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*

NATURAL PRODUCTS ISOLATION

Separation Methods for Antimicrobials, Antivirals and Enzyme Inhibitors

edited by **GERALD H. WAGMAN** and **RAYMOND COOPER**,
Schering-Plough Research, Bloomfield, NJ, USA

(Journal of Chromatography Library, 43)

This new book encompasses, in great detail, the most recent progress made in the isolation and separation of natural products. Written by experts in their respective fields, it covers antibiotics, marine and plant-derived substances, enzyme inhibitors and interferons. The book has extensive isolation schemes, tables, figures and chemical structures. In many instances a short summary of the producing organism, brief chemical description and structure and biological activity of the compounds is presented. Detailed information of extraction, separation and purification techniques follow. Each chapter has an extensive bibliography and, where applicable, an appendix showing sources of materials and equipment. A detailed index to the subject matter is also provided.

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Contemporary Practice of Chromatography

by **C.F. Poole** and **S.A. Schuette**, Dept. of Chemistry, Wayne State University, Detroit, MI, USA

"A tremendous amount of work has gone into the writing of this book, and it may be considered to be one of the few volumes of its kind that could be used both as a text by senior students in the field and by analysts in many industries. All academic, research association and appropriate industrial laboratories should buy at least one copy (preferably more, as borrowers will probably wish to keep it on loan for some time)."
(The Analyst)

Written for everyone who uses chromatography as an analytical tool, this book covers all areas of gas, liquid, and thin-layer chromatography; **no other book offers the same scope.** The authors have had considerable experience in teaching graduate-level courses and the material presented here has been tried and tested, having formed the basis for short courses taught to groups of industrial chemists. Emphasis is on the practice of chromatographic methods, including "how to" sections and numer-

ous examples of calculation methods. Extensively illustrated, the book contains numerous tables of all useful constants, materials and formulas frequently used by chromatographers. Valuable features are the chapters on sample preparation for chromatographic analysis, on instrumental methods for sample identification, and the comprehensive literature review.

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Automatic Methods of Analysis

by **M. VALCÁRCEL** and **M.D. LUQUE DE CASTRO**,
Department of Analytical Chemistry, University of Córdoba,
Córdoba, Spain

(Techniques and Instrumentation in Analytical Chemistry, 9)

This new book gives a comprehensive overview of the state of the art of the automation of laboratory processes in analytical chemistry. The topics have been chosen according to such criteria as the degree of consolidation, scope of application and most promising trends.

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The text is supplemented by 290 figures and 800 literature references. It is written primarily for those directly involved in laboratory work or responsible for industrial planning and control, research centres, etc. It will also be useful to analytical chemists wishing to update their knowledge in this area, and will be of especial interest to scientists directly related to environmental sciences or clinical chemistry.

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INTERPRETATION OF NORMAL-PHASE SOLVENT STRENGTH SCALES BASED ON LINEAR SOLVATION ENERGY RELATIONSHIPS USING THE SOLVATOCHROMIC PARAMETERS π^* , α AND β

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(First received August 29th, 1988; revised manuscript received November 24th, 1988)

SUMMARY

An analysis of normal-phase solvent eluotropic strength (ϵ^0) and solute adsorption energy (S^0) was carried out by use of linear solvation energy relationships using solvatochromically based measurements of molecular dipolarity/polarizability (π^*), hydrogen bond acidity (α) and hydrogen bond basicity (β). It is demonstrated that in normal-phase chromatography acceptable regressions of the ϵ^0 parameter vs. the solvatochromic parameters (π^* , α , β) are obtained. The dependence of ϵ^0 on the solvatochromic parameters indicates that silica and alumina are virtually equally retentive towards dipoles, whereas silica surfaces are definitely stronger hydrogen bond donors than are alumina surfaces. In contrast, the surface of alumina is a stronger hydrogen bond base than is that of silica. Unlike reversed-phase liquid chromatography, where the dominant solute properties are cavity formation and a solvent (as acid) to solute (as base) hydrogen bond interaction, normal-phase processes are sensitive to dipole-dipole interactions and to hydrogen bond formation to the solvent acting as both an acid and a base.

INTRODUCTION

A knowledge of the eluotropic strength of a pure or mixed solvent is vital in all forms of liquid chromatography in order to establish the initial separation conditions and to optimize the final separation¹. Normal-phase adsorption chromatography, which is most often implemented using silica or alumina as the adsorbent, unquestionably entails the use of a much wider range of solvent classes than does reversed-phase liquid chromatography. Solvents varying in strength and type from exceedingly non-polar, non-polarizable perfluorinated alkanes to highly dipolar hydrogen bond acceptor/donor alcohols are in common use in normal-phase chromatography. The parameter ϵ^0 was developed by Snyder^{2,3} to quantify the overall strength of a solvent on a particular adsorbent. This parameter is related to the adsorption energy of a solvent molecule per unit area occupied by that molecule on

a specified adsorbent of standard activity. It can be shown, based on the assumption of a displacement mechanism^{3,4}, that the adsorption coefficient, *i.e.*, the equilibrium constant (K_{ads}) for the process defined by eqn. 1, is given by eqn. 2.



$$\log K_{\text{ads}} = \log V_a + \alpha(S^0 - A_s \varepsilon^0) \quad (2)$$

where X and M denote the solute and solvent molecules and the subscripts n and a refer to a molecule of either type (X or M) in the non-adsorbed and adsorbed states, respectively; V_a is the adsorbent surface volume (*i.e.*, the volume of an adsorbed solvent monolayer per gram of adsorbent) and α is the surface activity which varies with the condition of the surface, *i.e.*, how it is thermally and chemically preconditioned with water or some other deactivating agent. In principle, both V_a and α are properties of the adsorbent and are not related to the solvent or the solute. Hence they are of no further interest in this work. The term S^0 is the dimensionless free energy of adsorption of the solute molecule on to a surface of unit activity using pentane as the mobile phase and A_s is the surface area occupied by a solute molecule.

It is most important to note that eqn. 2 is based on the simplifying assumption that mobile phase interactions between the solute and the solvent can be neglected². This condition will obtain when solute-solvent and solvent-solvent interactions are inherently small compared with the energies of adsorption. Eqn. 2 will also be accurate when the energy gained by the formation of the solute-solvent interactions, on displacement of the solute from the adsorbed state, is cancelled by the corresponding solvent-solvent interactions which are lost. Related work involving solubility parameter-based models of liquid chromatographic processes indicate that this approximation is likely to be most realistic when the solute and solvent are unable to interact significantly via hydrogen bonding⁵.

With the above assumptions, the ε^0 scale is independent of the solute and of the specific activity of the surface (α); ε^0 does depend on the chemical nature of the surface. Therefore, values of ε_0 for a particular solvent, *e.g.*, methanol, will be different for different adsorbents such as silica, alumina and activated carbon. The sequence of solvents in order of their ε^0 values is termed an eluotropic series. The most recent comprehensive collection of eluotropic data for a number of adsorbents is given in ref. 3. The major thrust of this work is to explore the chemical and physical differences between silica and alumina as normal-phase adsorbents and determine how these differences influence the solvent (and secondly the solute) adsorption energy.

Experimentally ε^0 cannot be separated from the test solutes used to establish it. Eqn. 2 indicates that for non-localizing solutes a plot of $\log k'$ (k' = capacity factor) vs. ε^0 will be independent of the solute adsorption energy but dependent on solute size (A_s) for non-localizing solutes. This has been demonstrated repeatedly. Snyder and Schunk⁶ did so using an amino-bonded phase as the column packing. The validity of eqn. 2 was further substantiated by Snyder and Glajch⁷, who have shown that for a non-localizing solvent mixture the measured ε^0 value of the strong solvent is independent of both the eluent composition (volume percent) and of the solute. This is not the case when the eluent contains a localizing component. In essence, ε^0 is an

empirical parameter obtained by measuring the k' value of a solute in a series of solvents on an adsorbent of known activity with a solute of known size. Eqn. 2 yields the following relationship which is used to calculate ε_2^0 :

$$\log k'_1/k'_2 = \alpha A_s(\varepsilon_2^0 - \varepsilon_1^0) \quad (3)$$

In order to calculate ε_2^0 it is necessary to define arbitrarily the ε^0 value of some solvent. For this purpose the value of ε^0 of *n*-pentane was defined as zero².

