIMIDOPROPYL-BONDED SILICA WITH PURE ISOOCTANE

Additive	*Function*	k'
MDTP[a]	Anti-oxidant	0.75
Zn di-2-octyldithiophosphate	Anti-wear	0.87
Thiophosphate[b]	Detergent	<2
Carbonate[b]	Detergent	<2
Phenol[b]	Anti-oxidant	<2
Polyisobutylene[b]	Viscosity improver	<2
Poly(ethylene–propylene)	Viscosity improver	<3
Alkyl polymethacrylate	Viscosity improver	<3
Chlorinated alkane	High-pressure resistance	≈ 0
Chlorinated hydrocarbons[c]	High-pressure resistance	<1.4
Sulphurized hydrocarbons[c]	High-pressure resistance	<1.4
Fat[c]	High-pressure resistance	<1.4
Sulphurized ester	High-pressure resistance	1.3

[a] MDTP = 4,4′-methylenebis(2,6-di-*tert.*-butylphenol).

[b] Additive mixture; the capacity factor of the most retained is less than 2.

[c] Additive mixture; the capacity factor of the most retained is less than 3.

The sulphur-containing compounds can represent up to 10% of the oil matrix[23] and, owing to their strong π-electron delocalization, thiophenes are retained similarly to PAHs (Table I).

The oxidation process has been fully described previously[22], and Fig. 1 shows its efficiency. For a used thermal oil, fluoranthene, benzofluoranthenes, benzo[*a*]pyrene and possibly benzo[*ghi*]perylene can be determined, but only after the separation of PAHs from thiophenes.

Separation of PAHs. The separation of PAHs was achieved by charge-transfer liquid chromatography on tetrachlorophthalimidopropyl-bonded silica with isooctane–methylene chloride as the mobile phase. An elution gradient programme from 30% to 100% methylene chloride was used. Fig. 2 shows the separation of six PAHs: fluoranthene, benzo[*k*]- and benzo[*b*]fluoranthene, benzo[*a*]pyrene, indeno-[1,2,3-*cd*]pyrene and benzo[*ghi*]perylene. These PAHs are the most toxic in the National Bureau of Standards list[24], and we decided to determine the concentration of only these six in order to simplify the analytical problem. The separation on tetrachlorophthalimidopropyl-bonded silica (Fig. 2a) and on C_{18}-bonded silica (Fig. 2b) can be compared. Although the selectivity was higher in charge-transfer

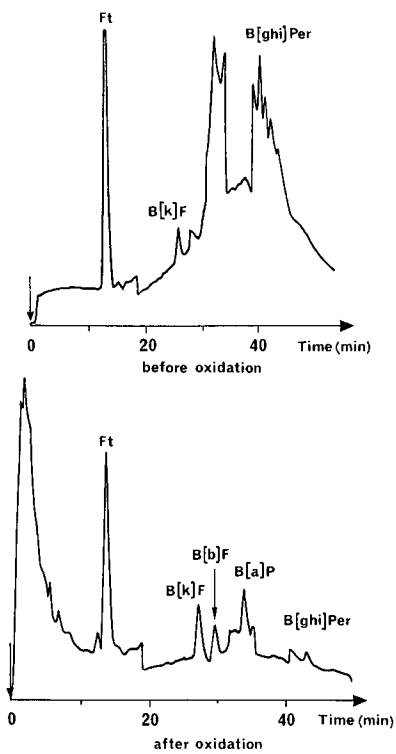

Fig. 1. Separation of PAHs in a used lubricating oil before and after oxidation of thiophenes. Chromatographic conditions as in Table I. Mobile phase: 0–13 min, isooctane–methylene chloride (70:30, v/v); 13–23 min, 70:30 to 50:50 (v/v); 23–37 min, 50:50 to 0:100 (v/v). Solutes: Ft = fluoranthene; B[k]F = benzo[*k*]fluoranthene; B[b] = benzo[*b*]fluoranthene; B[a]P = benzo[*a*]pyrene; B[ghi]Per = benzo[*ghi*]-perylene; ? = unidentified.

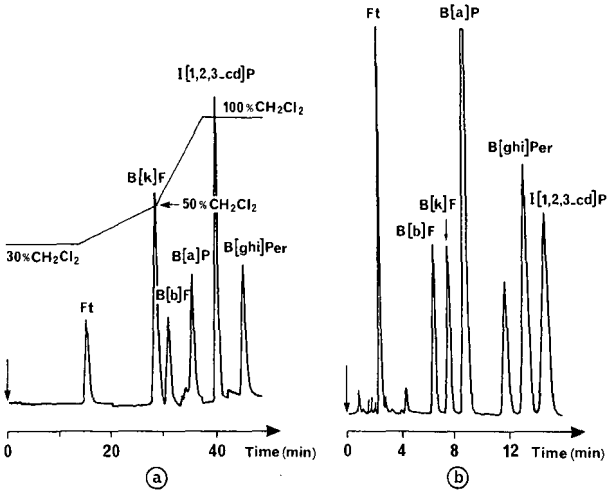

Fig. 2. Separation of six PAHs, (a) by charge-transfer liquid chromatography on tetrachlorophthalimidopropyl-bonded silica and (b) by reversed-phase liquid chromatography. (a) Chromatographic conditions as in Table I. Detection: spectrofluorimetry: 0–20 min, λ_{ex} = 278 nm, λ_{em} = 440 nm; 20–30 min, λ_{ex} = 308 nm, λ_{em} = 411 nm; 30–32 min, λ_{ex} = 302 nm, λ_{em} = 437 nm; 32–37 min, λ_{ex} = 285 nm, λ_{em} = 410 nm; 37–42 min, λ_{ex} = 277 nm, λ_{em} = 504 nm; 42–50 min, λ_{ex} = 273 nm, λ_{em} = 412 nm. (b) Column, 5 cm × 4.6 mm I.D.; stationary pase; C_{18}, particle diameter 3 μm; mobile phase, acetonitrile–water (75:25, v/v); flow-rate, 1 ml min^{-1}; detection, UV absorbance at 254 nm. Solutes as in Fig. 1; I[1,2,3-cd]P = indeno[1,2,3-*cd*]pyrene.

liquid chromatography, the resolutions were comparable and the duration of analysis was shorter using reversed-phase liquid chromatography. In addition, the separation was obtained with isocratic elution, so no column regeneration was needed. However, the solubility of the PAHs is very low in an aqueous medium, and is even lower for real samples. Consequently, charge-transfer liquid chromatography was used for the efficient and reproducible separation of PAH.

Detection was effected with a spectrofluorimetric detector that was time-programmable with respect to the excitation and emission wavelengths in order to improve both the selectivity and sensitivity simultaneously. Fig. 2a shows the wavelength programme and Table IV gives the detection limits. PAHs at concentrations lower than 1 $\mu g\ l^{-1}$ (ppb) can be identified and determined.

TABLE IV
DETECTION LIMITS FOR SIX PAHs
Chromatographic conditions as in Fig. 2. Abbreviations as in Table I.

PAH	*Detection limit ($\mu g\ l^{-1}$)*
Ft	10
B[k]F	0.26
B[b]F	15
B[a]P	1.5
I[1,2,3-cd]P	14
B[ghi]Per	29

TABLE V

RECOVERIES FOR SIX PAHs FOR THE FULL DETERMINATION PROCEDURE (WITH A 95% CONFIDENCE INTERVAL)

Abbreviations as in Table I.

PAH	*Recovery (%)*
Ft	72 ± 4
B[k]F	96 ± 8
B[b]F	97 ± 5
B[a]P	71 ± 28
I[1,2,3-cd]P	28 ± 19
B[ghi]Per	82 ± 2

Recoveries

The recoveries were determined by adding known amounts of the target PAHs to a new lubricating oil (this oil was new in order to reduce the total amount of PAHs, *i.e.*, to be close to the detection limits). For each PAH, a linear relationship between the added and measured amounts was observed, the recovery being equal to the slope.

Table V gives the recoveries determined with a 95% confidence interval. The results obtained for benzofluoranthene are excellent and for fluoranthene, benzo[*a*]pyrene and benzo[*ghi*]perylene they are satisfactory. However, for indeno[1,2,3-*cd*]pyrene the recovery is very low. An explanation could be degradation of this PAH during the oxidation of thiopenes. For both indeno[1,2,3-*cd*]pyrene and benzo[*a*]pyrene the calculated recovery is imprecise owing to an unstable baseline during their detection.

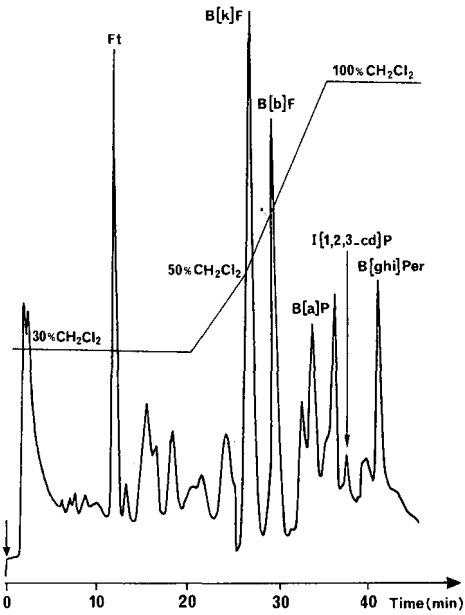

Fig. 3. Determination of PAHs in regenerated oil. Chromatographic conditions as in Fig. 2.

TABLE VI

MEASURED PAH CONCENTRATIONS IN A NEW LUBRICATING OIL, IN TWO USED LUBRICATING OILS AND IN A REGENERATED OIL (WITH A 95% CONFIDENCE INTERVAL)

Abbreviations as in Table I.

Oil	*Concentration (ppb)*					
	Ft	*B[k]F*	*B[b]F*	*B[a]P*	*I[1,2,3-cd]P*	*B[ghi]Per*
New lubricating oil	0.90 ±0.003	0.063 ±0.0011	0.017 ±0.003	0.011 ±0.003	0.0264 ±0.0119	3.8 ±3.6
Used lubricating oil A	336 ±58	0.24 ±0.015	43 ±4	8.64 ±1.69	16.0 ±5.0	7.91 ±1.02
Used lubricating oil B	164 ±23	0.097 ±0.017	23 ±3	2.51 ±0.6	18.6 ±5.6	4.64 ±0.26
Regenerated oil	108 ±27	5.15 ±0.22	84.0 ±8.7	14.55 ±2.25	44.4 ±21.7	83.94 ±12.67

Sensitivity

With a signal-to-noise ratio of 3, the sensitivity ranged from 50 ppt to 5 ppb, *i.e.*, fluoranthene 1 ng l^{-1}, benzo[*k*]fluoranthene 0.05 ng l^{-1}, benzo[*b*]fluoranthene 2 ng l^{-1}, benzo[*a*]pyrene 0.3 ng l^{-1}, indeno[1,2,3-*cd*]pyrene 5 ng l^{-1} and benzo[*ghi*]perylene 5 ng l^{-1}.

PAH concentrations in oils

PAHs were determined in four oils: a new lubricating oil, used lubricating oils A and B and a regenerated oil. For economic reasons, oil regeneration is becoming increasingly common, but leads to an increase in PAH concentrations. Hence the determination of PAHs in regenerated oils is essential.

Fig. 3 shows the separation of the PAHs detected in the regenerated oil and Table VI gives the measured PAH concentrations. The peaks were mainly identified by comparison with the retention times of standards.

Although in the new oil the PAH concentrations were very low and determined only inaccurately, they were much higher in the used oils and could be measured with good accuracy. Several concentrations are particularly high and justify the interest in this study. The PAH concentrations in regenerated oils are particularly disturbing, and, from Fig. 3, various PAHs could be identified and determined.

CONCLUSION

Charge-transfer liquid chromatography appears to be a satisfactory method for the determination of PAHs in oils: the extraction is selective and quantitative (very high capacity factors) owing to the mechanism based on the π–π interactions between the PAHs and the tetrachlorophthalimidopropyl-bonded silica; and the high selectivity and adequate efficiency allow chromatographic separation on the same

chromatographic support, *e.g.*, with an organic mobile phase (isooctane–methylene chloride) in which the PAH extract is soluble.

The problem of the simultaneous extraction and concentration of thiophenes was solved by their oxidation to polar sulphones, which were separated from the apolar PAHs by adsorption chromatography.

PAH concentrations in lubricating and regenerated oils were determined and the results show that these oils are unsafe.

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DETERMINATION OF AMPHETAMINE-RELATED COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH CHEMILUMINESCENCE AND FLUORESCENCE DETECTIONS

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SUMMARY

High-performance liquid chromatography with chemiluminescence detection has been established for the determination of trace levels of amphetamine-related compounds (APs) after fluorigenic derivatization. Bis(2,4,6-trichlorophenyl) oxalate and hydrogen peroxide in acetonitrile was used as a post-column chemilumigenic reagent. As derivatization reagents, dansyl chloride (Dns-Cl), 4-fluoro-7-nitrobenzoxadiazole (NBD-F) and naphtalene-2,3-dicarbaldehyde (NDA) were compared. Dns-Cl was the most suitable of the three for the simultaneous determination of both primary and secondary amino APs. The on-column detection limits for Dns-derivatives were $3 \cdot 10^{-15} - 4 \cdot 10^{-15}$ mol. NDA gave the most sensitive derivatives (cyanobenz[*f*]isoindole, CBI derivatives) with only primary amino APs. The detection limits for CBI derivatives were as low as $2 \cdot 10^{-16}$ mol. The present method was applied to the determination of methamphetamine in human urine. Only diethyl ether extraction was necessary as a clean-up treatment before Dns derivatization, because diethyl ether extracted methamphetamine quantitatively from urine at strong alkaline pH and the extract showed few interfering peaks around the retention time of methamphetamine after the derivatization. Methamphetamine concentrations as low as $1 \cdot 10^{-7}$ *M* in urine were determined after the above treatments.

INTRODUCTION

The abuse of amphetamine-related compounds (APs) has led to thorough studies of their metabolism, distribution and excretion. Many chromatographic methods were developed for the sensitive determination of APs in urine and plasma. Gas chromatography (GC) with either nitrogen selective or flamethermionic detection can detect sub-ng amounts of APs[1,2]. Gas chromatography–mass spectrometry (GC–

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MS) method can detect as little as several pg of APs[3]. High-performance liquid chromatography (HPLC) methods for the determination of APs have an advantage over GC and GC–MS in that the preparations of aqueous samples such as biological fluids are easy. The sensitivities of the HPLC methods reported were not as high as those of GC and GC–MS, although some of them used fluorescence or electrochemical detection[4–6].

HPLC with chemiluminescence detection was reported to be highly sensitive and selective for fluorescent compounds[7]. The chemiluminescence detection limits of several fluorescent compounds were 10 – 100 times lower than those by fluorescence detection. The selectivity of the method reduced clean-up treatments for the determination of polynuclear aromatic hydrocarbons (PAHs) in environmental samples[8–10]. Moreover, both primary and secondary amines were also chemilumigenically detected after derivatization to fluorescent compounds with such reagents as dansyl chloride (Dns-Cl)[11,12]. It was suggested that HPLC with chemiluminescence detection might be useful in the sensitive and selective determination of APs. However, there has been no application of this method to the determination of APs.

The purpose of this study was to find the best derivatizing agent for the determination of APs by HPLC with chemiluminescence detection and to compare the sensitivity of chemiluminescence detection with that of fluorescence detection. Furthermore, the present method was applied to the determination of low levels of methamphetamine in human urine.

EXPERIMENTAL

Chemicals

Acetonitrile, acetone, methanol, diethyl ether and imidazole of reagent grade were obtained from Nakalai (Kyoto, Japan). Bis(2,4,6-trichlorophenyl) oxalate (TCPO) and 30% hydrogen peroxide solution of electronic grade were from Tokyo Kasei (Tokyo, Japan) and Kanto (Tokyo, Japan), respectively. Dns-Cl and naphthalene-2,3-dicarbaldehyde (NDA) were from Wako (Osaka, Japan) and Molecular Probes (Eugene, U.S.A.), respectively. 4-Fluoro-7-nitrobenzoxadiazole (NBD-F) was kindly supplied by Dr. K. Imai, Pharmacy of Branch Hospital, University of Tokyo. Other chemicals used were all commercially available.

APs and their standard solutions

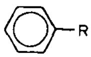
The structures and sources of APs used are listed in Table I. A standard solution ($1.0 \cdot 10^{-4}$ *M*) was prepared by dissolving the appropriate amount of each compound in water.

Apparatus

The HPLC system consisted of two Shimadzu (Kyoto, Japan) LC-6A high-pressure pumps, a 7125 injector with a loop of 20 μl (Rheodyne, Cotati, CA, U.S.A.), an MPLC guard column (30 mm × 4.6 mm I.D.) packed with Spheri-5 RP-18 (Brownlee, Santa Clara, U.S.A.), a separation column (250 mm × 4.6 mm I.D.) packed with Inertsil ODS-2 (Gasukuro Kogyo, Tokyo, Japan), a reaction coil (300 mm × 0.25 mm I.D.), a damper coil (2000 mm × 0.1 mm I.D.), a KZS-1 mixing device (Kyowa Seimitsu, Mitaka, Japan), a Shimadzu RF-530 fluorescence detector,

TABLE I

FORMULAE OF APs USED



Compound	*–R*	*Source*[a]	*Peak number*
Benzylamine	$-CH_2NH_2$	1	2
Phenylethylamine	$-CH_2CH_2NH_2$	1	4
Phenylpropylamine	$-CH_2CH_2CH_2NH_2$	2	5
Phenylbutylamine	$-CH_2CH_2CH_2CH_2NH_2$	2	6
N-Methylphenethylamine	$-CH_2CH_2NH$ CH_3	2	7
Methamphetamine[b]	$-CH_2CH-NH$ CH_3CH_3	3	8
Phenylpropanolamine	$-CH-CH-NH_2$ $OH\ CH_3$	1	1
Ephedrine[b]	$-CH-CH-NH$ $OH\ CH_3\ CH_3$	3	3
N-Isopropylbenzylamine	CH_3 $-CH_2NH-CH$ CH_3	2	9

[a] 1 = Nakarai (Kyoto, Japan); 2 = Aldrich (Milwaukee, WI, U.S.A.); 3 = Dainippon Pharmacy (Osaka, Japan).
[b] Hydrochloride form.

an AC-2220 luminomonitor (spiral flow cell of 60 μl) (Atto, Tokyo, Japan) and two Shimadzu C-R3A integrators. A schematic diagram of the system is shown in Fig. 1.

The fluorescence spectra were measured by a 650-10S fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

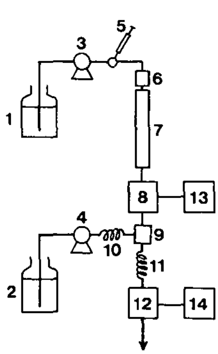


Fig. 1. Schematic diagram of the HPLC system. 1 = Reservoir (eluent); 2 = reservoir (chemilumigenic reagent); 3, 4 = pumps; 5 = injector; 6 = guard column; 7 = separation column; 8 = fluorescence detector; 9 = mixing device; 10 = damper coil; 11 = reaction coil; 12 = luminomonitor; 13, 14 = integrators.

HPLC operating conditions

The mobile phase for HPLC was prepared by dissolving imidazole in acetonitrile–water to a final concentration of $1.0 \cdot 10^{-3}$ *M*. The mixture was adjusted to pH 7.0 with nitric acid before adjusting the final volume. The ratios of acetonitrile–water were 8:2 (v/v) for the separation of CBI (cyanobenz[*f*]isoindole) derivatives, 7:3 for Dns derivatives and 6:4 for NBD derivatives. Each mobile phase was treated with a Fuji Film FR-40 membrane filter (pore size 0.4 μm) and supersonicated before use. The chemilumigenic reagent solution was prepared before use as follows. TCPO (0.112 g) was dissolved in 500 ml of acetonitrile, and 8.6 ml of 30% hydrogen peroxide solution were added. The mixture was supersonicated.

Other conditions were as follows: Flow-rate of the mobile phase, 1.0 ml/min; flow-rate of the chemilumigenic reagent solution, 1.0 ml/min; column temperature, ambient; injection volume, 20 μl; excitation (Ex) and emission (Em) wavelengths of the fluorescence detector, 343 nm (Ex) and 530 nm (Em) for Dns derivatives, 418 nm (Ex) and 483 nm (Em) for CBI derivatives and 470 nm (Ex) and 530 nm (Em) for NBD derivatives. The attenuation of the integrator was adjusted to give similar height of corresponding peaks in the comparison of chromatograms obtained by chemiluminescence and fluorescence detections.

Extraction

Methamphetamine hydrochloride was dissolved in human urine at the concentration of $1.0 \cdot 10^{-7}$ *M*. To 2.0 ml of this solution in a test-tube, 2.0 ml of sodium hydroxide solution (2.0% in water) and 2.0 ml of diethyl ether were added successively. The tube was capped and shaken vigorously for 5 min. After centrifuging at 1000 *g* rpm for 10 min, the ether phase was collected. The extraction procedure was repeated twice.

Derivatization

The procedure of Dns derivatization was as follows. To 0.1 ml of the solution of APs (the mixture of $1.0 \cdot 10^{-4}$ *M* of each compound) in a test-tube, 0.4 ml of carbonate buffer (sodium bicarbonate dissolved in water at a concentration of $1.0 \cdot 10^{-2}$ *M*, pH adjusted to 9.0 with sodium hydroxide solution) and 0.5 ml of Dns-Cl solution ($1.0 \cdot 10^{-3}$ *M* in acetone) were added successively. After mixing, the capped tube was kept at 45°C in a water-bath in the dark for 1 h. When methamphetamine in human urine was to be determined, 0.1 ml of a diethyl ether extract was used instead of the amine solution.

The procedure for NBD derivatization was as follows. To 0.1 ml of the solution of APs (as used for Dns derivatization) in a test-tube, 0.4 ml of borate buffer (boric acid dissolved in water at a concentration of $1.0 \cdot 10^{-1}$ *M*, pH adjusted to 8.0 with sodium hydroxide solution) and 0.5 ml of NBD-F ($8.0 \cdot 10^{-2}$ *M* in ethanol) were added successively. After mixing, the capped tube was kept at 60°C in a water-bath in the dark for 1 min.

The procedure for CBI derivatization was as follows. To 0.1 ml of the solution of APs (as used for Dns derivatization) in a test-tube, 0.3 ml of borate buffer (boric acid was dissolved in water at a concentration of $5.0 \cdot 10^{-2}$ *M*, pH adjusted to 9.0 with sodium hydroxide solution), 0.4 ml of acetonitrile, 0.1 ml of sodium cyanide solution ($1.0 \cdot 10^{-2}$ *M* in water) and 0.1 ml of NDA solution ($2.0 \cdot 10^{-3}$ *M* in

methanol) were added successively. After mixing, the capped tube was kept at room temperature in the dark for 2 h.

Each derivatized solution was mixed and diluted in acetonitrile–water (1:1). An aliquot of the solution was injected into the chromatographic system.

RESULTS AND DISCUSSION

In our previous work concerning the determination of PAHs, we found that a mixture of TCPO and hydrogen peroxide was stable enough in acetonitrile to be used by a one-pump post-column chemilumigenic reaction system of HPLC for several hours[13]. The same system was used in the present work. The concentration of acetonitrile in the mobile phase was optimized to give the best resolution in each series of derivatives of APs. In a batchwise operation with the TCPO–hydrogen peroxide solution and the mobile phases described above, chemiluminescence intensities of the three derivatives tested reached their maxima in 2 s and lasted for more than 10 s after mixing. The size of the reaction coil of the present system was adjusted to detect the chemiluminescence between 0.5 and 2.3 s after mixing. The other conditions were the same as used for PAHs.

Dns-Cl, which reacts with both primary and secondary amines, is a popular fluorigenic labelling reagent. It has also been used as a chemilumigenic labelling reagent[11,12]. Fig. 2 shows chromatograms of nine Dns derivatives of APs with chemiluminescence (A) and fluorescence (B) detections by the injection of $1.0 \cdot 10^{-12}$ mol of each derivative. All APs were separately determined by both detection methods in 22

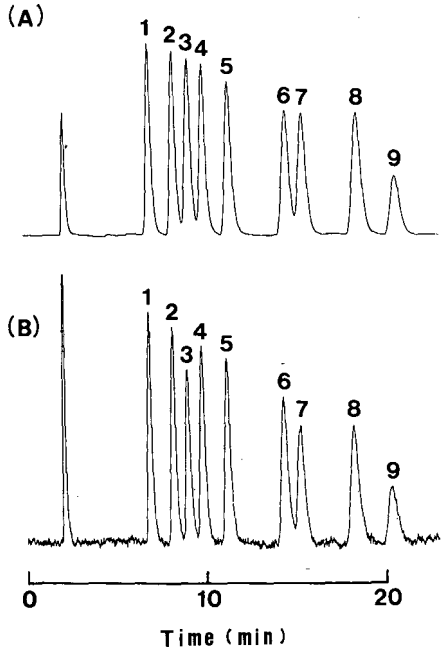


Fig. 2. Typical chromatograms of Dns-APs with chemiluminescence (A) and fluorescence (B) detections. Peak numbers as in Table I. For conditions see text.

min. The noise level by chemiluminescence detection was much smaller than that by fluorescence detection, and the corresponding peaks showed similar heights in the two detection methods. This result indicated that the detection sensitivity of the former method was better than that of the latter in the determination of Dns derivatives of APs. The peak areas of the nine APs were almost constant except for that of N-isopropylbenzylamine which may have a steric effect on the tertiary propyl group next to nitrogen on the derivatization yield or the quantum yield. The constant response is advantageous in the simultaneous determination of metabolites of APs.

NBD-F reacts with both primary and secondary amines to give fluorescent compounds. The reaction is very rapid under mild conditions, which is superior to NBD-Cl[14]. NBD derivatization has not been applied for the HPLC determination of amines with chemiluminescence detection, although the chemiluminescence intensities of NBD-proline have been compared to those of other compounds[15]. Fig. 3 shows chromatograms of NBD derivatives of nine APs with chemiluminescence (A) and fluorescence (B) detections. The separation of phenylpropanolamine and N-methylphenethylamine was not achieved under these conditions. The amount of primary amino APs injected was $1.0 \cdot 10^{-12}$ mol of each, while that of the secondary amino APs was $1.0 \cdot 10^{-11}$ mol of each, because of the poorer response of the latter. The noise level by chemiluminescence detection was much larger than that by fluorescence detection in Fig. 3. The main disadvantage of NBD derivatives was that the sensitivity of APs by chemiluminescence detection was less than that by fluorescence detection.

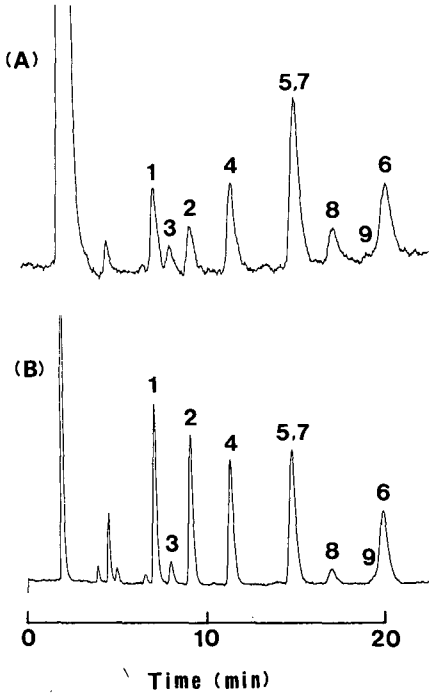


Fig. 3. Typical chromatograms of NBD-APs with chemiluminescence (A) and fluorescence (B) detections. Other details as in Fig. 2.

NDA is a fluorigenic reagent which reacts only with primary amines in the presence of cyanide[16]. Recently, it has been reported that NDA derivatives (also called CBI derivatives) of catecholamines were very sensitive to chemiluminescence detection[17]. Fig. 4 shows chromatograms of CBI derivatives of primary amino APs with both chemiluminescence (A) and fluorescence (B) detections by the injection of $1.0 \cdot 10^{-13}$ mol. Five APs were separately determined in 14 min. The areas of the corresponding peaks for five APs were not very different by each detection mode as shown in Fig. 3. The important fact is that the large signals of the CBI derivatives were observed with a very stable baseline by chemiluminescence detection even with the injection of 1/10 of the amount of Dns derivatives in Fig. 2. This suggests that NDA might be a very useful derivatization agent in the sensitive chemilumigenic determination of such primary amino APs as amphetamine.

The detection limits for Dns-, NBD- and CBI derivatives of five APs are compared in Table II. They are defined as that mass of analyte which provides a signal equal to two times the peak-to-peak noise by using mobile phases corresponding to the three series. The most important result is the high sensitivity of chemiluminescence detection. The detection limits for CBI derivatives were $2 \cdot 10^{-16}$ mol, which is the smallest in the three series. CBI derivatives might be more sensitive to chemiluminescence detection than to fluorescence detection even if excited at 252 nm which gave detection limits 10 times better than those at 418 nm[15]. The disadvantage of CBI derivatives is that they are applicable only for primary amino APs. If both primary and secondary amino APs are to be determined, Dns derivatives give higher sensitiv-

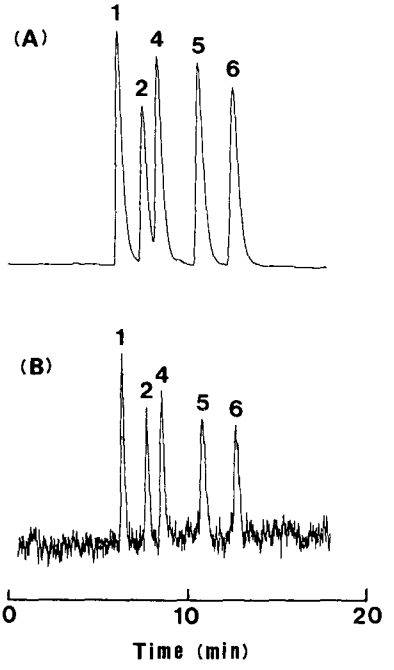


Fig. 4. Typical chromatograms of CBI-APs with chemiluminescence (A) and fluorescence (B) detections. Other details as in Fig. 2.

TABLE II

DETECTION LIMITS FOR DNS-, NBD- AND CBI-APs BY HPLC WITH BOTH CHEMILUMINESCENCE AND FLUORESCENCE DETECTIONS

Derivatization	*Detection method*	*Detection limit[a] of analyte (10^{-15} mol)*				
		PPA	*BA*	*EPH*	*PEA*	*MA*
Dns	Chemiluminescence	4	3	4	4	4
	Fluorescence	50	40	50	50	50
NBD	Chemiluminescence	6000	900	30 000	400	20 000
	Fluorescence	2000	200	4000	200	30 000
CBI	Chemiluminescence	0.2	0.2		0.2	
	Fluorescence	20	30		20	

[a]PPA = Phenylpropanolamine; BA = benzylamine; EPH = ephedrine; PEA = phenylethylamine; MA = methamphetamine. Signal-to-noise ratio = 2.

ity than NBD derivatives. This result is in accord with a report concerning the comparison of NBD, Dns and *o*-phthalaldehyde (OPA) derivatives of aliphatic amines[12]. The detection limits of Dns derivatives were $3 \cdot 10^{-15} - 4 \cdot 10^{-15}$ mol. To date, GC–MS is the most sensitive determination method for APs. The detection limits for heptafluorobutyryl derivatives of amphetamine and methamphetamine by GC–MS were $1.5 \cdot 10^{-14}$ and $1.4 \cdot 10^{-14}$ mol, respectively[3]. The present HPLC method with chemiluminescence detection is more sensitive than GC–MS by a factor of 70 for CBI derivatives and 3.5 for Dns derivatives.

Methamphetamine is a major abuse drug in Japan. A large fraction of methamphetamine administered is excreted in urine as methamphetamine itself[18]. Therefore, the present method was applied to the determination of methamphetamine added in human urine. It was necessary to clean up the urine sample before derivatization in order to reduce interfering peaks. Diethyl ether had the advantages that it easily

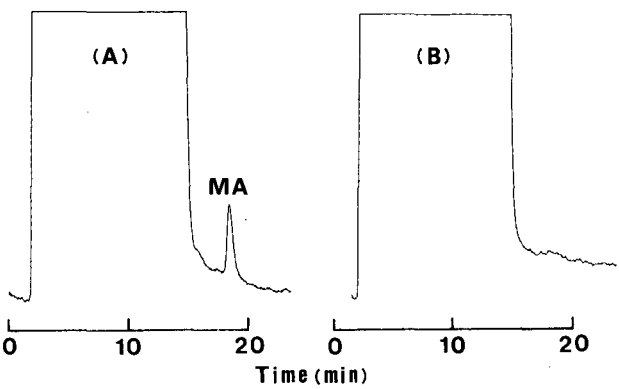


Fig. 5. Typical chromatograms of diethyl ether extracts from methamphetamine-spiked (A) and control (B) human urine samples after Dns-derivatization. The concentration of methamphetamine (MA) was $1.0 \cdot 10^{-7}$ *M* in human plasma. For other conditions see text.

extracted methamphetamine from urine at strong alkaline pH[19] and that the extract gave less interfering peaks than other organic solvents tested by GC[20]. Table II shows that Dns-Cl was the best derivatization agent for methamphetamine. Consequently, a diethyl ether extract from urine was directly derivatized by Dns-Cl, and an aliquot of the mixture was injected onto the column. Fig. 5 shows chromatograms of methamphetamine-spiked human urine (A) and control human urine (B) after the treatment described above. Methamphetamine as low as $1.0 \cdot 10^{-7}$ *M* in urine was detected without any interfering peak at the corresponding retention time. Lower detection limits may be obtained by concentration of the ether extracts. The large peak eluted before 15 min interferes with the detection of other analytes which are eluted in 15 min or less, although this peak was almost negligible on diluting the reaction mixture as shown in standard chromatograms. The contribution of the control urine sample to this peak was not significant. It might be decreased by purification of the reagents used or by the extraction of degradation products such as Dns-OH, because it was observed upon injection of the reagent blank. The recoveries of methamphetamine from human urine by the diethyl ether extraction were $99.3 \pm 0.7\%$ when the concentration of methamphetamine was $5.0 \cdot 10^{-7}$ *M*. These results indicate that the present method with diethyl ether extraction and Dns derivatization is promising for the determination of trace levels of methamphetamine in human urine. Optimization of the clean-up and derivatization will increase the practical value of the method, and will be reported elsewhere.

ACKNOWLEDGEMENTS

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ANALYSIS OF DRUG RESIDUES IN TISSUE BY COMBINED SUPERCRITICAL-FLUID EXTRACTION–SUPERCRITICAL-FLUID CHROMATOGRAPHY–MASS SPECTROMETRY–MASS SPECTROMETRY

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SUMMARY

The combination of supercritical-fluid extraction–supercritical-fluid chromatography–tandem mass spectrometry has been evaluated for the detection of residues of a small group of veterinary drugs in freeze-dried pig's kidney. During extraction with supercritical CO_2 the drugs were retained by the column while non-polar endogenous material was not retained and thus passed to waste. Subsequent changes to the mobile phase composition eluted the drugs which were detected with high specificity by tandem mass spectrometry. Although the sensitivity in this preliminary study was not adequate for surveillance or enforcement, there is potential for further development of the approach.

INTRODUCTION

Supercritical-fluid extraction (SFE) has been used for the industrial-scale separation and isolation of a variety of compounds[1,2]. The relatively low critical temperatures of fluids such as CO_2 (T_c 31.1°C) enable extractions to be performed under mild conditions and this, coupled with low cost, miscibility, non-toxicity, non-flammability and ease of removal of the solvent has made SFE with this fluid especially attractive[3]. Many aspects of SFE have been reviewed in detail[4].

On the analytical scale SFE has also attracted attention[5,6] and a variety of techniques have been used for on-line analyses. Unger and Roumeliotis[7] have described the combination of SFE with high-performance liquid chromatography (HPLC) applied to the analysis of valtrate and didrovaltrate from *Radix valerianae*. The combination of SFE with gas chromatography (GC) has been applied to the analysis of polycyclic aromatic compounds in model matrices and air particulates[8], and also to the analysis of flavour and fragrance compounds in a variety of matrices[9]. The latter work also included the use of SFE–GC on-line to mass spectrometry (MS).

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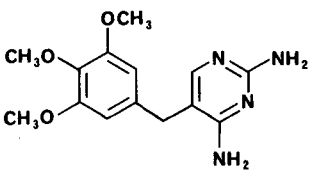
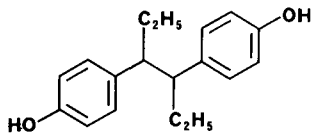
Direct fluid injection of supercritical-fluid extracts into a mass spectrometer has been explored by Kalinoski *et al.*[10]. In this work, dealing with the determination of trichothecene mycotoxins in wheat, tandem mass spectrometry (MS–MS) was employed to achieve adequate specificity.

Supercritical-fluid chromatography (SFC) has also been coupled to SFE[11–13]. Sugiyama *et al.*[11] have demonstrated this approach in the analysis of caffeine in coffee beans, and present a detailed exploration of the effects of varying the SFE conditions. Engelhart and Gross[12] have used the combination with flame ionization detection for the study of plant materials. McNally and Wheeler[13] have used SFE–SFC with ultraviolet (UV) detection for the analysis of sulphonylurea herbicides in various matrices including soil and plant material. Although these workers all used packed column SFC, capillary column SFC can also be used on-line with SFE and a prototype apparatus has recently been described[14].

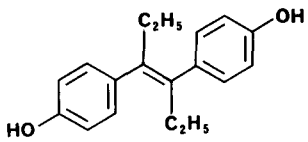
In this laboratory we have paid great attention to the potential of modern instrumental methods for rapid trace analysis, and especially to the use of automated methods or on-line combinations of techniques. We have previously reported the use of MS–MS[15] and HPLC–MS–MS[16] for the detection of veterinary drug residues in animal tissues following simple extraction methods and minimal clean-up. We now report a preliminary investigation of SFE–SFC coupled to MS–MS for the detection, in pig's kidney, of a small group of veterinary drugs including trimethoprim (molecular weight, MW 290), hexestrol (MW 270), diethylstilbestrol (MW 268) and dienestrol (MW 266).



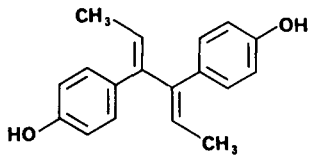
Trimethoprim



Hexestrol



Diethylstilbestrol



Dienestrol

EXPERIMENTAL

Materials

HPLC-grade methanol was obtained from Rathburn (Walkerburn, U.K.). Instrument-grade liquid carbon dioxide supplied in a cylinder with a syphon tube was obtained from British Oxygen (London, U.K.). Trimethoprim, hexestrol, diethylstilbestrol, dienestrol and sulphamethazine were obtained from Sigma (Poole, U.K.).

Sample preparation

A whole pig kidney, purchased from a local supplier, was blended to a smooth paste in a food processor. The paste (167 g) was spread onto trays and freeze dried using an Edwards EF6(S) shelf freeze drier (Edwards High Vacuum, Crawley, U.K.). The dried sample (43.4 g) was sieved through a 0.710-mm aperture sieve (Endecotts, London, U.K.). Portions of the powdered kidney (1 g) were mixed with methanol (10 ml), spiked with the appropriate quantity of each drug, allowed to equilibrate for one hour whilst being sonicated and then evaporated to dryness in a nitrogen stream. All samples were thoroughly mixed prior to being loaded into the extraction cell.

Supercritical-fluid chromatography

Throughout these studies supercritical fluid was supplied by a Hewlett-Packard packed column SFC apparatus based on a 1084B liquid chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.). The liquid carbon dioxide supply line and the pump heads were cooled to −25°C using a Neslab RTE-42 refrigerated bath. Carbon dioxide–methanol was used as mobile phase, and was supplied at a flow-rate of 4 ml/min. The mobile phase composition was initially 100% carbon dioxide for 8 min and was then programmed linearly to carbon dioxide–methanol (80:20) in 0.5 min. The final composition was maintained for 10 min. The 100 × 4.6 mm I.D. column used in these studies was packed in our own laboratory with amino-bonded Spherisorb 5 (Phase Separations, Queensferry, U.K.). The column pressure was 302 bar and oven temperature was 75°C. For sample introduction a Rheodyne 7125 valve was used with a 20-μl loop.

Supercritical-fluid extraction

A schematic of the SFE–SFC system is shown in Fig. 1. An Uptight 20 × 2 mm I.D. HPLC precolumn (Anachem, Luton, U.K.) was used as the extraction cell and was packed with freeze-dried material retained by 2 μm frits. The cell was connected in place of the fixed volume loop of the Rheodyne valve. The inject and load positions of the valve were used to allow the contents of the cell to be extracted or isolated as the elutropic strength of the mobile phase was varied during the SFC gradient programme. Samples were extracted for the first 8.5 min of the SFC gradient programme. Simple SFE experiments were conducted with a length of 0.15 mm bore stainless-steel tubing replacing the analytical column. For SFE–SFC the column and chromatographic conditions were as above.

Mass spectrometry

All MS studies were carried out using a VG 7070EQ tandem mass spectrometer (VG Analytical, Wythenshawe, U.K.) equipped with a standard VG water cooled

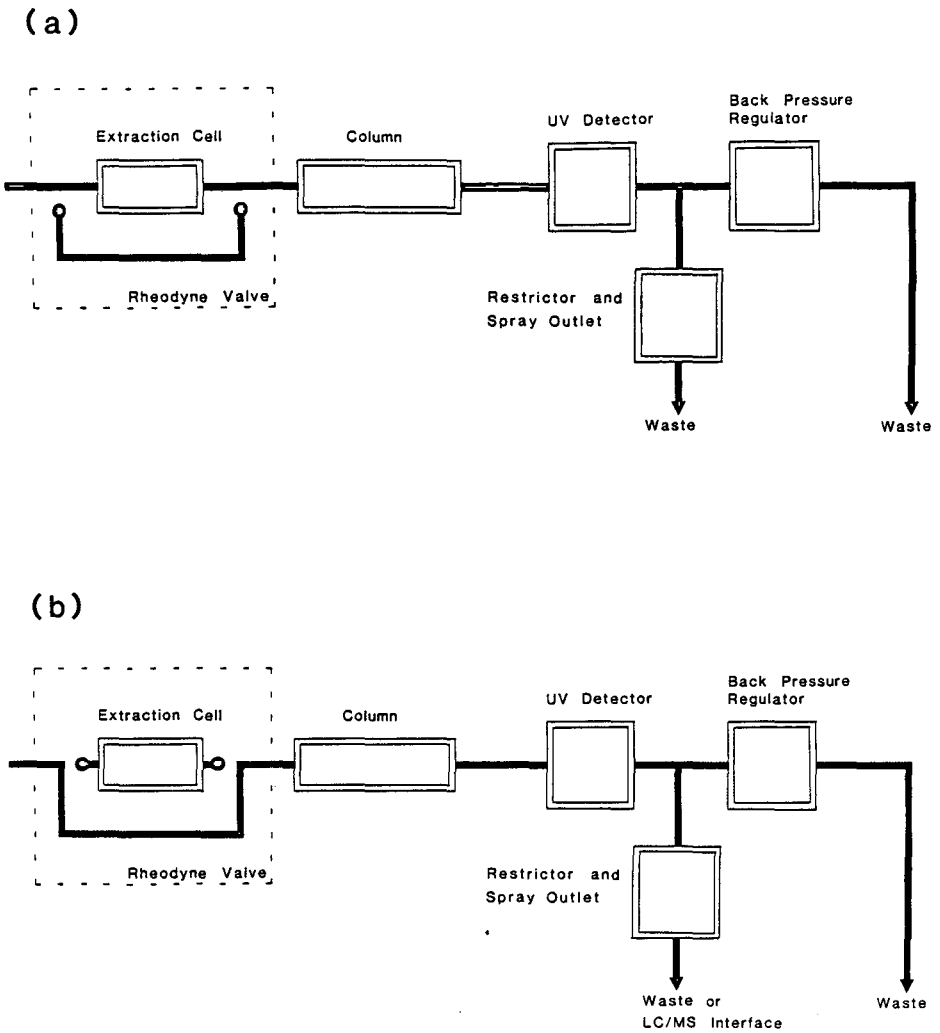


Fig. 1. Schematic of SFE–SFC–MS–MS system (a) during extraction, (b) during post extraction chromatography.

temperature-programmable probe and moving belt HPLC interface. A VG 11-250 datasystem was used for instrument control, and for data acquisition and analysis.

Electron ionization mass spectra were recorded at a source temperature of 240°C with an electron energy of 70 eV and a trap current of 200 μA. For normal mass spectra the double focussing part of the instrument was set to operate at a resolution of 1000 (10% valley definition) and ions were detected at the intermediate collector. For MS–MS experiments the parent ion resolution was set to 500 and the daughter ion resolution was unit mass. The collision gas was argon and the collision gas pressure was adjusted to give a housing pressure indication of $8 \cdot 10^{-6}$ mbar. The collision energy was set to 15 eV. Reference mass spectra were obtained using the direct insertion probe with a temperature programme of 40 to 350°C at 2°C/s.

Supercritical fluid chromatography–mass spectrometry

Combined SFC–MS was carried out using the moving belt interface in the manner described by Berry[17]. The eluent was applied to the belt with a modified Finnigan MAT (San Jose, CA, U.S.A.) thermospray deposition device. The spray deposition device was connected by a 60-cm length of 0.15 mm bore stainless-steel tubing to a tee-piece situated between the UV detector exit and the back-pressure regulator of the chromatograph. In order to maintain SFC conditions to the point at which the eluent left the system, the end of the thermospray jet was slightly crimped. This arrangement resulted in a 1:1 split of the column eluent between UV and MS detection and also facilitated use of the full density range of supercritical carbon dioxide for SFC. A 6-V power supply and rheostat adjustment was used to provide direct heating of the thermospray jet in order to prevent solute precipitation and freezing of the eluent at the tip. The belt speed was 1.2 cm/s resulting in a 40-s lag between responses at the UV detector and the mass spectrometer. The sample evaporator was set at 240. During SFE–SFC–MS and SFE–SFC–MS–MS experiments the eluent was directed to the mass spectrometer only during the elution period corresponding to the compounds of interest (approximately 10–12 min).

During MS–MS experiments the datasystem was used to control the instrument so that the quadrupole comprising MS-2 was scanned repetitively about once per second while MS-1 was used, by accelerating voltage switching at constant magnet current, to select the desired parent for each scan.

RESULTS AND DISCUSSION

In order to establish the feasibility of direct SFE–MS or SFE–MS–MS analyses, initial experiments were performed to determine the quantity of endogenous material extracted from the loaded extraction cell. The SFC gradient programme described above was used during these preliminary investigations and the amino bonded column was replaced with a length of 0.15 mm bore stainless-steel tubing. The average cell loading was 28.4 mg and these preliminary experiments showed that approximately 20% of this material was removed during the initial 8.5 min extraction period.

Even if MS detection were able to provide sufficient specificity, the use of direct SFE–MS or SFE–MS–MS would have been impractical due to the rapid contamination of the mass spectrometer which would have occurred. To avoid this problem an SFE–SFC procedure was developed to provide a degree of clean-up. This approach was designed to exploit the fact that most drugs are rather polar while the major part of the co-extracted endogenous material is relatively non-polar, and involved the use of a polar amino phase SFC column. During the period of extraction with unmodified CO_2, the extracted drugs were strongly retained at the inlet of the column whilst the majority of extracted endogenous components were eluted rapidly and directed to waste. The drugs and more polar endogenous components were then eluted by adding modifier to the mobile phase. The mass of extracted endogenous material present in the same elution window as the drugs was estimated by collecting and weighing this fraction. This indicated that approximately 100–200 μg of material was deposited onto the moving belt interface per analysis.

The SFC–UV chromatogram of standards shown in Fig. 2 was obtained by replacing the extraction cell with a 20-μl valve loop prior to analysing a standard

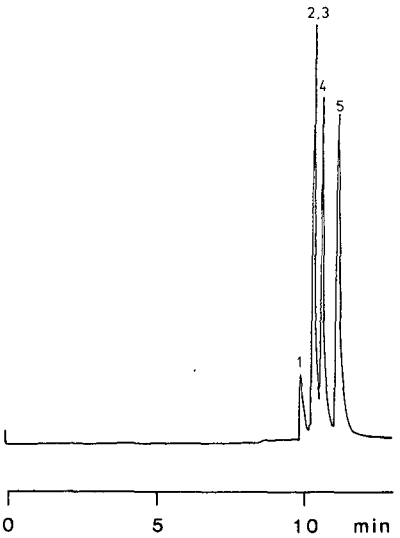


Fig. 2. SFC analysis of a standard drug mixture: 1 = trimethoprim; 2 = hexestrol; 3 = diethylstilbestrol; 4 = dienestrol; and 5 = sulphamethazine. Of each drug 20 μg was injected on column. UV detection at 254 nm. Range 0.4 a.u.f.s.

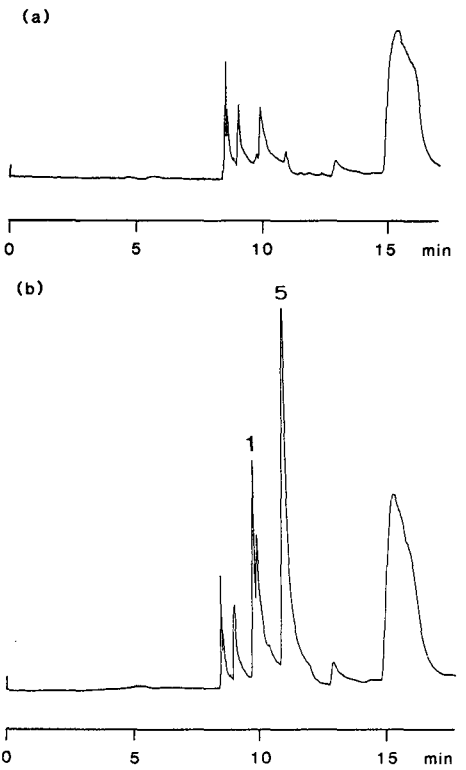


Fig. 3. SFE–SFC analysis (UV detection at 254 nm, range 0.4 a.u.f.s.) of (a) blank kidney sample (b) kidney sample spiked with 150 mg/kg each of the marker drugs trimethoprim (1) and sulphamethazine (5).

solution of the drugs and demonstrates the chromatographic separation obtained with the SFE–SFC gradient programme. As shown in Figs. 3 and 4, SFE–SFC with UV detection at either 254 or 215 nm did not afford sufficient resolution and specificity to allow detection of the drugs spiked at the 10 mg/kg level. Although our ultimate aim was to develop an approach using MS–MS to provide specificity, and in which a high degree of chromatographic separation was not required, the initial attempts to develop an SFE–SFC programme were hampered by the high background of endogenous components. This problem was overcome by the preparation and subsequent analysis of a kidney sample spiked with high levels of trimethoprim (the earliest eluting drug) and sulphamethazine (eluting after the last drug of interest). Sulphamethazine was introduced as a retention range marker because it exhibited a slightly longer retention time that dienestrol, but gave a sharp chromatographic peak in contrast to the tailing obtained with dienestrol. The SFE–SFC chromatogram obtained for a kidney sample spiked with 150 mg/kg of the marker drugs is shown in Fig. 3.

Although precise extraction efficiencies for the drugs have not been established during these preliminary investigations, re-analysis by SFE–SFC–MS–MS of the dried residue of a sample initially analysed after spiking at 10 mg/kg suggested that all extractable drugs had been recovered in the first cycle.

We have previously discussed[15] some of the mass spectral properties desirable for the development of an MS–MS procedure for target compound detection. Direct probe introduction of standards was used during preliminary mass spectral studies since this permitted control of the evaporation rate to give moderately persistent spectra and thus facilitated adjustment of the mass spectrometer tuning. The electron impact (EI) mass spectra obtained for trimethoprim, hexestrol, diethylstilbestrol and

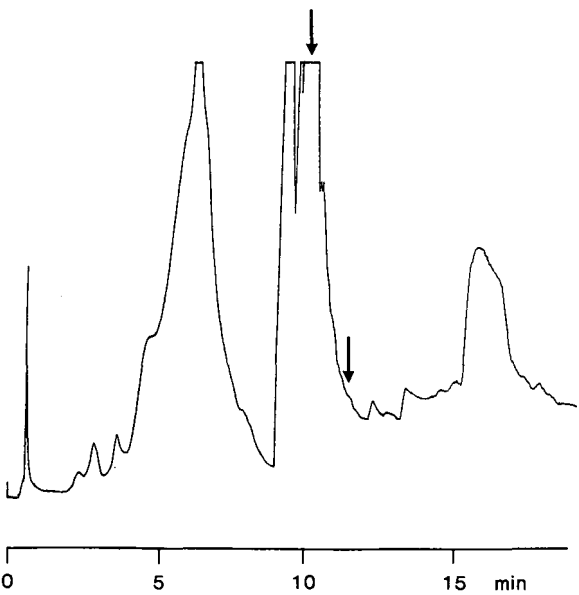


Fig. 4. SFE–SFC analysis of a blank kidney sample. UV detection at 215 nm. Range 1.6 a.u.f.s. Elution range of drugs indicated by arrows.

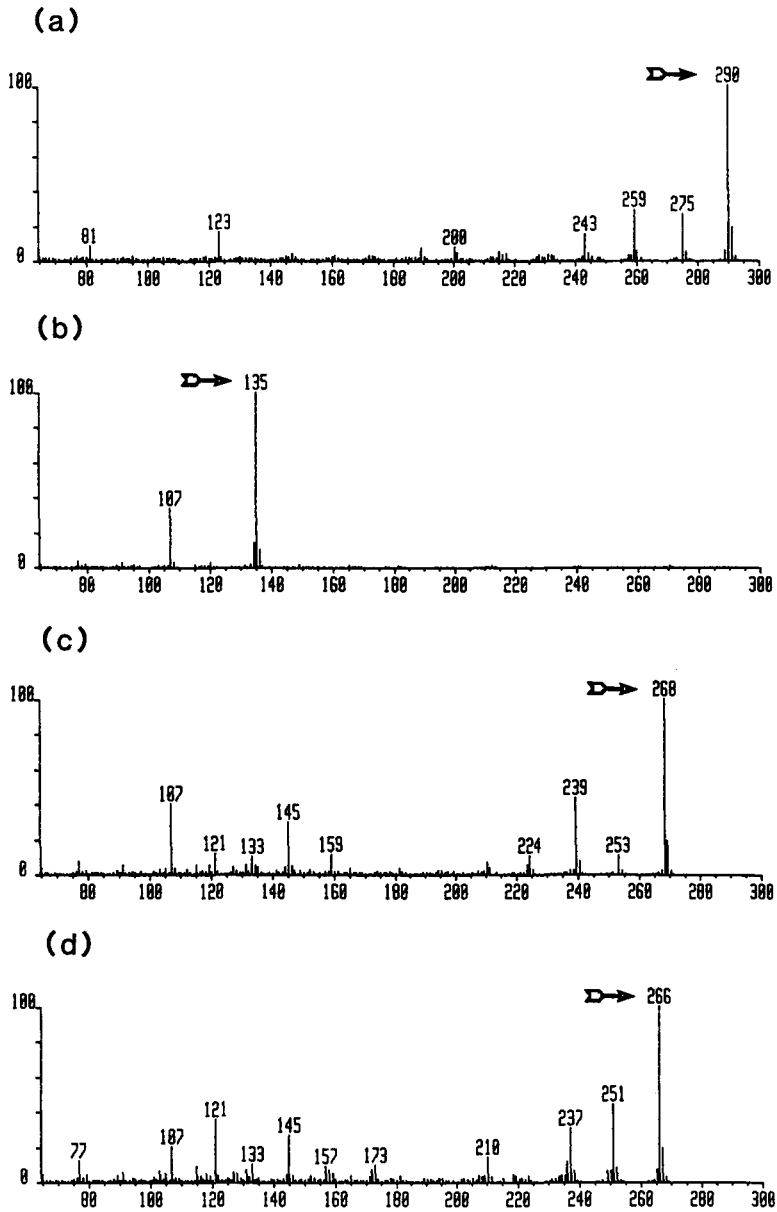


Fig. 5. Direct insertion probe EI mass spectra obtained for (a) trimethoprim, (b) hexestrol, (c) diethylstilbestrol, (d) dienestrol.

dienestrol are shown in Fig. 5. With the exception of hexestrol, the molecular ion of each drug is the most abundant species in the mass spectrum and was selected as the parent ion for collision-induced dissociation (CID) MS–MS experiments. In the case

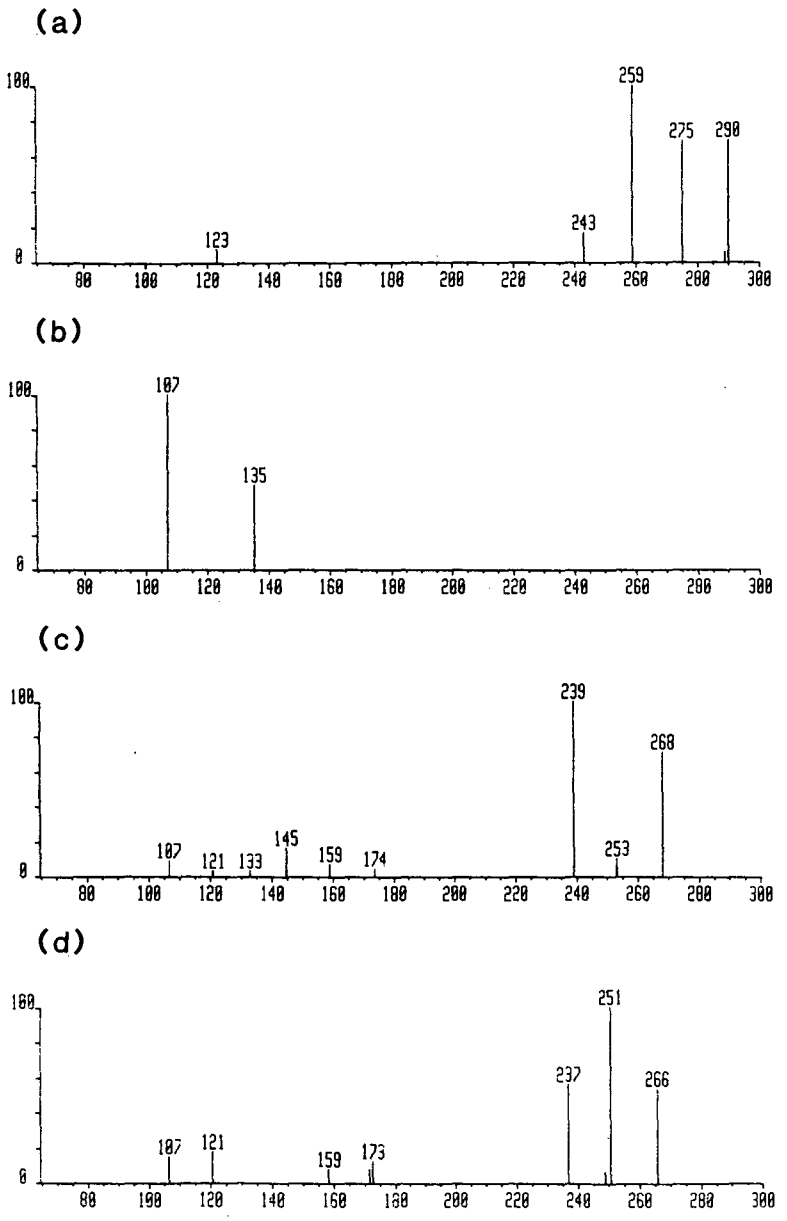


Fig. 6. Direct insertion probe CID daughter ion mass spectra obtained for (a) $M^{+\cdot}$ (m/z 290) trimethoprim, (b) m/z 135 hexestrol, (c) $M^{+\cdot}$ (m/z 268) diethylstilbestrol, (d) $M^{+\cdot}$ (m/z 266) dienestrol.

of hexestrol the fragment ion at m/z 135 was selected. The CID daughter ion mass spectrum of each drug is shown in Fig. 6.

In experiments using SFE–SFC–MS the intense background mass spectra of the

co-chromatographing endogenous components prevented the analysis of the drugs. The mass spectrum obtained at the retention time corresponding to trimethoprim is shown in Fig. 7, where the mass spectrum of the drug is completely obscured. SFE–SFC–MS–MS analysis provided daughter ion spectra which were virtually free of interferences (Fig. 8) and permitted the unambiguous detection of the drugs at the 10-mg/kg level.

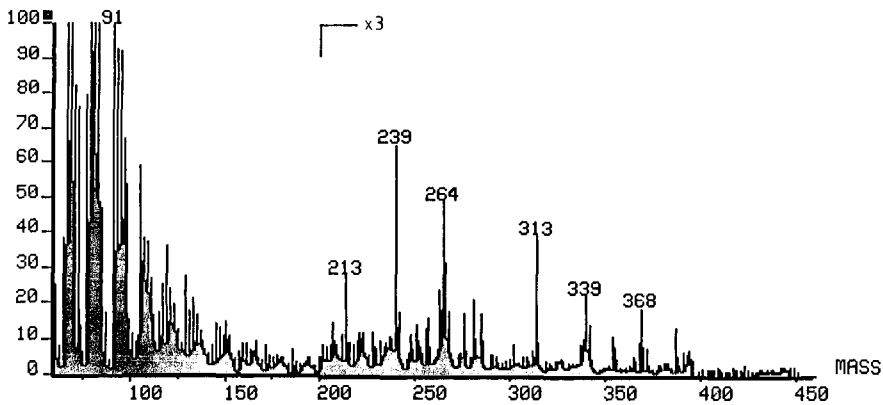


Fig. 7. EI mass spectrum obtained at retention time of trimethoprim. Result from SFE–SFC–MS analysis of kidney sample spiked with 10 mg/kg of each drug.

The technique of selected-reaction monitoring (SRM) seeks to extend the sensitivity of MS–MS in an analogous manner to selected-ion monitoring in conventional MS, and records information about selected daughter ions formed from selected parents. Although we have previously reported[16] that the neutral noise effect sometimes observed on our instrument has precluded SRM analyses of some matrices, in the present study SRM was found to be a useful means of extending the sensitivity of SFE–SFC–MS–MS and analysis of blank kidney samples did not indicate problems associated with neutral noise effects or chemical interference. The results of an SRM SFE–SFC–MS–MS analysis, using two reactions per drug, for a kidney sample spiked with 1 mg/kg of each of the three drugs trimethoprim, dienestrol and diethylstilbestrol are shown in Fig. 9. Hexestrol could also be detected at the 1-mg/kg level with this approach but the simplicity of the CID daughter ion spectrum under the specified collision conditions, meant that only one reaction was suitable for monitoring.

CONCLUSIONS

This preliminary study demonstrates considerable potential for the combination of SFE–SFC–MS–MS in the detection of trace levels of contaminants in foods and other matrices. Although in the current study the detection limits are not sufficiently low to meet the stringent controls on drug residues in meat for human consumption, we believe that there is considerable scope for further optimisation in this respect.

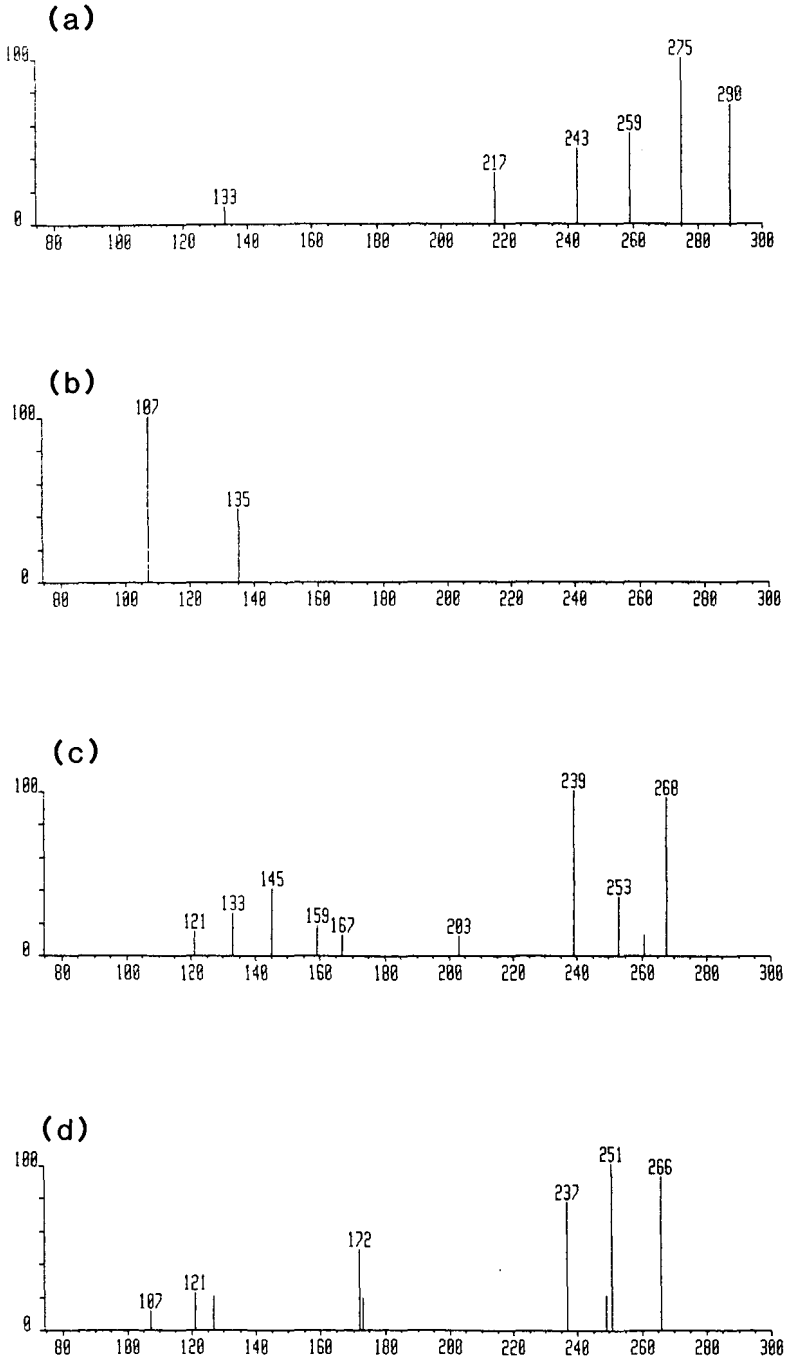


Fig. 8. CID daughter ion mass spectra obtained for (a) trimethoprim, (b) hexestrol, (c) diethylstilbestrol, (d) dienestrol. Results from SFE–SFC–MS–MS analysis of kidney sample spiked with 10 mg/kg of each drug.

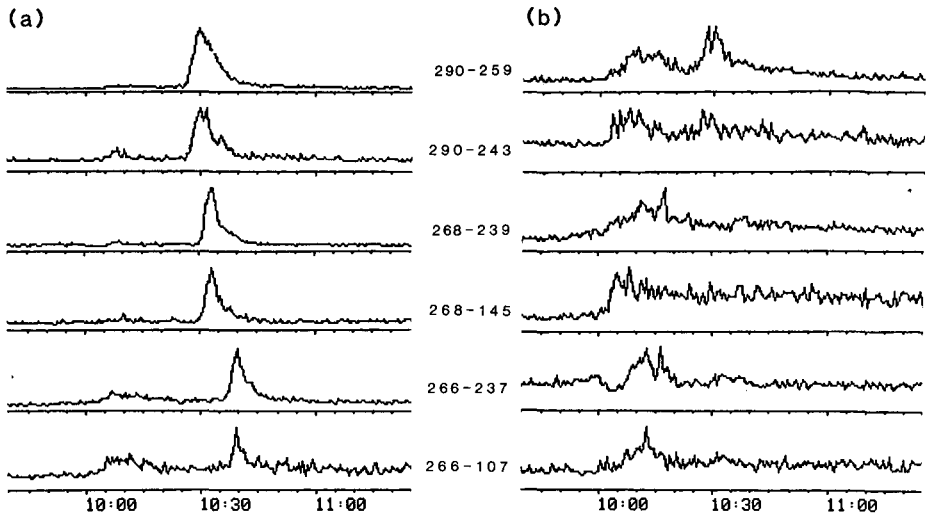


Fig. 9. SRM chromatograms obtained from SFE–SFC–MS–MS analysis of (a) kidney spiked with 1 mg/kg each of trimethoprim (*m/z* 290–259; *m/z* 290–243), diethylstilbestrol (*m/z* 268–239; *m/z* 268–145) and dienestrol (*m/z* 266–237; *m/z* 266–107), (b) blank kidney.

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HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY OF HOMOGENEOUS D-GLUCO-OLIGOSACCHARIDES AND -POLYSACCHARIDES (POLYMERIZATION DEGREE $\geqslant$ 50) WITH PULSED AMPEROMETRIC DETECTION

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SUMMARY

High-performance anion-exchange chromatography under alkaline conditions with pulsed amperometric detection was applied to the analyses of (1→2)-, (1→3)-, (1→4)- and (1→6)-linked homogeneous α- or β-D-gluco-oligosaccharides and -polysaccharides up to a degree of polymerization (DP) of $\geqslant$ 50. Each series of homogeneous D-gluco-oligomers and -polymers showed a linear relationship between log k' and DP in isocratic elution using 150 m*M* sodium hydroxide solution containing 100 m*M* sodium acetate as the eluent. An effective separation of individual members of an homologous series of linear glucans was achieved using gradient elution, accomplished by maintaining the sodium hydroxide concentration at 150 m*M* and increasing the sodium acetate concentration during the analysis. The detector response per HCOH group in D-gluco-oligomers (DP 2–7) was almost the same.

INTRODUCTION

In polysaccharide structural studies, partial hydrolysis of polysaccharides followed by identification of the resulting oligosaccharides is a powerful aid. We found previously that high-performance liquid chromatography (HPLC) on a 3-μm NH_2-bonded silica column by using a pump minimized pulsating flow, and a refractive index (RI) detector with high sensitivity is effective for analyses of homogeneous D-gluco-oligosaccharide and -polysaccharide mixtures with a degree of polymerization (DP) up to about 30[1]. However, this method suffers serious disadvantages. One of these is that the RI detector does not easily provide a stable baseline at high sensitivity and, moreover, cannot be used with a gradient. In addition, as the silica-based column must be used at pH < 7.5 and therefore only neutral solutions of samples can be applied, saccharides with higher DPs which are soluble only in alkaline solutions cannot be analyzed on the NH_2-bonded silica column.

Recently, we applied high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection, introduced by Rocklin and Pohl[2], to

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the determination of cyclic glucans (DP 6–25) and obtained excellent resolution and high sensitivity[3]. As this new method is expected to overcome the aforesaid limitations of HPLC of oligo- and polysaccharides on NH_2-bonded silica with RI detection, we studied HPAEC of (1→2)-, (1→3)-, (1→4)- and (1→6)-linked homogeneous D-gluco-oligo- and -polysaccharides using pulsed amperometric detection (PAD).

EXPERIMENTAL

Apparatus and column

HPAEC was conducted with a Dionex BioLC Model 4000i system and a Model PAD II pulsed amperometric detector consisting of an amperometric flow-through cell with a gold working electrode, a silver–silver chloride reference electrode and a potentiostat. The sample loop size was 50 μl. The column used was a Dionex HPIC-AS6 (250 mm × 4 mm I.D.) equipped with an AG6 guard column (50 mm × 4 mm I.D.) (all from Dionex, Sunnyvale, CA, U.S.A.). A Chromatopac C-R3A digital integrator (Shimazu Kyoto, Japan) was used to calculate peak areas.

Chromatographic conditions and measurements

The following pulse potentials and durations were used for analysis of homogeneous linear D-gluco-oligo- and -polysaccharides at range 2 (sampling period, 200 ms): $E_1 = 0.10$ ($t_1 = 300$); $E_2 = 0.60$ ($t_2 = 120$); $E_3 = -0.80$ V ($t_3 = 300$ ms). The response time of the PAD II detector was set to 1.0 s. Eluents prepared daily were degassed by sonication under bubbling of helium gas and kept under a stream of nitrogen. The sample solutions were prepared using 18 MΩ cm deionized water and filtered through a 0.2-μm membrane filter. All separations were carried out at ambient temperature with a flow-rate of 1 ml/min.

Materials

Cyclosophoraose [Cys, cyclic (1→2)-β-D-glucan]-P (DP 32) was isolated from a culture filtrate of *Rhizobium meliloti* IFO 13336 according to the method described previously[4]. (1→3)-α-D-glucan was extracted from the fruit body of *Laetiporus sulphureus* with 1 *M* sodium hydroxide for 3 h at room temperature[5]. Short-chain amylose EX-1 ($\overline{DP} \approx 17$) [a mixture of (1→4)-α-D-glucans] was a gift from Hayashibara Biochemical Lab. (Okayama, Japan). Absorbent cotton, of Japanese Pharmacopoeial standard, was used as cellulose [(1→4)-β-D-glucan]. Luteose [(1→6)-β-D-glucan] and curdlan [(1→3)-β-D-glucan)] were gifts. Dextran [(1→6)-α-D-glucan], maltose and gentiobiose were commercially available. Malto- (DP 3–7), sophoro- (DP 2–5), laminara- (DP 2–6) and gentio-oligomers (DP 3–6) were all gifts. These oligomers were individually purified by HPLC on a YMC-Pack PA-03 (250 mm × 4.6 mm I.D.) column (Yamamura Chemical, Kyoto, Japan) with acetonitrile–water as the eluent. All reagents were of analytical reagent grade. The eluent A was 150 m*M* sodium hydroxide solution which was prepared by dilution of carbonate-free 50% sodium hydroxide solution in 18 MΩ cm deionized water. The eluent B was 150 m*M* sodium hydroxide solution, containing 500 m*M* sodium acetate; 300 m*M* sodium hydroxide solution, prepared from carbonate-free 50% sodium hydroxide solution, was diluted in the same volume of 1000 m*M* sodium acetate solution, prepared with 18 MΩ cm deionized water and filtered through a 0.2-μm membrane filter.

Preparation of homologous series of linear glucans

(1→2)-β-D-Glucosaccharides. Cyclosophoraose-P (2.5 mg) was hydrolysed in 1 ml of 0.1 *M* trifluoroacetic acid (TFA) at 100°C for 60 min. After removing TFA by evaporation, the hydrolysate was dissolved in 0.5 ml of deionized water.

(1→6)-α- and (1→6)-β-D-glucosaccharides. Dextran (10 mg) and luteose (10 mg) were individually hydrolysed in 5 ml of 0.3 *M* TFA at 100°C for 30 and 40 min, respectively. Their hydrolysates were dissolved in 1 and 2 ml of deionized water, respectively.

(1→3)-α- and (1→3)-β-D-glucosaccharides. (1→3)-α-D-Glucan (10 mg) of *Laetiporus sulphureus* and curdlan (10 mg) were individually formolysed with 90% formic acid (10 ml) at 90°C for 60 min, and after evaporation of the excess of formic acid the formates were hydrolysed at 100°C under the following conditions; 0.05 *M* TFA (10 ml) for 45 min for the former and 0.1 *M* TFA (10 ml) for 60 min for the latter. The hydrolysates were dissolved in 2 ml of deionized water.

(1→4)-β-D-Glucosaccharides. After formolysis with 90% formic acid at 100°C for 30 min, cellulose was subjected to partial acetolysis using acetic acid–acetic anhydride–sulphuric acid (10:10:1) at 40°C for 10 h. A part of the acetolysate obtained was deacetylated in the usual way. A 0.3% deionized water solution of the deacetylation product was used for an HPAEC analysis. The acetolysate was also used after deacetylation by stirring in 150 m*M* sodium hydroxide solution immediately before HPAEC.

(1→4)-α-D-Glucosaccharides. A sample of short-chain amylose EX-1 (3 mg) was dissolved in 1 ml of deionized water or 150 m*M* sodium hydroxide solution.

RESULTS AND DISCUSSION

Relationship between log k′ and DP in isocratic elution

HPAEC of each homologous series of D-gluco-oligo- and -polysaccharides on an HPIC-AS6 column was investigated with an isocratic elution using 150 m*M* sodium hydroxide solution containing 100 m*M* sodium acetate as the eluent. Of all D-glucans examined, (1→6)-α-D-glucans were most rapidly eluted and (1→3)-β-D-glucans were retained longest on the column. In each series, a plot of log k' against DP gave a straight line (Fig. 1A). Although these homologous series of D-glucans had an individual linear relationship between log t_R and DP on an amino column (ERC-NH-1171) with acetonitrile–water (Fig. 1B), and between log k' and DP on a reversed-phase column (Asahipak ODP-50) with sodium hydroxide solution (pH 11)[6], too, the elution order of glucans, having different types of linkages and the same DP, on an HPIC-AS6 column was different relative to that not only on an amino column but also on a reversed-phase column. According to Rendleman's review[7], carbohydrates are weak acids having ionization constants in the range of 10^{-12}–10^{-14}; in aqueous media the acidity of the hydroxyl groups in methyl D-glucopyranoside decreases in the order 2-OH ≫ 6-OH > 3-OH > 4-OH, and substitution for an hydroxyl group at C-2 should increase the acidities of the 3- or 4-hydroxyl groups. As it is thought that substitution of the hydroxyl group having stronger acidity in the molecule results in less retention, some of the retention behaviour of D-glucans can be explained on the basis of the acidity of the hydroxyl groups in methyl D-glucopyranoside mentioned above. However, it was not explicable why (1→6)-α-D-glucans are eluted first and much more

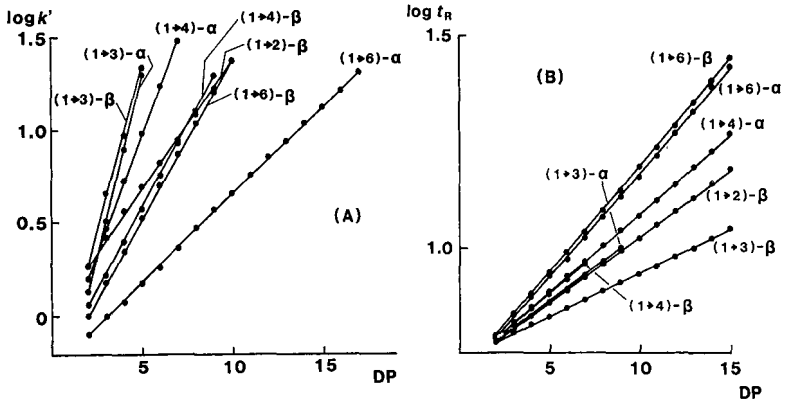


Fig. 1. Relationship between log k' and DP on an HPIC-AS6 column with 150 mM sodium hydroxide solution containing 100 mM sodium acetate (A), and as a reference relationship between log t_R and DP on an ERC-NH-1171 column with acetonitrile–water (57:43) (B). (1→2)-β, Sophoro-saccharides; (1→3)-α, nigero-saccharides; (1→3)-β, laminara-saccharides; (1→4)-α, malto-saccharides; (1→4)-β, cello-saccharides; (1→6)-α, isomalto-saccharides; (1→6)-β, gentio-saccharides.

rapidly than corresponding (1→6)-β-D-glucans and why (1→4)-β-D-glucans move faster than corresponding (1→4)-α-D-glucans on the column. Further, the acidity of 4-OH seems to be stronger than that of 3-OH.