It should be evident from the above discussion that with a typical normal-phase adsorbent the chief properties which establish the strength of the interaction of a molecule with an adsorbent are its dipolarity and hydrogen bonding donor (acidity) and acceptor (basicity) strength.

Over the past decade Kamlet, Taft and co-workers have developed a methodology for quantifying such interactions and the influence of pure bulk solvents on a wide variety of solution-phase processes. They have made use of the phenomena of solvatochromism⁸⁻¹⁰, that is, the effect of a solvent on a spectroscopic property, to establish three carefully constructed scales representing solvent dipolarity (π^*) and hydrogen bond acidity (α) and basicity (β). Most of these parameters have been measured for over 200 solvents¹¹.

Taft and co-workers^{12,13} have applied these measures of interaction strength to about 600 processes, including a large number of systems of immediate relevance to chromatography. For example, Rohrschneider's gas-liquid partition coefficients¹⁴, retention of McReynold's solutes on polymeric silicone oil gas chromatographic phases¹⁵ and reversed-phase liquid chromatography^{16,17} have been studied. As originally developed by Taft and co-workers, π^* , α and β are properties of the bulk liquid. Therefore, it would seem that they should not be used to characterize a species when it is a solute or to characterize a solvent-surface interaction as is proposed here. Recent work has shown that for species which self-associate (e.g., alcohols) in the bulk liquid state the values of α and β require modification¹⁸. In this work we rationalize the use of these parameters based on the findings of Abraham *et al.*¹⁹ that gas-solid adsorption coefficients on several types of carbon can be rationalized based on the solvatochromic properties of the adsorbate.

In this work we examined the ε^0 scale and solute retention in normal-phase liquid chromatography by use of linear solvation energy relationships (LSER) based on the above-mentioned parameters. In previous studies of solvent effects on chemical and physical properties of a solute we considered a free energy parameter with a linear combination of the solvent cohesiveness as measured by the Hildebrand solubility parameter (δ_H^2), the solvent polarizability-dipolarity parameter (π_1^*), hydrogen bonding acidity (α_1) and hydrogen bonding basicity (β_1). For several reasons which will be given below we believed that the elutropic strength (ε^0) for normal-phase chromatography would not depend on the solvent's solubility parameter, so an equation of the following form was used:

Solvent studies:

$$\varepsilon_1^0 = \varepsilon_0^0 + s(\pi_1^* + d\delta) + a\alpha_1 + b\beta_1 \quad (4)$$

where δ is a polarizability correction term²⁰ ($d = -0.2$, $\delta = 0.00$ for non-chlorinated

aliphatic solvents, 0.50 for polychlorinated aliphatics and 1.00 for aromatics), the subscript 1 denotes a solvent property and ϵ_0^0 is the intercept of the regression.

Similarly in the past we represented the variation of a free energy property with solute type by a linear equation which depended on the solute size as measured by its molar volume, in addition to its dipolarity (π_1^*), and for solutes which self-associate in the pure liquid state on their monomer hydrogen bond acidity (α_m) and basicity (β_m). Because we felt that cavity formation would not be a significant factor in normal-phase chromatography, we delete the dependence on solute size to give an equation of the following form:

Solute studies:

$$S_2 = S_0 + s\pi_2^* + a(\alpha_m)_2 + b(\beta_m)_2 \quad (5)$$

where S is the solute adsorption energy, subscript 2 denotes the solute and the additional subscripts indicate the use of the monomer value for the solute as explained previously^{16,17}. The terms α_m and β_m indicate the α and β values of a substance when it acts as a monomer. For all but non-associating solutes $\alpha_m = \alpha$ and $\beta_m = \beta$. When a compound self-associates, owing to strong intermolecular hydrogen bond formation, α_m is less than α and β_m is less than β . This is based on the experimental findings of Abboud *et al.*¹⁸, who have shown convincingly that dimeric alcohols are both stronger hydrogen bond acids and bases than are the monomers.

RESULTS AND DISCUSSION

All the solvent parameters used here are given in Table I. These were taken from the most recent compilations of both types of parameters³.

As the outset it should be understood that the solvent strength scale on alumina is closely related to that on silica (see Fig. 1). A linear regression shows that

$$\epsilon_{\text{alumina}}^0 = -0.003 (\pm 0.06) + 1.19 (\pm 0.09) \epsilon_{\text{silica}}^0 \quad (6)$$

$$n = 19; r = 0.958; sd = 0.07$$

where n is the number of solvents tested, r is the correlation coefficient and sd is the average residual of the fit. This is not a particularly good regression relative to what we have been able to obtain regularly when other solvent properties were correlated with the solvatochromic parameters and indicates that there may be chemical differences in the interactions of silica and alumina with the set of solvents included in the regression. Clearly the eluotropic strength scale on silica is compressed relative to alumina.

Solvatochromic study of silica

Based on previous experiences, it is important to build up the regression in a stepwise fashion in order to avoid addition of unnecessary parameters. For all available solvents we obtain

$$\epsilon_{\text{silica}}^0 = 0.08 (\pm 0.06) + 0.62 (\pm 0.13) (\pi^* - 0.2\delta) \quad (7)$$

$$n = 19; r = 0.753; sd = 0.13$$

TABLE I
SOLVENT PARAMETERS FOR LIQUID-SOLID CHROMATOGRAPHY

<i>Solvent</i> ^a	$\epsilon_{alumina}^0$ ^b	ϵ_{silica}^0 ^b	π^{*c}	α^c	β^c
Hexane (S)	0	0	-0.04	0	0
Heptane (S)	0	0	-0.05	0	0
Tetrachloromethane	0.17	0.11	0.29	0	0
Diisopropyl ether (S)	0.28	0.32	0.27	0	0.49
Isopropyl chloride (S)	0.31	0.28	0.37	0	0
Toluene	0.30	0.22	0.55	0	0.11
<i>n</i> -Propyl chloride (S)	0.31	0.28	0.37	0	0
Chlorobenzene	0.31	—	0.71	0	0.07
Benzene	0.32	0.25	0.59	0	0.10
Bromoethane	0.34	—	0.60	0	0
Triethylamine	0.36	—	0.14	0	0.71
Diethyl ether (S)	0.38	0.43	0.27	0	0.47
Chloroform	0.36	0.26	0.58	0.44	0
Dichloromethane	0.40	0.30	0.82	0.30	0
Tetrahydrofuran (S)	0.51	0.53	0.58	0	0.55
1,2-Dichloroethane	0.44	0.34	0.81	0	0
Acetone (S)	0.58	0.53	0.72	0.08	0.48
<i>p</i> -Dioxane	0.61	0.51	0.55	0	0.37
Ethyl acetate (S)	0.60	0.48	0.55	0	0.45
Acetonitrile (S)	0.55	0.52	0.75	0.19	0.31
Pyridine	0.70	—	0.87	0	0.64
Isopropyl alcohol	0.82	0.60	0.48	0.76	0.95
Methanol	0.95	0.70	0.60	0.93	0.62

^a S denotes select solvents.

^b Data from ref. 3.

^c Data from ref. 11.