Separation of each series of homogeneous D-*glucans*

An effective separation of individual members of an homologous series of D-glucans was achieved using gradient elution, which was accomplished by maintaining the sodium hydroxide concentration at 150 mM and increasing the sodium acetate concentration during the analysis. In Table I, gradient programmes used for the separation of (1→2)-, (1→3)-, (1→4)- and (1→6)-linked homologous D-gluco-oligosaccharides and -polysaccharides are summarized.

Fig. 2 shows the HPAEC elution profile of short-chain amylose EX-1 ($\overline{DP} \approx 17$). The number on each peak, indicating its DP, was confirmed by adding malto-oligosaccharides of known DPs. Amylose tends to precipitate from the solution and to retrograde on the column during liquid chromatography, but these phenomena are avoidable in high pH eluents such as 150 mM sodium hydroxide solution. Although amylose EX-1 dissolved more easily in 150 mM sodium hydroxide solution and the alkaline sample solution gave essentially the same chromatogram as that of the aqueous solution, after storage for 3 days in a refrigerator small peaks of epimerization products appeared in the chromatogram of the alkaline sample solution.

In the chromatogram of partial hydrolysates of cyclic (1→2)-β-D-glucan (Cys-P, DP 32), the residual cyclic glucan appears just behind the linear glucan of DP 21 (Fig. 3). This phenomenon suggested that the separation mode on an HPIC-AS6 column was not only simple anion exchange, but also involved some hydrophobic interactions. In the case of cyclic (1→4)-α-D-glucan (cyclodextrin, CD), α-CD (DP 6) and γ-CD (DP 8) were eluted together with maltotetraose (DP 4) and maltooctaose (DP 8), respectively, and β-CD (DP 7) appeared just prior to maltodecaose (DP 10). On an amino column, cyclic glucans moved together with the linear glucans having one or

TABLE I

GRADIENT PROGRAMMES USED FOR THE SEPARATION OF (1→2)-, (1→3)-, (1→4)- AND (1→6)-LINKED HOMOGENEOUS D-GLUCO-OLIGOSACCHARIDES AND -POLYSACCHARIDES

Eluents: A, 150 m*M* sodium hydroxide solution; B, 150 m*M* NaOH solution containing 500 m*M* sodium acetate. Eluent composition is changed linearly over a specified time.

Programme for	*Time (min)*	*Eluent A (%)*	*Eluent B (%)*	*Concn. of sodium acetate in eluent (mM)*
(1→2)-β-D-Glucans	0	75	25	125
	15	60	40	200
	40	50	50	250
(1→3)-α-D-Glucans	0	80	20	100
	1	40	60	300
	15	0	100	500
(1→3)-β-D-Glucans	0	70	30	150
	2	40	60	300
	20	0	100	500
(1→4)-α-D-Glucans	0	60	40	200
	10	40	60	300
	30	20	80	400
(1→4)-β-D-Glucans	0	80	20	100
	10	50	50	250
	30	40	60	300
(1→6)-α-D-Glucans	0	90	10	50
	1	80	20	100
	10	70	30	150
	30	60	40	200
(1→6)-β-D-Glucans	0	80	20	100
	3	70	30	150
	30	50	50	250

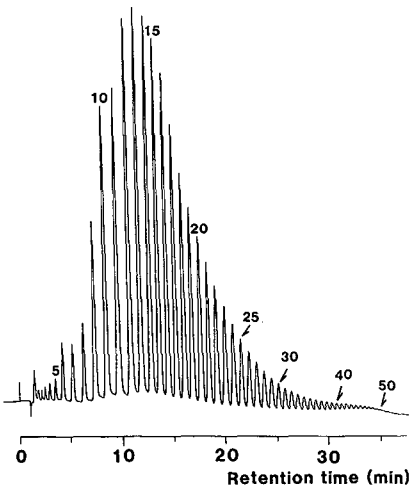
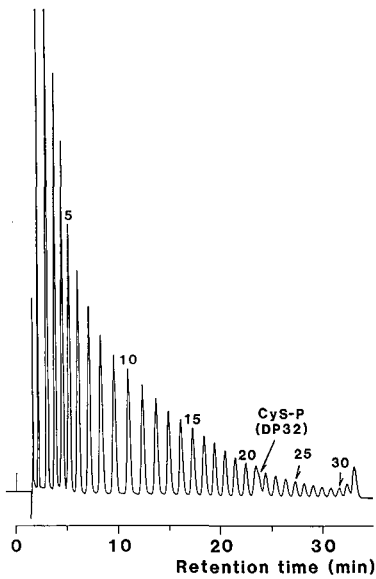


Fig. 2. Chromatogram of (1→4)-α-D-glucans [short-chain amylose EX-1 ($\overline{DP} \approx 17$)]. The number on each peak indicates its DP. Chromatographic conditions: column, HPIC-AS6 (250 mm × 4 mm I.D.); eluent and gradient programme as shown in Table I; flow-rate, 1 ml/min; detector, PAD II; meter scale, 10 K nA; temperature, ambient.



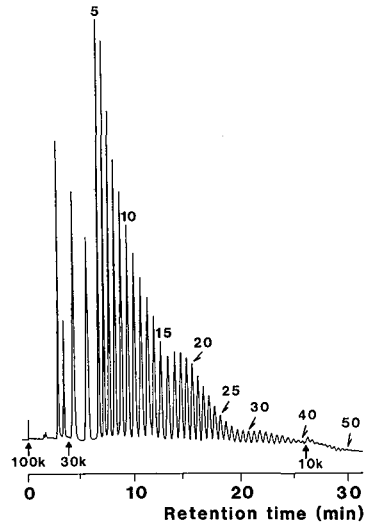
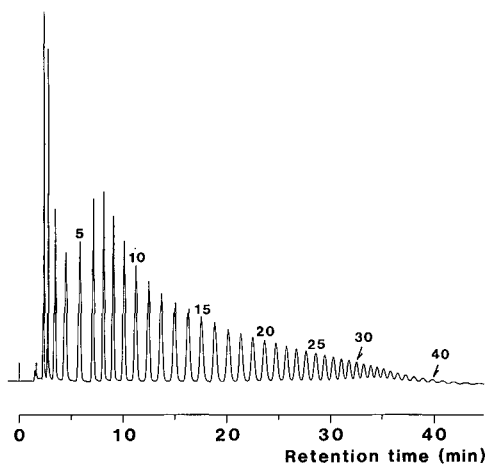


Fig. 3. Chromatogram of (1→2)-β-D-glucans [partial hydrolysates of CyS-P (DP 32)]. Chromatographic conditions: meter scale, 30 K nA; other conditions as in Fig. 2.

Fig. 4. Chromatogram of (1→6)-α-D-glucans (partial hydrolysates of dextran). Chromatographic conditions: meter scale, 100, 30 and 10 K nA; other conditions as in Fig. 2.



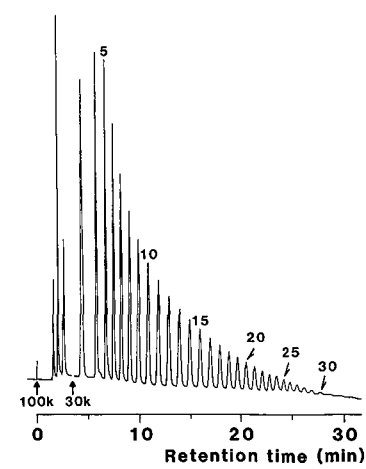


Fig. 5. Chromatogram of (1→6)-β-D-glucans (partial hydrolysates of luteose). Chromatographic conditions as in Fig. 3.

Fig. 6. Chromatogram of (1→3)-β-D-glucans (partial hydrolysates of curdlan). Chromatographic conditions: meter scale, 100 and 30 K nA; other conditions as in Fig. 2.

two DP units less[1], because the cyclic glucan has two hydroxyl groups less than the corresponding linear glucan.

Figs. 4–7 illustrate separations of the partial hydrolysates of dextran [α-(1→6)], luteose [β-(1→6)] and curdlan [β-(1→3)], and partial acetolysates of cellulose [β-(1→4)]. Around 45–50 distinct peaks can be seen in Figs. 4 and 5. By previous HPLC on NH_2-bonded silica with acetonitrile–water, using a sensitive RI detector, (1→6)-linked glucans were detected only up to about DP 30. Furthermore, no distinct peaks of higher polymers with DP > 19 were observed in the chromatogram of the hydrolysates of curdlan, because the β-(1→3)-linked higher polymers are not sufficiently soluble in water, and the β-(1→4)-linked D-glucans are more insoluble and hence only ten distinct peaks were observed in the HPLC chromatogram of the partial acetolysates of cellulose[1]. On the other hand, by HPAEC on an HPIC-AS6 column with alkaline eluents, using PAD, β-(1→3)-linked D-glucans of DP up to 30 and β-(1→4)-linked D-glucans of DP up to 22 were observed as distinct peaks in each chromatogram (Figs. 6 and 7). When the hydrolysates of curdlan were dissolved in 150 m*M* sodium hydroxide solution, the number of distinct peaks in the chromatogram increased up to 35. Fig. 7 shows a chromatogram of an aqueous mixture of β-(1→4)-linked D-glucans, prepared by deacetylation of partial acetolysates of cellulose. When the partial acetolysates of cellulose were dissolved in 150 m*M* sodium hydroxide solution by stirring for 30 min, the resulting deacetylates mixture in alkaline

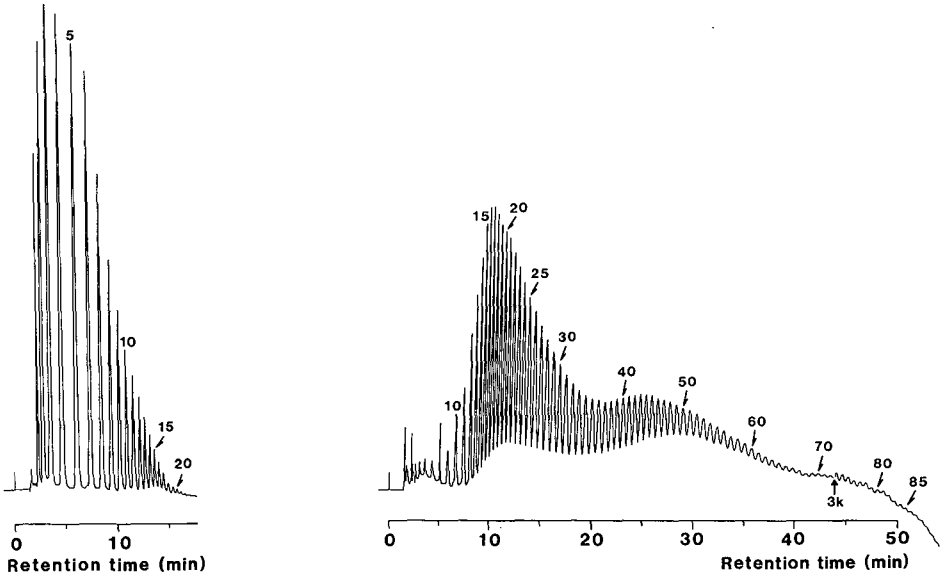


Fig. 7. Chromatogram of (1→4)-β-D-glucans (partial acetolysates of cellulose). Chromatographic conditions as in Fig. 3.

Fig. 8. Elution profile of a mixture of maltodextrins prepared from starch of Amylo-Waxy maize. Chromatographic conditions: gradient programme (% of eluent B), 40 at 0 min; 60 at 5 min, 70 at 20 min, 80 at 45 min and 90 at 70 min. Attenuation rose from 10 to 3 K nA full scale for the last 10 min; other conditions as in Fig. 2.

TABLE II

RELATIVE DETECTOR RESPONSES OF D-GLUCO-OLIGOMERS

Chromatographic conditions as in Fig. 2. The amounts of D-gluco-oligomers used were 5 nmol each.

DP	*No. of HCOH*	*Relative detector response*[a]			
		(1→4)-α-	*(1→2)-β-*	*(1→3)-β-*	*(1→6)-β-*
2	8	1.00	1.00	1.00	1.00
3	11	1.39	1.36	1.38	1.21
4	14	1.72	1.70	1.63	1.51
5	17	2.06	2.04	2.03	1.78
6	20	2.33		2.28	2.07
7	23	2.59			

[a] (1→4)-α-, Malto-oligomers; (1→2)-β-, sophoro-oligomers; (1→3)-β, laminara-oligomers; (1→6)-β-, gentio-oligomers.

solution was filtered and was directly chromatographed, 28 distinct peaks being detectable.

These results indicate that if polymers having higher DPs and soluble in alkaline solution are available, the numbers of detectable peaks should be increased. Fig. 8 shows an elution profile of a mixture of maltodextrins prepared from starch of Amylo-Waxy maize[8] by debranching with isoamylase and removing most of the water-soluble components by repeated extraction with hot water. The sample was dissolved in a small amount of 1 *M* sodium hydroxide solution and diluted five times in deionized water. The elution programme for the short-chain amylose (Table I) was modified for an effective separation of the maltodextrins (see the legend of Fig. 8).

Relative detector response

From the individual chromatogram of malto- (DP 2–7), sophoro- (DP 2–5), laminara- (DP 2–6) and gentio-oligomers (DP 2–6) (5 nmol each), obtained by the respective elution programme shown in Table I, the relative detector response was evaluated. The results indicated that the sensitivity of detection does not decrease with increasing molecular weight. The detector response per HCOH group in those oligosaccharides was almost the same (Table II). This is favourable for analysis of higher oligomers and polymers, and one of the principal advantages of the new method with PAD.

ACKNOWLEDGEMENTS

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DETERMINATION OF LIMONIN IN GRAPEFRUIT JUICE AND OTHER CITRUS JUICES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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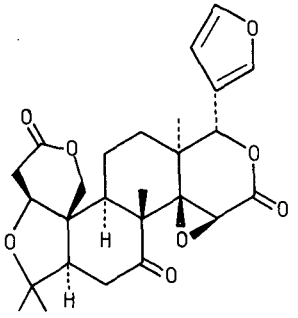
SUMMARY

A method has been developed for the quantitation of the bitter component limonin in grapefruit juice and other citrus juices. The sample clean-up consisted of centrifugation, filtration and a selective, rapid and reproducible purification with a C_2 solid-phase extraction column. The limonin concentration was determined by high-performance liquid chromatography on a C_{18} column with UV detection at 210 nm. A linear response was obtained from 0.0 to 45 ppm limonin. The minimum detectable amount was 2 ng. The minimum concentration which was detected without concentration with good precision was 0.1 ppm. The method was also used for the determination of limonin in different types of oranges, including navel oranges, mandarins, lemons, limes, pomelos and uglis.

INTRODUCTION

Limonoids, a group of oxygenated tetranortriterpenoids, are of general occurrence in the genus *Citrus*. Two of these compounds, limonin (**1**) and nomilin (**2**), have a very bitter taste which is still detectable in concentrations of 2–5 ppm[1]. As they occur in the economically important oranges and grapefruits their exact concentration is of much interest to the citrus industry. Too high a concentration of limonin causes excessive bitterness which is not acceptable to the consumer.



Limonin (**1**)

0021-9673/89/$03.50

Of nomilin and limonin the latter is the more important because of its more widespread occurrence and its usually higher concentration. Limonin occurs in two forms: the bitter dilactone **1** and the non-bitter monolactone **3**. The monolactone, which is stored separately in carpellary membrane tissues in the intact fruit[2], is however converted into the bitter dilactone within hours once it comes in contact with the acidic juice during processing[1,3]. This explains the delayed bitterness which is sometimes observed in processed citrus juices, especially in early season grapefruits and navel oranges. Recently several investigations have been carried out on the reduction of the concentrations of bitter principles in juices of early season oranges and grapefruits[4–8]. To check the effect of the various techniques it is necessary to be able to measure the concentrations before and after treatment.

Due to the interest of the citrus industry in the limonin concentration a large number of quantitations have been published. Limonin has been determined by thin-layer chromatography (TLC)[9,10], chemical derivatization followed by spectrometry[11,12] or fluorimetry[13], gas–liquid chromatography (GLC)[14], high-performance liquid chromatography (HPLC)[15–20], radioimmunoassay (RIA)[21] and enzyme immunoassay (EIA)[22,23]. Two articles have summarized and discussed the different methods for the determination of limonin[1,24].

Several HPLC determinations have been published using either reversed (C_{18}, C_8, CN) or straight-phase (CN) materials. The major problem of the HPLC determinations is the absence of any selective chromophore in limonin ($\lambda_{max.}$ 207 nm) and the low concentration in the citrus juices (1–20 ppm). The sample clean-up has to be very thorough. This results in time-consuming partitions, usually with chloroform. The actual HPLC run takes from 10 to 20 min. A recently published method uses small solid-phase extraction columns instead of partitions, which resulted in a considerable time gain[18,25]. This advantage was however partially offset by the diminished quality of the resulting chromatograms. Especially in grapefruit juice extracts, the limonin is only a minor peak when compared with several impurities. Also the analysis time was much longer due to the long retention time of some major impurities when compared with for instance the straight-phase system of Rouseff and Fisher[17].

In this article we report on an improved method for the determination of limonin in various citrus juices using solid-phase extraction columns with a selective purification step and a C_{18} column with UV detection.

EXPERIMENTAL

Solvents

The acetonitrile, methanol and tetrahydrofuran (THF) used were of HPLC quality. Water was doubly distilled in an all-glass apparatus. All solvents were filtered (0.45 μm) and ultrasonically degassed before use.

Standards

The limonin used for recovery experiments and calibration graphs was supplied by Dr. R. L. Rouseff. Its purity was checked by HPLC in various solvents, TLC and 300-MHz ^{1}H NMR. The limonin and nomilin used for comparison of retention times were supplied by Dr. J. H. Tatum. A purified extract of grapefruit seeds was also used for this purpose. This extract was prepared by crushing several seeds, extracting them

in acetonitrile for 1 h with ultrasonic agitation, diluting the crude extract in four parts of water and purifying the turbid solution over a C_2 column as described below for the HPLC samples.

Plant materials

All fruits were obtained locally from Dutch greengrocers or supermarkets. When known, the type and the country of origin are given in the text.

Instrumentation

All separations were carried out isocratically at room temperature (20°C) with a Gilson 303 HPLC pump, equipped with a Rheodyne Model 7125 injector with a 20-μl or a 100-μl loop. The detection was carried out at 210 nm with a Gilson 116 variable-wavelength detector. Integration was done with a Shimadzu CR3A integrator. The column (150 mm × 4.6 mm) and guard (15 mm × 4.6 mm) were manufactured by Rainin-U.S.A. and packed with 5-μm C_{18} material (Microsorb, Cat. No. C_{18} 80-215-C5).

Chromatographic conditions

Three different solvent systems were used: (1) acetonitrile–methanol–water (28.5:13:58.5), flow-rate 2.0 ml/min (standard solvent). (2) acetonitrile–methanol–water (31.8:22.7:45.5), flow-rate 1.0 ml/min; (3) methanol–water (65:35), flow-rate 1.0 ml/min.

Identification and purity of peaks

Limonin and nomilin were identified in the chromatogram by comparing the retention times of the various peaks with those of authentic reference samples.

The identity and purity of limonin and the two compounds corresponding to the adjacent peaks in the HPLC chromatogram of grapefruit juice with solvent system 1 was also checked by recording UV, mass and 300-MHz ^{1}H NMR spectra of collected HPLC fractions. For this purpose 600 ml of grapefruit juice were processed on a larger scale but in exactly the same way as described below for the quantitation of limonin (column 25 cm × 1 cm, 5-μm C_{18}). The purity and identity of the limonin fraction was additionally investigated by HPLC with solvent system 3 and TLC (ready-made silica gel 60F_{254} plates, Merck, Cat. No. 5719). The following two TLC systems were used in saturated chambers: (1) toluene–ethanol–water–acetic acid (76:18:5.7:0.5) (limonin, R_F 0.21; nomilin, R_F 0.18); (2) 100% ethyl acetate (limonin, R_F 0.61; nomilin, R_F 0.48). Detection on the TLC plates was carried out by viewing under UV light at 254 and 366 nm followed by spraying with 5% *p*-dimethylaminobenzaldehyde in ethanol and placing the plates in a warm hydrogen chloride atmosphere[26]. The purity of the limonin peak in the chromatograms of citrus juices other than grapefruit juice was checked by recording the chromatogram both at 210 and 254 nm. When only a baseline was observed at the retention time of limonin in the chromatogram at 254 nm the limonin peak was considered pure.

Extraction and purification procedure

A grapefruit or other citrus fruit was cut in two and immediately extracted by hand on a lemon squeezer. The juice was centrifuged for 30 min in an Heraeus labofuge

I at 2100 *g* and the supernatant filtered over a filter paper (Schut, V259, diameter 5.5 cm for Büchner).

A Bond Elut® (Analytichem International) 500-mg C_2 solid-phase extraction column was washed with 4 ml acetonitrile and equilibrated with 4 ml acetonitrile–water (2:98). Next 2.00 ml of the clear, filtered juice were introduced on the column, the column was washed with 4 ml acetonitrile–water (30:70) and finally the limonin was eluted with 2 ml acetonitrile–water (60:40). This fraction was collected in a 2-ml volumetric flask, and water was added to 2.00 ml. The column was never allowed to run dry. The flow-rate through the column was 2.5 ml/min with the wash solvent and 4.7 ml/min with the extraction solvent (constant vacuum). The limonin-containing solution in the volumetric flask was directly injected into the HPLC column.

To determine the concentration of both limonin and its precursor limonin monolactone (total limonin), the pressed juice was heated at 80°C for 15 min after centrifugation.

Calibration graph and reproducibility of integration

A calibration graph for limonin was prepared by injecting 20 μl of ten known concentrations (0.000, 0.430, 1.09, 3.12, 5.45, 7.84, 11.36, 18.18, 27.27 and 45.45 ppm) and plotting the integration value from 0.0 to 45 ppm against the concentration. A linear detector response from 0.0 to 45 ppm limonin was observed ($r = 0.99993$). All injections were done five times. The reproducibility of the integration process was determined for five standard solutions. Each solution was injected five times and standard deviations were calculated. The results are given in Table I.

TABLE I

STANDARD DEVIATIONS OF INTEGRATION RESULTS OF LIMONIN SOLUTIONS

For conditions see Experimental. $n = 5$.

Limonin concentration (ppm)	*Standard deviation (ppm)*	*Relative standard deviation (%)*
1.160	0.045	3.91
3.136	0.079	2.52
5.454	0.083	1.52
12.35	0.113	0.92
24.96	0.293	1.17

Recovery experiments

Known amounts of limonin in water were submitted to the purification procedure and the relative recovery determined. Each fraction was tested five times on the C_2 column. The resulting extracts were each injected four times for HPLC analysis. Four different concentrations (1.160, 3.136, 12.35 and 24.96 ppm) were investigated. The results are given in Table II.

Standard addition experiments

To grapefruit juice with a known limonin concentration (determined on the same day using the same procedure), various but exactly known quantities of limonin were

TABLE II

RESULTS OF RECOVERY EXPERIMENTS

For conditions see Experimental. $n = 5$.

Initial limonin concentration (ppm)	*Concentration after C_2 column (ppm)*	*Recovery (%)*	*Standard deviation (ppm)*	*Relative standard deviation (%)*
1.16	1.11	95.5	0.026	2.24
3.14	3.12	99.4	0.075	2.40
12.35	12.02	97.3	0.137	1.11
24.96	25.19	100.9	0.362	1.45

added. The resulting enriched solutions were processed according to the described procedure. The limonin concentration of the grapefruit juice, the limonin-containing solution and the spiked grapefruit juice were each purified five times over the C_2 column. Each of the resulting fifteen extracts was injected four times for HPLC analysis. The results are given in Table III.

Quantitation

The citrus fruits to be investigated were worked up in triplicate according to the above purification procedure. Each resulting extract was injected in triplicate for HPLC. The limonin concentration of the juice was calculated by comparing the integration values with the calibration graph. The results are given in Table IV.

TABLE III

RESULTS OF STANDARD ADDITION EXPERIMENTS

For conditions see Experimental. $n = 5$.

Grapefruit sample no.	*Limonin concentration (ppm)*	*Limonin added (ppm)*	*Expected concentration (ppm)*	*Concentration found (ppm)*	*Recovery (%)*	*Stand. dev. (ppm)*	*Rel. stand. dev. (%)*
1	6.25	10.33	16.58	16.50	99.5	0.09	0.54
2	8.95	5.53	14.48	14.14	97.7	0.17	1.17
3	8.96	10.78	19.74	19.35	98.0	0.27	1.37

RESULTS AND DISCUSSION

Our goal was to develop a simple, rapid, sensitive and accurate determination of limonin in grapefruit and preferably other citrus juices. As the final separation and quantitation technique, reversed-phase HPLC (standard C_{18}) with UV detection was chosen. Many laboratories have HPLC apparatus and such columns available for other analysis so additional costs remain limited to solvents. As UV detectors have become much more sensitive in the last 5 years, the difference in sensitivity between HPLC and the immuno techniques has become very small. A simple and rapid method means no tedious and time-consuming partitions but the use of rapid and reproducible

TABLE IV

LIMONIN CONCENTRATIONS OF VARIOUS CITRUS FRUITS

For conditions see Experimental. n.d. = Not determined.

Fruit	*Type or brand*	*Country of origin*	*Limonin concentration (ppm)*	
			Before heating	*After heating*
Grapefruit	Yellow (Jaffa)	Israel	10.4	n.d.
Grapefruit	Red (Pride)	U.S.A.	0.60	1.17
Orange	Navel	Spain	0.78	5.61
Orange	Grandiosa	Spain	0.66	2.15
Mandarin	Unknown	Argentina	0.74	3.53
Lemon	Unknown	Spain	4.77	4.77
Lime	Unknown	Brazil	20.8	n.d.
Pomelo	Jaffa	Israel	33.1	n.d.
Ugli	Unknown	Jamaica	0.5	1.21

solid-phase precolumns. Several different RP precolumns were investigated, *i.e.*, C_{18}, C_8, C_2, cyclohexyl, phenyl and CN, to determine whether they showed any selective retention of the limonoids or the major impurities (coumarins, flavonoids) present in grapefruits. Surprisingly this was not the case; only the elution strength of the washing and extraction solvents varied.

The composition of the washing and extraction solvent had however a big influence on the purity of the final extract. Acetonitrile–water mixtures gave much better results than methanol–water mixtures. Finally a C_2 column was selected with acetonitrile–water (30:70) as the washing solvent and acetonitrile–water (60:40) as the extraction solvent. The extracts were initially investigated with several different HPLC solvents on a C_{18} column: acetonitrile–water mixtures, methanol–water mixtures and methanol–acetonitrile–water (21.5:26.5:52), according to Shaw[25] the optimum solvent for the determination of limonin in citrus juices. However, none of these solvents showed good results with our purified extract due to either coelution of limonin with impurities, large negative peaks or very late eluting impurities. Because of the low absorption maximum of limonin at 207 nm, only methanol, acetonitrile and water can be used for the HPLC solvent. Many different HPLC solvents consisting of these three solvents were subsequently tried. Finding the optimum solvent was greatly hampered by the fact that water was not the non-selective solvent which it is supposed to be in RP-HPLC. When the water content was changed not only the retention time changed but also the elution order of limonin and two of the most disturbing remaining impurities in the purified sample changed.

Finally two solvents were found to be suitable for the analysis of limonin. The first, acetonitrile–methanol–water (28.5:13:58.5), can be used for all citrus juices investigated by us and is the standard solvent used for all the analyses described in this publication. For a characteristic chromatogram of a purified extract of a yellow grapefruit see Fig. 1a. Due to the high water content the k' value is high, 12.5. For reasonable analysis times (*ca.* 10 min per experiment) the flow-rate has therefore to be quite high, 2 ml/min. If necessary the solvent consumption can be reduced by 80% without loss of resolution, speed or sensitivity by using a 2.0 mm I.D. instead of a 4.6

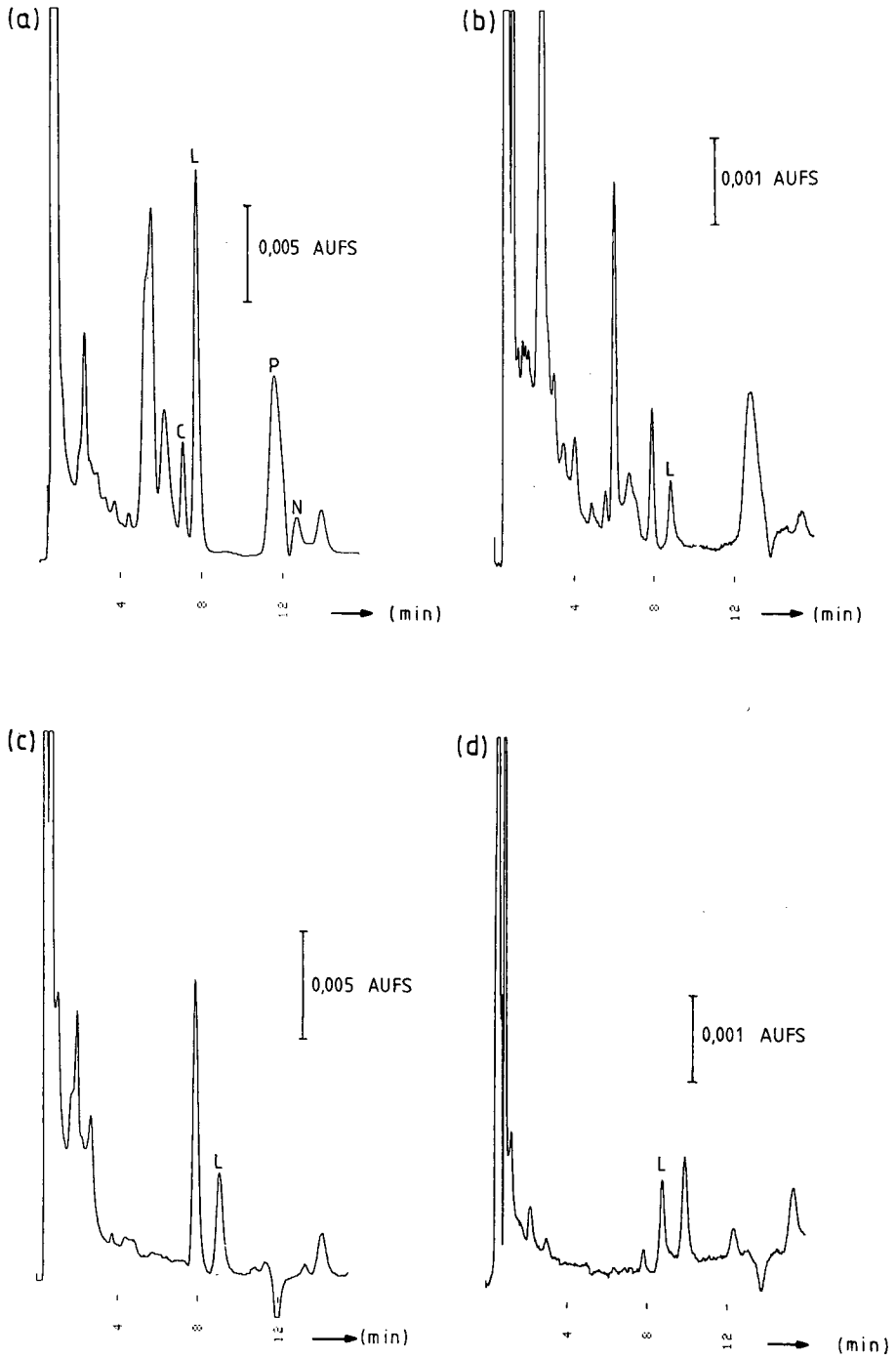


Fig. 1.

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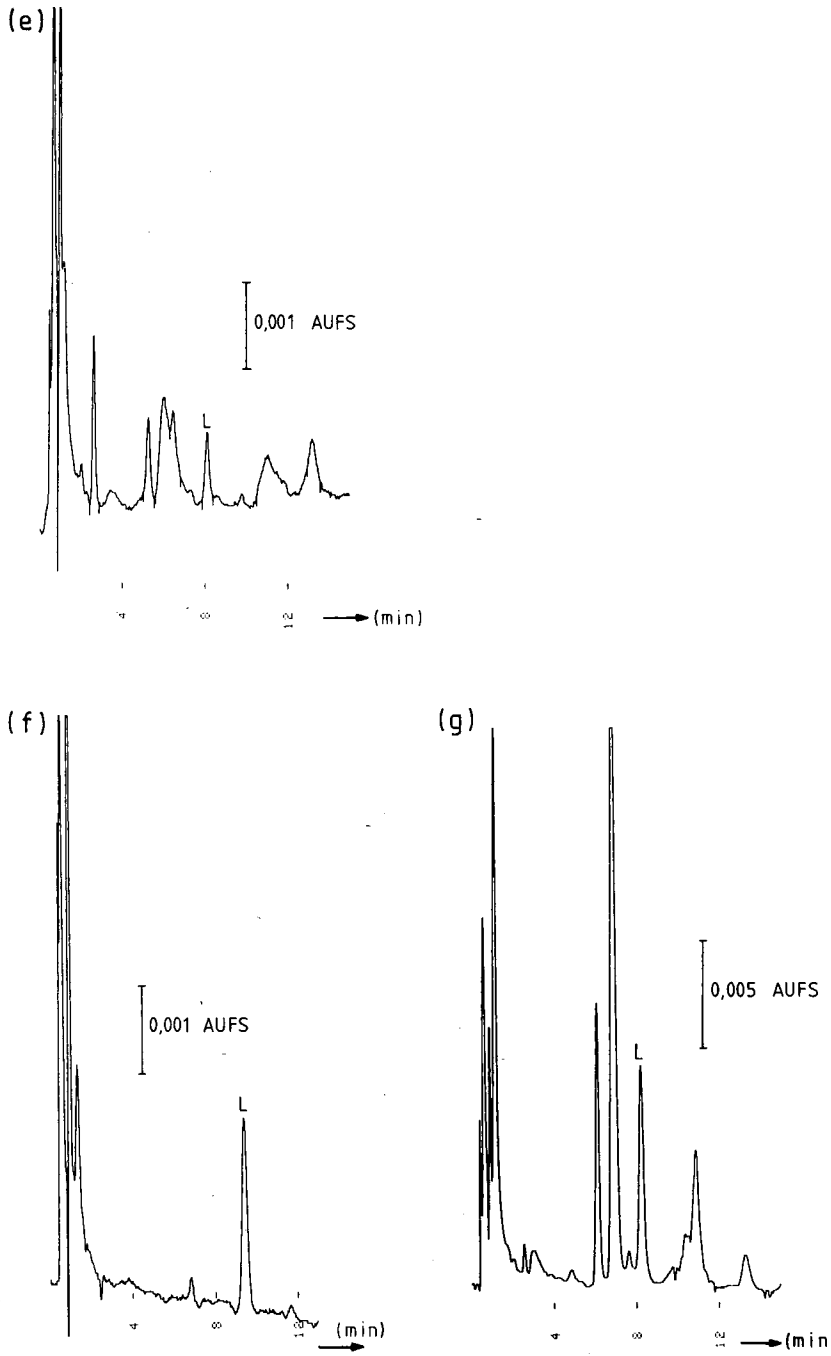
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Fig. 1.

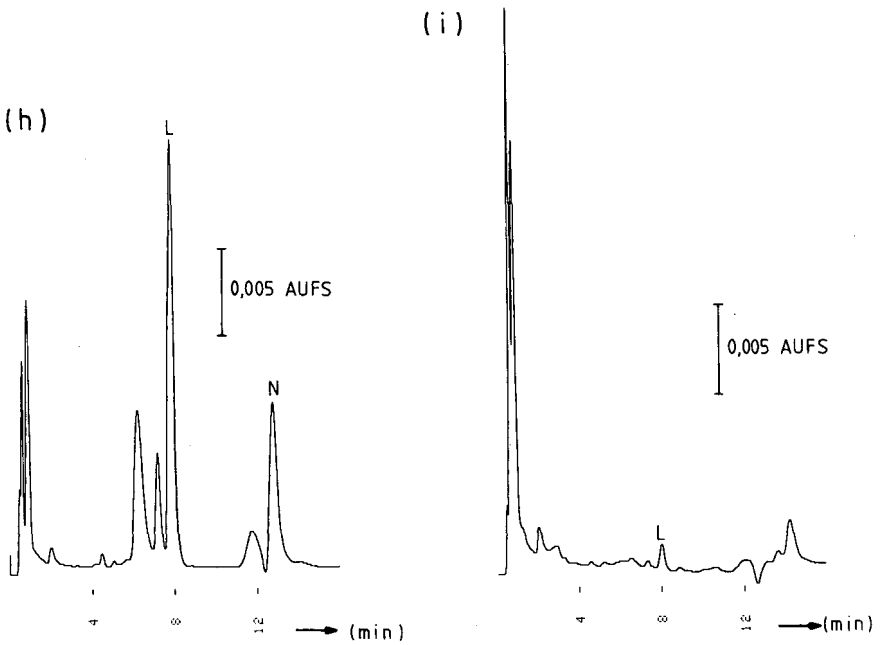

Fig. 1. HPLC traces of purified extracts of various citrus juices. Solvent 1, flow-rate 2.0 ml/min. C = isoaurapten; L = limonin; N = nomilin; P = psoralen. (a) Yellow grapefruit, unheated, 100-μl loop; (b) red grapefruit, heated, 20-μl loop; (c) navel orange, heated, 100-μl loop; (d) Grandiosa orange, heated, 20-μl loop; (e) mandarin, unheated, 20-μl loop; (f) lemon, unheated, 20-μl loop; (g) lime, unheated, 20-μl loop; (h) pomelo, unheated, 100-μl loop; (i) ugli, unheated, 100-μl loop.

mm I.D. column. In grapefruit juice, nomilin (k' = 21.2) cannot be determined simultaneously because it almost coelutes with a major impurity.

The other useful solvent was acetonitrile–methanol–water (31.8:22.7:45.5). Limonin has a much lower k' value (3.5) in this solvent resulting in shorter analysis times with a 50% reduction in solvent consumption. Also nomilin can be determined simultaneously (k' = 5.2). A disadvantage of this solvent system is that the limonin peak is very close to two other peaks in the chromatogram of grapefruit juice (see Fig. 2). When the limonin concentration is relatively low this results in less accurate quantitations. In many cases however this solvent may be of much benefit.

The following amounts of time are necessary for the determination of one juice sample: extraction, 3 min; centrifugation and filtration, 32 min; purification, 5 min and one actual HPLC run, 10–15 min depending on the time of injection. Centrifugation can be carried out for many samples at the same time and thus the centrifugation time per sample will be small. Purification can be done manually for 12 to 20 samples at the same time. Automation is nowadays possible, *e.g.*, with the Gilson ASPEC system. HPLC can be done manually or with an autosampler. With an autosampler it should be possible to analyse routinely 100 juice samples per day.

Detection was carried by UV at 210 nm. Although the reported maximum for limonin is at 207 nm no difference in integration values for a standard solution of

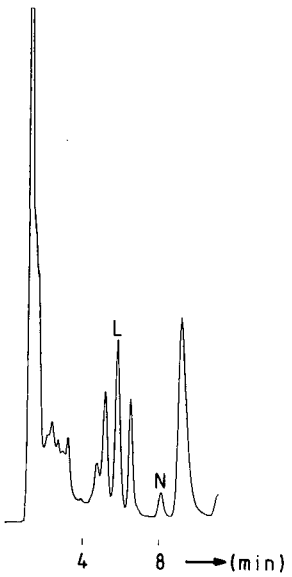

Fig. 2. HPLC trace of a purified yellow grapefruit juice extract. Solvent system 2, flow-rate 1.0 ml/min, 20-μl loop. L = Limonin; N = nomilin.

limonin was found between a detection wavelength of 207 or 210 nm. At 210 nm problems with solvent absorption will be less.

The purity of the limonin peak was not only assessed by comparing the retention time with a reference and by recording the chromatogram at 254 nm, but also by recording mass and 300-MHz ^{1}H NMR spectra off-line. For this purpose, 600 ml of grapefruit juice were worked up in the same manner as for an analytical sample. The resulting extract was concentrated and separated into the individual components by means of semipreparative HPLC with solvent 1. According to the NMR and mass spectra, the limonin fraction consisted solely of limonin. The purity was additionally investigated by analytical TLC in two solvent systems and using a spray reagent selective for limonoids, and by HPLC in solvent system 3. Again no other compounds were detected.

Fractions corresponding to the peaks marked C and P in Fig. 1a were also collected and investigated by UV, mass and NMR spectroscopy. Peak C was identified as 7-methoxy-8-(3-methyl-2-butenyloxy)coumarin (**4**) (synonym isoaurapten). Peak P was identified as 5-[(6,7-dihydroxy-3,7-dimethyl-2-octenyl)oxy]psoralen (**5**). A minor component present in the preparative juice sample which eluted just before **4** was identified as 7-[(6,7-dihydroxy-3,7-dimethyl-2-octenyl)oxy]coumarin (**6**) (synonym marmin).

After determining the major experimental conditions, the method was investigated with respect to its reproducibility and recovery. The relative standard deviations of the detector response for standard solutions of limonin are given in Table I. Next the reproducibility and the recovery of the purification procedure were determined. Results are given in Table II. Standard addition experiments in which known amounts of limonin were added to known quantities of limonin in grapefruit juice were also

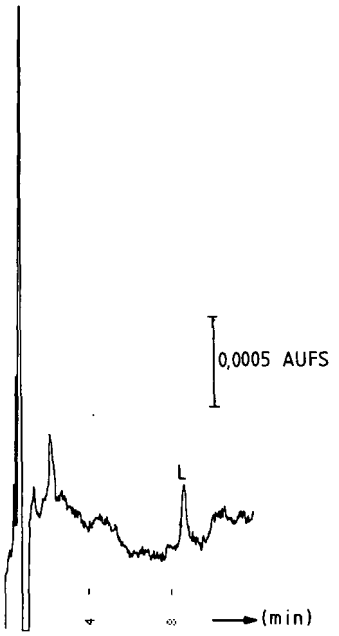

Fig. 3. HPLC trace of a 0.1-ppm standard solution of limonin. Solvent system 1, flow-rate 2.0 ml/min, 100-μl loop. L = Limonin.

performed. The results are similar to those obtained in the recovery experiments and are presented in Table III. Limonin concentrations below 1 ppm were determined with adequate precision.

The minimum detectable quantity of limonin was approximately 2 ng (limonin peak twice as big as the short term noise amplitude, 20-μl loop). This value resulted in a minimum detectable concentration of 0.1 ppm for qualitative purposes and of 0.5 ppm for quantitative purposes (20-μl loop). With the 100-μl loop these values were 0.03 and 0.1 ppm respectively. A chromatogram of a 0.1-ppm standard solution of limonin is given in Fig. 3 (100-μl loop).

In order to find out whether this sample clean-up is also applicable to other citrus juices apart from yellow grapefruit juice, it was tested on the following fruits: red grapefruit (Pride, Florida), navel orange (Spain, Genesis), orange (Spain, Grandiosa), mandarin (Argentina), lemon (Spain), lime (Brazil), pomelo (Israel, Jaffa) and ugli (Jamaica). The resulting chromatograms are presented in Fig. 1b–1i. In all cases the proposed procedure was used. Limonin concentrations were calculated with the calibration graph and are given in Table IV. The limonin concentration of some juices was determined both before and after heating. The peak purity of limonin was determined by recording a chromatogram at 210 and 254 nm. In all instances only a baseline was observed at 254 nm at the retention time of limonin.

CONCLUSION

Solid-phase extraction columns in combination with standard C_{18} HPLC

columns can be used with good results for the quantitation of the bitter compound limonin in various citrus juices. The present method combines speed with acceptable resolution and sensitivity. The purification, injection and analysis procedure is suitable for automation.

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CHROM. 21 109

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FREE FORMALDEHYDE IN COSMETICS

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SUMMARY

An improved, sensitive method for the determination of formaldehyde in cosmetics and other commercial products is reported. The procedure is based on dilution of the sample with tetrahydrofuran–water (9:1), followed by precolumn derivatization with 2,4-dinitrophenylhydrazine and direct reversed-phase high-performance liquid chromatography. The formaldehyde derivative is stabilized in the reaction medium by addition of phosphate buffer and neutralization and detected in less than 10 min by the standard additions method. The method also appears to be suitable for the direct evaluation of the formaldehyde donors used in cosmetics as preservatives.

INTRODUCTION

An EEC Council Directive[1] allows the use of formaldehyde as a preservative in cosmetic products at a maximum concentration of 0.2%; if the concentration exceeds 0.05%, the addition of formaldehyde must be declared on the label. In order to ascertain whether cosmetic products conform to this regulation and to prevent undesirable effects, rapid and reliable analytical methods are required. The official EEC method is based on condensation of free formaldehyde with ammonium acetate and acetylacetone to form fluorescent 3,5-diacetyl-1,4-dihydrolutidine, which is selectively detectable[2]. Although this method is sensitive, it is not suitable when formaldehyde donors are present in the cosmetic formula because additional formaldehyde is released during analysis.

One approach, based on headspace diffusion and direct reaction with 2,4-dinitrophenylhydrazine (2,4-DNPH) followed by high-performance liquid chromatography (HPLC), allows the detection of free formaldehyde[3] in the presence of formaldehyde donor preservatives[4]. With this method, however, the 2,4-DNPH derivative must be extracted and each analysis takes several hours. Other methods involving UV or fluorescence spectroscopy, HPLC and gas chromatography after

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derivatization with different reagents have been reported[5–21]. However, they all require sample pretreatments that are not suitable for routine control and stability studies.

The aim of this study was to overcome these problems by developing a rapid and reliable method based on direct reversed-phase HPLC of untreated samples after precolumn derivatization with 2,4-DNPH using the standard additions method. The method has the following advantages: (i) treatment and extraction steps are avoided through sample dilution with tetrahydrofuran (THF)–water (9:1)[22]; (ii) the formaldehyde 2,4-DNPH derivative which is formed is compatible with the medium and the mobile phase and is stable at neutral pH; (iii) standard additions before derivatization allow the evaluation of the matrix effect and the equilibrium rate of decomposition of formaldehyde donors during sample preparation, derivatization and HPLC steps; (iv) free formaldehyde can be determined in less than 15 min in any complex matrix[23].

EXPERIMENTAL

Materials and reagents

Bronopol (2-bromo-2-nitropropane-1,3-diol) was obtained from Formenti (Milan, Italy), formaldehyde (40% RPE) from Carlo Erba (Milan, Italy), Germall 115 {N,N′-methylenebis[N′-(1-hydroxymethyl)-2,5-dioxo-4-imidazolidinylurea]} from Medolla (Milan, Italy) and 2,4-DNPH from Carlo Erba. Reagents and solvents were of analytical-reagent grade from Merck (Darmstadt, F.R.G.).

A 0.1% solution of 2,4-DNPH was prepared by dissolving 0.25 g of 2,4-DNPH in 100 ml of 32% hydrochloric acid, heating until dissolved and then diluting to 250 ml with water in a volumetric flask.

Apparatus

A Perkin-Elmer Series 410 liquid chromatograph equipped with a Rheodyne 7125 valve, UV LC-95 detector and 3700 data station was used. The column was LiChrosorb RP-8 (10 μm, 250 mm × 4 mm I.D.) from Merck with acetonitrile–water (1:1) as eluent at a flow-rate of 1 ml/min and UV detection at 345 nm.

Standard solutions

Formaldehyde solution (40%, measured iodimetrically) was diluted to 0.004–0.0001% with THF–water (9:1). The solutions were freshly prepared and stored in a refrigerator.

Samples

About 1 g of each cosmetic sample, accurately weighed, was diluted to 10 ml in a screw-capped tube with THF–water (9:1) or THF and stirred in a vortex mixer until completely homogeneous.

Derivatization procedure

A 1-ml volume of standard or sample solution was added to 0.4 ml of 0.1% 2,4-DNPH solution, stirred for 60 s in a vortex mixer and allowed to stand for 2 min at room temperature. The solution was then stabilized by adding 0.4 ml of 0.1 *M* phosphate buffer (pH 6.8) and 0.7 ml of 1 *M* sodium-hydroxide solution NaOH. Aliquots of 6 μl were injected into the HPLC system.

RESULTS AND DISCUSSION

Derivatization procedure

The use of an acidic solution of 2,4-DNPH as the derivatizing agent for carbonyl groups is well known and widely used; the reaction occurs rapidly at room temperature, yielding a UV-absorbing derivative detectable by HPLC after extraction with an organic medium.

To avoid this step, the reaction yield obtained using the same aqueous–organic medium for sample preparation and for the mobile phase was investigated. The results in Fig. 1 show that the THF–water and acetonitrile–water mixtures allow the formaldehyde derivative obtained by derivatization with 2,4-DNPH to be evaluated without memory effects. Consequently, the combined use of a THF–water mixture and reversed-phase HPLC can be successfully employed for the direct determination of formaldehyde without need for sample pretreatment and extraction steps.

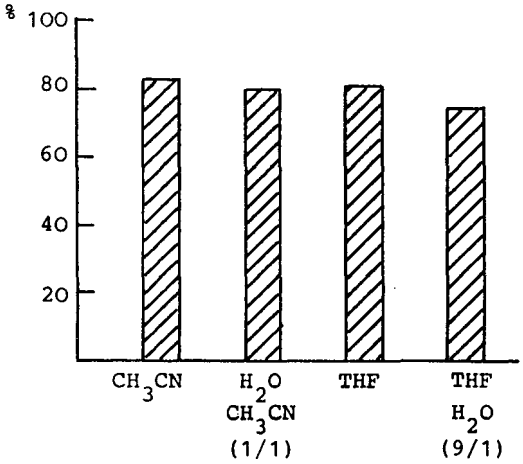

Fig. 1. Reaction yield of 4.0 μg/ml formaldehyde standard solution derivatized in the aqueous–organic medium used for sample preparation and as the mobile phase and injected directly into the HPLC system.

The stability of the 2,4-DNPH derivative is a function of reaction time, as shown in Fig. 2. The product formation reaches a maximum within 3 min (a), after which its concentration decreases as a function of time because of its instability in the acidic medium (b). Stability of the reaction over a period of 60 min was obtained by addition of phosphate buffer and by neutralization with 1 *M* sodium hydroxide solution.

Fig. 3 shows the progress of the reaction during the first 3 min of reaction with the standard solution of formaldehyde in THF–water (4 μg/ml). After only 30 s the yield is substantial and the maximum is attained within 3 min. This period appears to ensure reproducible measurements.

HPLC

Fig. 4 compares the chromatograms of (a) a THF–water (9:1) blank, (b) a standard solution of formaldehyde in THF–water (9:1), (c) a commercial cosmetic emulsion diluted with THF–water (9:1) and (d) the same cosmetic sample to which

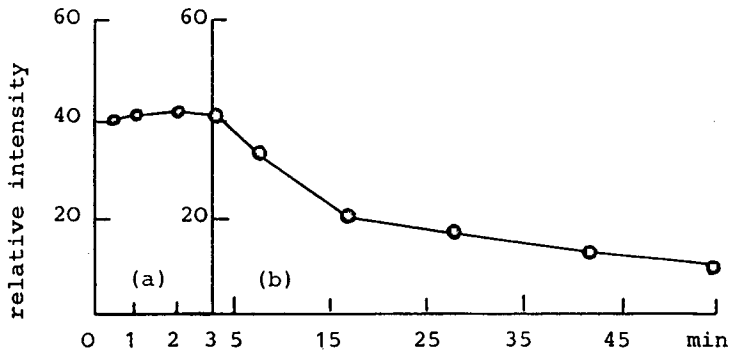

Fig. 2. Effect of addition of phosphate buffer and neutralization on stability of 2,4-DNPH derivative as a function of reaction time using a solution of formaldehyde in THF–water (9:1).

a known amount of formaldehyde standard had been added followed by derivatization and stabilization as described above. The patterns show the resolution of the method, with capacity factors of $k' = 2.35$ for the formaldehyde derivative and 1.67 for the unreacted 2,4-DNPH, respectively. Under the chromatographic conditions used there is no interference from other carbonyl compounds that also react with 2,4-DNPH.

Peaks were characterized by the absorbance ratio method using the stop flow technique or by measuring the peak-area ratio at two different wavelengths (345 and 254 nm).

For quantitative determinations, the standard additions method before derivatization was employed for the simultaneous evaluation of both the matrix effect and the derivatization rate. Calibration graphs and correlations for concentrations in the range 2–40 μg/ml with an average coefficient of variation of less than 1.5% can easily be obtained. Fig. 5 shows the calibration graphs for formaldehyde in the standard solution and added to a cosmetic emulsion sample; the identical slopes confirm the usefulness of the method and the absence of a matrix effect. The detection limit was 0.2 μg/ml (twice the signal-to-noise ratio).

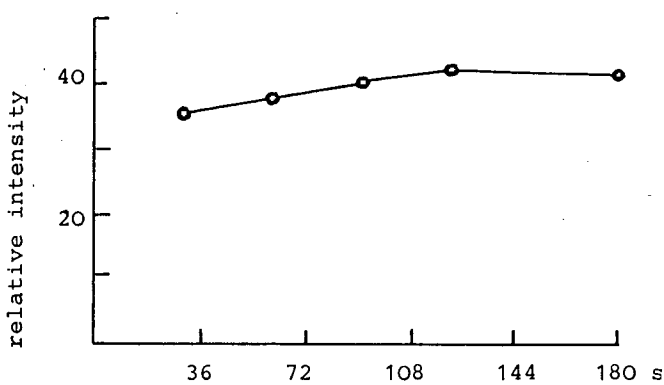

Fig. 3. Effect of increasing time of reaction on reaction rate and yield using a standard solution of formaldehyde in THF–water (9:1).

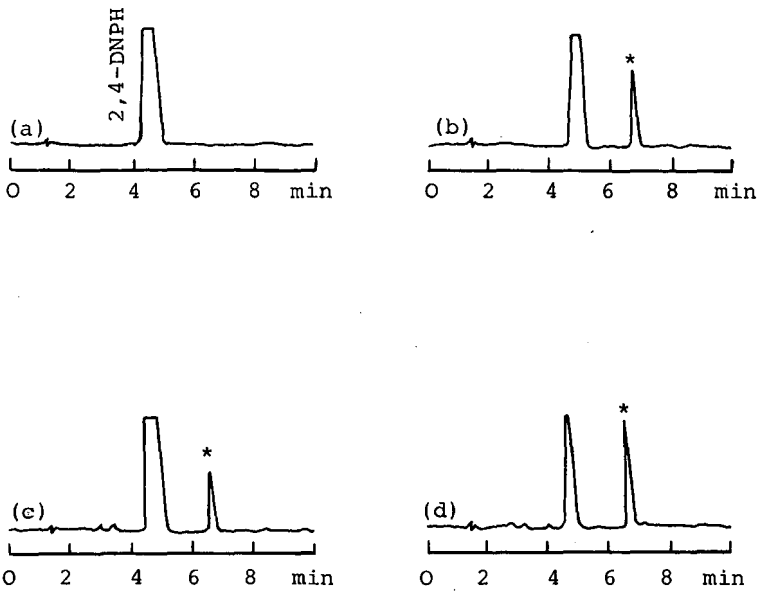

Fig. 4. Chromatographic patterns of (a) THF–water (9:1) blank, (b) formaldehyde standard, (c) cosmetic emulsion and (d) cosmetic emulsion after standard additions. The 2,4-DNPH peak represents the excess of derivatization agent and can be considered as a marker for reproducible measurements. (★), formaldehyde derivative.

Application

Recovery trials, carried out on typical commercial products diluted 1:10 or 1:50 with THF–water (9:1) depending on their formulative complexity and formaldehyde content, showed the reproducibility and flexibility of the method, which appears to be suitable for rapid and sensitive formaldehyde investigations (Table I).

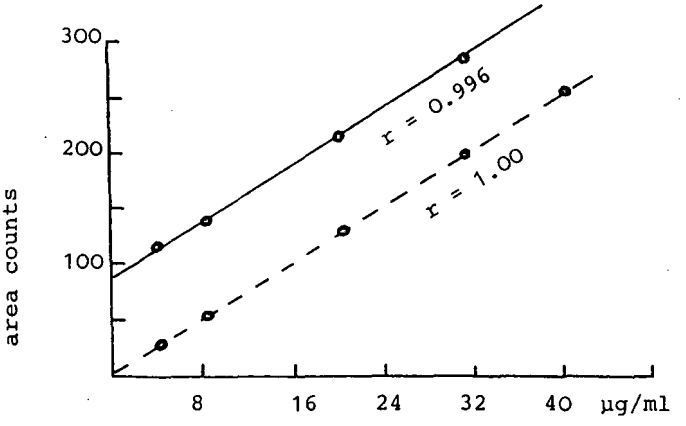

Fig. 5. Calibration graphs and correlations for formaldehyde standard solution (broken line) and added to cosmetic emulsion sample (solid line) after 2,4-DNPH precolumn derivatization.

TABLE I

RECOVERY TRIALS ON FORMALDEHYDE ADDED TO COSMETIC PRODUCTS BEFORE THE DERIVATIZATION STEP

Cosmetic product	*Formaldehyde added (%)*	*Recovery (%)*[a]
Emulsion	0.04	94 ± 3.8
Detergent	0.04	97 ± 2.7
Shampoo	0.04	99 ± 2.2
Disinfectant	0.04	97 ± 2.3
Toothpaste	0.04	99 ± 1.9

[a] ± Relative standard deviation (n = 10).

TABLE II

STABILITY OF FORMALDEHYDE DONOR PRESERVATIVES IN REACTION MEDIUM AND RELEASED FORMALDEHYDE LEVELS IN STANDARD SOLUTIONS

Formaldehyde donor preservative	*Compatibility with method*	*Maximum dose authorized*[1] *(%)*	*Formaldehyde released (%)*
Germall 115	+	0.6	0.043
Germall II	+	0.3	0.047
Bronopol	+	0.1	0.00054
Bronidox	+	0.1	0.000006
MDMHydantoin	+	0.2	0.025
Quaternium 15	−	0.2	N.D.
Benzylformal	+	0.2	0.06
Monochloroacetamide	+	0.3	0.00035
Dimethoxane	+	0.2	N.D.

The method can be used to study preservatives known to be formaldehyde donors[24,25]. Table II reports the stability of these compounds in the reaction medium and released free formaldehyde levels in standard solutions. Quaternium 15 appears to be the only compound that cannot be directly determined because of its instability with respect to the pH required for the 2,4-DNPH derivatization reaction.

Fig. 6 shows the formaldehyde released from a Bronopol cosmetic preservative[26] in an emulsion sample after standing for 5 weeks.

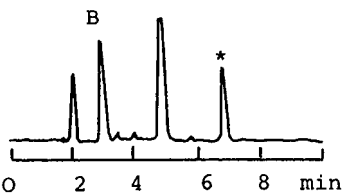

Fig. 6. Chromatographic pattern (at 216 nm) of formaldehyde (★) released from Bronopol (B) preservative in an emulsion sample after standing for 5 weeks.

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Note

Sorption of *n*-alkanes on Tenax

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In our recent paper[1] an empirically modified Langmuir isotherm equation was derived for the description of the relationship between the gas-phase and the sorbent-phase benzene concentrations in a benzene–nitrogen–Tenax system.

The resulting equation

$$\log V_g = a + b/T + c \log(1 + ec_s) + d/T \log(1 + ec_s) \quad (1)$$

where V_g (ml/g) is the solute specific retention volume, c_s (mol/g) is the solute sorbent-phase concentration and T is the absolute temperature of the system, contains five adjustable parameters a, b, c, d and e. Under the conditions of infinite dilution ($c_s \rightarrow 0$), eqn. 1 reduces to

$$\log V_g = a + b/T \quad (2)$$

which is the usual expression for the temperature dependence of the specific retention volume.

If the gas-phase solute concentration, c_g, has dimensions mol/ml, the following equations apply

$$K = c_s/c_g \quad (3)$$

$$V_g = K \cdot \frac{273.15}{T} \quad (4)$$

where K is the solute partition coefficient.

This work was undertaken to test the applicability of eqn. 1 to a description of non-polar solute sorption on Tenax. A series of *n*-alkanes (C_5–C_8) was chosen because it makes possible verification of the assumption of linear dependence of the thermodynamic functions of sorption on the number of methylene groups for the non-linear part of the sorption isotherm. The validity of this concept for the infinite dilution region was demonstrated by Novák *et al.*[2].

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EXPERIMENTAL

The methods for the preparation of the model gaseous mixtures, the measurements and the calculation of the partition coefficients were described earlier[3,4]. The purity of *n*-alkanes was better than 99.5% (Fluka, Switzerland) and they were used without further purification.

n-Alkane partition coefficients (155 values) were measured at temperatures in the range 19.9–50.3°C and at the gas-phase concentrations $6 \cdot 10^{-12}$–$7.5 \cdot 10^{-7}$ mol/ml.

THEORETICAL

The change in the standard Gibbs function of sorption, ΔG_s^0, is related to the specific retention volume, V_g, by

$$\Delta G_s^0 = -2.3\ RT \log (k^0 V_g) \tag{5}$$

where k^0 is a constant dependent on the choice of the standard state[5].

Since

$$\Delta G_s^0 = \Delta H_s^0 - T\Delta S_s^0 \tag{6}$$

where ΔH_s^0 and ΔS_s^0 are the changes in the standard enthalpy and entropy of sorption, respectively, eqn. 5 can be rewritten as:

$$\log V_g = -\frac{\Delta H_s^0}{2.3\ RT} + \frac{\Delta S_s^0}{2.3\ R} - \log k^0 \tag{7}$$

From a comparison of eqns. 2 and 5 it follows that:

$$a = (\Delta S_s^0/2.3\ R) - \log k^0 \tag{8}$$

$$b = -\Delta H_s^0/2.3\ R \tag{9}$$

Assuming additivity[6] of the contributions of the individual parts of the molecule to the values of ΔH_s^0 and ΔS_s^0, we can write for *n*-alkanes

$$\Delta H_s^0 = 2\ \Delta H_s^0(CH_3) + n\Delta H_s^0(CH_2) \tag{10}$$

$$\Delta S_s^0 = 2\ \Delta S_s^0(CH_3) + n\Delta S_s^0(CH_2) \tag{11}$$

where *n* is the number of methylene groups in the *n*-alkane molecule. Combining eqns. 8–11

$$a = c_2 n + c_1 \tag{12}$$

$$b = c_4 n + c_3 \tag{13}$$

where the parameters c_1, c_2, c_3 and c_4 have the following physical meanings

$$c_1 = 2\Delta S_s^0(CH_3)/2.3\ R - \log k^0 \quad (14)$$

$$c_2 = \Delta S_s^0(CH_2)/2.3R \quad (15)$$

$$c_3 = -2\Delta H_s^0(CH_3)/2.3R \quad (16)$$

$$c_4 = -\Delta H_s^0(CH_2)/2.3R \quad (17)$$

and hence we can finally write

$$\log V_g = c_1 + c_2 n + c_3/T + c_4 n/T \quad (18)$$

which is valid under the condition of infinite dilution or, in other words, in the linear parts of the sorption isotherms.

Eqn. 1 can be rewritten with the use of the additivity assumption if we suppose the same linear dependence of parameters c, d and e on the number of methylene groups:

$$c = c_6 n + c_5 \quad (19)$$

$$d = c_8 n + c_7 \quad (20)$$

$$e = c_{10} n + c_9 \quad (21)$$

By combining eqns. 1, 12, 13 and 19–21 we obtain

$$\log V_g = c_1 + c_2 n + c_3/T + c_4 n/T + (c_5 + c_6 n)Y + (c_7 + c_8 n)Y/T \quad (22)$$

which describes the sorption properties of the whole homologous series of *n*-alkanes. The function Y reflects the extent of the deviations from linearity of the sorption isotherm:

$$Y = \log [1 + (c_{10} n + c_9)c_s] \quad (23)$$

In the case of $c_s \rightarrow 0$ the function Y is zero and eqn. 22 is transformed into eqn. 18.

RESULTS AND DISCUSSION

The whole set of experimental data was correlated by eqn. 22 with the use of the simplex method on a Hewlett-Packard 9845 A/S computer. The optimized values of parameters c_1–c_{10} are summarized in Table I. For comparison, the values of parameters c_1–c_4 of eqn. 18 from ref. 2 are also presented. The agreement between these two sets of parameters is only qualitative, obviously as a consequence of the different temperature intervals (75–95°C). In Figs. 1–4 the experimental data are plotted in the coordinates $\log V_g$ *vs.* $\log c_s$ together with the courses calculated from

TABLE I

SUMMARY OF ADJUSTABLE PARAMETERS

c_i	*Eqn. 22*	*Eqn. 18 from ref. 2*
1	−2.614858	−5.4550
2	−1.214159	−0.42981
3	1236.3472	2052.74
4	579.9602	340.363
5	0.480481	
6	0.383629	
7	−253.461	
8	−212.9841	
9	15309.77552	
10	16077.803	

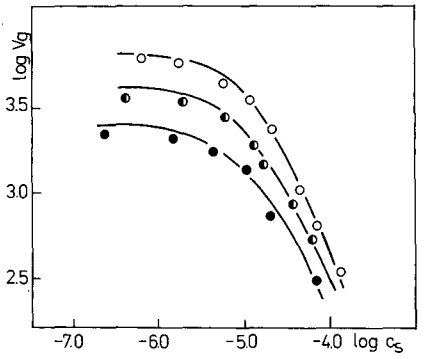


Fig. 1. Relationship of log V_g *vs.* log c_s for *n*-pentane on Tenax calculated by eqn. 22 (full lines) and experimental points (○ = 19.9; ◐ = 27.1; ● = 34.7°C).

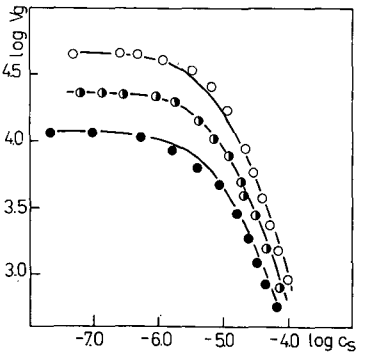
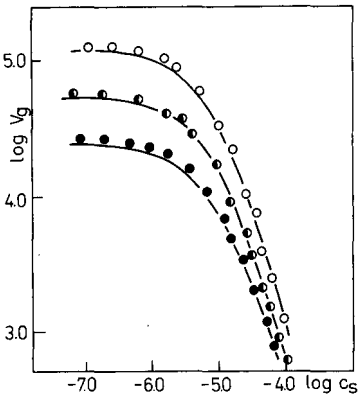


Fig. 2. Relationship of log V_g *vs.* log c_s for *n*-hexane on Tenax calculated by eqn. 22 (full lines) and experimental points(○ = 19.9; ◑ = 27.1; ● = 35.1°C).



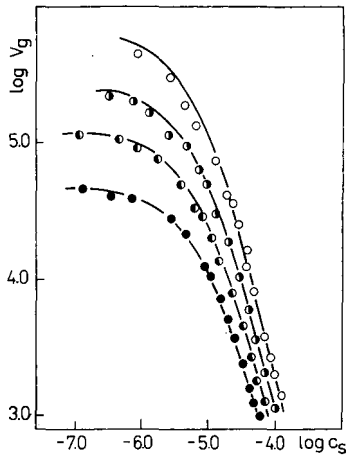


Fig. 3. Relationship of log V_g *vs.* log c_s for *n*-heptane on Tenax calculated by eqn. 22 (full lines) and experimental points (○ = 27.1; ◐ = 35.0; ● = 43.15°C).

Fig. 4. Relationship of log V_g *vs.* log c_s for *n*-octane on Tenax calculated by eqn. 22 (full lines) and experimental points (○ = 27.1; ◑ = 35.0; ◐ = 41.7; ● = 50.3°C).

eqn. 22. The average deviation between the calculated and the experimental values of the specific retention volume is 8.5% relative, more than twice that in ref. 1. This is partly due to the systematic deviations in the regions of low gas-phase concentrations.

In the non-linear parts of the sorption isotherms, eqn. 2 can still be used to describe the temperature dependence of the specific retention volume, of course under the condition of a constant c_s value. In this case

$$\log V_g = A + B/T \qquad (c_s = \text{constant}) \tag{24}$$

and the following relationships apply:

$$a = \lim_{c_s \to 0} A \tag{25}$$

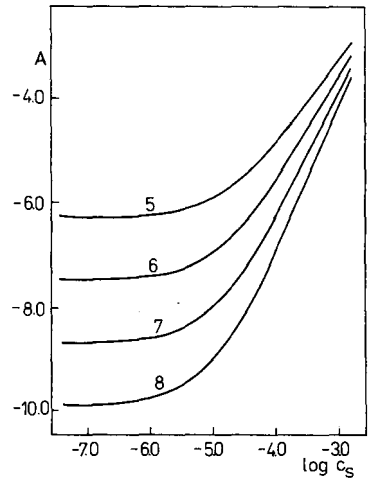
$$b = \lim_{c_s \to 0} B \tag{26}$$

$$A = n(c_2 + c_6 Y) + (c_1 + c_5 Y) \tag{27}$$

$$B = n(c_4 + c_8 Y) + (c_3 + c_7 Y) \tag{28}$$

The physical meanings of the expressions in parentheses are the same as those defined by eqns. 14–17.

In Figs. 5 and 6 the dependences of parameters A and B on the sorbent-phase solute concentration, c_s, are shown. They confirm the conclusions about the non-homogeneity of the Tenax surface[1]. These phenomena can also be caused by the



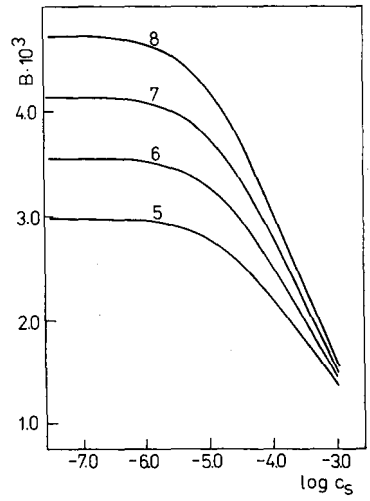


Fig. 5. Dependence of parameter A on the sorbent-phase concentration, c_s, for the series of n-alkanes (C_5–C_8).

Fig. 6. Dependence of parameter B on the sorbent-phase concentration, c_s, for the series of n-alkanes (C_5–C_8).

presence of different pores in a porous adsorbent (micropores, transition pores, as well as macropores) and by an unequal use of the internal surfaces of the adsorbent throughout a range of adsorbates possessing different molecular areas.

The values of the gas-phase solute concentrations up to which the courses of the sorption isotherms are linear were determined: n-pentane (9.4), n-hexane (1.0), n-heptane (0.11) and n-octane (0.012 ppm).

CONCLUSION

The applicability of eqn. 1 to the description of the sorption behaviour of non-polar solutes on Tenax was demonstrated. With the use of the additivity concept, eqn. 22 was derived, which permits simultaneous correlation of logarithms of the specific retention volumes of homologous compounds with the number of methylene groups, temperature and sorbent-phase solute concentration. The extrapolated values of the specific retention volumes can also be obtained for other members of homologous series of n-alkanes. The concept was confirmed to be valid also for homologous series of n-alcohols[7].

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Note

Prediction of alkane–water partition coefficients using a C_{18} derivatized polystyrene–divinylbenzene stationary phase

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Octanol–water partition coefficients have been utilized as a reference system in many fields, due primarily to the vast literature base developed by Hansch and co-workers[1,2]. Unfortunately, the hydrogen bonding capability of octanol reduces the intrinsic usefulness of the octanol–water system. Rytting *et al.*[3] and Anderson[4] have suggested that alkanes provide more information on intermolecular forces than octanol due to the lack of hydrogen bonding and dipole–dipole interactions. Furthermore, alkane–water partition coefficients are more relevant in biomembrane transport since the interior region of phospholipid membranes present an alkane barrier to transport[3]. The objective of this report is to disclose a rapid method for determining alkane–water partition coefficients using high-performance liquid chromatography (HPLC) on a new C_{18} bonded support, for future application to transport and structure–activity studies.

Several articles have appeared in the literature which review the use of HPLC retention times to estimate octanol–water partition coefficients[5–7]. Compared to the traditional "shake flask" method, the HPLC method has the advantage of speed, lower expense, less sensitivity to solute degradation, lower requirement for amount of solute, and less sensitivity to impurity interactions. Silica-based columns are generally utilized for the stationary phase, however, polystyrene–divinylbenzene[8,9] and glyceryl-coated glass[10] stationary phases have recently been employed. These columns do not have the mobile phase pH limitations of the silica-based columns[8–11]. Ideally, specific interactions between the stationary phase and the solute of interest should not occur. While silanol groups are typically masked by chemical modification[12], the addition of a lipophilic amine to the mobile phase[13], or by saturation of the stationary phase with octanol[14], outliers due to specific interactions between the free silanol groups and various solutes are commonly seen[6,7,13,14]. Trace metals in silica columns provide an additional site for specific interactions[15]. It has been shown that polystyrene–divinylbenzene stationary phases are also capable of specific interactions[8,9], possibly due to the electron rich pi orbitals which are present[11,16,17].

A C_{18} derivatized polystyrene–divinylbenzene column (Act-I) has been utilized

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in the present study. Benson and Woo[11] have suggested that C_{18} derivatization virtually eliminates solute interaction with the aromatic portion of the stationary phase through steric hinderance. This report shows that this assumption appears to be correct, and alkane–water partition coefficients can be predicted with a high degree of confidence.

EXPERIMENTAL

The chromatographic system utilized in the present study included a Milton Roy/LDC (Riviera Beach, FL, U.S.A.) SM4000 UV detector, a Waters Assoc. (Milford, MA, U.S.A.) WISP 710B autosampler, a Beckman Instruments (Fullerton, CA, U.S.A.) 110A Pump and a Nelson Analytical (Cupertino, CA, U.S.A.) 760 interface with Series 2600 chromatography software. A specially prepared 5-cm Act-I column (normally available as a 15-cm column) was received from Interaction Chemicals (Mountain View, CA, U.S.A.). A methanol–water (60:40) mobile phase was utilized at a flow-rate of 1 ml/min. The pH of the mobile phase was adjusted for acidic and basic compounds. The low ionic strength ($I = 0.01$) buffers of Perrin[18], 0.01 *M* hydrochloric acid, or 0.01 *M* sodium hydroxide were used to control the apparent pH (prepared in 60% methanol). The Perrin buffers were chosen to be at least 2 pH units below the pK_a of the acid (or above the pK_a of the base), and two different apparent pH values were utilized for each compound to demonstrate that the maximum retention was achieved. The 30 compounds used as standards in the present study and their sources are listed in Table I. Samples were prepared in the mobile phase at a concentration of approximately 1 mg/ml, as has been previously recommended[7]. An injection volume of 20 μl and a detection wavelength of 230 nm were utilized. Capacity factor (k') was calculated as $k' = (t_R - t_0)/t_0$ where t_R and t_0 are the retention times of the sample (in duplicate) and an unretained solute (methanol), respectively.

Hexane–water partition coefficients for nitroethane and nitrobutane were determined at 25°C (in duplicate) by the traditional "shake flask" method, using a Fisher (Springfield, NJ, U.S.A.) Model 236 shaker bath. The volume ratio of hexane to water was 1, and the initial aqueous concentration of the test solute was 0.5–0.7 mg/ml. The concentration of solute in the aqueous phase was determined using the above HPLC system, except a 25-cm Whatman (Clifton, NJ, U.S.A.) Partisil 10 ODS-3 column and a Brownlee (Santa Clara, CA, U.S.A.) RP-18 Spheri 10 (3 cm × 4.6 mm I.D.) guard column were used in place of the Act-I column. All other partition coefficients (PC) were taken from the literature (see Table I). The alkane–water partition coefficients used were limited to *n*-pentane through *n*-decane and cyclohexane, and did not include those estimated from HPLC methods or those thought questionable by the authors of the reference. In cases where multiple values were available, the mean was utilized.

RESULTS AND DISCUSSION

In estimating partition coefficients by HPLC, a choice must be made between using a capacity factor determined at a particular mobile phase organic volume fraction, or by linearly extrapolating the capacity factor to 0% organic. The latter

TABLE I

STANDARD COMPOUNDS

Suppliers: a = Aldrich (Milwaukee, WI, U.S.A.); e = Eastman Kodak (Rochester, NY, U.S.A.); f = Fisher Scientific (Fair Lawn, NJ, U.S.A.); k = Fluka Chemie (Buchs, Switzerland); l = Lancaster Synthesis (Windham, NH, U.S.A.); m = Mallinckrodt (Paris, KY, U.S.A.); s = Sigma (St. Louis, MO, U.S.A.). Types (hydrogen acceptor if not specified): 1 = non-hydrogen bonding; 2 = acid–alcohol; 3 = base.