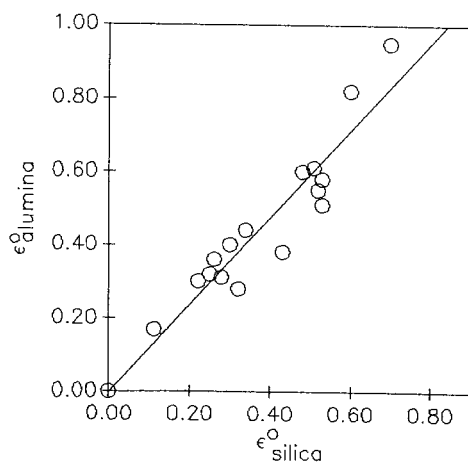


Fig. 1. Plot of $\epsilon_{alumina}^0$ vs. ϵ_{silica}^0 . The straight line indicates the least-squares line. For data see Table I.

By any standard this is an extremely poor result. It is the expected result as this regression equation completely omitted any solvatochromic representation of hydrogen bonding interactions, which are known to be important owing to the presence of hydrogen-bond active silanol groups on the surface of silica².

The original list of 23 solvents contains twelve 'select' solvents. Select solvents are defined as aliphatic, monofunctional, non-polyhalogenated, aprotic solvents and are denoted in Table I with an S in parentheses following the solvent name. For the select solvents:

$$\epsilon_{\text{silica}}^0 = 0.08(\pm 0.04) + 0.67(\pm 0.10) (\pi^* - 0.2\delta) \quad (8)$$

$$n = 10; r = 0.928; d = 0.08$$

This is clearly an improvement over eqn. 7. Although the select solvents are by definition aprotic and therefore have no acidity, the select solvent set does contain many strong hydrogen bond bases ($\beta > 0$), so we now include the solvatochromic hydrogen bond basicity parameter (β) and carry out a two-parameter regression:

$$\epsilon_{\text{silica}}^0 = 0.06(\pm 0.03) + 0.51(\pm 0.07) (\pi^* - 0.2\delta) + 0.32(\pm 0.08)\beta \quad (9)$$

$$n = 10; r = 0.979; sd = 0.05$$

Incorporation of a solvatochromic representation of HB basicity obviously improves the regression very considerably. The Ehrenson test²¹ indicates that the β parameter is significant at the 99% confidence level. The entire data set, including the hydrogen bond donor solvents, is now examined and we obtain

$$\epsilon_{\text{silica}}^0 = 0.04(\pm 0.03) + 0.46(\pm 0.06) (\pi^* - 0.2\delta) + 0.43(\pm 0.05)\beta \quad (10)$$

$$n = 19; r = 0.963; sd = 0.06$$

When the solvent hydrogen bond acidity parameter (α) is incorporated in the regression, we find there is no improvement in the goodness of the fit and the α coefficient is very small (statistically zero):

$$\epsilon_{\text{silica}}^0 = 0.04(\pm 0.03) + 0.45(\pm 0.06) (\pi^* - 0.2\delta) + 0.03(\pm 0.06)\alpha + 0.41(\pm 0.05)\beta \quad (11)$$

$$n = 19; r = 0.964; sd = 0.06$$

If the surface of silica were substantially basic, that is, able to accept a hydrogen bond from an acidic solvent, then we could expect to see a significant dependence of ϵ^0 on the solvent acidity (α). As this does not occur (the coefficient of α in eqn. 11 is trivial), we conclude that the surface of silica is not a good hydrogen bond base.

The quality of the fit is demonstrated in Fig. 2. First, as expected, the intercept term in the final regression (eqn. 11) is extremely small. Based on the definition of ϵ^0 as 0.00 for *n*-pentane and the very small π^* value (-0.087) for this solvent²², the

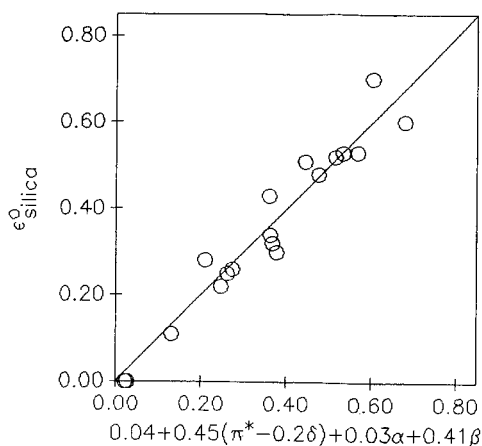


Fig. 2. Plot of $\epsilon_{\text{silica,expt}}^0$ vs. $\epsilon_{\text{silica,calcd}}^0$. Results calculated from eqn. 11.

calculated value of ϵ^0 for *n*-pentane is 0.009, which is well within the standard deviation of the fit. Second, the two factors, *viz.*, dipolarity and HB basicity, lead to an increase in solvent strength on silica. All of these results are in good agreement with the chemistry of silica, *i.e.*, it is known to be a very strong hydrogen bond acid and therefore should interact strongly with hydrogen bond bases. Basic solvents are known to be very strong displacing agents on silica.

In general, we prefer to deal with data sets in which there are a minimum of four or five data points per parameter, including the intercept. The paucity of solvents for which a set of ϵ^0 values is available on both silica and alumina is a real impediment to achieving precise regressions in this work, particularly as ϵ^0 values of the more polar solvents are certainly not more precise than ± 0.02 units. Nonetheless, the regression results are reasonably robust, that is, the coefficients are insensitive to the data set within the standard deviation of the coefficients. This is shown most clearly in comparing the *s* and *b* coefficients in eqns. 9, 10 and 11. It is incorrect to compare the coefficients in eqns. 7 and 8 with those in eqns. 9, 10 and 11 as the models used in eqns. 7 and 8 are incomplete owing to the lack of a β term and the inclusion of basic solvents in the data set.

Alumina

A similar set of correlations were carried out for alumina. For all solvents:

$$\epsilon_{\text{alumina}}^0 = 0.11 (\pm 0.07) + 0.68 (\pm 0.14) (\pi^* - 0.2\delta) \quad (12)$$

$$n = 23; r = 0.722; sd = 0.16$$

For just the select solvents:

$$\epsilon_{\text{alumina}}^0 = 0.11 (\pm 0.05) + 0.65 (\pm 0.11) (\pi^* - 0.2\delta) \quad (13)$$

$$n = 12; r = 0.882; sd = 0.10$$

For the same solvents including a β dependence:

$$\epsilon_{\text{alumina}}^0 = 0.05(\pm 0.03) + 0.57(\pm 0.06) (\pi^* - 0.2\delta) + 0.30(\pm 0.06)\beta \quad (14)$$

$$n = 12; r = 0.967; sd = 0.06$$

The entire data set is examined incorporating π^* and an HB acidity term instead of a HB basicity term:

$$\epsilon_{\text{alumina}}^0 = 0.11(\pm 0.05) + 0.57(\pm 0.11) (\pi^* - 0.2\delta) + 0.43(\pm 0.10)\alpha \quad (15)$$

$$n = 23; r = 0.860; sd = 0.12$$

There is a significant improvement in the goodness of the fit relative to the regression incorporating only π^* term (eqn. 12). This indicates that the HB basicity of alumina is important. For the same solvents incorporating π^* and a HB basicity term, the resulting regression equation is:

$$\epsilon_{\text{alumina}}^0 = 0.04(\pm 0.04) + 0.55(\pm 0.08) (\pi^* - 0.2\delta) + 0.45(\pm 0.07)\beta \quad (16)$$

$$n = 23; r = 0.919; sd = 0.09$$

There is also a significant improvement in the goodness of the fit relative to the regression incorporating only π^* term (eqn. 12). This indicates that the HB acidity of alumina is important. For the regression including all three terms we obtain

$$\epsilon_{\text{alumina}}^0 = 0.06(\pm 0.03) + 0.51(\pm 0.06) (\pi^* - 0.2\delta) + 0.28(\pm 0.06)\alpha + 0.37(\pm 0.05)\beta \quad (17)$$

$$n = 23; r = 0.961; sd = 0.07$$

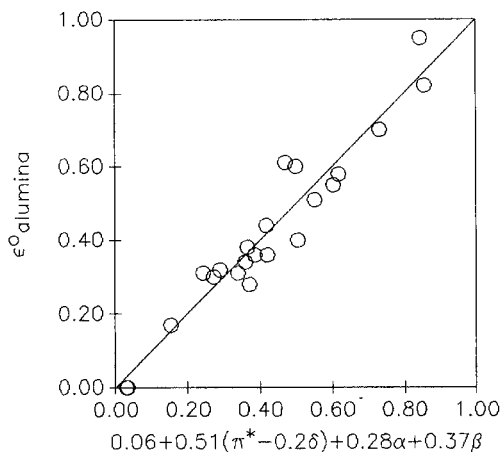


Fig. 3. Plot of $\epsilon_{\text{alumina,expt}}^0$ vs. $\epsilon_{\text{alumina,calc}}^0$. Results calculated from eqn. 17.

The triple regression equation (eqn. 17) is an improvement over both eqns. 15 and 16 at the 99% confidence level according to the Ehrenson test²¹. The quality of the fit is demonstrated in Fig. 3. We again note that the intercept is small, as predicted from the π^* value for *n*-pentane, and an increase in any of the solvatochromic parameters correctly leads to an increase in eluotropic strength.

Despite the poor robustness of the results on alumina, it is clear that a complete description of an alumina surface requires the presence of both an α and a β term. We conclude that the surfaces of alumina and silica are acidic but alumina is a considerably stronger HB base than silica.

In this context it should be noted that previous discussions of the acid-base chemistry of these surfaces has focused primarily on their Brønsted acidity and basicity²³. While there is often a parallelism between proton transfer strength and proton sharing strength, the relationships are by no means general. In some instances an inverse relationship exists. Further, there are enormous medium effects on Brønsted basicity such that a complete change in sequence of basicity between solution and gas phase process is common²⁴.

Many other correlations of ϵ^0 parameters with solvatochromic and non-solvatochromic parameters have been reported. Some time ago Keller and Snyder²⁵ showed the existence of a rough correlation between ϵ^0 for both alumina and silica and the Hildebrand solubility parameter of the solvent (δ_H). Their correlations explained about only 65–70% of the variation in ϵ^0 . As discussed in their paper, this results because the Hildebrand parameter is roughly proportional to the ability of the solvent molecule to interact with surrounding molecules of any type. An increase in δ_H would lead to an increase in the strength of a solvent's interaction with any neighboring molecule or surface group and therefore would be subject to preferential adsorption of materials with large δ_H values.

In our view this is not entirely correct. It is well known that the Hildebrand solubility parameter can be profitably subdivided into component parts. Karger *et al.*⁵ expressed the total solubility parameter as the following sum of four distinct types of interactions:

$$\delta_H^2 = \delta_d^2 + \delta_o^2 + \delta_{in}\delta_d + \delta_a\delta_b \quad (18)$$

where the subscripts d, o, in, a and b denote dispersive, orientational, inductive, hydrogen bond acid and hydrogen bond base contributions, respectively. Karger *et al.*⁵ reported a multi-variable regression of ϵ^0 for alumina *vs.* the expanded solubility parameters with a much improved correlation. We believe that the relative weightings of the contributions of the various physical processes represented in eqn. 18 probably differ depending on whether one is considering the total solubility parameter or the eluotropic strength. Although it is possible to arrive at measures of the component solubility parameters by various schemes, *e.g.*, based on the use of homologs²⁶, the solvatochromic methodologies which provide the values of π^* , α and β are far more accurate measures of the relative dipolarity and hydrogen bonding properties. However, it should be mentioned that Karger *et al.*⁵ reported a fairly good correlation of ϵ^0 for alumina with the component solubility parameters of solvents.

We opted not to include any correlations between ϵ^0 , δ_H and the solvatochromic parameters here because δ_H is strongly correlated by a multi-variable equation with the

solvatochromic parameters, π^* and α . Inclusion of two strongly correlated explanatory parameters in a regression must lead to variance inflation and can generate regression coefficients which are entirely deceptive.

Finally, we must mention that Krygowski *et al.*²⁷ reported a multi-variable regression of ϵ^0 for alumina vs. the normalized $E_T(30)$ parameter of Dimroth and Reichardt²⁸ and a modified β parameter. We do not report related correlations here for the same reason as stated above, *ie.*, E_T is demonstrably very strongly correlated with a linear combination of π^* and α , so it is not truly a measure of a single specific solvent characteristic.

The above results focus on interpreting the solvent strength parameter on very polar adsorbents in order to understand the surface chemistry better. We can, of course, gain an insight into the chemical factors which control intermolecular interactions by examining solute retention processes. This approach is very similar to that used in our previously reported studies of reversed-phase liquid chromatography¹⁶. Further, the ϵ^0 parameter is actually the energy of adsorption per unit area occupied (see eqn. 2), whereas the solute parameter (S^0) is an adsorption energy parameter *per se*. Snyder² showed that S^0 results from the sum of the interactions between all of the functional groups (i) in a solute molecule with the surface and presented an extensive set of group interaction parameters (Q_i):

$$S^0 = \sum Q_i \quad (19)$$

His values of Q_i were arrived at by averaging over data obtained with many different solutes, so in general Q_i values are likely to be more reliable than a set of S^0 values for a series of solutes under one set of mobile phase conditions. In order to be specific we have generated a data set for a group of solutes on both alumina and silica using at least one compound for each functional group in Snyder's compilations². These data, together with the appropriate solvatochromic parameters, are presented in Table II. In all instances the solvatochromic parameters are for the monomer form of the solute. In general $\alpha_m = \alpha$ and $\beta_m = \beta$, except for those compounds which self-associate in the pure liquid phase¹⁸. Although the database is overwhelmingly aromatic and polyhalogenated materials are not represented, the primary goal of this study was to examine the relative hydrogen bond acidity and basicity of silica and alumina. Addition of aliphatic and halogenated materials would be of little value.

As in previous solute studies^{16,17}, we found that the molar volume (V_2) must be included in the regression equation to obtain satisfactory correlations (see below). The following regression results were obtained:

$$S_{\text{alumina}}^0 = -1.21(\pm 0.43) + 1.51(\pm 0.32)V_2/100 + 4.59(\pm 0.45)\pi^* + 8.22(\pm 0.68)\alpha_2 + 5.82(\pm 0.58)\beta_2 \quad (20)$$

$$n = 31; r = 0.993; sd = 0.27$$

$$S_{\text{silica}}^0 = -1.06(\pm 0.58) + 0.95(\pm 0.43)V_2/100 + 3.96(\pm 0.61)\pi^* + 3.04(\pm 0.92)\alpha_2 + 8.05(\pm 0.78)\beta_2 \quad (21)$$