Compound	*Supplier*	*Type*	*log PC*[a]
Acetanilide	a		−1.70
Acetophenone	a		1.16
Aniline	m	3	−0.01
Anisole	s		2.19
Benzaldehyde	a		1.19
Benzamide	l		−2.30
Benzene	f	1	2.30
Benzoic acid	e	2	−1.06
Benzonitrile	f		1.04
Benzophenone	e		3.29
Benzylalcohol	e	2	−0.62
Benzylamine	s	3	−0.21
Biphenyl	k	1	4.10[b]
Chlorobenzene	f		2.95
N,N-Dimethylaniline	s	3	2.32
Ethylbenzene	s	1	3.08[b]
Ethylbenzoate	a		1.40
Methylbenzoate	e		2.08
Nitrobenzene	s		1.52
Nitrobutane	k		1.14[c]
Nitroethane	k		−0.38[c]
Nitromethane	k		−0.93[b]
Phenol	m	2	−0.81
Phenylacetic acid	e	2	−1.23
Phenylacetone	s		0.98
Phenylacetonitrile	e		1.31
Propiophenone	a		2.02
n-Propylbenzene	e	1	4.11[b]
Pyridine	a	3	−0.31
Toluene	f	1	2.86

[a] Ref. 26 if not specified.
[b] Ref. 27.
[c] This study.

method has several drawbacks. First, it is well known, both theoretically and experimentally, that the logarithm of capacity factor is related quadratically to the organic volume fraction[19–21]. Thus, a linear extrapolation can only be performed over a limited (and impractical) range. Second, the quadratic relationship is dependent on the organic solvent used[19,21,22]. Finally, the extrapolation method requires much more time, which defeats a primary advantage of the HPLC method over the shake flask method. Therefore, a 60% methanolic mobile phase was chosen for the present study. This choice was not entirely arbitrary. Methanol is known to produce a less dramatic curvature in plots of log k' *versus* volume fraction when compared to other

commonly used HPLC solvents[19,21,22]. This is most likely due to the fact that the solubility parameter of methanol is closer to that of water than the other solvents. Furthermore, the physical properties of methanol–water mixtures have been well studied[23,24], which accounts for why methanol–water mixtures are often used for pK_a determination of compounds with low aqueous solubility[25]. A volume fraction of 60% was chosen since it was found to give reasonable retention times for the solutes used in the present study (which is reasonable for many pharmaceutically relevant compounds as well).

A plot of log PC(alkane–water) *versus* log k' and the linear regression line is shown in Fig. 1. The slope, intercept, and correlation coefficient of the regression line are 2.11 (11.7%), −0.998 (30.7%) and 0.953, respectively (95% confidence intervals are in parentheses). This correlation is considered very good due to the wide range of lipophilicities of the solutes (partition coefficients ranging over six orders of magnitude). The deviation from the regression line appears to be most dramatic for compounds with a log PC less than 0. This is most likely due to the error in quantitating k' for solutes with retention times approaching t_0. Therefore, investigators may wish to alter the methanol percentage to match the lipophilicities of the compounds under study.

The compounds in Fig. 1 were classified as non-hydrogen bonding, acid or alcohol, base, or hydrogen bond acceptor (see Table I). The inclusion of various classes of solutes demonstrates the lack of any significant specific interaction between the stationary phase and the solutes. The lack of specific interactions will be treated quantitatively in a future report.

In conclusion, a significant correlation was found between log PC(alkane–water) and log k'. There appears to be no significant specific interaction between the solutes and the polymeric stationary phase, thus, providing a distinct advantage over methods using traditional reversed-phase HPLC columns. Finally, the system can be

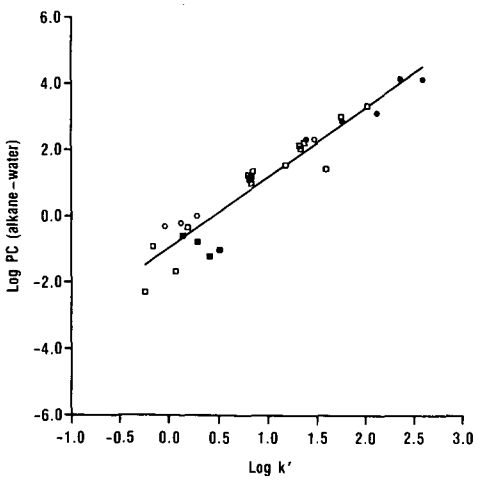

Fig. 1. Relationship between the alkane–water partition coefficient and the HPLC capacity factor [methanol–water (60:40)]. Key: ● = non-hydrogen bonding; ■ = acid–alcohol; ○ = base; □ = hydrogen bonding acceptor.

utilized for the rapid screening of lipophilicity of compounds, including those with acidic and basic functionalities.

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Note

Optimization of mobile phase composition for high-performance liquid chromatographic analysis of eleven priority substituted phenols

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Substituted phenols are of great environmental concern particularly as water pollutants[1]. Eleven phenols, listed by the United States Environmental Protection Agency (U.S.E.P.A.) as priority pollutants[2], have been subjected to many previous investigations[3–10]. One of the more widely used techniques is high-performance liquid chromatography (HPLC) of which both reversed-phase isocratic and gradient elution analyses were reported. In this paper, the isocratic HPLC separation of these phenols is investigated. The separation of the eleven compounds is optimized by the use of the overlapping resolution mapping (ORM) scheme proposed by Glajch *et al.*[11]. The aim of the optimization scheme is to predict the best mobile phase composition consisting of mixtures of water with various proportions of three common organic modifiers, methanol, acetonitrile and tetrahydrofuran[12].

EXPERIMENTAL

Analyses were carried out using a Shimadzu LC-6A isocratic instrument, equipped with a Model SPD-6A variable-wavelength UV spectrophotometric detector set at 280 nm throughout this work. A reversed-phase Whatman Partisil-5 ODS-3 column (particle size 5-μm, 100 mm × 4.6 mm I.D.) was used. The chromatographic data were collected and analyzed on a Chromatopac C-R3A data processor. A flow-rate of 1 ml/min was used. The void volume, V_0, was determined using methanol as the unretained component.

The phenols mixture was supplied by Alltech Associates whereas the individual compounds used to identify peaks in the mixture chromatograms were obtained from Aldrich. These chemicals were the purest available. The solvents used were of HPLC grade supplied by J. T. Baker and the mobile phases were prepared according to the A + B (*quantum sufficit*) addition method[13]. All solvents were filtered through a Millipore membrane filter and then thoroughly degassed in an ultrasonic bath. The phenols were dissolved in the mobile phases, and filtered before injection with a Rheodyne 7125 injector. For each injection, 1.5 μl of the solution were used. This injection volume corresponds to 7.5 ng of each phenol.

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RESULTS AND DISCUSSION

The first step of the optimization scheme was to define the three vertices of the solvent selectivity triangle[14–16] which correspond to the compositions of three binary (organic modifier + water) solvents. The first vertex was established using a binary mixture of methanol–water as the mobile phase[14]. A total analysis time of 10 min was selected as a time constraint. Two preliminary experiments using different mixtures of methanol and water were performed. The results of these are given in Table I. The mobile phase composition of methanol–water (58.5:41.5, v/v) which achieved elution of all the peaks within the time constraint was selected as the first vertex[14]. The solvent strength of this mixture was then calculated using eqn. 1[14]

$$ST = S_a\varphi_a + S_b\varphi_b + \ldots\ldots \quad (1)$$

where ST represents the total solvent strength of the mixture, S_a, S_b are the individual solvent strengths and φ_a, φ_b are the volume fractions of components *a* and *b* respectively. Based on the total solvent strength found, the compositions of the other two binary mixtures which have equal solvent strength were then calculated using eqn. 1. The three binary mixtures were used as the vertices of the solvent selectivity triangle[14] and were denoted as A = methanol–water (58.5:41.5, v/v), B = acetonitrile–water (56.0:44.0, v/v) and C = tetrahydrofuran–water (40.0:60.0, v/v) respectively. Subsequently, a simplex design approach[11] was employed to select the mobile phases to be used in a set of seven HPLC experiments. The seven mobile phases are listed in Table II. Since the solvent compositions given in this table are based on the binary mixtures A, B and C, the corresponding compositions based on the individual solvents can be calculated easily from these values. The latter compositions are listed in Table III and the retention times of the phenols obtained when using them are listed in Table IV.

From the experiments using these seven different mobile phases, the resolutions, *R*, between every pair of peaks in the chromatograms obtained for each solvent composition were calculated using eqn. 2[17]

$$R = \frac{1}{4}(\alpha - 1)N^{1/2} \cdot \frac{K'}{(1 + K')} \quad (2)$$

TABLE I

RESULTS OF PRELIMINARY EXPERIMENTS USING BINARY MIXTURES OF METHANOL–WATER

Solvent composition (%, v/v)		*Capacity factor for last component eluted*	*Retention time (min) for last component eluted*
Methanol	*Water*		
50.0	50.0	23.65	12.96
58.5	41.5	19.68	10.78

TABLE II

SOLVENT COMPOSITION AS PERCENTAGE OF BINARY MIXTURES IN THE MOBILE PHASE

A = Methanol–water; B = acetonitrile–water; C = tetrahydrofuran–water.

Eluent mixture	*A*	*B*	*C*
1	100	0	0
2	0	100	0
3	0	0	100
4	50	50	0
5	0	50	50
6	50	0	50
7	33.3	33.3	33.3

TABLE III

SOLVENT COMPOSITIONS AS PERCENTAGE OF PURE SOLVENTS IN THE MOBILE PHASE

Eluent mixture	*Methanol*	*Acetonitrile*	*THF*	*Water*
1	58.50	0.00	0.00	41.50
2	0.00	56.00	0.00	44.00
3	0.00	0.00	40.00	60.00
4	29.25	28.00	0.00	42.75
5	0.00	28.00	20.00	52.00
6	29.25	0.00	20.00	50.75
7	19.50	18.65	13.30	48.55

TABLE IV

RETENTION TIMES (IN MIN) OF PHENOLS IN THE SEVEN ELUENT MIXTURES LISTED IN TABLE III

Compounds: 1 = 2,4-dinitrophenol; 2 = 2-methyl-4,6-dinitrophenol; 3 = phenol; 4 = *p*-nitrophenol; 5 = *o*-chlorophenol; 6 = *o*-nitrophenol; 7 = 2,4-dimethylpenol; 8 = 4-chloro-3-methylphenol; 9 = 2,4-dichlorophenol; 10 = pentachlorophenol; 11 = 2,4,6-trichlorophenol.

Compound	*Mobile phase*						
	1	*2*	*3*	*4*	*5*	*6*	*7*
1	1.775	1.605	2.615	1.972	1.728	2.038	1.707
2	2.000	1.628	2.632	2.400	1.777	2.138	1.745
3	5.050	4.538	11.182	4.620	5.700	7.558	5.392
4	5.950	4.643	13.497	5.355	4.903	10.365	6.308
5	7.667	6.033	16.580	6.483	8.122	12.697	8.158
6	8.295	6.965	15.427	7.233	6.212	10.667	7.600
7	11.950	7.640	20.402	8.825	10.190	17.280	10.575
8	15.707	8.225	23.297	10.398	2.040	24.767	14.053
9	19.508	9.880	31.723	12.892	15.517	34.763	18.688
10[a]	15.658	3.192	4.442	15.398	9.295	7.528	4.892
11	28.937	8.073	24.142	21.033	8.217	31.383	19.142

[a] Standard phenol mixture was spiked with this component to improve detection.

where R is the resolution for a pair of adjacent peaks, α is the relative retention ratio, N is the number of theoretical plates and K' is the capacity factor for one of the peaks.

The calculated resolutions were then fitted by a second order polynomial equation

$$R = a_1x_1 + a_2x_2 + a_3x_3 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 + a_{123}x_1x_2x_3 \quad (3)$$

where a_i are coefficients and x_i are volume fractions of the binary mixtures A, B and C.

A minimum resolution of unity between each pair of peaks is specified. With the aid of a BASIC program[14] and the use of the minimum resolution criterion, a Venn diagram[11] was generated for each pair of peaks. Subsequently, by overlapping all the Venn diagrams, areas which satisfy the desired resolution for all the peaks were determined. The overlapping resolution diagram for the eleven phenols is illustrated in Fig. 1. The region that is shown with # represents the mobile phase compositions that give the best separation.

To confirm the success of the optimization procedure, a mobile phase composition from this region corresponding to 55.0% of A and 45% of B was chosen for a further experiment. Fig. 2 illustrates the chromatogram obtained using this mobile phase, *i.e.*, methanol–acetonitrile–tetrahydrofuran–water (32.2:25.2:0:42.6, v/v). The order of elution and retention times of the eleven phenols are listed in Table V. The chromatogram shows that all the eleven phenols are satisfactorily separated. Notably, the analysis time of 9 min is much shorter than the sequential procedure proposed by Buckman *et al.*[4], which involves actual times of 25 min for the first eluent mixture and 17 min for the second eluent mixture. The present method is also a significant improvement over the previous best isocratic separation of these

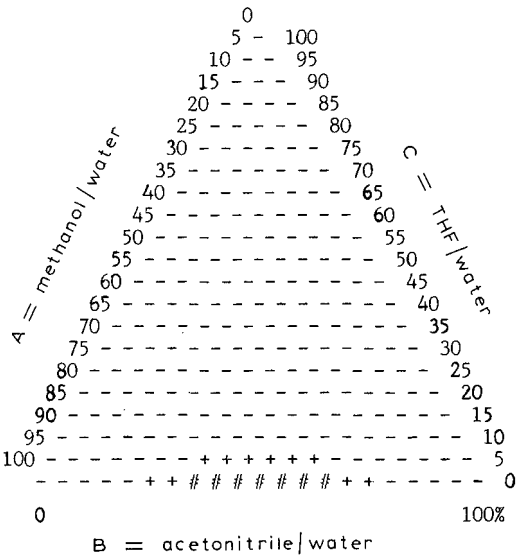

Fig. 1. Overlapping Venn diagram for the ten pairs of peaks: – –, $R < 0.5$; + +, $0.5 < R < 1.0$; # #, $R \geqslant 1.0$.

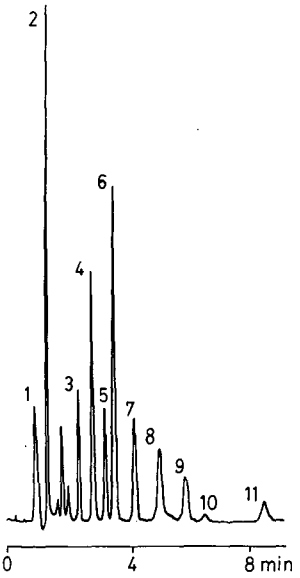

Fig. 2. Chromatogram of a mixture of eleven substituted phenols. Eluent: methanol–acetonitrile–THF–water (32.2:25.2:0:42.6, v/v). Peak numbers refer to Table IV. Chromatographic conditions as described in the text.

compounds obtained by Lee *et al.*[7] which requires an analysis time of 25 min, and is therefore suitable for adaptation to routine analysis of the phenols in environmental samples. Furthermore, the present work is the first application of a systematic approach to the optimization of mobile phase composition for the separation of these phenols. The method can readily be extended to the analysis of other compounds, which necessitates the use of ternary or more complicated eluent mixtures.

The results obtained in this work have successfully demonstrated the application

TABLE V

THE ELEVEN PRIORITY PHENOLS AND THEIR RETENTION TIMES FOR THE ELUENT MIXTURE METHANOL–ACETONITRILE–THF–WATER (32.2:25.2:0:42.6, v/v)

Peak No.	*Phenol*	*Retention time (min)*
1	2,4-Dinitrophenol	0.935
2	2-Methyl-4,6-dinitrophenol	1.313
3	Phenol	2.404
4	*p*-Nitrophenol	2.867
5	*o*-Chlorophenol	3.280
6	*o*-Nitrophenol	3.578
7	2,4-Dinitrophenol	4.258
8	4-Chloro-3-methylphenol	5.073
9	2,4-Dichlorophenol	5.948
10	Pentachlorophenol	6.595
11	2,4,6-Trichlorophenol	8.567

of a systematic approach to the optimization of mobile phase composition for HPLC. Optimization of the separation of the eleven phenols using an ORM scheme was achieved quite easily even though a quaternary mobile phase was considered.

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CHROM. 21 101

Note

High-performance liquid chromatographic separation of enantiomers on axially chiral binaphthalene dicarboxylic acid–chiral phenylethylamine bonded to silica gel

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A variety of chiral stationary phases(CSPs) derived from amino acids and amines have been developed for the direct separation of enantiomers by high-performance liquid chromatography (HPLC)[1,2]. Recently, chiral stationary phases involving two stereogenic centres have been reported to be more effective for the chromatographic separation of the various types of enantiomers[3–7].

In a previous paper, we described the preparation of several axially chiral binaphthalene-derived phases and their effectiveness for the separation of a wide range of enantiomers[8]. Thus, in this paper, we describe the preparation and effectiveness of novel chiral stationary phases involving two different stereogenic centres, axially chiral 1,1′-binaphthalene-2,2′-dicarboxylic acid coupled with chiral 1-phenylethylamine.

EXPERIMENTAL

Materials

1,1′-Binaphthalene-2,2′-dicarboxylic acid (compound **1**)[9,10] and 11-aminoundecyl silanized silica gel (spherical 5-μm particles, amino group 0.66 mmol/g) (compound **2**)[11] were prepared according to the reported procedures.

Preparation of (*aS*, 1*S*)-(−)- and (*aR*, 1*S*)-(+)-2-(N-1-phenylethylcarbamoyl)-1,1′-binaphthalene-2-carboxylic acid (**3**-*a*SS and **3**-*a*RS) was accomplished according to Fig. 1. Compound **1** was treated with dicyclohexylcarbodiimide (DCC), followed by (1*S*)-(−)-1-phenylethylamine to yield diastereomeric mixture **3**, which was recrystallized from acetonitrile to afford **3**-*a*SS–acetonitrile (1:1 inclusion complex)(m.p. 190.5°C; $[\alpha]_D^{20} = -123.3°$, $c = 1.038$, $CHCl_3$. Found: C, 79.27; H, 5.67; N, 5.29%. Calc. for $C_{30}H_{23}NO_3 \cdot C_2H_3N$, C, 78.99; H, 5.39; N, 5.76%). The filtered acetonitrile solution was evaporated and the residue was recrystallized from ethanol

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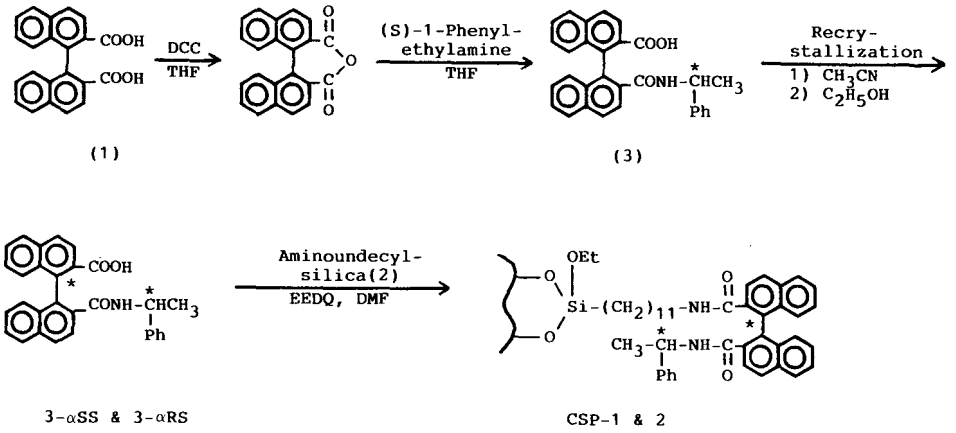

Fig. 1. Preparation of CSP 1 and 2. THF = tetrahydrofuran; Et = ethyl; Ph = phenyl.

to afford **3**-*a*RS–ethanol (1:1 inclusion complex)(m.p. 260.0–261.0°C; $[\alpha]_D^{20}$ = 183.2°, c = 1.01, $CHCl_3$. Found: C, 78.25; H, 6.09; N, 2.91%. Calc. for $C_{30}H_{23}NO_3 \cdot C_2H_6O$, C, 78.19; H, 5.95; N, 2.85%). Total yields of **3**-*a*SS and **3**-*a*RS starting from compound **1** were 86 and 81% respectively. The optical purities of **3**-*a*SS and **3**-*a*RS were determined to be 100% by HPLC as their methyl esters. The configuration of the binaphthalene carboxylic acid moiety in each diastereomer **3** was determined by hydrolysis of the amides to the corresponding acids. The experimental details will be described elsewhere[12].

Preparation of stationary phases

CSP1. To a solution of 3.30 g of compound **2** in 60 ml of dry dimethylformamide (DMF) were added 3.00 g of compound **3**-*a*SS and 3.30 g of N-ethoxycarbonyl-2-ethoxy-1,3-dihydroquinone (EEDQ). The slurry was irradiated under a nitrogen atmosphere in the water-bath of an ultrasound laboratory cleaner (35 W, 41 kHz) which was maintained at 70°C. After 8 h of irradiation, the modified silica gel was collected and washed exhaustively with DMF, methanol, acetone and diethyl ether and then dried under reduced pressure to afford CSP 1 (Found: C, 15.05; H, 2.20; N, 1.02%. Calc. for. **3**-*a*SS: 0.18 mmol/g based on % C).

CSP 2. Phase CSP 2 was prepared similarly using compound **3**-*a*RS instead of **3**-*a*SS (Found: C, 14.88; H, 2.18; N, 1.02%. Calc. for **3**-*a*RS: 0.18 mmol/g based on % C).

Liquid chromatography

The experiments were carried out using a Shimadzu LC-5A or a JASCO Trirotor III high-performance liquid chromatograph equipped with a Shimadzu SPD-6A or a JASCO UVIDEC-100-III ultraviolet detector (254 nm). Stainless-steel columns (250 mm × 4.6 mm I.D.) were slurry packed using conventional techniques.

Various derivatized compounds for use as solutes were prepared by employing reagent-grade chemicals.

TABLE I

SEPARATION OF THE ENANTIOMERS OF AMINO ACID, AMINE AND ALCOHOL DERIVATIVES ON CHIRAL STATIONARY PHASES

Mobile phases: 2-propanol–*n*-hexane, 10:90 (A); 20:80 (B). Flow-rate: 1 ml/min. k' = Capacity factor for the initially eluted enantiomer. The configuration of the more strongly retained enantiomer is indicated in parentheses. The separation coefficient of the enantiomers, α, is the ratio of the capacity factors of the enantiomers. 3,5-DNB = 3,5-Dinitrobenzoyl; 3,5-DNPC = 3,5-dinitrophenylcarbamate.

Racemate	*CSP 1*			*CSP 2*		
	Mobile phase	k'	α	*Mobile phase*	k'	α
N-3,5-DNB-alanine butyl ester	A	6.39	1.14(*S*)	A	5.26	1.27(*S*)
N-3,5-DNB-valine butyl ester	A	3.42	1.08(*S*)	A	2.91	1.24(*S*)
N-3,5-DNB-leucine butyl ester	A	3.88	1.05(*R*)	A	3.23	1.13(*S*)
N-3,5-DNB-phenylglycine butyl ester	A	4.64	1.24(*R*)	A	4.30	1.13(*R*)
N-3,5-DNB-phenylalanine butyl ester	A	6.12	1.09(*R*)	A	5.94	1.42(*S*)
N-3,5-DNB-1-phenylethylamine	B	6.20	1.40(*S*)	B	7.22	1.40(*S*)
N-3,5-DNB-1-(1-naphthyl)ethylamine	B	7.00	2.12	B	9.93	1.84
1-Phenylethanol 3,5-DNPC	A	9.25	1.09(*R*)	A	11.03	1.26(*S*)
1-Phenylpropanol 3,5-DNPC	A	8.08	1.11	A	9.72	1.29
1-Phenylbutanol 3,5-DNPC	A	7.37	1.11	A	8.94	1.27

RESULTS AND DISCUSSION

The preparation of the diastereomers **3**-*a*SS and **3**-*a*RS was easily accomplished according to Fig. 1, starting from racemic compound **1** and (1*S*)-1-phenylethylamine, whereby the troublesome optical resolution of compound **1**[9,13] was excluded.

Chromatographic results for the derivatives of amino acids, amines and alcohols are summarized in Table I. Both CSPs have the same configuration in the amine moiety, but different configurations in the binaphthalene unit. Better separation was achieved on CSP 2 than on CSP 1 except for phenylglycine and naphthylethylamine, which shows that both chiral sites of CSP 2, *aR*-binaphthalene and *S*-phenylethyl-

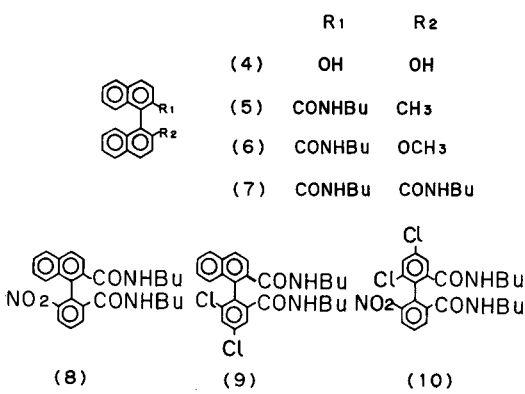

Fig. 2. Structures of biaryls. Bu = Butyl.

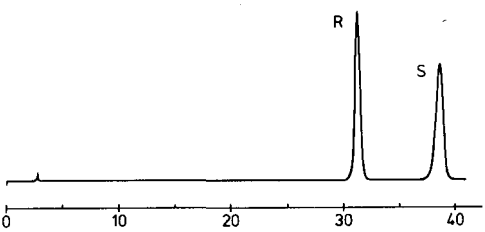

Fig. 3. Chromatographic separation of enantiomers of 1-phenylethanol 3,5-dinitrophenylcarbamate on CSP 2. Chromatographic conditions as in Tabel I. Elution time in min.

amine, cooperate with each other in separating enantiomers. In the case of CSP 1, the chiral recognition ability of the inverted configuration of the binaphthalene site may be opposite to that of the *S*-phenylethylamine site and the separation factors were reduced or the elution order was reversed for leucine, phenylalanine and 1-phenylethanol.

Axially disymmetric biaryls (Fig. 2) are separated as shown in Fig. 3 and Table II. Better separation was also achieved on CSP 2 than on CSP 1. The configuration of the more strongly retained enantiomers is inverted between CSP 1 and 2, which suggests the major site for chiral recognition is the binanpthalene moiety and the contribution of *S*-phenylethylamine moiety is small for the enantiomer separation of biaryls.

TABLE II

SEPARATION OF THE ENANTIOMERS OF BIARYLS ON CHIRAL STATIONARY PHASES

Chromatographic conditions, α and k' as in Table I. Mobile phases: 2-propanol–*n*-hexane, 10:90 (A), 20:80 (B), 5:95 (C).

Racemate	*CSP 1*			*CSP 2*		
	Mobile phase	k'	α	*Mobile phase*	k'	α
4	B	7.36	1.09(*R*)	B	6.76	1.09(*S*)
5	C	1.81	1.00	C	1.80	1.06
6	C	4.16	1.12(*R*)	C	4.19	1.13(*S*)
7	B	3.61	1.30(*R*)	B	3.71	1.50(*S*)
8	A	6.50	1.13(*R*)	A	6.50	1.22(*S*)
9	A	6.43	1.23	A	6.07	1.32
10	A	6.64	1.11(*R*)	A	5.45	1.17(*S*)

We consider that these novel phases are potentially very useful for the separation of a wide range of enantiomers as they have two stereogenic centres of different types in the same molecule.

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CHROM. 21 187

Note

Application of gas chromatography for the study of precipitated calcium carbonate

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Over the past twenty years gas chromatography (GC) has become a well established technique for studying adsorption equilibria at very low surface coverage[1,2]. Thermodynamic data obtained in this manner have been reported for the adsorption of a wide range of organic vapours on graphitised carbons[1,3,4], zeolites[1,2,5] and silicas[1,2,6]. On the other hand much less attention has been given to catalysts, fillers and other materials of industrial importance. However, the few exploratory studies made with inorganic solids such as barium sulphate[7,8] and calcium carbonate[9] do provide an indication of the potential value of the technique.

Precipitated calcium carbonate is now widely used as an inexpensive white filler[10,11]. To improve its dispersibility and mechanical performance the filler is generally subjected to surface treatment, *e.g.* by coating with stearate[11]. Little is known about the changes brought about by surface treatment. Papirer *et al.*[9] reported a marked decrease in surface energy as the calcium carbonate surface was progressively coated with stearic acid and concluded that the modified surface became more uniform and less polar. The work of Schreiber *et al.*[12] also revealed that $CaCO_3$–polymer interaction could be considerably improved by surface modification.

No systematic study appears to have been made of the energetics of adsorption of selected organic vapours before and after the surface treatment of precipitated calcium carbonate. In principle, isosteric heats of adsorption can be calculated from GC retention measurements conducted at different temperatures[1,2]. It is the aim of the present paper to establish the conditions under which reproducible and reliable retention data can be obtained. Hydrocarbons of different molecular weight and polarity have been used as molecular probes. The GC retention data reported here were determined on laboratory samples of precipitated calcium carbonate. The results obtained with modified and coated samples will be presented and discussed in a subsequent paper[13].

EXPERIMENTAL

Gas chromatographic (GC) measurements were made using a Pye Unicam Model 104 gas chromatograph equipped with a flame ionisation detector. The carrier gas was nitrogen (zero grade, supplied by the British Oxygen Co.) and had a maximum

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water content of < 5 vpm (volumes per million). The carrier gas was further dried and purified by passing through two molecular sieve traps and a bed of calcium carbonate before passage to the flow controller. These extreme precautions were found to be necessary after initial experiments had indicated large and random fluctuations of peak retention times for the various eluted species. The gas module was used to keep the gas pressure at the column inlet constant throughout the course of the experiments.

Glass columns of 50 cm length and internal diameters ranging from 2 to 7 mm were packed with coarse particles (44–72 mesh size) of calcium carbonate. Uniform packing was achieved by slow addition of the coarse powder into a continuously evacuated column which was at the same time subjected to gentle vibration. Before use each column was conditioned at 200°C by passing dry nitrogen gas at 20 ml min^{-1}. The pressure of the carrier gas at the column inlet was measured by a septum pressure gauge and the outlet was assumed to be at atmospheric pressure. The flow-rate of the carrier gas at the column outlet, at each temperature, was measured by a soap-bubble flow meter kept at room temperature. The measured flow-rates of the carrier gas were corrected for the gas compressibility factor, j, the saturation vapour pressure of water and the temperature difference between the ambient atmosphere and the column.

It was found necessary to inject the hydrocarbon in the form of vapour into the carrier gas stream rather than as the liquid. To generate a known amount of vapour 1 μl of the liquid hydrocarbon was introduced into a litre Pyrex glass flask, which had been purged with dry nitrogen and contained a small quantity of 3A molecular sieve (to remove any traces of water vapour). The flask was sealed with a stopper incorporating a silicone rubber injection septum. A Hamilton syringe was then used to transfer 0.02–0.5 ml of the vapour from the flask into the carrier gas stream. The amount of vapour injected was equivalent to $2 \cdot 10^{-5}$–$5 \cdot 10^{-4}$ μl of liquid hydrocarbon. The hydrocarbons were all of high purity grade supplied by Aldrich. Methane was used to determine the gas hold-up time.

The results in Fig. 1 confirm the reproducibility of the retention data when this

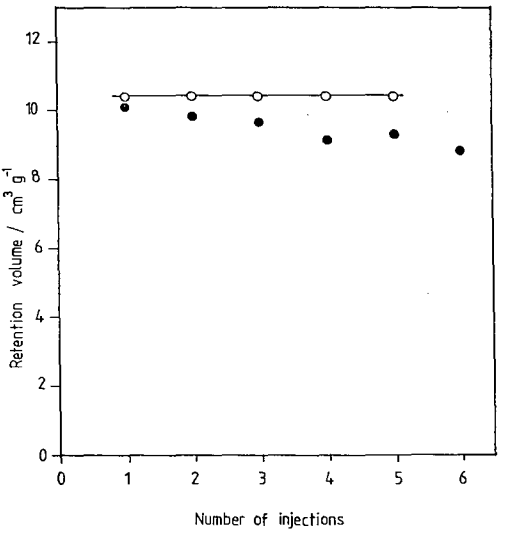

Fig. 1. Variation of retention volume with the number of injections for heptane at 100°C on pure calcium carbonate. ○ = n-Heptane vapour; ● = n-heptane liquid < 0.05 μl.

procedure was followed —in contrast to the lack of reproducibility found with the liquid injection (*ca.* 0.05 μl) technique.

Preliminary experiments were conducted at different temperatures (over the range 130–190°C) to establish the conditions required to achieve acceptable reproducibility in the retention measurements (*i.e.* to within 2%). It was found necessary to restore the column temperature to the original level (*i.e.* 200°C) before each determination and minimum times of around 40 min and 2 h were required to remove all adsorbed non-polar molecules and polar molecules, respectively.

Values of specific retention volume, V_g, were calculated from the equation

$$V_g = (t_R - t_0)F_c \cdot \frac{273}{T} \cdot \frac{1}{\omega} \quad (1)$$

where t_R and t_0 are the retention times of the eluted hydrocarbon and of methane respectively, F_c is the corrected flow-rate, T is the column temperature and ω is the mass of calcium carbonate.

Laboratory samples of calcium carbonate were prepared by the passage of carbon dioxide through aqueous suspensions of calcium hydroxide. The reaction conditions used to prepare the pure material were as follows: pure calcium oxide was added to water to give an equivalent concentration of 0.9 *M*; the suspension was cooled to an initial temperature of 10°C and 40% (v/v) carbon dioxide in air passed through the suspension (total gaseous flow rate *ca.* 200 l h^{-1}) with vigorous stirring until pH 7 was attained. The product was filtered and dried at 115°C for 16 h. The levels of impurities in the calcium carbonate (w/w percentage) were: Cl, 0.01; Na, 0.026; SO_4, 0.024; P, 0.08. The oven-dried material had a BET nitrogen area of 29–30 m^2 g^{-1}.

RESULTS AND DISCUSSION

This work has brought to light a number of problems associated with the application of GC for surface characterisation. First, since the presence of small amounts of water vapour caused a pronounced change in the adsorptive retention time and peak shape, it was found necessary to scrupulously dry both the carrier gas and the hydrocarbon probe. Furthermore, to avoid a progressive decrease in retention time, it was necessary to recondition the column (*i.e.* by sweeping out with carrier gas at 200°C) before each measurement.

Another problem was the lack of reproducibility when small amounts of liquid adsorptive were injected into the column. This behaviour indicated that effective infinite dilution of hydrocarbon in the gas stream was not being approached —even when volumes as low as 0.05 μl were injected. This problem was overcome by the injection of small amounts of vapour equivalent to *ca.* $2 \cdot 10^{-5}$ μl of liquid.

When the above procedures were followed it was found that at a given temperature the experimentally determined values of specific retention volume were independent of flow-rate and of hydrocarbon sample size. Under these conditions it could be assumed that adsorption occurred at very low surface coverage so that use could be made of the equation

$$q_0^{st} = R \frac{d(\ln V_g)}{d(1/T)} \quad (2)$$

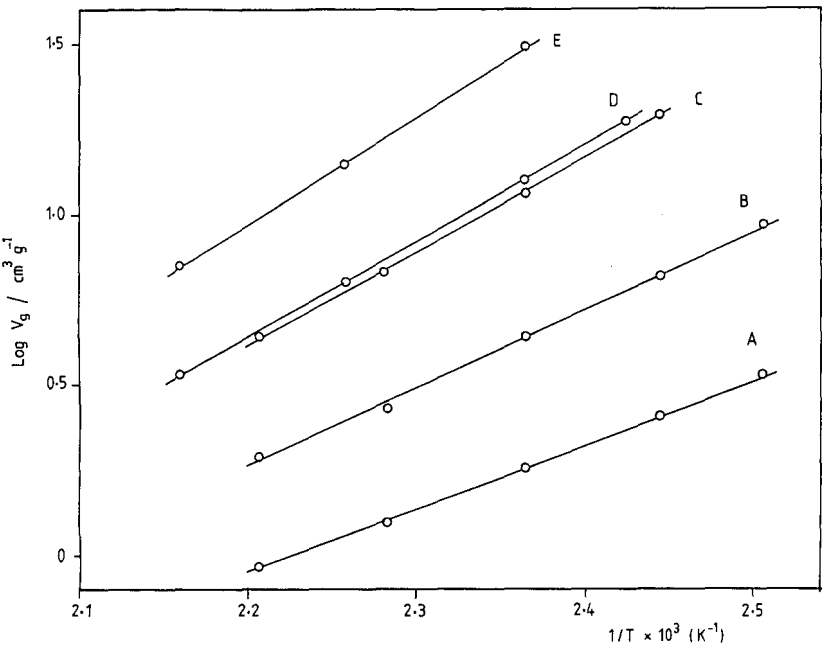

Fig. 2. Variation of specific retention volume with temperature for various hydrocarbons on pure calcium carbonate. Plots A, B, C, D and E are for *n*-hexane, *n*-heptane, *n*-octane, hex-1-ene and hept-1-ene, respectively.

in order to calculate q_0^{st}, the isosteric heat of adsorption at zero uptake (R is the universal gas constant).

Representative plots of log V_g against $1/T$ for the pure calcium carbonate are shown in Fig. 2 and the corresponding values of q_0^{st} are given in Table I. The log V_g *vs.* $1/T$ plots were all linear over the temperature range studied in spite of the fact that the asymmetry of the alkene peaks was pronounced at the lowest temperature (150°C). Values of skew ratio (*i.e.* the ratio of the horizontal distances at half peak height from the perpendicular at the peak maximum to the leading and trailing edges of the peak) are given in Table II. Since there was no detectable effect of sample size on the retention time we may attribute the band asymmetry to slow desorption from high energy sites.

It is evident from the values of q_0^{st} recorded in Table I that there is a fairly steady

TABLE I

ISOSTERIC HEATS OF ADSORPTION FOR VARIOUS HYDROCARBONS ON PURE CALCIUM CARBONATE

Adsorptive	q_0^{st} *(kJ mol^{-1})*
Hexane	36.4
Heptane	44.1
Octane	52.6
Hex-1-ene	53.7
Hept-1-ene	60.3

TABLE II

SKEW RATIOS OF THE CHROMATOGRAPHIC BANDS FOR VARIOUS HYDROCARBONS AT DIFFERENT TEMPERATURES ON PURE CALCIUM CARBONATE

Adsorptive	*Skew ratio*			
	190°C	*180°C*	*170°C*	*150°C*
n-Hexane	–	0.86	0.85	0.80
n-Heptane	–	0.78	0.75	0.70
n-Octane	–	0.77	0.74	0.68
Hex-1-ene	0.78	–	0.79	0.63
Hept-1-ene	0.71	–	0.69	0.61

increase (*ca.* 8 kJ mol^{-1} per CH_2 group) in the adsorbent–adsorbate interaction energy with increase in chain length of the *n*-alkanes. This level of increment is somewhat larger than that observed by Kiselev and co-workers[1,3,6] for the interaction of *n*-alkanes with the surface of graphitised carbon black or macroporous silica, although the *n*-hexane values for the $CaCO_3$ and SiO_2 are very similar.