$$n = 31; r = 0.987; sd = 0.36$$

TABLE II

SOLUTE PARAMETERS FOR STUDY OF LIQUID-SOLID CHROMATOGRAPHY

<i>Solute</i>	S^0_{alumina}	S^0_{silica}	$V_2/100$	π_m^*	α_m	β_m
Benzene	1.86	1.50	0.989	0.59	0	0.1
Toluene	1.92	1.61	1.163	0.54	0	0.11
Ethylbenzene	1.90	1.58	1.324	0.48	0	0.12
Propylbenzene	1.92	1.53	1.494	0.44	0	0.12
Butylbenzene	1.94	1.48	1.661	0.42	0	0.12
<i>tert.</i> -Butylbenzene	1.94	1.72	1.649	0.42	0	0.12
<i>m</i> -Xylene	1.98	1.72	1.328	0.47	0	0.13
<i>p</i> -Xylene	1.98	1.72	1.333	0.43	0	0.12
Fluorobenzene	1.97	1.35	1.039	0.62	0	0.07
Bromobenzene	2.19	1.33	1.150	0.79	0	0.06
Chlorobenzene	2.06	1.30	1.118	0.71	0	0.07
Methoxybenzene	3.60	3.40	1.186	0.73	0	0.22
Ethoxybenzene	3.62	3.41	1.364	0.69	0	0.30
<i>n</i> -Propoxybenzene	3.64	3.42	1.524	0.65	0	0.32
Benzonitrile	5.11	4.83	1.120	0.90	0	0.36
Benzyl cyanide	6.93	6.78	1.253	0.75	0	0.48
4-Methylbenzonitrile	5.13	4.91	1.294	0.85	0	0.35
Ethyl benzoate	5.25	4.97	1.530	0.74	0	0.41
Methyl benzoate	5.23	5.02	1.351	0.76	0	0.39
Benzyl acetate	6.90	6.85	1.524	0.53	0	0.54
Ethyl acetate	4.96	5.36	0.978	0.55	0	0.45
Ethyl propionate	4.98	5.31	1.146	0.47	0	0.42
Acetophenone	5.57	6.26	1.269	0.90	0	0.48
Ethyl phenyl ketone	5.59	6.21	1.429	0.88	0	0.43
Benzyl alcohol	8.43	7.11	1.138	0.45	0.33	0.55
1-Propen-3-ol	7.14	6.05	0.680	0.45	0.33	0.45
1-Phenylpropen-3-ol	9.00	7.55	1.385	0.45	0.33	0.55
Nitrobenzene	4.61	4.27	1.129	1.01	0	0.25
<i>o</i> -Nitrotoluene	4.65	4.37	1.279	0.90	0	0.25
Nitropropane	5.41	5.68	0.896	0.75	0	0.25
Benzaldehyde	5.21	4.98	1.116	0.92	0	0.44

The results for all solutes and the input data are shown in Figs. 4 and 5. The necessity to include the molar volume dependence and the accuracy of the coefficients recovered by the regression can be checked to a limited extent by estimating the S^0 value of *n*-pentane ($V_2 = 115 \text{ cm}^3/\text{mole}$, $\pi^* = -0.087$), which is defined as zero. The two regression equations lead to estimates of S^0 for *n*-pentane of 0.13 and -0.19 for alumina and silica, respectively. Given the standard deviations in the intercept, these are acceptable. In view of the fact that no solute for which S^0 is less than 1.3 is included for either adsorbent, the extrapolation involved is impressively accurate. Of course, this does not address the reliability of the hydrogen bonding terms, as α and β are zero for *n*-alkanes.

The signs of all regression coefficients are as expected (see below for a discussion of the V_2 term), that is, as in the study of ϵ^0 an increase in π^* , α and β increases S^0 . In agreement with the study of ϵ^0 , the coefficients of π^* are similar for both adsorbents, so both are equally effective in retaining dipolar materials. The precision of the hydrogen

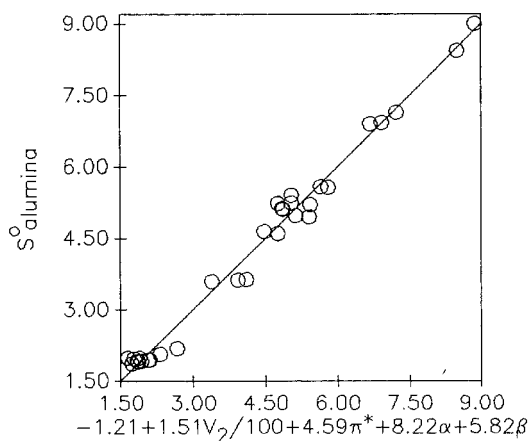


Fig. 4. Plot of $S^0_{\text{alumina,expt}}$ vs. $S^0_{\text{alumina,calcd}}$. Results calculated from eqn. 20.

bonding terms is good enough for us to state that alumina is decidedly more basic than silica ($a_{\text{alumina}} = 8.2$ vs. $a_{\text{silica}} = 3.0$), whereas silica is definitely more acidic than alumina ($b_{\text{silica}} = 8.0$ vs. $b_{\text{alumina}} = 5.8$). As no Brønsted acids or bases were included in the data sets, the basicity and acidity refer specifically to hydrogen bond interactions. From these results we may infer the practically significant result that silica will be a more selective adsorbent for the separation of materials which differ in their hydrogen bond basicity whereas alumina will be better able to discriminate between hydrogen bond acids. The above is predicted from the idea that the two solutes differ only in their hydrogen bond basicity. In practice, a change in a substituent alters all three of the solvatochromic parameters (π^* , α and β) and it is therefore not possible to predict whether silica or alumina will be more selective for a particular separation. We do believe that it is safe to say that if the two solutes have a significant difference in β (0.2–0.3) then silica will produce a greater chromatographic selectivity.

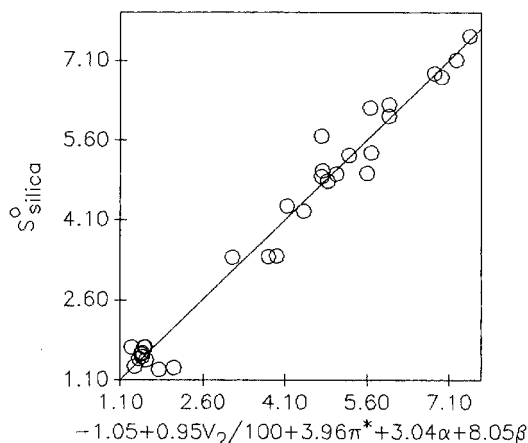


Fig. 5. Plot of $S^0_{\text{silica,expt}}$ vs. $S^0_{\text{silica,calcd}}$. Results calculated from eqn. 21.

As a cautionary note, we point out that alumina manifests decidedly superior selectivity towards polynuclear aromatic solutes than does silica²⁹. These solutes differ in their Lewis basicity, that is, in terms of the "soft" basicity, whereas hydrogen bond interactions are considered to be "hard" processes³⁰. It is well known that normal-phase chromatography on solid surfaces such as silica and alumina is much more sensitive to solute structure than is liquid-liquid partition chromatography¹. As Snyder³¹ pointed out, alumina has a highly crystalline surface and consequently it has a high selectivity for linear molecules. It may be that the aromatic selectivity of alumina results from a combination of its Lewis acidity and its ordered surface.

The above regression equations can be compared with those obtained in many other studies. Before doing so, some comment on the term containing V_2 must be made. As expected, we found no necessity to incorporate a cavity term in analyzing the values of ϵ^0 as it is a pure solvent parameter. It seems that the cavity term is compensated for by the displaced solvent which forces the formation of a cavity of the same size, that is, it cancels out. In all previous solute studies we invariably needed a solute parameter which complements the measure of cavity formation energy in the solvent. As described, the displacement model of normal-phase chromatography neglects mobile phase solute-solvent interactions at the first level of analysis. Why, then, is the term V_2 required? In comparison with other studies, the magnitude of the coefficient of this term is much less important. For the present purpose we believe that this term crudely approximates an increase in dispersive interactions between the adsorbent and the solute as the solutes increase in size and hence polarizability¹⁹. Dipole-dipole and dipole-induced dipole interactions are reflected in the π^* term.

In comparison with reversed-phase chromatography, the hydrogen bond acidity of the solute is important, as is its dipolarity. Hence the prediction of the retention of solutes in normal-phase chromatography will require far more accurate information than in reversed-phase chromatography, where a knowledge of solute size and hydrogen bond basicity suffices.

CONCLUSION

Based on the studies of ϵ^0 and S^0 , alumina and silica are concluded to be similarly dipolar and both are reasonably strong hydrogen bond acids, silica being the stronger. In contrast, alumina is definitely a stronger hydrogen bond base than silica.