The most striking feature of the results in Table I is the large difference between the values of q_0^{st} for the corresponding alkenes and alkanes (*i.e.* for hex-1-ene and hexane, Δq_0^{st} = 17.3 kJ mol^{-1}; for hept-1-ene and heptane, Δq_0^{st} = 16.2 kJ mol^{-1}). Since the equivalent values of Δq_0^{st} have been found[3] to be negative and close to zero for the adsorption of these vapours on graphitised carbon, we may conclude that this contribution of *ca.* 30% to the adsorption energy is due to a strong specific interaction between the alkene molecules and certain sites on the $CaCO_3$ surface. The scale of this specific contribution is of the same order of magnitude as has been reported for NaX zeolite[1,5] and $BaSO_4$[7] and these and other findings indicate that the active sites are probably exposed cations. The evidence for this conclusion will be discussed in detail in a subsequent paper.

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We wish to thank the Science and Engineering Research Council (SERC) for the provision of a research studentship (to T.A.) and ICI Chemicals and Polymer Group for financial assistance. In addition we thank Roger Rothon and David Harrison for help and advice.

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Note

Sampling and determination of carbon disulphide in air by gas chromatography with electron-capture detection

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Carbon disulphide is used in the viscose industry as a reactive solvent. It reacts with the hydroxy groups of cellulose to form cellulose xanthate, which dissolves in the alkali to form a viscous colloidal dispersion called viscose. When viscose is forced through a spinnerette into an acid bath, cellulose is regenerated in the filaments, which yield threads of rayon and carbon disulphide is liberated. Carbon disulphide causes severe damage to the nervous system and heart disease[1]. The threshold limit value (TLV) of carbon disulphide is 30 mg/m^3 (10 ppm)[2].

The first report of the measurement of carbon disulphide was based on the reaction between an amine, carbon disulphide and copper acetate. The reaction product shows UV absorption at 435 nm[3]. McCammon *et al.*[4] subsequently developed a sampling and gas chromatographic (GC) method with flame photometric detection. This method was adopted by NIOSH[5] and the HSE[6].

In this paper we report the determination of carbon disulphide by GC with electron-capture detection after its collection from air using different absorption tubes.

EXPERIMENTAL

Carbon disulphide and toluene were purchased from EGA Chemie. Toluene was doubly distilled and dried with magnesium sulphate. The gas chromatograph was a Hewlett-Packard 5890 A equipped with a nickel-63 electron-capture detector. The column (25 m × 0.32 mm I.D.) contained Nordibond OV-1701 with a film thickness of 1.0 μm (Nordion Instrument). The carrier gas was helium at a flow-rate of 1 ml/min. The analysis was performed isothermally at 85°C with split injection. The splitting ratio was 1:20 and the injector temperature 180°C.

The charcoal tubes tested were MSA 57 (Mine Safety Appliances), Orbo 32 (Supelco), SKC 226-38, SKC 226-65 and SKC 226-01 (SKC). The other sorbent tubes tested were Florisil 226-39, silica gel 226-51, XAD-2 226-30-04, Tenax 226-35-03 and Porapak Q 226-59-30-03, all from SKC. All the tubes contained 100 mg of sorbent in the front section and 50 mg in the back section, except Porapak Q, which contained 150 and 75 mg, respectively. Air was aspirated with the aid of an SKC Universal 224-PC7 air sample pump. At the low flow-rates of air adjustable flow holders were used (SKC

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224-26-04). The volume of the desorption solvent was 1 ml and the desorption time was 1 h at room temperature. The sample was used for GC analysis without percolation.

RESULTS

Standards were prepared by aspirating air at a flow-rate of 50 ml/min from an atmosphere containing 21 mg/m^3 (7 ppm) of carbon disulphide. The sampling times used were 30 min and 1 h, which theoretically produce 32 and 63 μg of carbon disulphide, respectively. The breakthrough was determined by analysing the two sections of the tubes separately. The results are presented in Table I. The minimum breakthrough was achieved with charcoal tubes. No marked difference in the breakthrough between the different types of charcoals was observed.

The desorption efficiency was measured using a phase equilibrium technique and toluene as solvent[7]. Eight concentrations of carbon disulphide were used. The best desorption efficiency was achieved with porous polymer tubes, and the best efficiency among the charcoal tubes was given by SKC 226-65. However, the coefficient of variation with SKC 226-65 was high. With all the charcoals tested the desorption efficiency increased strongly with increasing carbon disulphide concentration (Fig. 1). The desorption time had no marked effect on the recovery and the time selected for desorption was 1 h.

More detailed experiments were performed with charcoal tube type SKC 226-01. With a 4-h sampling time the effect of flow-rate on the breakthrough was studied. No

TABLE I

BREAKTHROUGH OF THE TESTED SORBENT TUBES

Carbon disulphide atmosphere, 7 ppm (21 mg/m^3); flow-rate 50 ml/min.

Sorbent	*Sampling time (min)*	*Breakthrough (%)*	*R.S.D. (%) (n=8)*
MSA 57	30	23	3.4
	60	32	3.9
Orbo 32	30	22	3.2
	60	29	3.6
SKC 226-38	30	23	3.6
	60	31	4.2
SKC 226-65	30	22	4.8
	60	27	5.0
SKC 226-01	30	18	2.8
	60	20	3.0
Tenax 226-35-03	30	49	5.6
	60	100	1.2
Porapak Q 226-59-30-03	30	50	5.7
	60	52	5.8
Silica gel 226-51	30	79	4.2
	60	94	3.2
Florisil 226-38	30	87	3.3
	60	100	2.4
XAD-2 226-30-04	30	46	3.9
	60	48	3.9

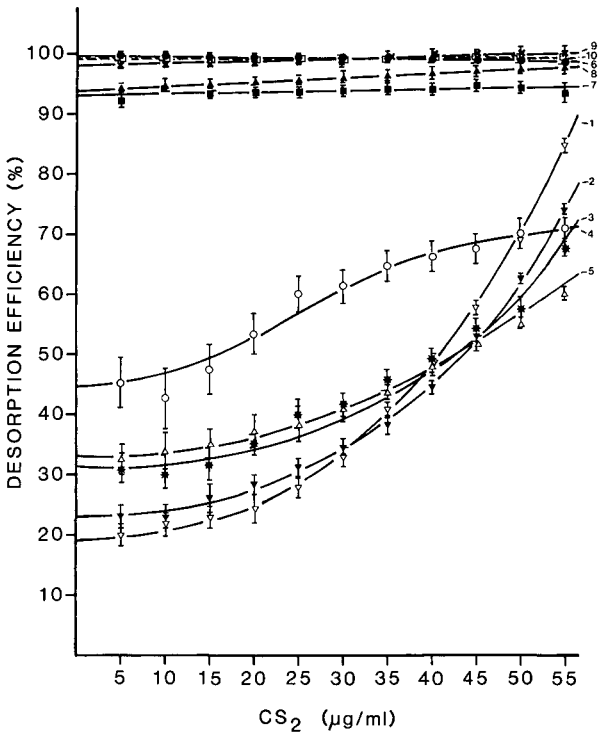

Fig. 1. Desorption efficiencies obtained using a phase equilibrium technique. 1 = SKC 226-01; 2 = SKC 226-38; 3 = SKC 226-65; 4 = MSA 57; 5 = Orbo 32; 6 = Tenax 226-35-03; 7 = Porapak Q 226-59-30-03; 8 = silica gel 226-51; 9 = Florisil 226-39; 10 = XAD-2 226-30-04.

breakthrough, even at relatively high concentrations, was observed using a flow-rate of 20 ml/min. Breakthrough as a function of flow-rate is shown in Table II.

Owing to the nature of the viscose process, brief "peak" concentrations of carbon disulphide occur. These may cause breakthrough and errors in the determina-

TABLE II

BREAKTHROUGH OF THE SKC 226-01 CHARCOAL TUBE (n=8) AS A FUNCTION OF FLOW-RATE AND COLLECTION TIME

Concentration		*Collection time (min)*	*Flow-rate (ml/min)*	*Breakthrough (%)*	*R.S.D. (%)*
ppm	*mg/m³*				
20	63	240	100	38	6.8
1	3.1	240	100	28	7.2
30	95	240	50	26	5.6
10	32	240	50	24	4.3
1	3.1	240	50	2.1	3.2
1	3.1	120	50	–	–
10	3.1	240	20	–	–
30	95	240	20	–	–

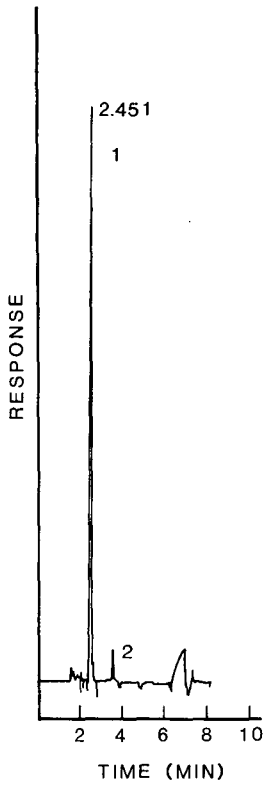

Fig. 2. Gas chromatogram with electron-capture detection of a sample collected from a Finnish viscose plant. 1 = Carbon disulphide; 2 = toluene.

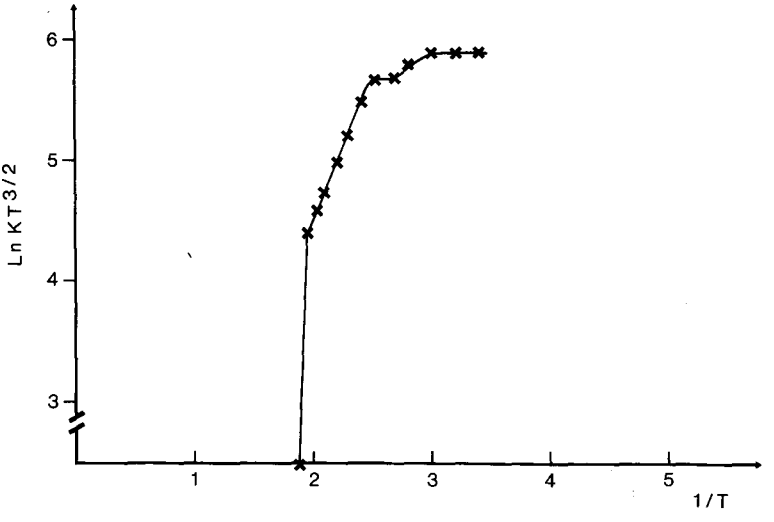

Fig. 3. Correlation of the electron-capture coefficient of the detection used towards carbon disulphide. The detector response was studied at a standard carbon disulphide concentration of 15 μg/ml.

tion of exposure during the whole working day. Therefore, SKC 226-01 charcoal was tested at a "peak" concentration of 42 mg/m^3 (100 ppm). The sampling time for 100 ppm was 20 min, which followed a 4-h collection of 15.5 mg/m^3 (5 ppm). The flow-rate was 20 ml/min. However, no breakthrough was observed.

The loss of sample in SKC 226-01 as a function of time was studied by storing the tubes in a refrigerator (6°C) for 3–14 days. The amount of carbon disulphide in each tube was 20 μg, which corresponds to 4.2 ppm for a 1.5-l sample. The observed sample loss was 2.3% [relative standard deviation (R.S.D.) 1.6%, $n=8$] and it was not dependent on the storage time.

Finally, in view of the relatively low desorption efficiency at low concentrations (Fig. 1), the desorption efficiency was studied with different solvents and solvent mixtures. No marked increase in desorption was observed after adding a polar modifier (methanol, ethanol, dimethylformamide) to the toluene or other solvents tested (acetone, methyl ethyl ketone, ethyl acetate or diisopropyl ether). The amount of the desorption solvent did not affect the desorption efficiency. Toluene was selected because of its good chromatographic and low electron-capture response properties.

The detection limit using the SKC 226-01 charcoal tube and toluene as desorption solvent was 1.5 ng per 1-μl injection. The desorption efficiency was 27% (R.S.D. 6.2%, $n=8$), which corresponds to 1.25 mg/m^3 (0.5 ppm) using a flow-rate of 20 ml/min and a collection time of 1 h.

A typical chromatogram for carbon disulphide in toluene is shown in Fig. 2. The peak was symmetrical and a good baseline separation was achieved at a column temperature of 85°C. The isothermal mode permits a fast analysis. The detector response was linear and the detection limit using pure carbon disulphide was 150 pg per 1-μl injection at a signal-to-noise ratio of 1:10. The ionization potential of carbon disulphide is 10.08 eV and the mechanism of the response of the electron-capture

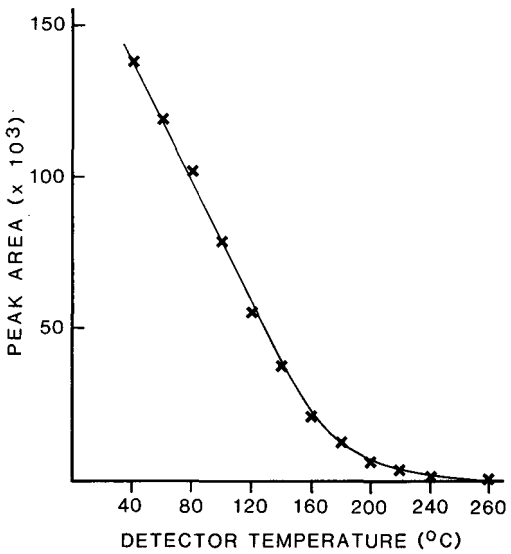

Fig. 4. Effect of detector temperature on detector response for the carbon disulphide.

detector seems to be a non-dissociative attachment to produce a stable negative molecular ion (Fig. 3). However, the electron-capture mechanism of carbon disulphide seems to be strongly dependent on temperature and the best response can be achieved at 40°C (Fig. 4).

CONCLUSION

The collection efficiency of the charcoal tubes is good, but the desorption efficiency at low concentrations is only moderate. Recommended sampling conditions are a charcoal tube, a flow-rate of 20 ml/min, a collection time of up to 4 h and desorption in toluene for 1 h, followed by GC with electron-capture detection.

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Note

Determination of sun-screen agents in cosmetic products by reversed-phase high-performance liquid chromatography

Part II.

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As part of a study concerning the determination by high-performance liquid chromatography (HPLC) of sun-screen agents in cosmetics[1,2] in order to verify their adherence to legislation within the European Economic Community (EEC), we have extended our investigation to a series of compounds commonly employed in suntan preparations as UV absorbers. Table I lists the compounds investigated with the code system proposed by Liem and Hilderink[3], and reports the maximum limits established under Italian legislation (law 713/86 and subsequent adjournments) in compliance with EEC Directive 76/768 on cosmetics.

The sun-screen agents BENZ 1, BENZ 6, BENZ 8, PABA 8 and CINA 11, already investigated[2], were considered again because of their wide application in cosmetic products. BENZ 1, BENZ 2, BENZ 4 and BENZ 5, which are not permitted as sun-screen agents for the protection of the skin, were examined owing to their frequent use in commercial products to protect them from UV radiation. This paper reports the results of an analysis performed on 85 samples of commercial suntan cosmetics.

EXPERIMENTAL

Apparatus

A model 5000 liquid chromatograph (Varian, Zug, Switzerland), equipped with a Valco AH 60 injection valve, a Varian Polychrom 9060 photodiode array detector and a Varian 4290 integrator, was used. The analytical column was made of stainless steel (250 mm × 4.6 mm I.D.), packed with 10-μm LiChrosorb RP-18 (Merck, Darmstadt, F.R.G.).

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Reagents

All reagents were of analytical-reagent grade, except methanol and acetonitrile (solvent for liquid chromatography grade). Water was deionized and doubly distilled from glass apparatus. All solvents and solutions for HPLC analysis were filtered through a Millipore filter (pore size 0.5 μm) and vacuum degassed by sonication before use. The sun-screen agents, available commercially, were obtained as listed in Table I.

Chromatographic conditions

The HPLC conditions were as follows: initial mobile phase, acetonitrile–water (5:95, v/v) containing 10^{-3} *M* perchloric acid and $5 \cdot 10^{-2}$ *M* sodium perchlorate; a linear gradient to 35% acetonitrile at 20 min; this composition for 5 min, and then another gradient to 80% acetonitrile at 40 min; flow-rate, 2.5 ml/min; column temperature, 30°C; injection volume, 10 μl; detector wavelength, 311 nm; detector sensitivity, 0.32 a.u.f.s.; and chart speed, 0.5 cm/min.

Calibration graphs

Stock solutions were prepared by dissolving the appropriate amount of the sun-screen agent in methanol. A set of standard solutions were produced by diluting aliquots of the stock solutions with methanol to give concentrations of each compound ranging from 5 to 100 μg/ml. The methanol solution was evaporated under reduced pressure and the residue extracted twice with 5 ml of 2 *M* sulphuric acid. The pooled acidic supernatants, containing BENZ 4, BENZ 8, SSCR 1 and PABA 8 (solution A) were submitted to HPLC. The residue containing the remaining sun-screen agents was dissolved in 10 ml of methanol (solution B) and injected into the chromatograph. Calibration graphs of peak area *versus* amount injected were constructed.

Extraction of sun-screen agents from cosmetics

The mixture of the compounds under investigation was added to samples of commercial cosmetic samples, known not to contain any sun-screen agent, to yield the concentrations given in Table III. About 1 g of cosmetic sample was weighed exactly into a centrifuge tube, 0.25 ml of 2 *M* sulphuric acid and 10 ml of methanol were added and the tube was immersed in an ultrasonic bath thermostated at 30°C for 30 min, then centrifuged at 900 *g* for 10 min. The extraction procedure was repeated and the pooled supernatants were diluted to volume (25 ml) with methanol. If PABA 8 and CINA 11 were both present, it was necessary to perform another extraction in order to separate the two sun-screen agents; 10 ml of the methanol solutions were withdrawn, evaporated under reduced pressure, and submitted to the extraction procedure described above to give solutions A and B.

RESULTS AND DISCUSSION

Table II reports the retention times, the purity parameter format values[4] and the limits of detection for the sun-screen agents investigated. All retention times were reproducible under the experimental conditions used, the average coefficient of variation being less than 3%. The UV spectral characteristics of the compounds were studied using a photodiode-array detector and the spectral data were interpreted by determining the purity parameter (Varian) format. This parameter, which utilizes an

TABLE I

SUN-SCREEN AGENTS STUDIED AND THEIR MAXIMUM LIMITS ACCORDING TO ITALIAN LAW 713/86 (ANNEX V, SECTION II)

Code name[a]	*Chemical name*	*Maximum admissible concentration (%)*	*Supplier*
BENZ 1[b]	2,4-Dihydroxybenzophenone	–	Sigma (I)
BENZ 2[b]	2,2′-Dihydroxy-4-methoxybenzophenone	–	Saipo (I)
BENZ 4[b]	2,2′-Dihydroxy-4,4′-dimethoxybenzophenone-5-sulphonic acid (sodium salt)	–	Saipo (I)
BENZ 5[b]	2,2′,4,4′-Tetrahydroxybenzophenone	–	Saipo (I)
BENZ 6	2-Hydroxy-4-methoxybenzophenone	10	Sigma (I)
BENZ 8	2-Hydroxy-4-methoxybenzophenone-5-sulphonic acid (sodium salt)	5 (expressed as free acid)	Sigma (I)
CAMP 4	3-(4′-Methylbenzilidene)-*d,l*-camphor	6	Merck (D)
SSCR 1	2-Phenylbenzimidazole-5-sulphonic acid	8 (expressed as free acid)	Saipo (I)
CINA 7	Diethanolammonium 4-methoxycinnamate	8 (expressed as free acid)	Saipo (I)
CINA 10	Isoamyl 4-methoxycinnamate	10	Haarman & Reimer (NL)
CINA 11	2-Ethylhexyl 4-methoxycinnamate	10	Givaudan (CH)
SALA 5	Homomenthyl salicylate	10	Quest International (NL)
SALA 9	Isopropylbenzyl salicylate	4	Vevy (I)
PABA 8	2-Ethylhexyl 4-dimethylaminobenzoate	8	Van Dyk (NL)

[a] According to ref. 3.
[b] Not covered by Italian law 713/86.

TABLE II

RETENTION TIMES, PURITY PARAMETER FORMAT VALUES (λm) AND LIMITS OF DETECTION

Compound	*Retention time (min)*	*λm (nm)*	*Detection limit (μg/ml)*
BENZ 1	27.05	284.75	0.20
BENZ 2	29.85	284.39	0.30
BENZ 4	3.27	263.06	0.40
BENZ 5	17.05	292.07	0.20
BENZ 6	33.84	284.00	0.20
BENZ 8	9.91	266.82	0.30
CAMP 4	38.61	294.02	0.10
SSCR 1	6.89	278.29	0.10
CINA 7	18.49	297.24	0.10
CINA 10	37.73	294.94	0.05
CINA 11	42.29	292.78	0.05
SALA 5	43.22	250.61	0.05
SALA 9	39.59	240.75	1.00
PABA 8	42.35	304.20	0.10

absorbance-weighted mean wavelength of a spectrum to reduce spectral data to a single value, can be used to confirm chromatographic peak identity and purity and to establish the class to which a compound belongs (*e.g.* to identify a compound as an amine). The detection limits were calculated as a response three times the noise level.

Fig. 1 shows the chromatogram of a standard mixture of sun-screen agents. PABA 8 and CINA 11 coelute with the mobile phase used, and to overcome this drawback a rapid screening of the cosmetic sample was carried out by thin-layer chromatography[5,6] to establish their presence. In this instance it was necessary to perform an extraction procedure capable of separating the compounds soluble in an aqueous solvent acidified with sulphuric acid, such as PABA 8, from the neutral and acidic compounds extractable into methanol, such as CINA 11. Fig. 2 shows the chromatograms of the aqueous and methanolic phases obtained after the extraction procedure described under Experimental.

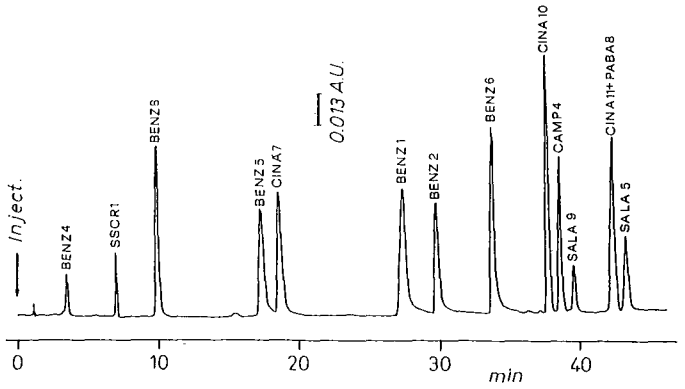

Fig. 1. Typical chromatogram of a standard mixture of sun-screen agents.

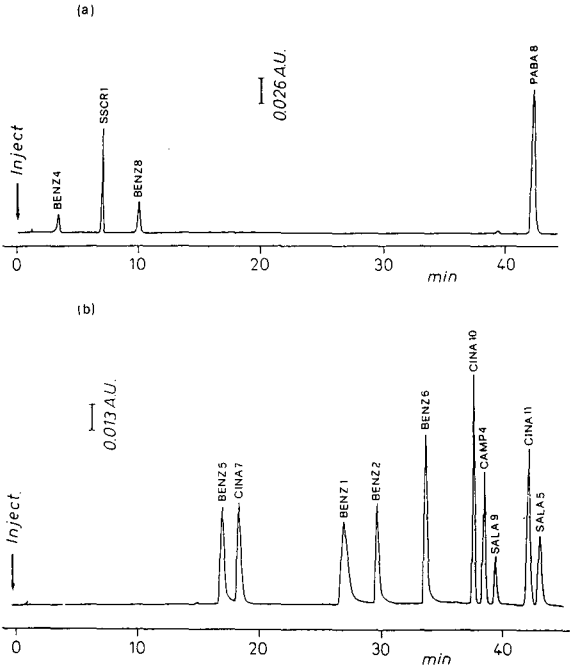

Fig. 2. Typical chromatograms of (a) solution A and (b) solution B obtained from a mixture of sun-screen agents subjected to the whole extraction procedure.

TABLE III

RECOVERIES OF SUN-SCREEN AGENTS FROM COSMETIC SAMPLES

Each value is the mean of five determinations.

Compound	*Amount added (%, w/w)*	*Recovery (%)*	*S.D. (%)*
BENZ 1	1.25	98.4	0.9
BENZ 2	1.25	98.2	1.6
BENZ 4	1.00	98.7	1.9
BENZ 5	1.25	99.6	1.4
BENZ 6	1.25	99.0	1.5
BENZ 8	1.00	97.9	2.0
CAMP 4	0.50	99.6	1.4
SSCR 1	0.50	97.6	1.6
CINA 7	0.50	98.1	1.6
CINA 10	0.37	97.6	1.8
CINA 11	0.37	98.7	1.2
SALA 5	2.00	97.9	1.0
SALA 9	1.75	97.6	1.9
PABA 8	0.50	99.1	0.9

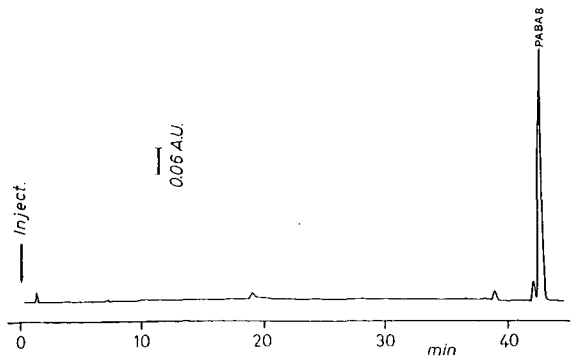

Fig. 3. Chromatogram obtained for a commercial suncream containing PABA 8.

Calibration graphs were constructed from six consecutive injections. Correlation coefficients obtained by linear regression analyses were in the range 0.9922–0.9999. Linearity was observed up to 1 μg of each sun-screen agent injected.

The applicability of the proposed method was demonstrated by determining the analytical recoveries of sun-screen agents added to some cosmetic samples known not to contain any of the compounds under investigation. Four proprietary suncreams were spiked with various amounts of the agents and submitted to the extraction procedure, and the recoveries obtained are shown in Table III. Good recoveries and precision were observed.

Fig. 3 shows the chromatogram obtained for a commercial suncream containing PABA 8. The minor peaks correspond to the excipients present in the cosmetic sample. Eighty-five commercial products were analysed for the presence of the sun-screen agents considered. In all instances the levels found were lower than the limits imposed by Italian law 713/86. Among the sun-screen agents examined the six most frequently used by the industry were found to be CINA 11 (47%), CAMP 4 (28%), SSCR 1 (25%), PABA 8 (18%), CINA 10 (13%) and BENZ 6 (10%). Frequently the cosmetic products contained a combination of two sun-screen agents, the most common being CINA 11 + CAMP 4 and CAMP 4 + SSCR 1.

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CHROM. 21 125

Note

High-performance liquid chromatographic determination of some psychotropic indole derivatives

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The indole derivatives psilocybin and psilocin were identified as active compounds of hallucinogenic mushrooms of the genus *Psilocybe*[1,2]. These compounds and their demethylated analogue baeocystin were later also found in many species of fungi[3–7]. A reliable determination of these compounds is very important in forensic analysis, toxicology and mushroom research. As the biosynthesis of psilocybin has not yet been satisfactorily clarified[8], it was of interest to monitor potential precursors and intermediates.

Thin-layer chromatography and/or high-performance liquid chromatography (HPLC) on silica or reversed-phase HPLC have often been used for the analysis of psychotropic indole derivatives[9–17]. In addition to conventional UV-photometric measurements, fluorimetric[10,12,15] and electrochemical[12,17] detection were used.

A simple method for determination of five hallucinogenic indole derivatives and serotonin is proposed in this study. The use of UV-photometric and electrochemical detection for "non-chromatographic separation" of non-readily separable compounds is described. The method was applied to the analysis of mycelium and fruit bodies of the fungus *Psilocybe bohemica* Šebek.

EXPERIMENTAL

Chemicals

Psilocybin and psilocin were from Sandoz (Basle, Switzerland), the other standard substances from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade from Lachema (Brno, Czechoslovakia).

Instrumentation

The liquid chromatograph comprised a 3 B high-pressure pump, injection valve Model 7105, LC-75 spectrophotometric detector and Chromatographics 2 data system (all from Perkin-Elmer, Norwalk, CT, U.S.A.). A voltammetric 641 VA-Detector (Metrohm, Herisau, Switzerland) was connected in series with the UV detector. The separation was performed on an analytical column (250 mm × 4 mm I.D.) packed with Separon SGX C_{18}, 7 μm (Tessek, Prague, Czechoslovakia). The mobile phase was a mixture of buffer pH 3.1 (5.1 g KH_2PO_4, 2.1 g KOH and 13.1 g citric acid monohydrate) with ethanol, flow-rate 1.0 ml/min.

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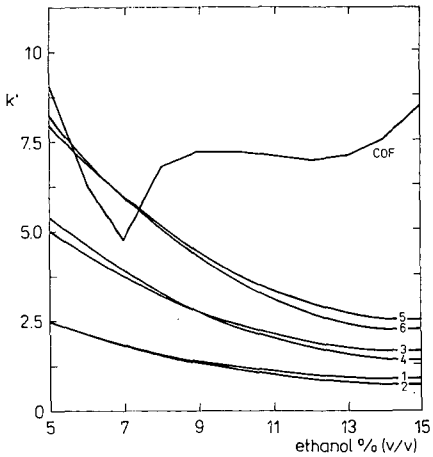

Fig. 1. Dependence of the capacity factor and the chromatographic optimization function (COF) on the ethanol content in the mobile phase. 1, Psilocybin; 2, serotonin; 3, tryptophan; 4, bufotenine; 5, tryptamine; 6, psilocin.

RESULTS AND DISCUSSION

For the optimization of the ethanol content in the mobile phase, we used a procedure based on computer processing of retention and detection data of the compounds studied[18]. Fig. 1 shows that, under the given conditions, it is not possible to separate chromatographically the pairs psilocybin–serotonin, tryptophan–bufotenine and tryptamine–psilocin. Separation of these pairs of compounds is optimal at 10% (v/v) ethanol in the mobile phase. A chromatogram of a mixture of standard substances in this mobile phase is shown in Fig. 2. All pairs of compounds are perfectly baseline separated.

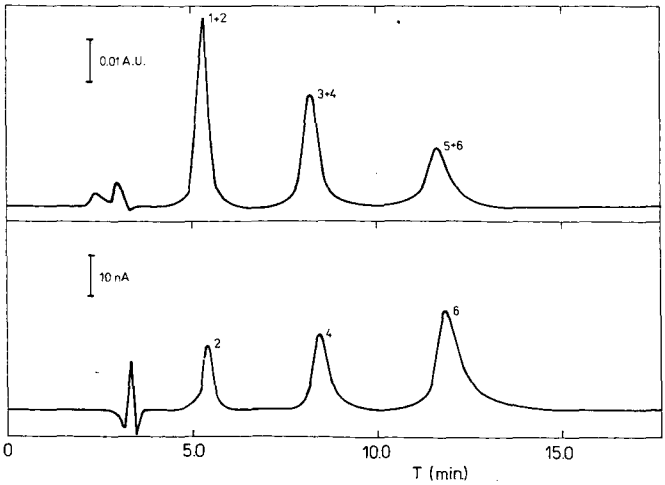

Fig. 2. Chromatogam of a mixture of hallucinogens with electrochemical (lower trace) and UV-photometric (upper trace) detection. Designation of substances as in Fig. 1.

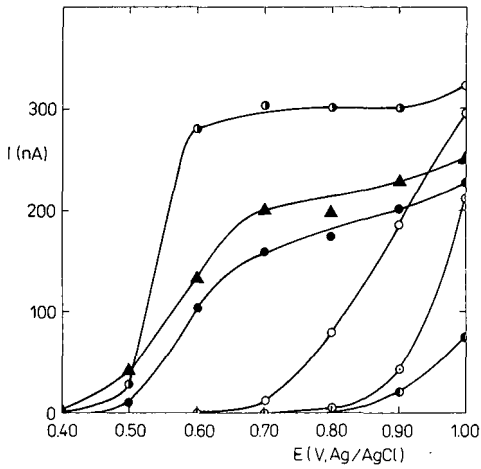

Fig. 3. Hydrodynamic voltammograms of psilocybin (○), serotonin (●), tryptophan (◐), bufotenine (◑), tryptamine (⊙) and psilocin (▲). Mobile phase: citrate–phosphate buffer pH 3.1 with 10% (v/v) ethanol. Flow-rate: 1.0 ml/min.

It is possible to separate chromatographically non-separable pairs by electrochemical selective detection. From the hydrodynamic voltammograms (Fig. 3) it follows that 4- and 5-hydroxylated derivatives are selectively detected at a working electrode potential of approximately +0.60 V (Ag/AgCl), whereas the half-wave potential of the other substances is higher than +0.80 V.

The external standard method was used for quantitation. The calibration must be performed with two standard mixtures of different compositions. The first mixture (M1) consisted of all compounds to be determined, the second mixture (M2) contained serotonin, bufotenine and psilocin. The signal of the electrochemical detector was evaluated directly; the concentration of the compounds detected only by the

TABLE I

CALIBRATION DATA FOR THE UV PHOTOMETRIC AND ELECTROCHEMICAL DETECTORS

Substance	*Detector type*	*Slope*[a]	*Correlation coefficient*	*Detection limit (ng)*	*Relative standard deviation*[b] *(%)*
Psilocybin	UV	0.3767	0.9963	40	6.3
Tryptophan		0.4344	0.9856	35	3.4
Tryptamine		0.1924	0.9851	79	3.8
Serotonin	ED	56.956	0.9470	4.3	9.1
Bufotenine		15.648	0.9581	15	7.4
Psilocin		26.432	0.9740	9.4	7.2

[a] For UV photometric detector at 267 nm in a.u. · 10^4/ng and for electrochemical detector at +0.60 V (Ag/AgCl) in pA/ng.

[b] Five parallel determinations.

UV-photometric detector were calculated according to

$$C = \frac{H(\mathrm{UV, S}) - H(\mathrm{EC, S})H(\mathrm{UV, M2})/H(\mathrm{EC, M2})}{H(\mathrm{UV, M1}) - H(\mathrm{EC, M1})H(\mathrm{UV, M2})/H(\mathrm{EC, M2})} \cdot \frac{V(\mathrm{M1})}{V(\mathrm{S})} \cdot C(\mathrm{M1})$$

where C is the concentration, H the peak height, UV and EC refer to the detector type, M1 and M2 to the calibration mixture used, S to the sample and V is the volume injected.

Table I presents the calibration data obtained by the method described. This method of chromatogram evaluation is less accurate than conventional methods. However, it is very advantageous in cases when a satisfactory chromatographic separation is impossible.

We used the method for the analysis of methanolic extracts of the fungus *Psilocybe bohemica*. Values of 0.93% of psilocybin, 0.01% of psilocin, 0.01% of tryptophan and 0.02% of tryptamine per dry mass of fruit bodies were detected. The concentration in the mycelium is lower by about an order of magnitude.

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CHROM. 21 136

Note

Rapid determination of isomer ratios of butylated hydroxyanisole by high-performance liquid chromatography

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(Received November 9th, 1988)

Butylated hydroxyanisole (BHA) is widely used as an antioxidant in food and cosmetic products. Commercially available BHA consists of two isomers (Fig. 1): 3-*tert*.-butyl-4-hydroxyanisole (3-BHA, major isomer, >85%) and 2-*tert*.-butyl-4-hydroxyanisole (2-BHA, minor isomer, <15%). 3-BHA has the stronger antioxidant properties[1].

BHA is both an efficient inhibitor and promoter of chemically induced carcinogenesis[2,3]. 2-BHA is the more effective inhibitor of benzo[*a*]pyrene-induced forestomach neoplasia in mice[4]. Further, BHA causes forestomach lesions, hyperplasia, papillomas and carcinomas in rodents after feeding of high levels in the diet[3,5]. This carcinogenic action of BHA is largely attributable to 3-BHA, the main component; 2-BHA appears to be much less effective[5].

Therefore, in experiments with BHA its isomer ratio should be known. Also, current pharmacopoeias require that the levels of 2- and 3-BHA in a batch be determined. We have previously reported a sensitive high-performance liquid chromatography (HPLC) method for measuring plasma BHA concentrations[6]. That method made no distinction between 2- and 3-BHA, however, as both analytes had the same retention time. We now report a rapid and simple method for the determination of the isomer ratio in BHA batches, applying normal-phase HPLC and UV or fluorescence detection, which is convenient with respect to simplicity, speed, column maintainance, precision and cost in comparison with other methods involving thin-layer chromatography[7,8], gas chromatography[8,9], HPLC[8,10,11], column chromatography[12] or non-chromatographic methods[8,13].

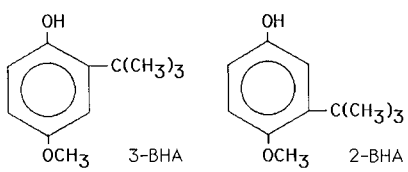

Fig. 1. Structures of 3-BHA (major isomer, >85%) and 2-BHA (minor isomer, <15%).

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EXPERIMENTAL

Reference standards were a gift from Rhône-Poulenc (Dagenham, U.K.) and contained 85.3, 88.8, 91.5 and 96.2% of 3-BHA respectively (determined by infrared spectroscopy[14]). Two batches of food-grade BHA were a gift from J. Dekker (Wormerveer, The Netherlands) and one batch of BHA was purchased from Sigma (St. Louis, MO, U.S.A.). *n*-Heptane (Fisons, Loughborough, U.K.) and ethanol (Merck, Darmstadt, F.R.G.) were of HPLC grade.

Solutions of 100 μg/ml of 2- and 3-BHA were prepared in *n*-heptane and 20-μl samples were injected on to a LiChrosorb Si 60-5 column (250 × 4.6 mm I.D.) (Chrompack, Middelburg, The Netherlands). The mobile phase was *n*-heptane–ethanol (400:5, v/v) at a flow-rate of 2.5 ml/min. The effluent was monitored by a Kratos Spectroflow 980 programmable fluorescence detector set at 290 nm with a cut-off filter at 320 nm in series with a Kratos Spectroflow 783 programmable UV detector set at 290 nm. For the determination of isomer ratios, peak heights were measured in addition to peak areas (by cutting and weighing).

RESULTS AND DISCUSSION

Preliminary experiments indicated that a mixture of 2- and 3-BHA can be separated on a LiChrosorb 5 RP-18 column (150 × 4.6 mm I.D.) and on a LiChrosorb RP-18 10-cm cartridge column when eluted with methanol–water–acetic acid (40:59:1, v/v/v) or an eluent of comparable strength with acetonitrile instead of methanol. These methods resulted in long retention times (up to 1 h), however, and produced very asymmetric peaks, 3-BHA eluting before 2-BHA.

Fast elution of separated isomers can be achieved, however, on a normal-phase LiChrosorb Si 60-5 column. Fig. 2 shows the effect of increasing concentrations of ethanol in *n*-heptane on the capacity factors (k') of the BHA isomers. Ethanol (1.25%) produced a fast and complete separation of isomers, with almost symmetrical peaks, within 11 min; 3-BHA and 2-BHA elute after 8.9 and 10.3 min, respectively. The limit of detection was about 0.5 ng for either isomer. Typical chromatograms are shown in Fig. 3.