ACKNOWLEDGEMENTS

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ION-EXCHANGE EQUILIBRIA OF LYSOZYME, MYOGLOBIN AND BOVINE SERUM ALBUMIN

EFFECTIVE VALENCE AND EXCHANGER CAPACITY

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SUMMARY

A series of ion-exchange equilibrium experiments were completed and analyzed by a mass action model. The isotherms at various counter-ion concentrations are regressed to determine the average number of binding sites, Z , the mass action constant, K , and the effective capacity of the exchanger, P_m . The estimates for P_m are 1–10 mmol of protein adsorbed per liter of packed Sepharose exchanger, in agreement with values determined from fitting the Langmuir isotherms. A geometric model, developed to relate the charge distribution of the exchanger and the size of the protein to the protein capacity, also confirms the estimates of P_m .

The Z values estimated (and confirmed with an independent slope method) range from 0.8 to 3.6, indicating that most of the ionized sites on the protein do not undergo ion exchange. Geometric model estimates of exchanger sites covered by a protein at maximum surface coverage show that only 1–32% of the sites actually undergo ion exchange. This result indicates that adsorbed protein molecules “block” many exchanger sites, preventing other molecules from ion exchanging at those sites. This high degree of blocking by proteins also indicates that there is an excess of exchanger sites, as evidenced by Z showing no dependence on charge separation distance over the range 6–10 Å.

Scatchard plots of the equilibrium data indicate that there is competition between at least two different binding forms of each protein studied. For the high-affinity systems, cooperative binding was observed at low exchanger loading; the character then changed to heterogeneous competitive binding as the exchanger loading increased. The effect on an isotherm plot is a subtle but systematic deviation from the Langmuir and mass action models. More important, the competition means that Z represents an average for competing binding forms and, as such, Z can be fractional.

INTRODUCTION

The separation of proteins by ion exchange usually requires changes in pH and salt (counter-ion) concentration. These changes affect the three-dimensional struc-

tures and charge distributions of the proteins, shifting protein affinity in ways which are difficult to predict. The type of exchanger, its charge density and structural characteristics can have equally profound effects on the separation. A model that could accurately account for all of these effects would be extremely complex and would require extensive information about the system.

What would be more reasonable is a model that requires a moderate amount of system information, yet still gives useful predictions of behavior for a wide range of conditions. In this research we have developed a mass action model which closely describes the ion exchange of proteins using only three fitted parameters (average binding number, Z , protein capacity of the exchanger, P_m , and equilibrium parameter, K). The regressed Z values are compared with those determined by a slope method¹. Myoglobin, lysozyme and bovine serum albumin all have known amino acid sequences, allowing us to determine the maximum number of charge sites available for binding.

To gain a better understanding of the physical interaction of the protein molecules with the exchangers, we have developed a geometric model for the interaction of charged surfaces. This model also allows comparison between the regressed value of protein capacity for the exchanger and the expected value based solely on geometric considerations. The exchangers (CM-, DEAE-, Q- and S-Sepharose) were selected to provide a range of surface charge densities for the geometric model.

Competition of various binding forms was explored by Scatchard plots. The proposed model is also compared with another mass action model, proposed by Mazsaroff *et al.*², that requires five fitted parameters and provides a sigmoidal dependence of Z on adsorbed protein concentration.

THEORY

The basic principles of ion exchange were discussed by Walton³. However, most of the cases considered were limited to small, inorganic ions. The basic ion-exchange relationship for this type of batch equilibrium experiment was used by Boardman and Partridge¹ in a study of proteins. It was also derived in terms of a mass action expression by Velayudhan and Horváth⁴. The idea has also been presented in a series of papers by Regnier's group⁵⁻⁷. In general terms, the ion-exchange process may be represented by the following "reaction":



where P and P_b are protein concentrations in the liquid and solid phases, respectively, and C and C_b are the analogs for the counter ion; Z represents the average number of binding sites between a protein molecule and the exchanger. For our experimental conditions we used only monovalent salts in order to focus on the behavior of the protein binding.

The model assumes that the ion-exchange process can be approximated by a single adsorption step with a constant number of binding sites (independent of concentration). From this "reaction" one obtains the following equilibrium expression (defining a binary selectivity coefficient in terms of concentrations):

$$K_b = \frac{P_b C^Z}{P C_b^Z} \quad (2)$$

Next, an empirical parameter, K_f is introduced to account for the fraction of charges on the exchanger that are actually accessible to the bulky protein molecule:

$$C_b = K_f q_m - Z P_b \quad (3)$$

Here C_b is actually the total concentration of counter ion that is accessible to the protein and q_m is the small ion-capacity of the exchanger in mN. This equation can be rearranged in terms of the protein capacity of the exchanger, $[P_m]$, in units of mM;

$$C_b = Z(P_m - P_b) \quad (4)$$

where $P_m = K_f q_m / Z$, representing the maximum number of moles of protein which can be bound to the exchanger. Substituting this expression into eqn. 2 and solving for P_b , one obtains

$$P_b = K_b Z^Z P \left(\frac{P_m - P_b}{C} \right)^Z \quad (5)$$

Combining constant terms gives

$$P_b = KP \left(\frac{P_m - P_b}{C} \right)^Z \quad (6)$$

where

$$K = K_b Z^Z \quad (7)$$

and C is approximately equal to C_0 , the initial counter-ion concentration in free solution, as the free solution volume is much greater than the exchanger volume under our experimental conditions. If Z is set equal to 1, the mass action expression may be rearranged to form a Langmuir expression:

$$P_b = \frac{aP}{1 + bP} \quad (8)$$

where

$$a = \frac{KP_m}{C} \quad (9)$$

and

$$b = \frac{K}{C} \quad (10)$$

The mass action model represents an isotherm over varying counter-ion levels for a specific pH. A similar model has been applied to the high-performance ion-exchange chromatography of proteins by Kopaciewicz *et al.*⁵ for very low surface coverage. Additional column evaluation of the model was presented by Rounds and Regnier⁶. The limitations of applying this model to proteins have been discussed elsewhere⁸.

Determination of ion-exchange capacity

The ion-exchange capacity per unit volume must be determined experimentally for each system. The exchange capacity is affected by the type and porosity of the exchanger, charge density and charge distribution on the sorbents. As each solute usually differs in size, shape and charge distribution, it will have access to a unique fraction of the binding sites. For a binary exchange system, competition occurs at the exchanger sites which are accessible to both compounds. For our experimental conditions, the species in competition are Cl^- or Na^+ and a protein. From size considerations alone, a given exchanger will have a smaller capacity for a protein than for an inorganic ion. We denote this protein capacity as P_m in terms of millimoles of protein per liter of packed exchanger.

Determination of apparent binding number

One complexity of protein ion exchange arises from the different ways in which an individual protein molecule may bind to an exchanger. This competition among various binding forms causes a change in the apparent (average) Z as a function of protein concentration⁸. The mass action model discussed in this paper assumes that the equilibrium behavior can be represented by an average Z value for the conditions of interest. Other researchers² have proposed an empirical model to describe the variation of Z with bound protein concentration. The merits of both methods will be discussed later.

The value of Z at low surface coverage can be estimated by a slope method. We begin with eqn. 6, written for low surface coverage (C approximately constant at the initial value, C_0 , and $P_m - P_b$ approximately equal to the exchanger capacity, P_m):

$$P_b = KP \left(\frac{P_m}{C_0} \right)^Z \quad (11)$$

Taking logarithms and rearranging gives

$$\log \left(\frac{P_b}{P} \right) = \log K + Z \log \left(\frac{P_m}{C_0} \right) \quad (12)$$

If the P_b versus P data are fitted to a Langmuir isotherm (eqn. 8), the Langmuir

coefficient, a , at low surface coverage is equivalent to the P_b/P term in eqn. 12. Plotting $\log a$ versus $\log (P_m/C_0)$ then gives Z as the slope. This relationship serves as a check of the Z values from the mass action model in the limiting case of low surface coverage.