Table I gives the results of the determination of isomer ratios of 2- and 3-BHA in

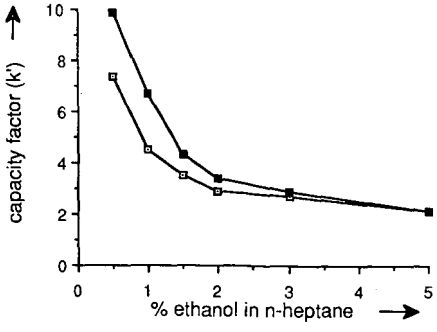

Fig. 2. Effect of increasing concentrations of ethanol in *n*-heptane on the capacity factors (k') of (■) 2-BHA and (⊡) 3-BHA.

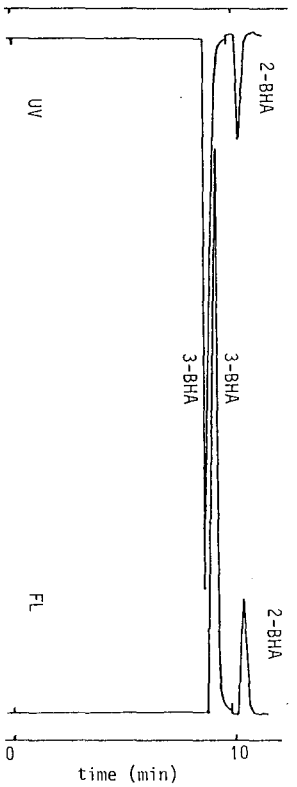

Fig. 3. Typical chromatograms for the separation of 2- and 3-BHA on a LiChrosorb Si 60-5 column using ethanol–*n*-heptane (400:5). For UV detection (up) the range was set at 0.05 a.u.f.s. for 0–9.5 min and at 0.01 a.u.f.s. thereafter; for fluorescence detection (down) the settings were 0.25 and 0.05 a.u.f.s., respectively.

TABLE I

DETERMINATION OF ISOMER RATIOS OF 2-BHA AND 3-BHA IN DIFFERENT BATCHES

Isomer ratios are expressed in terms of the percentage of 3-BHA (mean ± S.D.).

Batch[a]	*Peak-area method*		*Peak-height method*	
	UV detection	*Fluorescence detection*	*UV detection*	*Fluorescence detection*
RP (85.3%)	85.8 ± 0.5	85.4 ± 0.5	87.4 ± 0.2	86.8 ± 0.2
RP (88.8%)	90.0 ± 0.2	89.7 ± 0.2	91.3 ± 0.1	90.8 ± 0.1
RP (91.5%)	91.8 ± 0.3	91.5 ± 0.3	92.9 ± 0.1	92.5 ± 0.1
RP (96.2%)	96.2 ± 0.2	96.0 ± 0.1	96.7 ± 0.1	96.5 ± 0.0
FG I	86.9 ± 0.3	86.5 ± 0.2	88.4 ± 0.1	87.8 ± 0.2
FG II	93.6 ± 0.3	93.0 ± 0.2	94.2 ± 0.1	93.8 ± 0.1
S	95.8 ± 0.2	95.5 ± 0.1	96.3 ± 0.1	96.1 ± 0.1

[a] RP, sample from Rhône-Poulenc containing known isomer ratios; FG, food-grade sample; S, commercially obtained sample.

several batches. Determinations based on peak-area measurement after UV or fluorescence detection gives a reliable index of isomer ratios as the values determined are in close agreement with those already known. Determinations based on peak heights give poorer results. The coefficients of variation (C.V.) for the peak-area and peak-height methods were 0.3% and 0.1% respectively, with no difference between UV and fluorescence detection. The larger C.V. of the peak-area method may be due to the weighing method.

In conclusion, 2- and 3-BHA are easily separated on a LiChrosorb Si 60-5 column using 1.25% ethanol in *n*-heptane as the eluent. The isomer ratios are determined by measuring peak areas based on UV or fluorescence detection.

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CHROM. 21 137

Note

Reversed-phase high-performance liquid chromatographic separation of lutein and lutein fatty acid esters from marigold flower petal powder

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Lutein is a common carotenoid in nature, occurring in all green structures of plants and also in many flower petals[1]. It is frequently used in industry as a natural food colorant, for instance in poultry feeds, to enhance pigmentation of the skin and egg yolk[2]. Currently, the commonest commercial source of lutein is the flower of the marigold plant *Tagetes erecta*[3], where it is found esterified with one or two fatty acids, and constitutes about 90% (w/w) of the petals[4].

Free xanthophylls and their esters not only have different stabilities but also differ widely in their ability to act as coloring agents in food technology[5]. Hence it is of great interest to have methods to separate and determine lutein and lutein esters in every step of the processing of this coloring agent (*i.e.*, in raw materials, during saponification procedures, etc).

The aim of this work was to devise a fast, sensitive and quantitative method to investigate the pigment composition of lutein sources and the pigment composition of these materials during processing. In recent years high-performance liquid chromatography (HPLC) has been shown to be a useful and accurate technique for separating and identifying carotenoids[6]. We have developed a new reversed-phase HPLC method for separating in a single step lutein and the different lutein fatty acid esters in colour sources and in other coloured products. This method could also be of general interest in the study and control of xanthophyll saponification processes.

EXPERIMENTAL

Extraction and sample preparation

Carotenoids from marigold (*Tagetes erecta*) petal powder were extracted with acetone overnight in a tightly closed flask according to the general method of Britton[7]. Carotenoids were transferred to *n*-hexane and the *n*-hexane fractions were dried over anhydrous sodium sulphate and evaporated to dryness in a stream of nitrogen. Samples were dissolved in ethyl acetate and passed through a Sep-Pak C_{18} cartidge (Millipore) in order to remove any substance that may stick non-reversibly to the octadecylsilane. Samples were then filtered twice through a 0.45-μm HVLP Millipore filter to remove insoluble particles before analysis.

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Saponification

Saponification was performed in order to hydrolyse carotenoid esters. After evaporation of the *n*-hexane the carotenoid extracts were dissolved in ethanol and sufficient potassium hydroxide solution (60%, w/v) was added to bring the final overall potassium hydroxide concentration to 10%. The mixture was allowed to stand at room temperature under nitrogen and in the dark for different times to allow saponification to progress to different extents. The ethanolic phase was re-extracted with *n*-hexane, and the *n*-hexane layer washed with water until free from alkali. Then the saponified extracts were prepared as indicated above before performing the analysis.

HPLC procedure

The HPLC system consisted of a high-pressure pump (LKB 2150), a low-pressure mixer driver (LKB Ultrograd 11300), a mixing valve to form gradients (LKB 2040-203) and a Rheodyne injection valve (7125). The mobile phase consisted of a linear gradient of ethyl acetate from 0 to 100% in acetonitrile–methanol (9:1, v/v) over 30 min, performed with an LKB 2125 programmable solvent delivery controller. Acetonitrile, methanol and ethyl acetate (HPLC grade, Scharlau) were filtered (Ultipor NX 0.45-μm membrane filter) and degassed with a stream of helium prior to use. The stationary phases used were two different octadecylsilane reversed-phase columns: LiChrosorb RP-18 (LKB UltroPac) and Zorbax ODS (Thames Chromatography) (both 250 × 4.0 mm I.D., with 5-μm spherical particles).

Samples were loaded on to the column via a Rheodyne injection valve (20-μl sample loop). The flow-rate was 1 ml/min and the pressure ranged from 66 to 87 bar in the LiChrosorb column and from 22 to 29 bar in the Zorbax column. Carotenoids in the effluent were continuously monitored with a photodiode array detector (Waters 990) covering the range 300–600 nm and connected to an NEC APC III computer for storing and processing of chromatograms and spectra.

Identification and quantitative evaluation

Peak identification was based on retention times and comparison with a lutein standard, and also on visible spectra of the chromatographic peaks obtained with the photodiode array detector. All the peaks present in the chromatograms were identified as xanthophylls (lutein and lutein esters) by UV–VIS spectrophotometry, having maxima at 425, 446 and 475 nm.

To carry out quantitative analysis of the data, peaks were monitored at the λ_{max} of the carotenoid and simultaneous integration was achieved at this wavelength using the NEC APC III think-jet integration facility. Calibration was carried out with the lutein standard, plotting the peak-area ratio *versus* concentration. The response factor of lutein was obtained from separate injections of the standard solution at several concentrations. Quantitation was achieved using the extinction coefficient of the standard because all the fractions eluted were lutein and lutein esters.

The relative quantitation of the peaks of diesterified and monoesterified lutein was based on partial integration of each group of these peaks in the chromatograms with the NEC APC III think-jet.

RESULTS AND DISCUSSION

Serious difficulties have previously been reported when separating carotenoid mixtures in a single-step chromatographic procedure owing to the need to cover the whole range of polar and non-polar carotenoids[8,9]. This difficulty is increased when separations between xanthophylls and their fatty acid esters are to be achieved, owing to the large difference in polarity between these compounds. Gau *et al.*[10] used HPLC on C_{18} with eluent mixtures of medium polarity (dichloromethane–acetonitrile) in order to separate xanthophyll esters, but this isocratic method needed very high flow-rates (3 and 15 ml/min) and did not resolve simultaneously diesterified, monoesterified and free xanthophylls.

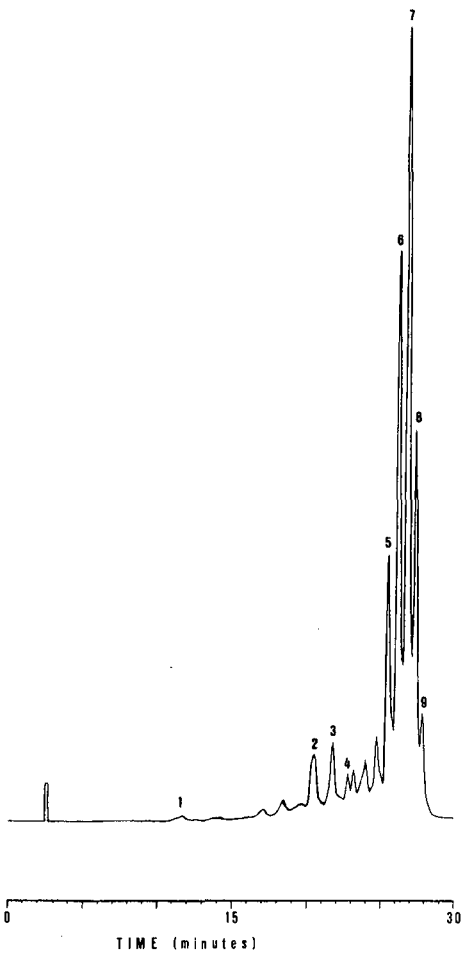

Fig. 1. HPLC of an unsaponified extract of marigold. Mobile phase, linear gradient of ethyl acetate from 0 to 100% in acetonitrile–methanol (9:1, v/v) over 30 min; flow-rate, 1.00 ml/min; column, Zorbax ODS, 5 μm (250 × 4.0 mm I.D.); detection, 450 nm; volume injected, 20 μl. Peaks: (1) lutein; (2) monomyristate of lutein; (3) monopalmitate; (4) monostearate; (5) dimyristate; (6) myristate–palmitate; (7) dipalmitate; (8) palmitate–stearate; (9) distearate.

The HPLC method proposed here separates in 30 min free, mono- and diesterified lutein with a simple linear gradient of ethyl acetate in acetonitrile–methanol. An example of the use of this method can be seen in Fig. 1, which shows a chromatogram run on a Zorbax column of the unsaponified extract from marigold petal powder. The chromatogram shows a lack of significant amounts of free lutein (peak 1). This confirms the notion that the xanthophyll esters constitute the majority of the carotenoid pool in marigold petal powder. In the chromatogram shown in Fig. 1, lutein esters eluted after 20 min, represented 95.5% of the total carotenoids in marigold petals, the last five peaks (5–9) being the major components (79.5%). Peaks not numbered in the figures are minor xanthophyll esters that were not identified.

The major peaks in the original unsaponified extract (Fig. 1) were identified as diesterified lutein (lutein esterified with two fatty acid chains). The two fatty acids can be the same (diesters) or different (mixed esters)[10–11]. These esters were associated with dimyristate (Fig. 2, peak 5), myristate–palmitate (peak 6), dipalmitate (peak 7), palmitate–stearate (peak 8) and distearate (peak 9) by comparing their relative retention times with those reported by Gau *et al.*[10] who separated xanthophyll fatty acid esters extracted from marigold flower petals by reversed-phase HPLC (LiChrosorb RP-18). These five fatty acid esters of lutein are the most abundant in marigold petals, and the quantitation (means for five samples) of the major ester peaks showed good agreement between the values found by Gau *et al.*[10] and our results. These data are presented in Table I.

Fig. 2 shows the carotenoid composition at three different times during the saponification of the lutein esters from marigold petal powder: immediately after the start of saponification (Fig. 2a), after 10 min of hydrolysis (Fig. 2b) and after 3 h of hydrolysis (Fig. 2c). This series of chromatograms shows the progressive hydrolysis of esters. Peaks corresponding to lutein esters decreased during the saponification process whereas the lutein peak exhibited a concomitant increase, until it became the major carotenoid in the final mixture (Fig. 2c, peak 1 = 91.3%).

LiChrosorb (Fig. 2) and Zorbax (Fig. 1) columns gave similar chromatograms, with lutein retention times of 9.33 ± 0.05 and 11.25 ± 0.16 min, respectively.

After partial saponification (Fig. 2a and b), two groups of peaks were present. One corresponds to diesterified lutein (peaks 6–9) and the other, with lower retention times, to monoesterified lutein (peaks 2–4). These monoesters originated from hydrolysis of one of the ester bonds.

TABLE I

PERCENT DETERMINATION OF DIESTERIFIED LUTEIN IN AN EXTRACT OF UNSAPONIFIED MARIGOLD PETALS

Peak No.	*Concentration (area-%)*		*Xanthophyll diesters and mixed esters*
	Gau et al.[10]	*This work (mean of 5 samples)*	
5	12.6	11.59 ± 0.39	Dimyristate
6	24.7	24.23 ± 0.87	Myristate–palmitate
7	35.5	37.57 ± 1.42	Dipalmitate
8	14.4	15.55 ± 0.47	Palmitate–stearate
9	2.4	3.63 ± 0.52	Distearate

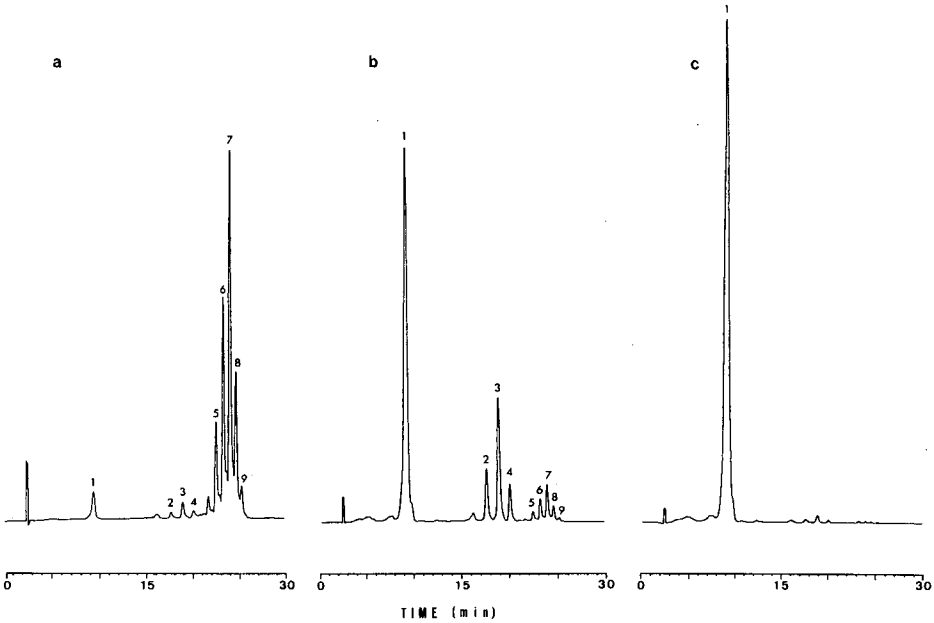

Fig. 2. HPLC absorbance (450 nm) chromatograms of marigold flower petal powder extracts saponified and semi-saponified: (a) immediately after the start of saponification; (b) after 10 min of hydrolysis; (c) after 3 h of hydrolysis. Chromatographic conditions as in Fig. 1, except the column was LiChrosorb RP-18,5 μm (250 × 4.0 mm I.D.).

The major fatty acid esters present in the hydrolysate of *Tagetes* are known to be myristate, palmitate and stearate[12]. In earlier work in our laboratory, the fatty acids isolated from marigold petals were identified by gas chromatography by comparison with pure standards; myristic, palmitic and stearic acids being the major components[13]. They correspond to the monoester peaks 2, 3 and 4, respectively. Moreover, the relative amounts of each of these three monoesters (*e.g.* monomyristate, peak 2: 24.0%) in the semi-saponified extract (Fig. 2b) was found to be equal to the sum of the corresponding diester (dimyristate, peak 5: 11.6%) plus half of the other mixed esters (myristate–palmitate, peak 6 24.2/2 = 12.1%) in the original unsaponified extract. This confirms the tentative identification, although it was not the original purpose of this work.

The HPLC method reported here is very suitable (simple, rapid and reproducible) for analysis of xanthophylls and their esters. This non-aqueous reversed-phase HPLC method surpasses thin-layer chromatography, as it allows the separation of mixed esters from the diesters of xanthophyll. Moreover, it is an improvement over other HPLC methods as it separates free xanthophylls from their fatty acid esters in a single step. Finally, this chromatographic system could be useful in the study and control of xanthophyll saponification processes.

ACKNOWLEDGEMENTS

I express my gratitude to Dr. José C. G. Milicua and Dr. Ramón Gómez for

critically evaluating, and to Dr. George Britton from Liverpool University for his kind collaboration to perform HPLC on carotenoids in our laboratory. This work was supported in part by a research grant from the "Ministerio de Educación y Ciencia"of Spain.

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Note

Analyse quantitative par chromatographie liquide haute performance du di-*tert.*-butyl-2,6-*para*-crésol dans les huiles de transformateurs

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(Reçu le 9 décembre, 1988)

Les huiles de transformateurs contiennent un inhibiteur d'oxydation: le di-*tert.*-butyl-2,6-*para*-crésol (DBPC). En service, cet additif se dégrade et dès son épuisement; le vieillissement de l'huile s'accélère et une modification importante de ses propriétés diélectriques est observée[1].

La spectroscopie infrarouge[2] et ultraviolette[3], la voltamétrie anodique[4], la cyclovoltamétrie impulsionnelle différentielle[5,6] et la chromatographie en phase gazeuse[7] sont les méthodes analytiques généralement utilisées pour quantifier le DBPC dans une huile neuve ou usée. Ces méthodes sont peu sensibles et non-spécifiques car les produits d'oxydation de l'inhibiteur interfèrent. Il y a quelques années, l'utilisation de la chromatographie liquide haute performance (CLHP)[8] a été proposée par notre laboratoire. Le procédé analytique était sensible, spécifique et reproductible mais il était long et fastidieux. Une pré-chromatographie d'absorption de l'huile neuve ou usée sur un gel de silice était nécessaire.

Cet article décrit l'analyse quantitative du DBPC dans une huile neuve par injection directe sur la colonne CLHP. Dans le cas d'une huile usée, une technique rapide de préparation de l'échantillon, faisant appel à l'utilisation de cartouches Sep-Pak de silice, a été optimisée. Elle permet d'obtenir une détermination du DBPC après CLHP en 18 min.

PARTIE EXPÉRIMENTALE

Appareils et conditions chromatographiques

Les études de CLHP ont été effectuées à l'aide d'un appareil Varian Série 5500 (Varian, Walnut Creek, CA, États-Unis). Il était équipé d'une vanne d'injection à boucle de 5 μl (Modèle 7126, Rheodyne, Cotati, CA, États-Unis) et d'un détecteur ultraviolet Modèle UV-200 de Varian. Les chromatogrammes ont été enregistrés et intégrés par le Modèle Varian DS 651 de la Série Vista.

Deux colonnes Hamilton PRP-1 montées en série (150 × 4.1 mm I.D., 10 μm) ont été utilisées (Hamilton, Reno, NE, États-Unis). La phase mobile était constituée par un mélange d'acétonitrile–eau (97,5:2,5%, v/v). Le débit a été fixé à 1,0 ml/min sous une pression de 20 atm. Les solvants ont été filtrés sur une membrane 0,45-μm (Millipore, Bedford, MA, États-Unis). L'analyse a été effectuée à la température ambiante et le DBPC a été détecté à 283 nm.

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Courbe de calibration

Des échantillons standards (25,1–1008,5 ppm) ont été obtenus par la dissolution de quantité précise de DBPC dans l'huile Voltesso 35 non inhibée (Pétroles Esso, Canada). Ces échantillons (5 μl) ont été injectés directement sur le système de CLHP et la surface (μV s) sous le pic d'élution du DBPC a été utilisée pour établir la courbe de calibration.

Analyse du DBPC dans une huile neuve ou usée

Huile neuve. Le contenu en DBPC est déterminé par l'injection directe de l'huile Voltesso 35 sur les colonnes CLHP.

Huile usée. Un échantillon de 100 μl d'huile est déposé sur une cartouche Sep-Pak de silice (Waters Assoc., Milford, MA, États-Unis) et 60 ml de pentane sont percolés en 30 s. La solution pentanique est évaporée sous vide et le résidu (5 μl) est injecté. La cartouche est, préalablement, conditionnée par le passage rapide de 100 ml de pentane.

Réactifs

Le DBPC est de qualité ACS et les solvants chromatographiques sont de qualité CLHP (Anachemia, Montréal, Canada).

RÉSULTATS ET DISCUSSION

Analyse d'huiles neuves

La Fig. 1 présente le chromatogramme de l'huile de transformateurs Voltesso 35 contenant 500 ppm de DBPC et le chromatogramme d'un blanc (0 ppm de DBPC).

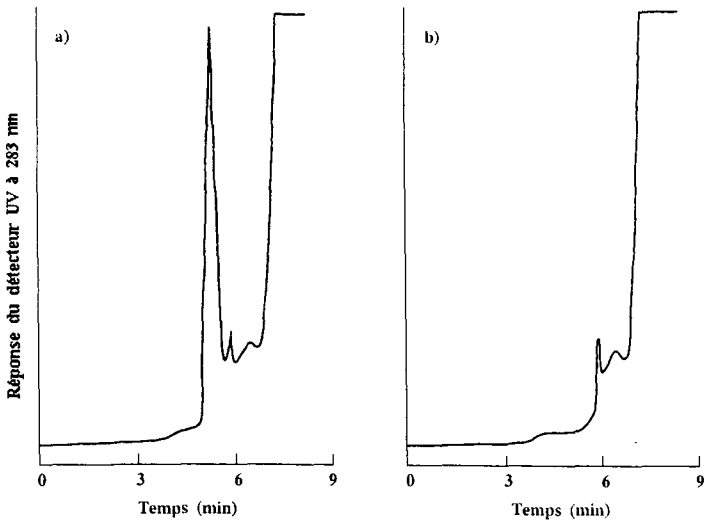


Fig. 1. Chromatogramme de l'huile de transformateurs Voltesso 35 contenant (a) 500 ppm de DBPC et (b) 0 ppm de DBPC. Conditions analytiques: colonnes Hamilton PRP-1, (10 μm, 300 × 4,1 mm I.D.); phase mobile, acétonitrile–eau (97,5:2,5); débit, 1,0 ml/min; détecteur, UV (283 nm).

Leur comparaison permet de constater que le DBPC est totalement séparé des composantes de l'huile et la présence d'interférences est presque nulle. Le temps d'élution du DBPC est de 5,3 min et une variation inférieure à 1% a été obtenue lorsque 50 échantillons sont injectés sur une période de cinq jours; ce qui dénote une excellente reproductibilité.

La limite de détection, la précision analytique et la linéarité de la méthode ont été examinées et les résultats sont présentés au Tableau I. La limite de détection du DBPC dans l'huile Voltesso 35 est 2,2 ppm lorsque cette huile est injectée directement sur les colonnes CLHP. Cette limite a été calculée, avec une probabilité de 95%, en utilisant l'approche statistique proposée par McAinsh *et al.*[9]. La précision analytique, exprimée par le coefficient de variation (C.V.) de l'aire du pic d'élution (cinq essais) est ± 1,04% pour des teneurs en DBPC comprises entre 25,1 et 1008,5 ppm. L'étude de la linéarité de la réponse du détecteur ultraviolet à 283 nm en fonction de la variation des concentrations en DBPC a permis d'obtenir une droite de régression dont le coefficient de corrélation (r) est supérieur à 0.9999. Ce résultat indique une très bonne linéarité de la méthode dans la gamme des concentrations examinées.

L'analyse d'huiles de transformateurs neuves (*i.e.* avant la mise en service de l'appareil) indique que les huiles isolantes Voltesso 35 utilisées à Hydro-Québec sont inhibées par l'addition de 700 pm de DBPC (n = 5).

Analyse d'huiles usées

En service, le vieillissement chimique de l'huile de transformateurs provoque l'altération de l'antioxydant et conduit à la formation des composés identifiés au Tableau II. Le temps d'élution en CLHP de la di-*tert.*-butyl-2,6-benzoquinone est de 5,3 min et il est identique à celui du DBPC. La présence de cette quinone dans une huile usée cause une interférence à l'analyse quantitative du DBPC. Une purification de l'huile est nécessaire et la chromatographie d'absorption sur des cartouches Sep-Pak de silice a été utilisée. Cette technique a été optimisée dans le but: (a) d'éliminer tous les composés d'altération du DBPC; (b) d'éliminer tous les produits polaires provenant de

TABLEAU I

LIMITE DE DÉTECTION, PRÉCISION ANALYTIQUE ET COURBE DE CALIBRATION DU DBPC

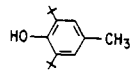
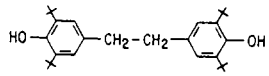
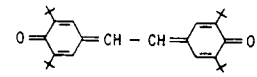
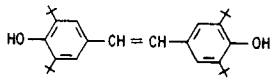
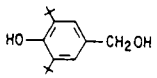
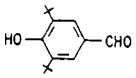
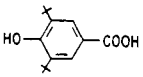
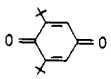
Régression linéaire: $y = 591{,}7x - 3481$. Coefficient de corrélation: $r = 0{,}9999$.

L.D.[a] *(ppm)*	*Précision analytique*		
	Teneur en DBPC (ppm)	*Réponse du détecteur ultraviolet: moyenne*[b] ± *écart type (μv s)* × 10^3	*C.V. (%)*
2,2	25,1	11,51 ± 0,12	1,04
	50,8	26,20 ± 0,13	0,49
	103,2	56,53 ± 0,47	0,83
	254,3	148,87 ± 0,31	0,21
	516,3	300,84 ± 1,86	0,60
	1008,5	593,04 ± 1,39	0,23

[a] Limite de détection.

[b] Moyenne de cinq essais.

TABLEAU II
TEMPS DE RÉTENTION (t_R) EN CLHP DES PRODUITS D'OXYDATION DU DBPC

Produits d'oxydation	t_R *(min)*
	5,3
	7,1
	12,0
	7,0
	3,5
	4,3
	3,4
	5,3

l'oxydation de l'huile; (c) de récupérer en quelques minutes la totalité du DBPC ainsi que les composantes de l'huile non affectées par le vieillissement.

Les résultats regroupés dans le Tableau III montrent que lorsqu'un échantillon d'huile (100 μl) contenant 378 ou 785 ppm de DBPC est déposé sur la silice d'une cartouche de Sep-Pak; l'élution de 60 ml de pentane en 30 s permet d'obtenir un pourcentage de récupération de l'antioxydant voisin à 100%.

TABLEAU III
ANALYSE DU DBPC DANS UNE HUILE APRÈS SA CHROMATOGRAPHIE D'ABSORPTION SUR SILICE

DBPC ajouté (ppm)	*DBPC trouvé*		*Récupération (%)*
	Moyenne[a] $\pm$ *écart type (ppm)*	*C.V. (%)*	
378	382,2 $\pm$ 10,04	2,62	101,1
785	784,9 $\pm$ 10,44	1,33	100,0

[a] Moyenne de dix essais.

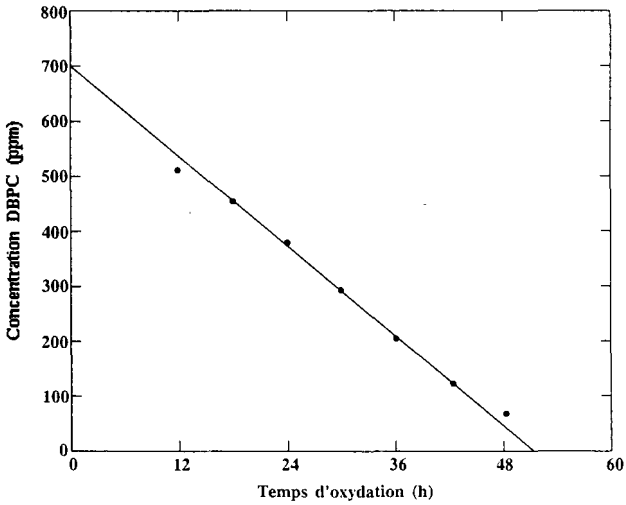


Fig. 2. Quantification par CLHP du DBPC dans une huile de transformateurs Voltesso 35 en fonction de son temps d'oxydation accélérée.

Application au dosage du DBPC dans l'huile Voltesso 35 oxydée

Les résultats du dosage du DBPC dans des échantillons d'huiles oxydées suivant la méthode ASTM D-2440[10] sont portés sur la Fig. 2. Nous observons un décroissement continu du DBPC jusqu'à l'obtention d'une valeur nulle à 54 h.

CONCLUSION

La méthode proposée est sensible, précise, reproductible et rapide. Elle permet une quantification du DBPC dans une huile neuve en 6 min et dans une huile usée en 18 min.

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Note

Reversed-phase thin-layer chromatography of homologues of antimycin A and related derivatives

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(First received July 29th, 1988; revised manuscript received December 19th, 1988)

During the course of recent studies[1–3] of the chemical analysis of antimycin A, a fish toxicant, we routinely employed thin-layer chromatographic (TLC) techniques for product isolation and sample purification. Since antimycin A was produced commercially from Streptomyces by fermentation, in every initial phase of the studies it was necessary to separate the pure antimycin A complex from other fermentation contaminants invariably present in the crude antibiotic products. The enrichment and further purification of these materials were readily accomplished by normal-phase TLC on silica gel plates. The methods utilizing conventional unmodified silica gel were found to be equally applicable in the isolation and purification of pure methylated and dansylated antimycins from the corresponding crude derivatization products.

However, numerous attempts to resolve antimycin A complex into its major homologous components A_1, A_2, A_3, and A_4 (Fig. 1) by normal-phase TLC have not been successful. In light of the general procedural simplicity of TLC techniques, adequate TLC resolution of the antibiotic homologues would provide a convenient means for studies dealing with subcomponents[2,3] of antimycin A or for product analysis entailing compositional determination of antibiotic mixtures derived from different batches of fermentation processes. We have explored the analytical potential of reversed-phase (RP) TLC methods for the quantification of antimycin A homologues and related methyl and dansyl derivatives. The first TLC separation of homologues of antimycin A is reported in this paper.

EXPERIMENTAL

Chemicals and reagents

Antimycin A mixtures and individual antimycin components A_1, A_2, A_3 and A_4 were either obtained from Sigma (St. Louis, MO, U.S.A.) or purified from the crude industrial materials[1,3]. Derivatives of antimycin A used in this study were synthesized at the National Fishery Research Center (La Crosse, WI, U.S.A.) following our published methods: methyl antimycins[3] were prepared by treating the parent antimycins with diazomethane in ethereal solutions; dansyl antimycins[1,3] were prepared from the corresponding antimycins by reactions with dansyl chloride in

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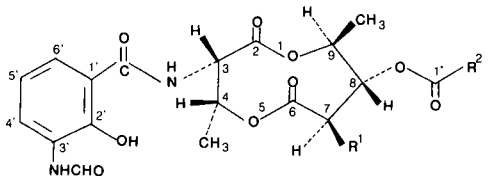

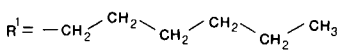

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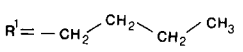

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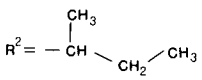

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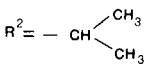

(IV)

Fig. 1. Structures of the homologues of antimycin A. A_1, $R^1 = I$, $R^2 = III$; A_2, $R^1 = I$, $R^2 = IV$; A_3, $R^1 = II$, $R^2 = III$; A_4, $R^1 = II$, $R^2 = IV$.

acetone in the presence of pyridine. TLC solvents and buffer reagents were acquired from J. T. Baker (Phillipsburgh, NJ, U.S.A.).

TLC plates were purchased from various commercial suppliers: (i) C_{18} RP-TLC and normal-phase TLC plates, 5 μm, 10 cm (length) × 20 cm (width) (with preadsorbent zone), E. M. Science (Cherry Hill, NJ, U.S.A.); (ii) C_{18} RP-TLC (bonded) plates, 10 μm, 20 × 20 cm, Whatman Chemical Separation (Clifton, NJ, U.S.A.); C_{18} RP-TLC (impregnated and not bonded), 10 μm, 20 × 20 cm, Analtech (Newark, DE, U.S.A.).

Thin-layer chromatography

In all experiments, plates with fluorescence indicators were used for visualization. Prior to sample applications, development chambers of suitable dimensions were saturated with solvents with the aid of saturation pads (Analtech) for 2 h. For qualitative analysis, 1-μl aliquot solutions (0.2%) of the antibiotic analytes in methylene chloride were spotted on a plate by means of Hamilton microliter syringes. After development of the plate, the solvent front was immediately measured when it reached a distance exactly 5 mm from the top edge of the plate. The chromatogram was then analyzed for the component spots. The unknown samples were identified by using known reference standards spotted alongside with the unknowns. Except for the methylated compounds, the antibiotics of interest were visible under short-wavelength or long-wavelength UV light generated from a mineralight lamp (Model UVSL-58 multiband UV-254/366 nm, Ultraviolet Products, San Gabriel, CA, U.S.A.). TLC spots of methyl antimycins could be seen only under 254 nm light.

Quantitative TLC analyses of antimycin samples in ethanol solutions were performed with a TLC scanning densitometer (Camag, Wrightsville Beach, NC, U.S.A.) equipped with a programmable integrator (Spectra Physics Model 4290). Calibration curves were constructed by plotting observed densities against the corresponding known amounts (0.1–10 μg) of samples spotted. The percent compositions of antimycin mixtures were determined automatically with the instruments. Minimum detection limits of the three types of antibiotic analytes were also determined at a signal-to-noise ratio of 4:1.

RESULTS AND DISCUSSION

Table I shows the results of the RP-TLC separation of homologous of the antimycin A complex under various chromatographic conditions. All the experiments listed in the table were performed with C_{18} RP-TLC plates of 5-μm particle size (10 × 20 cm with preadsorbent zone, E.M. Science). Examination of the R_F values indicated that the major antimycin components A_1, A_2, A_3 and A_4 were resolved only in solvent systems (A–H, and N) in which either acetonitrile or methanol was used as the organic modifier. The solvent pH and the presence of a buffer salt in a solvent system bore little significance on the resolution of the homologues. In contrast, mobile

TABLE I

RP-TLC HOMOLOGUES OF ANTIMYCIN A

All experiments were performed with E.M. Science RP-TLC plates, 5 μm, 10 × 20 cm with preadsorbent zone. Solvent systems: (A) methanol–water (9:1), 0.2 *M* sodium acetate, pH 2.5; (B) methanol–water (4:1), 0.2 *M* sodium acetate, pH 2.5, developed twice; (C) first with methanol–water (7:3), 0.2 *M* sodium acetate, pH 5, then with methanol–water (4:1), 0.2 *M* sodium acetate, pH 5; (D) methanol; (E) methanol–water (9:1); (F) methanol–water–acetic acid (18:1:1); (G) methanol–acetonitrile–water (9:9:2); (H) acetonitrile–water (9:1); (I) ethanol–water (9:1); (J) acetone–water (9:1); (K) tetrahydrofuran–water (9:1); (L) dimethyl formamide; (M) dimethyl formamide–water (4:1); (N) methanol–water (9:1), plate length 20 cm. For experiments with solvent systems A–M, plate length 10 cm.

Solvent system	R_F *values*[a]			
	A_1	A_2	A_3	A_4
A	0.286	0.356	0.419	0.481
B	0.031	0.053	0.088	0.129
C	0.181	0.291	0.401	0.527
D	0.601	0.654	0.709	0.709
E	0.253	0.316	0.376	0.437
F	0.459	0.522	0.579	0.662
G	0.347	0.412	0.473	0.545
H	0.396	0.466	0.538	0.592
I	0.586	0.586	0.586	0.586
J	0.698	0.698	0.698	0.698
K	0.932	0.932	0.932	0.932
L	0.716	0.716	0.716	0.716
M	0.152	0.152	0.152	0.152
N	0.329	0.389	0.449	0.546

[a] Mean values of three determinations; coefficients of variation ranged 1.57–2.89%.

phase buffers played crucial roles in the high-performance liquid chromatographic (HPLC) separation of these antimycin components[1,3]. The data in Table I clearly demonstrate that the antibiotic analytes were more strongly retained on the C_{18} RP-TLC phase as the percentage of an organic modifier decreased. Generally, the R_F values tended to increase with solvents of different solvent strength in the following order: methanol < acetonitrile < ethanol < acetone < tetrahydrofuran. The effect of distance travelled by the solvent on the degree of component resolution appeared to be small (experiment E *vs.* experiment N).

Examples of TLC results obtained with other types of silica-based stationary phases are presented in Table II. In the RP-TLC experiments (O–R) where C_{18} RP-bonded or impregnated stationary phases of 10 μm particle size and low carbon content (12.5–14.0%) were used, the homologues of antimycin A complex remained unresolved in all cases investigated. Apparently, the selection of a TLC stationary phase of high carbon content (20%, 5 μm, EM Science plates) was the determining factor for achieving the separation of antimycin homologues. In this regard, it seemed immaterial whether the hydrocarbonaceous phase was bonded or impregnated to silica (O, P *vs.* Q, R, Table II).

Normal-phase TLC on 5-μm silica, 10 × 10 cm (E. M. Science, this material was used in the manufacture of C_{18} RP-TLC plates) failed to separate the antimycin components as shown in experiments S and T, Table II. Apparently, the homologues were not differentiable by the adsorption mechanism that involved separations based on the polarity of antimycin solutes.