Numerical techniques

The non-linear least-squares regression was performed on eqn. 6 as part of a Fortran program; the data consisted of $P_b = f(P, C_0)$. The IMSL (International Mathematical and Statistical Libraries, edition 10, Houston, TX, U.S.A.) routine DUNLSF uses a finite difference, Levenberg-Marquardt algorithm (using strict descent) to fit Z , K and P_m . After convergence, fitted values of P_b were calculated by the IMSL routine ZBREN, which uses a combination of linear interpolation, inverse quadratic interpolation and bisection and usually gives better than linear convergence.

To aid in quantifying the accuracy of the parameter estimations, a computer program was developed to examine the sum of squares of error for perturbations in any two of the three regressed parameters for a constant value of the third parameter. This program reads the regressed estimates and generates a grid of perturbed estimates of two of the parameters within a given percentage. The sum of squares of error ($P_b - \hat{P}_b$), SSE, is determined for each of these grid points. The contouring package of DI3000 (Precision Visuals, Boulder, CO, U.S.A.) then uses that information to generate the constant-error curves for the particular data set. Departure of the orientation of the oval error curves from parallel to the axes gives a measure of parameter correlation. As the parameters become more correlated, the individual confidence intervals have less meaning and combined intervals should be used.

EXPERIMENTAL

Materials

The ion exchangers used, Q-Sepharose Fast Flow (strong anion), DEAE-Sepharose Fast Flow (weak anion), S-Sepharose Fast Flow (strong cation), CM-Sepharose Fast Flow (weak cation), were purchased from Pharmacia (Uppsala, Sweden). Sepharose ion exchangers are composed of agarose, a macroporous gel with good stability. The three different proteins used for the study [bovine serum albumin (BSA), horse heart myoglobin (MYO) and lysozyme (LYO)] were purchased from Sigma (St. Louis, MO, U.S.A.). The properties of these ion exchangers and proteins are given in Table I and Table II, respectively. The experimental conditions are

TABLE I
TOTAL SMALL ION CAPACITIES

<i>Ion exchanger</i>	<i>pH</i>	<i>Capacity</i> ($\mu\text{equiv./ml}$)
DEAE-Sepharose FF	6.0	112
	8.0	84
Q-Sepharose FF	6.0	210
	8.0	210
CM-Sepharose FF	4.0	55
	5.0	99
S-Sepharose FF	4.0	210
	5.0	210

TABLE II
PROPERTIES OF SELECTED PROTEINS

<i>Protein</i>	<i>MW</i>	<i>pI^a</i>	<i>Residues</i>	<i>Size (Å)</i>
BSA	67 000	4.98, 5.07, 5.18	582	141 × 42
MYO	17 500	6.9, 7.3	153	45 × 35 × 25
LYO	13 930	11.0	129	45 × 30 × 30

^a Isoelectric points from Righetti and Caravaggio¹⁸.

abbreviated using the convention protein/exchanger/pH, *e.g.*, MYO/CM/5 refers to myoglobin on CM-Sepharose Fast Flow at pH 5.

To avoid problems associated with a counter ion with multiple binding sites, the counter ion selected for all experiments was singly charged. Tris(hydroxymethyl)-aminomethane (Tris) and bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris) buffers were selected for the anion exchangers, while the acetate buffer was used for the cation exchange system. The buffers were purchased from Sigma.

Batch experiments

Because proteins can be denatured at the air-liquid interface, foaming and aeration of the solutions were minimized. The buffer concentration for all of the experiments was 0.1 *M*, sufficient to maintain a constant pH over the concentration range studied. Before an exchanger was used, it was loaded with the counter ion of the experiment. The exchanger was then equilibrated by washing with six aliquots of a buffered salt solution under experimental conditions as suggested by Pharmacia⁹.

A series of concentrations of protein solution was selected for each isotherm. For each concentration, a test-tube was filled with 5 ml of the buffered protein solution and 1 ml of the exchanger suspension (yielding about a 0.3-ml packed bed volume of exchanger). The tubes were gently rotated end-over-end for 25 h at 25°C, then the UV absorbance was measured at 280 nm. The exchangers have a particle void volume of 95%, making quantification by dry weight difficult (the degree of drying is not reproducible). We therefore selected the packed bed volume as the best measure to represent the amount of exchanger. This volume is determined by taking the exchanger from a test-tube after equilibration and packing it into a small, graduated column. The volume of packed exchanger is read, while the column is wetted, to a precision of ± 0.05 ml. The accuracy of this measurement was in line with that of other measurements in the experiments.

RESULTS AND DISCUSSION

Experimental isotherms

The resulting isotherms appear in Figs. 1-8. The best-fit curves are from the Langmuir model applied at each salt concentration. Each isotherm was regressed to obtain the Langmuir coefficients and their 95% confidence intervals (Table III). The Langmuir model fits the individual isotherms fairly well but does not allow for the dependence on salt concentration.

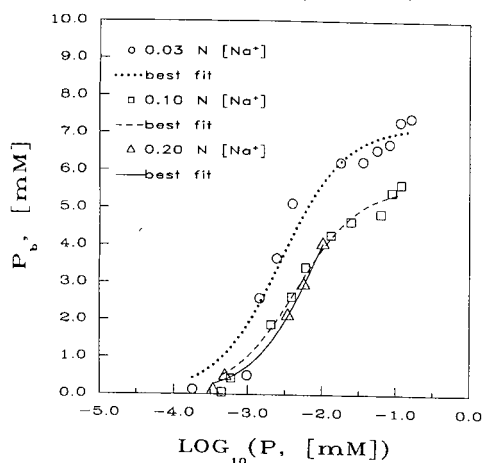


Fig. 1. Equilibrium binding isotherms for myoglobin on CM-Sepharose Fast Flow at pH 4.

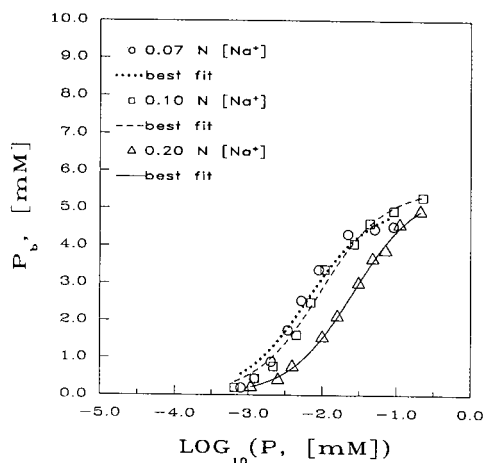


Fig. 2. Equilibrium binding isotherms for myoglobin on CM-Sepharose Fast Flow at pH 5.

The independent axis was plotted as $\log P$ in order to expand the low-concentration region. Some general trends can be noted immediately. Most of the curves show an elongated "S" shape as a result of the logarithmic scaling. In most instances the isotherms show at least the beginnings of monolayer saturation, which is indicated by the decrease in slope at higher P . The protein concentrations for these isotherms are well below saturation of the small ion capacities, indicating that, at most, 32% of the total exchanger capacity is used by the proteins.

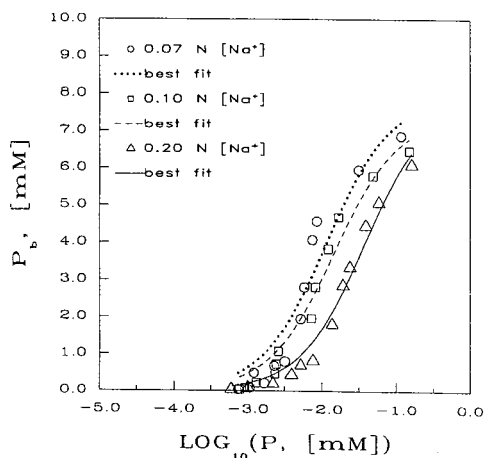


Fig. 3. Equilibrium binding isotherms for myoglobin on S-Sepharose Fast Flow at pH 5.