Table III compares the TLC data for homologues of antimycin A (AT) and the corresponding methylated (AT-ME) and dansylated (AT-DNS) derivatives. The plates used in these experiments were either C_{18} RP-bonded phases (A–D) or normal-phase silica (E) (all of 5-μm particle size, E.M. Science). These derivatives of

TABLE II

TLC OF ANTIMYCIN A COMPLEX ON VARIOUS SILICA-BASED PLATES

TLC systems: Whatman RP bonded plates with preadsorbent zone, 10 μm, 20 × 20 cm, (O) methanol–water (7:3); (P) methanol–water (1:1), 0.2 *M* sodium acetate, pH 5. Analtech RP impregnated plates, 10 μm, 20 × 20 cm, (Q) methanol–water (9:1); (R) methanol–water (7:3). E.M. Science normal-phase silica plates, 5 μm, 10 × 10 cm, (S) benzene–chloroform–methanol–acetatic acid (30:5:1:1); (T) chloroform–methanol–acetic acid (13:4:1).

TLC system	R_F *values*[a]			
	A_1	A_2	A_3	A_4
Reversed-phase				
O	0.364	0.364	0.364	0.364
P	0.000	0.000	0.000	0.000
Q	0.893	0.893	0.893	0.893
R	0.742	0.742	0.742	0.742
Normal phase				
S	0.276	0.276	0.276	0.276
T	0.769	0.769	0.769	0.769

[a] Mean values of three determinations; coefficients of variation ranged 2.51–3.32%.

TABLE III

COMPARISONS OF TLC DATA FOR HOMOLOGUES OF ANTIMYCIN A AND DERIVATIVES

Solvent systems: (A) acetonitrile; (B) acetonitrile–water (9:1); (C) methanol–water (9:1); (D) tetrahydrofuran–water (7:3); (E) benzene–chloroform–methanol–acetic acid (8:5:1:1). E.M. Science RP-TLC (5 μm) plates (10 × 20 cm) were used in A–D; E.M. Science normal-phase TLC (5 μm) plates were used in E. AT = Antimycin A; AT-ME = methylated antimycin A; AT-DNS = dansylated antimycin A.

Solvent system	R_F *values*[a]			
	A_1	A_2	A_3	A_4
A				
AT	0.520	0.573	0.607	0.667
AT-ME	0.507	0.560	0.613	0.640
AT-DNS	0.427	0.480	0.527	0.573
B				
AT	0.357	0.429	0.490	0.557
AT-ME	0.414	0.486	0.536	0.586
AT-DNS	0.314	0.386	0.457	0.529
C				
AT	0.233	0.293	0.360	0.407
AT-ME	0.240	0.307	0.373	0.427
AT-DNS	0.147	0.187	0.227	0.280
D				
AT	0.669	0.669	0.669	0.669
AT-ME	0.712	0.712	0.712	0.712
AT-DNS	0.718	0.714	0.718	0.718
E				
AT	0.482	0.482	0.482	0.482
AT-ME	0.439	0.439	0.439	0.439
AT-DNS	0.488	0.488	0.488	0.488

[a] Mean values of three determinations; coefficients of variation ranged 2.67–3.08%.

antimycins were useful in analytical studies[1,3] and in a structural investigation of its subcomponents[4]. In each case (Table III), the three types (the parent and the two derivatives) of antimycin compounds were chromatographed simultaneously on the same plate to eliminate the possible influence of different chromatographic variables. The chromatographic behavior of the derivatives under various TLC conditions was strikingly similar to that of the parent compounds. It was of interest to note that the elution order of the compounds seemed to be solvent-dependent. Thus, in the RP modes, TLC in acetonitrile–water (9:1) [or methanol–water (9:1)] afforded the analyte spots in a decreasing order of R_F values; AT-ME > AT > AT-DNS as found in solvent systems B and C. No definite trend of elution order could be delineated from TLC experiments in other solvents (A and D). On the other hand, normal phase TLC of the same sets of samples resulted in a reversal of the above elution order (B and C) as illustrated in (E) of Table III.

Unknown samples were quantified using calibration curves of all four individual antimycins A_1, A_2, A_3 and A_4. Determination of minimum detection limits by absorption scanning densitometry at a signal-to-noise ratio of 4:1 gave 20, 30 and 55 ng for AT, AT-ME, and AT-DNS, respectively. By virtue of their fluorescence properties,

TABLE IV

RP-TLC DETERMINATION OF THE COMPOSITION OF HOMOLOGUES IN DIFFERENT BATCHES OF ANTIMYCIN A COMPLEX

The HPLC method described in ref. 1 was used. The five samples represent materials derived from different batches of commercial fermentation processes.

Component	*Composition (%)[a] of batch No.*				
	1	*2*	*3*	*4*	*5*
A_1					
TLC	36.10	35.21	33.05	45.93	37.54
HPLC	36.42	34.99	33.26	46.10	37.20
A_2					
TLC	26.96	30.08	17.60	12.01	10.25
HPLC	27.10	29.67	17.42	11.75	10.34
A_3					
TLC	24.31	21.50	37.96	36.44	43.78
HPLC	24.27	21.56	38.09	36.23	43.81
A_4					
TLC	12.27	14.01	10.99	5.82	8.63
HPLC	12.21	13.78	11.23	5.92	8.65

[a] Mean values of three determinations; coefficients of variation for respective TLC and HPLC methods ranged 2.01–2.35% and 1.56–1.88%.

the detection sensitivities were somewhat improved in the cases of AT and AT-DNS. Both of these fluorescent compounds were detectible at a lower limit of 50 pg by fluorescence scanning densitometry. Table IV gives the results of compositional analyses of five antimycin mixtures by RP-TLC–UV and HPLC–UV. The two methods yielded comparable results.

In conclusion, the major components of antimycin A homologues can be separated by RP-TLC. Using the TLC method in conjunction with absorption and fluorescence scanning densitometry, quantification of unknown homologues as well as the composition of antimycin A complex can be achieved with good reproducibility.

REFERENCES

1 S. L. Abidi, *J. Chromatogr.*, 234 (1982) 187; and references cited therein.
2 S. L. Abidi and B. R. Adams, *J. Magnet. Reson.*, 25 (1988) 1078.
3 S. L. Abidi, *J. Chromatogr.*, 447 (1988) 65.
4 S. Ha, C. L. Wilkins and S. L. Abidi, *Anal. Chem.*, in press.

Author Index

Erratum

J. Chromatogr., 456 (1988) 444
Page 444, 3rd paragraph, 1st line, "0.01 *M*" should read "0.1 *M*".

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journal of chromatography news section

NEW BOOKS

Analytical artifacts: GC, MS, HPLC, TLC and PC (*Journal of Chromatography Library,* Vol. 44), by M.S. Middleditch, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1989, XXIV+1028 pp., price Dfl. 495.00, US$ 241.50, ISBN 0-444-87158-6.

Chiral separations by HPLC. Applications to pharmaceutical compounds, edited by A.M. Krstulovic, Ellis Horwood, Chichester, 1989, *ca.* 384 pp., price *ca.* US$ 116.30, ISBN 0-7458-0331-8.

Preparative-scale chromatography, edited by E. Grushka, Marcel Dekker, New York, Basel, 1989, XIV+324 pp., price US$ 99.75 (U.S.A. and Canada), US$ 119.50 (rest of world), ISBN 0-8274-8061-2.

High-performance liquid chromatography of biopolymers and biooligomers, Part B: Separation of individual compound classes (*Journal of Chromatography Library,* Vol 41B), by O. Mikeš, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1988, XXII+722 pp., price Dfl. 360.00, US$ 189.50, ISBN 0-444-43034-2.

Environmental analysis using chromatography interfaced with atomic spectroscopy, by R.M. Harrison and S. Rapsomanikis, Ellis Horwood, Chichester, 1989, *ca.* 420 pp., price *ca.* US$ 114.30, ISBN 0-8531-2979-X.

Chromatographie und Überkritischen verdichteten Gasen zur Trennung von Oligomeren und Polymeren, by F.P. Schmitz, Profil Verlag, München, 1988, VIII+235 pp., price DM 59.00, ISBN 3-89019-214-9.

AWARDS

1989 EAS AWARD IN CHROMATOGRAPHY

Harold McNair, professor of chemistry at Virginia Polytechnic Institute and State University (VPI&SU), will receive the EAS Award in Chromatography. Established in 1986, this award recognizes an individual who has helped to shape the fields of chromatography. McNair received his B.S. degree magna cum laude (1955) from the University of Arizona and his Ph.D. (1959) from Purdue University. He studied at The Technical University, Eindhoven, The Netherlands as a Fullbright Postdoctoral Fellow in 1959/1960. In 1960 he joined Esso Research and Engineering as a research chemist. From 1961 to 1964 he was employed by Hewlett-Packard in Holland and from 1964 to 1968 by Varian associates. In 1968 he joined the faculty of VPI&SU as an associate professor and was promoted to full professor in 1971. Over 30 students have received graduate degrees under the direction of Professor McNair.

McNair's research interests involve GC, LC, SFC and TLC, focusing on trace organic analysis. He teaches undergraduate courses in general and analytical chemistry and graduate courses dealing with GC, LC, SFC and TLC. McNair received the Alumni Teaching Award from VPI&SU in 1984, and the COLACRO Medal for Chromatographic Contributions in Latin America in 1986.

ANNOUNCEMENTS OF MEETINGS

WORKSHOP ON ION CHROMATOGRAPHY, FREIBURG i. Br. SWITZERLAND, APRIL 13–14, 1989

The workshop will follow the three-day symposium in Sils-Maria, Switzerland, and the workshop leader will be Professor P.R. Haddad (University of New South Wales, Australia). Participation of other speakers from the preceding symposium in Sils-Maria is envisaged.

Topics covered will include: sample handling, enrichment, clean-up, automation; dual and single column concepts; theory and practice of ion separations (isocratic and gradients); detection principles; applications in the pharmaceutical, environmental, biomedical, alimentation fields etc.

The registration fee (DM 690) will include luncheons, refreshments, reception, manual and company information. Upon registration you will receive further information on the course.

For further details contact: Workshop Office, IAEAC, M. Frei-Häusler, Postfach 46, CH-4123 Allschwill 2, Switzerland.

8th INTERNATIONAL BIOANALYTICAL FORUM: ANALYSIS FOR DRUGS AND METABOLITES, INCLUDING ANTI-INFECTIVE AGENTS, GUILDFORD, U.K., SEPTEMBER 5–8, 1989

The 8th International Bioanalytical Forum will be held at the University of Surrey, Guildford, U.K., September 5–8, 1989.

The focus is the setting up of sensitive methods, especially chromatographic, for drugs in blood, with some emphasis on anti-infectives including antivirals and antiparasitics, on immunosuppressives, and on relevant metabolites. State-of-the-art topics include supercritical-fluid chromatographic, high-performance liquid chromatographic advances, column switching, thin-layer chromatography–mass spectrometry, biosensors, and enantioselective radioimmunoassay. There will be attention to detection limits, statistics, and other aspects pertinent to producing results that will stand up to scrutiny. The subject-matter is relevant to therapeutic monitoring as well as drug development.

The fee (£ 104; possible rebates) includes the post-forum book. On-site living costs are about £ 27 per day. Attendance is limited to 100 persons.

For further details contact: Dr. E. Reid (Chief Organizer), Guildford Academic Associates, 72 The Chase, Guildford GU2 5UL, U.K. Tel.: (0483) 65324.

ION-EX 90, INTERNATIONAL CONFERENCE AND INDUSTRIAL EXHIBITION ON INDUSTRIAL, ANALYTICAL AND PREPARATIVE APPLICATIONS OF ION-EXCHANGE PROCESSES, WREXHAM, U.K., JULY 9–11, 1990.

Ion-Ex '90 will be held at the North East Wales Institute, Wrexham, Clwyd, U.K., July 9–11, 1990. This is the second in the series of Ion-Ex conferences and follows the successful Ion-Ex '87 Conference, on the "Recent Developments in Ion Exchange", the Proceedings of which have been published by Elsevier Applied Science Publishers.

The proposed scientific programme for Ion-Ex '90 will cover the following areas: instrumental developments; environmental analysis; inorganic analysis; organic analysis; development of new materials; biological analysis; ion-exchange membranes; ion-exclusion chromatography; ion chromatography and associated techniques; fundamental studies of ion-exchange processes and materials; water analysis; applications in the nuclear industry; and pharmaceutical analysis.

One evening of the meeting will be devoted to an exhibition by several of the major companies involved in the field. Organisations involved in the areas associated with the overall theme of the conference are invited to participate.

For further information on Ion-Ex '90, please contact: Ion-Ex '90, Conference Secretariat, Faculty of Research and Innovation, The North East Wales Institute, Connah's Quay, Deeside, Clwyd, CH5 4BR, U.K.

18th INTERNATIONAL SYMPOSIUM ON CHROMATOGRAPHY, AMSTERDAM, THE NETHERLANDS, SEPTEMBER 23–28, 1990

The 18th International Symposium on Chromatography (ISC) belongs to a series of distinguished international symposia, biannually held in Europe. Like the previous symposia — Nürnberg (1984), Paris (1986) and Vienna (1988) — it will cover all fundamental aspects, instrumental developments and applications of the various chromatographic techniques, *viz.* liquid chromatography, supercritical-fluid chromatography, (capillary) gas chromatography and thin-layer chromatography, as well as related topics such as field flow fractionation and column electrophoresis.

The scientific programme will comprise oral presentations in plenary and parallel sessions, poster presentations and tutorial and discussion sessions dealing with aspects of special current interest. In the plenary lectures, recognized authorities will report on trends and future developments over a broad range of subjects.

An exhibition of equipment and accessories will be held in the lounges around the lecture halls. The location ensures a close integration of this part with the scientific activities. The symposium site is the well-known Internationaal Congrescentrum RAI, in Amsterdam. Its full conference accommodation includes lecture halls, a spacious exhibition area, various restaurants and parking areas.

An attractive social programme, and a special programme including tours and excursions which feature characteristics of Amsterdam and the Netherlands are in preparation.

For more details (the second circular is available in July 1989) contact: 18th International Symposium on Chromatography, RAI Organisatie Bureau Amsterdam bv, Europaplein 21, 1078 GZ Amsterdam, The Netherlands. Tel.: (020) 5491212; telex: 13499 raico nl; telefax: (020) 464469.

COURSES

3rd EUROPEAN SPRING SCHOOL IN CHEMOMETRICS, BRISTOL, U.K., APRIL 2–7, 1989

Following the 1987 and 1988 courses, the University of Bristol announces the 3rd European Spring School in Chemometrics jointly organized by the School of Chemistry and Department of Extra-Mural studies, which will take place over the week of April 2–7, 1989.

The course will be taught by an internationally recognised team of tutors, from The University of Bristol (U.K.). The Free University of Brussels (Belgium), The University of Bergen (Norway), Glaxo Research (Durham, U.K.), BP Research (Sunbury-on-Thames, U.K.), Janssen Pharmaceutica (Beerse, Belgium) and AFRC Food Research Institute (Bristol, U.K.). The tutors will include a mixture of research active chemists and statisticians, industrialists and academics. The course will aim to show chemists what software tools are available to improve the performance of and analyse laboratory instrumental data. Special features will include extensive course notes which will eventually form the basis of internationally published texts and papers; extensive opportunities for hands-on use of software packages many of which have been developed by course tutors and their colleagues; and collaboration with the EEC funded COMETT scheme for continuing education. There will be a mixture of theoretical and applied tutorials, and most material will de divided into "Simple" and "Advanced" options to suit delegates prior experience.

The academic course organizer is Dr. R.G. Brereton of the School of Chemistry, University of Bristol. The COMETT scheme is co-ordinated by Prof. D.L. Massart of the Free University of Brussels. For enquiries please write to Dr. S.M. Pringle of the Department of Extra-Mural Studies, Wills Memorial Building, Queen's Road, Bristol BS8 1HR, U.K. Tel: (0272) 303611.

AMSTERDAM SUMMERCOURSE ON HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, AMSTERDAM, THE NETHERLANDS, JULY 10–14, 1989

High-performance liquid chromatography (HPLC) has now been accepted as a viable and routine technique for the separation and analysis of non-volatile compounds. It is widely applied in pharmaceutical, biomedical, environmental, biochemical, and food analysis.

The Amsterdam HPLC Summercourse is an international course which is organized every two years at the Laboratory for Analytical Chemistry of the University of Amsterdam. The course language will be English. The aim of the course is to educate newcomers in the field with the basic concepts of the technique and to inform them about the latest developments in HPLC. This is realized in overview lectures and informal seminar sessions about general, as well as specialized topics, given by experts in the field and by means of illustrative practical experiments. The participants can, within certain limits, compose their own programme by selecting those seminar topics and practical experiments which fit their interests. The course organizers are: Dr. J.C. Kraak and Dr. J. Kragten of the Laboratory for Analytical Chemistry, University of Amsterdam.

The tuiton fee will be approximately Dfl. 1750.00 which includes the course manual, lunches and refreshments. The number of participants is restricted and admittance will occur on basis of the order of receipt of the registration formular and payment.

For further details contact: Secretariat Amsterdam HPLC Summercourse, Laboratory for Analytical Chemistry, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands. Tel.: (31-20) 5256515; Telex: 16460 anal-chem.

CALENDAR OF FORTHCOMING MEETINGS

March 21–23, 1989
Reading, U.K.

Joint Meeting of the Royal Entomological Society of London and the Chromatographic Society — 1st International Symposium and Isolation of Insect Hormones, Pheromones and Related Compounds
Contact: Dr. A.R. McCafferty, Department of Pure and Applied Zoology, University of Reading, Whiteknights, Reading, RG6 2AJ, U.K.

April 2–7, 1989
Bristol, U.K.

3rd European Spring School in Chemometrics
Contact: Dr. S.M. Pringle, Department of Extra-Mural Studies, Wills Memorial Building, Queen's Road Bristol, BS8 1HR, U.K. Tel.: (0272) 303611.

April 7–9, 1989
Austin, TX, U.S.A.

2nd International Symposium on Polymer Analysis and Characterization
Contact: Dr. Howard G. Barth, Du Pont Co., Experiment Station, P.O. Box 80228, Wilmington, DE 19880-0228, U.S.A. Tel. (302) 695–4354.

April 9–12, 1989
Sils-Maria, Switzerland

6th Symposium on Ion Chromatography
Contact: Workshop Office IAEAC, Ms. M. Frei-Hausler, Postfach 46, CH-4123 Allschwil 2, Switzerland. (Further details published in Vol. 447, No. 2.)

April 10–12, 1989
Boston, MA, U.S.A.

1st International Symposium on High Performance Capillary Electrophoresis
Contact: Shirley E. Schlessinger, Symposium Manager, HPCE '89, 400 East Randolph Drive, Suite 1015, Chicago, IL 60601, U.S.A. Tel.: (312) 527-2011. (Further details published in Vol. 447, No. 2.)

April 11, 1989 Washington, DC, U.S.A.	**Chromexpo 1989, Chromatography Exhibition and Poster Session** Contact: Janet E. Cunningham, Barr Enterprices, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772.
April 11–14, 1989 Loughborough, U.K.	**7th International Symposium on Electroanalysis in Biomedical, Environmental and Industrial Sciences** Contact: Dr. A.G. Fogg, Chemistry Department, University of Loughborough, Leics. LE11 3TU, U.K.
April 12–14, 1989 Paris, France	**The International Chemical Industry Conference: Alternative Futures** Contact: The Customer Service Department, Management Centre Europe, P.O. Box 95, 3417 ZH Montfoort, The Netherlands.
April 13–14. 1989 Freiburg i. Br., F.R.G.	**Workshop on Ion Chromatography** Contact: Workshop Office, IAEAC, M. Frei-Häusler, Postfach 46, CH-4123 Allschwill, Switzerland.
May 7–12, 1989 Nürtingen, F.R.G.	**3rd International Conference on Fundamentals of Adsorption** Contact: DECHEMA, Abteilung Tagungen, P.O. Box 970146, Theodor-Heuss-Allee 25, D-6000 Frankfurt am Main, F.R.G.
May 8–10, 1989 Washington, DC, U.S.A.	**6th International Symposium on Preparative Chromatography** Contact: Mrs. Janet Cunningham, Prep-89 Symposium Manager,Barr Enterprices, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772. (Further details published in Vol. 447, No. 2.)
May 8–10, 1989 Minneapolis, MN, U.S.A.	**11th Annual Spring Symposium of the Minnesota Chromatography Forum** Contact: Kowler Associates, 4948 Lyndale Avenue South, Minneapolis, MN 55409, U.S.A. Tel.: (612) 823-6034. (Further details published in Vol. 456, No. 2.)
May 8–12, 1989 Colorado Springs, CO, U.S.A.	**11th Symposium on Biotechnology for Fuels and Chemicals** Contact: Elias Greenbaum, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831-6194, U.S.A.
May 15–17, 1989 Chicago, IL, U.S.A.	**Modern Methods in the Analysis and Purification of Biopolymers: Capillary Electrophoresis and High-Performance Liquid Chromatography** Contact: Janet Cunningham, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772.
May 16–19, 1989 Antwerp, Belgium	**3rd International Symposium on Drug Analysis** Contact: Dr. Apr. G. Laekeman, 3rd International Symposium on Drug Analysis, Universitaire Instelling Antwerpen, Departement Farmaceutische Wetenschappen, Universiteitsplein 1, B-2610 Wilrijk, Belgium. (Further details published in Vol. 438, No. 2.)
May 22–25, 1989 Baltimore, MD, U.S.A.	**3rd Annual Seminar on Analytical Biotechnology** Contact: Barr Enterprices, P.O. Box 279, Wallkersville, MD 21793, U.S.A. Tel.: (301) 898-3772. (Further details published in Vol. 450, No. 3.)

May 22–25, 1989 Riva del Garda, Italy	**10th International Symposium on Capillary Chromatography** Contact: Dr. P. Sandra, Laboratory for Organic Chemistry, University of Ghent, Krijgslaan 281 (S4), B-9000 Ghent, Belgium. (Further details published in Vol. 450, No. 3.)
May 23–26, 1989 Ghent, Belgium	**3rd International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences** Contact: Dr. Willy R.G. Baeyens, Symposium Chairman, State University of Ghent, Pharmaceutical Institute, Laboratory of Pharmaceutical Chemistry and Drug Quality Control, Harelbekestraat 72, B-9000 Ghent, Belgium. (Further details published in Vol. 447, No. 2.)
May 28–June 2, 1989 Riva del Garda, Italy	**9th International Conference on Computers in Chemical Research and Education** Contact: Professor Dr. Mario Marsili, 9th ICCCRE, Piazza Gondar 14, 00199 Roma, Italy. (Further details published in Vol. 450, No. 3.)
June 6–8, 1989 Windsor, U.K.	**3rd International Laboratory Information Management Systems Conference** Contact: Dr. C.J. Jackson, Health and Safety Executive, Broad Lane, Sheffield, S3 7HQ, U.K.
June 13–15, 1989 Snowbird, UT, U.S.A.	**1989 Workshop on Supercritical Fluid Chromatography** Contact: Dr. Milton L. Lee, Department of Chemistry, Brigham Young University, Provo, UT 84602, U.S.A. Tel.: (801) 378-2135. (Further details published in Vol. 447, No. 2.)
June 14–16, 1989 Salt Lake City, UT, U.S.A.	**1st International Symposium on Field-Flow Fractionation and FFF Workshop** Contact: Julie Westwood, Department of Chemistry, University of Utah, Salt Lake City, UT 84112, U.S.A. Tel.: (801) 581-5419. (Further details published in Vol. 447, No. 2.)
June 25–30, 1989 Montreux, Switzerland	**Transducers '89, 5th International Conference on Solid-State Sensors and Actuators & Eurosensors III** Contact: COMST S.A., Conference Organizers in Medicine, Science and Technology, P.O. Box 415, 1001 Lausanne 1, Switzerland. Tel.: (021) 234 886, Telefax: (021) 234 972. (Further details published in Vol. 445, No. 1.)
June 25–30, 1989 Stockholm, Sweden	**13th International Symposium on Column Liquid Chromatography** Contact: 13th International Symposium on Column Liquid Chromatography, The Swedish Academy of Pharmaceutical Sciences, P.O. Box 1136, S-111 81 Stockholm, Sweden. Tel.: (468) 24 50 85. (Further details published in Vol. 404, No. 2. and Vol. 448, No. 3.)
July 2–7, 1989 Bratislava, Czechoslovakia	**8th International Symposium on Advances and Application of Chromatography in Industry** Contact: Department of Analytical Chemistry, "Symposium on Chromatography", Faculty of Chemical Technology, Radlinského 9, 812 37 Bratislava, Czechoslovakia. (Further details published in Vol. 456, No. 2.)

July 2–9, 1989
Sofia, Bulgaria

XXVI Colloquium Spectroscopium Internationale
Contact: XXVI CSI '89, Sofia University, Faculty of Physics, Department of Optics and Spectroscopy, 5, A. Ivanov Blvd., 1126-30 Sofia, Bulgaria. Tel.: (3592) 627475, Telex: SUKO 23296 R BG. (Further details published in Vol. 445, No. 1.)

July 10–14, 1989
Amsterdam, The Netherlands

Amsterdam Summercourse on High-Performance Liquid Chromatography
Contact: Amsterdam HPLC Summercourse, Laboratory for Analytical Chemistry, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands. Tel.: (31-20) 5256515, Fax: (31-20) 5255802 CCITT 3.

July 30–August 5, 1989
Cambridge, U.K.

SAC 89, International Conference on Analytical Chemistry
Contact: SAC 89, Royal Society of Chemistry, Analytical Division, Burlington House, London W1V 0BN, U.K. Tel.: (01) 437-8656. (Further details published in Vol. 407.)

Aug. 2–7, 1989
Lund, Sweden

32nd IUPAC Congress
Contact: IUPAC, c/o Stockholm Convention Bureau, P.O. Box 6911, S-102 39 Stockholm, Sweden. Tel.: (46) 8230990, telex: 11556, FAX: 46 8 34 84 41. (Further details published in Vol. 450, No. 3.)

Aug. 21–25, 1989
Leipzig, G.D.R.

7th Danube Symposium on Chromatography
Contact: 7th Danube Symposium on Chromatography, Karl-Marx-Universität Leipzig, Sektion Chemie, Talstrasse 35, Leipzig, G.D.R. (Further details published in Vol. 411.)

Aug. 21–25, 1989
Amsterdam, The Netherlands

5th International Conference on Particle Induced X-Ray Emission and its Analytical Applications
Contact: 5th PIXE Conference, Department of Physics and Astronomy, Free University, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands. Tel.: (020) 5486224. (Further details published in Vol. 445, No. 1.)

Aug. 28–Sept. 1, 1989
Wiesbaden, F.R.G.

11th International Symposium on Microchemical Techniques
Contact: Gesellschaft Deutscher Chemiker, Abt. Tagungen, P.O. Box 900440, D-6000 Frankfurt/Main 90, F.R.G. Tel.: (069) 79 17-366/360, telex: 4170497 gdch d. (Further details published in Vol. 456, No. 2.)

Sept. 1–3, 1989
Leiden, The Netherlands

2nd International Symposium on Disposition and Delivery of Peptide Drugs
Contact: Dr. J. Verhoef, Center for Bio-Pharmaceutical Sciences, P.O. Box 9502, 2300 RA Leiden, The Netherlands.

Sept. 4–8, 1989
Colymbari, Crete
Greece

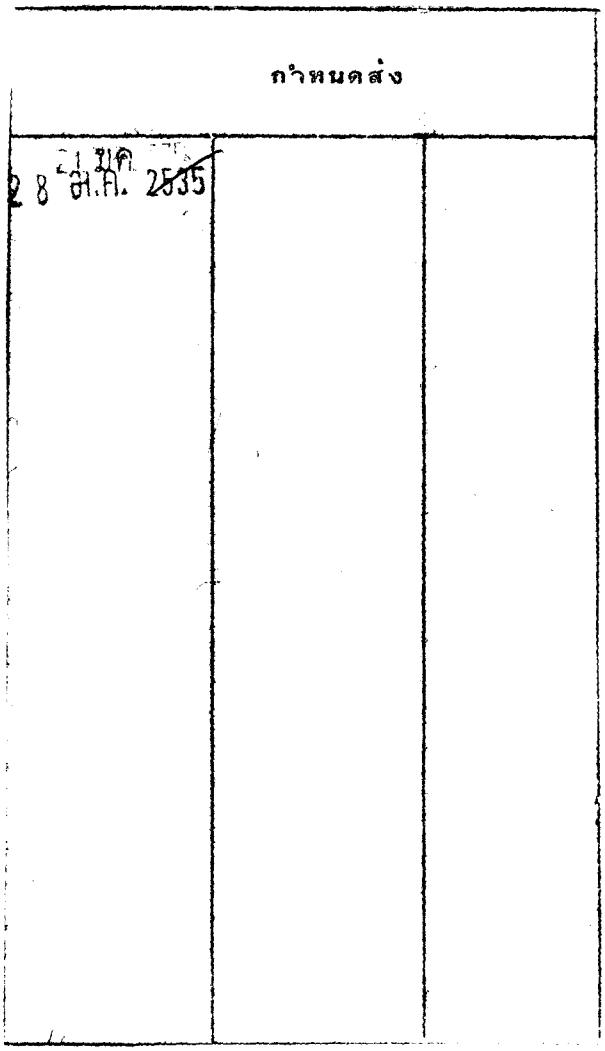
Pesticides and Alternatives, International Conference on Innovative Chemical and Biological Approaches to Pest Control
Contact: Professor John Casida, Department of Entomological Sciences, University of California, Berkeley, CA 94720, U.S.A. Tel.: (415) 642-5424.

Sept. 5–8, 1989 Guildford, U.K.	**8th International Bioanalytical Forum: Analysis for Drugs and Metabolites, Including Anti-infective Agents** Contact: Dr. E. Reid, Guildford Academic Associates, 72 The Chase, Guildford, GU2 5UL, U.K. Tel.: (0483) 65324.
Sept. 10–15, 1989 Antwerp, Belgium	**International Symposium on Gas Separation Technology** Contact: Dr. R. Dewolfs, University of Antwerp, Department of Chemistry, Universiteitsplein 1, B-2610 Antwerp-Wilrijk, Belgium. Tel.: (32) 3-828 25 28, ext. 204 or 215; telex: 336 46 UIA B; telefax: (32) 3-827 08 74. (Further details published in Vol. 438, No. 2.)
Sept. 19–21, 1989 Birmingham, U.K.	**5th BOC Priestly Conference** Contact: Dr. B.D. Crittenden, School of Chemical Engineering, Claverton Down, Bath BA2 7AY, U.K. Tel.: (0225) 826826, telex: 449097.
Sept. 19–22, 1989 Antwerp, Belgium	**International Symposium on the Analysis of Nucleoside, Nucleotide and Oligonucleotide Compounds** Contact: Dr. E.L. Esmans or Mr. J. Schrooten, University of Antwerp (R.U.C.A.), Laboratory for Organic Chemistry, Groenenborgerlaan 171, B-2020 Antwerp, Belgium. Tel.: (03) 2180233 or (03) 2180496, telex: 33362 rucabi, Fax: (03) 2180217. (Further details published in Vol. 448, No. 3.)
Sept. 20–22, 1989 Cordoba, Spain	**Symposium on Detection in Flow Injection Analysis and High-Performance Liquid Chromatography** Contact: Workshop Office IAEAC, Ms. M. Frei-Hausler, Postfach 46, CH-4123 Allschwill 2, Switzerland. (Further details published in Vol. 448, No. 3.)
Sept. 24–29, 1989 New York, NY, U.S.A.	**28th Eastern Analytical Symposium** Contact: David S. Klein, 642 Cranbury Cross Road, North Brunswick, NJ 08902, U.S.A.
Sept. 25–28, 1989 St. Louis, MO, U.S.A.	**103rd AOAC International Meeting and Exposition** Contact: Margaret Ridgell, AOAC, 1111th North 19th Street, Suite 210, Arlington, VA 22209, U.S.A. Tel.: (703) 522-3032.
Sept. 27–29, 1989 Paris, France	**Euro Food Chem V, 5th European Conference on Food Chemistry** Contact: G.A.M.S., 88 Boulevard Malesherbes, 75008 Paris, France. Tel.: (161) 45639304.
Oct. 1–4, 1989 Hamilton, Canada	**2nd International Conference on Separation Science and Technology** Contact: V. Lakshmanan, Ontario Research Foundation, Mississauga, Ontario, L5K 1B6 Canada.
Oct. 1–5, 1989 Toronto, Canada	**3rd International Congress on Chemical Sensors** Contact: Dr. M. Thompson, Department of Chemistry, University of Toronto, 80 St. George Street, Ontario, M5S 1A1 Canada.
Oct. 17–20, 1989 Tokyo, Japan	**10th International Symposium on Chromatography, CIS '89** Contact: Tadao Hoshino, Pharmaceutical Institute, School of Medicine, Keio University, 35-Shinanomachi, Shinjuku-ku, Tokyo 160, Japan. (Further details published in Vol. 456, No. 2.)

Oct. 22–27, 1989 Knoxville, TN, U.S.A.	**6th Symposium on Separation Science and Technology for Energy Applications** Contact: Dr. J.T. Bell, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831-6268, U.S.A. Tel.: (615) 574-4934 or 574-6795. (Further details published in Vol. 456, No. 2.)
Oct. 29–Nov. 3, 1989 Rehovot, Israel	**8th International Symposium on Affinity Chromatography and Biological Recognition** Contact: E.A. Bayer and F. Kohen, AC&BR Secretariat, The Weizmann Institute, Institute of Science, Rehovot 76100, Israel. (Further details published in Vol. 448, No. 3.)
Nov. 6–8, 1989 Philadelphia, PA, U.S.A.	**9th International Symposium on High-Performance Liquid Chromatographic Separation of Proteins, Peptides and Polynucleotides** Contact: Janet E. Cunningham, Barr Enterprices, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772. (Further details published in Vol. 462.)
Nov. 8–10, 1989 Baden-Baden, F.R.G.	**2nd International Symposium on Microcolumn Separation Methods** Contact: Workshop Office IAEAC, Ms. M. Frei-Hausler, Postfach 46, CH-4123 Allschwil, Switzerland. (Further details published in Vol. 462.)
April 3–5, 1990 Noordwijkerhout, The Netherlands	**ANATECH '90, 2nd International Symposium on Applications of Analytical Chemical Techniques to Industrial Process Control** Contact: Professor Dr. Willem E. van der Linden, Laboratory for Chemical Analysis-CT, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands
May 22–25, 1990 Dijon, France	**3rd European Meeting on Bio-Chromatography and Molecular Affinity** Contact: J.-P. Dandeu, Groupe Français de Bio-Chromatographie, Institut Pasteur, Unité d'Immuno-Allergie, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France. Tel.: (1) 45688000. (Further details published in Vol. 456, No. 2.)
July 9–11, 1990 Wrexham, U.K.	**Ion-Ex 90, International Conference and Industrial Exhibition on Industrial, Analytical and Preparative Applications of Ion-Exchange Processes** Contact: Ion-Ex 90, Conference Secretariat, Faculty of Research and Innovation, The North East Wales Institute, Connah's Quay, Deeside, Clwyd CH5 4BR, U.K. Tel. (0244) 817531, ext. 276 or 234, telex: 61629 NEWI G, fax: (0244) 822002.
Aug. 26–31, 1990 Vienna, Austria	**Euroanalysis VII, 7th European Conference on Analytical Chemistry** Contact: Professor Dr. M. Grasserbauer, c/o Interconvention, Austria Center Vienna, A-1450 Vienna, Austria. Tel.: (43) 222-2369/647; telex: 111803 icos a, Fax: (43) 222-2369/648. (Further details published in Vol. 445, No. 1.)
Sept. 23–28, 1990 Amsterdam, The Netherlands	**18th International Symposium on Chromatography** Contact: 18th International Symposium on Chromatography, RAI Organisatie Bureau Amsterdam bv, Europaplein 12, 1078 GZ Amsterdam, The Netherlands. Tel.: (31-20) 549 1212; telex: 13499 raico nl; Fax: (31-20) 464469.

Oct. 28–31, 1990
San Francisco, CA,
U.S.A.

ANABIOTEC '90, 3rd International Symposium on Analytical Methods in Biotechnology
Contact: Shirley Schlessinger, ANABIOTEC '90, 400 E. Randolph Drive, Chicago, IL 60601, U.S.A. (Further details published in Vol. 448, No. 3.)



PUBLICATION SCHEDULE FOR 1989

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

MONTH	J	F	M	A	M	
Journal of Chromatography	461 462 463/1	463/2 464/1	464/2 465/1 465/2	466 467/1	467/2	The publication schedule for further issues will be published later
Bibliography Section		486/1				
Biomedical Applications	487/1	487/2	488/1 488/2	489/1 489/2 490/1	490/2	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 445, pp. 453–456. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

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

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

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

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

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

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

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

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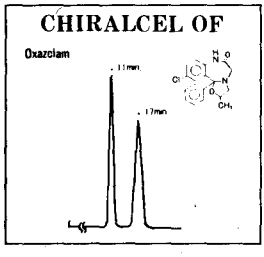
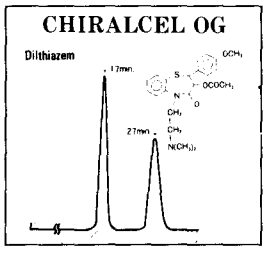
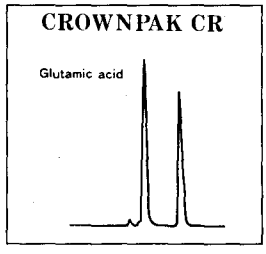
Baclofen	1.39	CR
Carbinoxamine	1.39	OD
Carteolol	1.86	OD
Chlophedianol	2.82	OJ
Chlormezanone	1.47	OJ
Cyclopentolate	2.47	OJ
Diltiazem	1.46	OD
	2.36	OF
	1.75	OG
Disopyramide	2.46	OF
Ethiazide	1.54	OF
Ethotoin	1.40	OJ
Fenoproten	1.35	OJ
Glutethimide	2.48	OJ

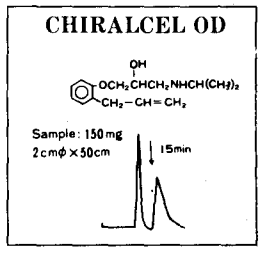
SUBSTANCE	α	column
Gauifenesin	2.40	OD
Hexobarbital	1.7	CA-1
Homatropine	3.13	OD
Hydroxyzine	1.17	OD
Indapamide	1.58	OJ
Ketamine	complete resolution	CA-1
Ketoprofen	1.46	OJ
Mephobarbital	5.9	OJ
	2.3	CA-1
Methaqualone	2.8	CA-1
	7.3	OJ
Methsuximide	2.68	OJ
Metoprolol	complete resolution	OD
Mianserin	1.75	OJ
Nilvadipine	complete resolution	OT

SUBSTANCE	α	column
Oxapadol	complete resolution	CA-1
Oxazepan	4.36	OC
Oxazolam	1.67	OF
Oxprenolol	6.03	OD
Perisoxal	1.33	OF
	1.27	OD
Pindolol	5.07	OD
Piprozolin	1.7	CA-1
Praziquantal	complete resolution	CA-1
Propranolol	2.29	OD
Rolipram	complete resolution	CA-1
Sulconazole	1.68	OJ
Suprofen	1.6	OJ
Trimebutine	1.81	OJ
Warfarin	1.96	OC









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