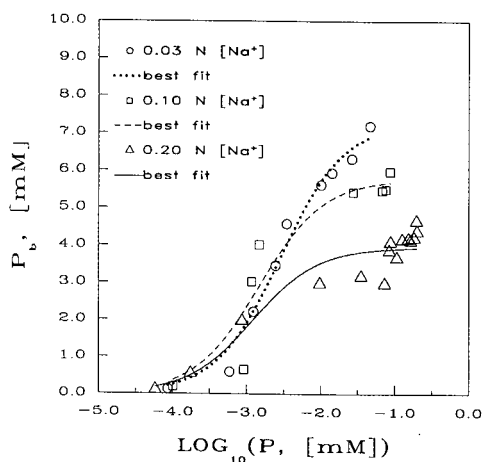


Fig. 4. Equilibrium binding isotherms for lysozyme on CM-Sepharose Fast Flow at pH 4.

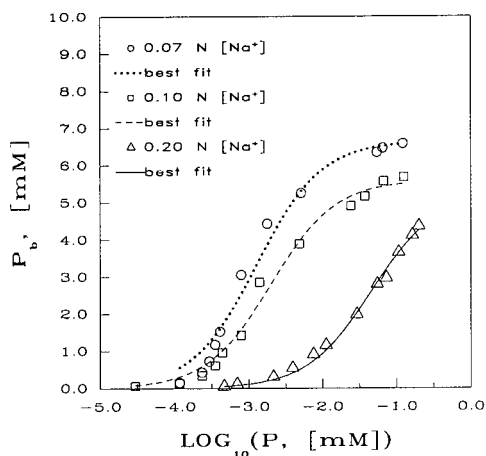


Fig. 5. Equilibrium binding isotherms for lysozyme on CM-Sepharose Fast Flow at pH 5.

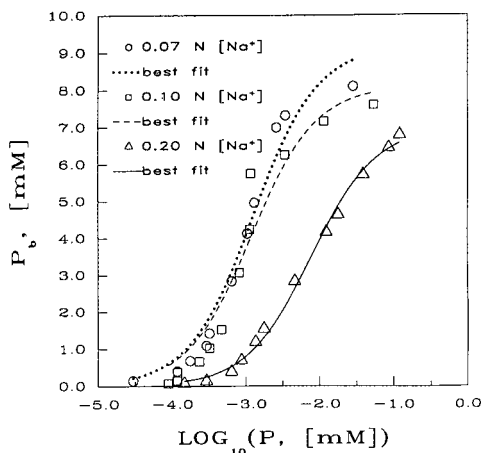


Fig. 6. Equilibrium binding isotherms for lysozyme on S-Sepharose Fast Flow at pH 5.

Determination of mass action model parameters

Before applying the model to batch data at different salt concentrations, we conducted a series of tests with four synthetic isotherms. These tests served both as a check on the program and as a gage of the model characteristics. The isotherms have $Z = 4$, $K = 1280$, $P_m = 21$ mM, $C_0 = 80, 100, 150$ and 200 mM, and a range of P values (0 – 1.5 mM). The first check was on the Z, K and P_m regression program. Runs on the four test sets returned $Z = 4$, $K = 1280$ and $P_m = 21$ mM for a wide series of initial guesses, and within the round-off error, had zero standard deviation.

In eqn. 6, if P_m is much greater than P_b , the model will predict a linear isotherm.

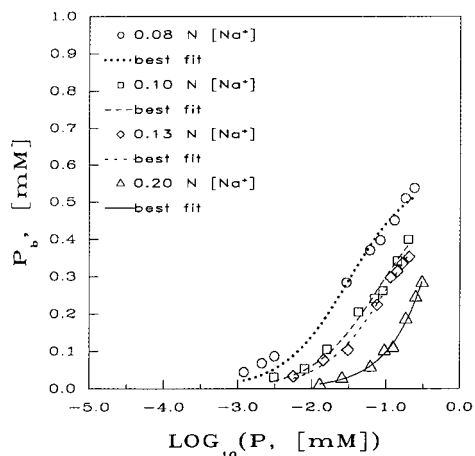


Fig. 7. Equilibrium binding isotherms for bovine serum albumin on DEAE-Sepharose Fast Flow at pH 8.

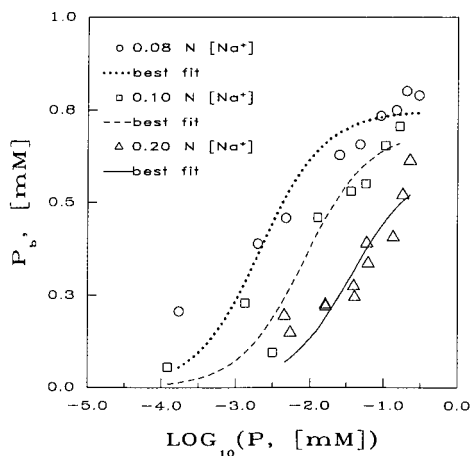


Fig. 8. Equilibrium binding isotherms for bovine serum albumin on Q-Sepharose Fast Flow at pH 8.

TABLE III
LANGMUIR COEFFICIENTS

Conditions ^a	$C_0(\text{mM})$	a	$b(\text{mM}^{-1})$	$a/b(\text{mM})$
MYO/CM/4	30.0	2581	358.7	7.19
	100.0	1256	224.6	5.59
	200.0	843.9	112.2	7.52
MYO/CM/5	70.0	811.4	158.5	5.12
	100.0	588.5	106.3	5.54
	200.0	209.9	37.9	5.53
MYO/SS/5	70.0	692.8	87.3	7.93
	100.0	510.9	68.9	7.42
	200.0	211.9	27.0	7.85
LYO/CM/4	30.0	2585	353.8	7.31
	100.0	3948	683.9	5.77
	200.0	3265	825.7	3.95
LYO/CM/5	70.0	5214	785.0	6.64
	100.0	2804	501.4	5.59
	200.0	114.0	21.9	5.21
LYO/SS/5	70.0	7311	795.6	9.19
	100.0	6820	841.9	8.10
	200.0	902.2	129.2	6.98
BSA/DS/8	80.0	18.4	31.7	0.58
	100.0	6.81	12.8	0.53
	130.0	5.05	9.18	0.55
	200.0	1.00	0.23	4.32
BSA/QS/8	80.0	345.5	462.3	0.75
	100.0	81.4	117.4	0.69
	200.0	16.8	28.0	0.60

^a Protein/exchanger/pH.

This is indeed what happens if the small ion capacity is used. Clearly, most of the isotherms in Figs. 1–8 show distinct curvature. For this reason and the molecule size consideration discussed earlier, P_m must be a fitted parameter, specific for a particular protein–exchanger combination.

Once the parameters had been determined, we wished to judge the resulting behavior of the model. As the plot of $P_b = f(P, C_0)$ is three-dimensional, we also wanted to check visually for any patterns in the difference between the data and the model prediction. In Fig. 9 the BSA/DS/8 isotherms are shown with the model surface. The vertical lines emanating from the data points represent the differences between the model estimates and the actual data points. The lower C_0 isotherms have fairly steep curvature at low P . This behavior cannot be well accommodated by the model and is probably due to a change in the value of Z as an inverse function of P . Fig. 10 shows this comparison for the MYO/CM/5 isotherms (three C_0 levels). Here, too, the initial slope is underestimated. Myoglobin binds fairly readily to the cation exchanger, so the initial slope is steep.

The characteristic curvature of the isotherms (constant C_0 in the three-dimensional plots) was not given by the model when the small ion capacities were used in the fit; it predicted a higher value of P_b for saturation. Using the lower, fitted values for P_m gives the expected curvature and a reduced standard deviation, indicating that

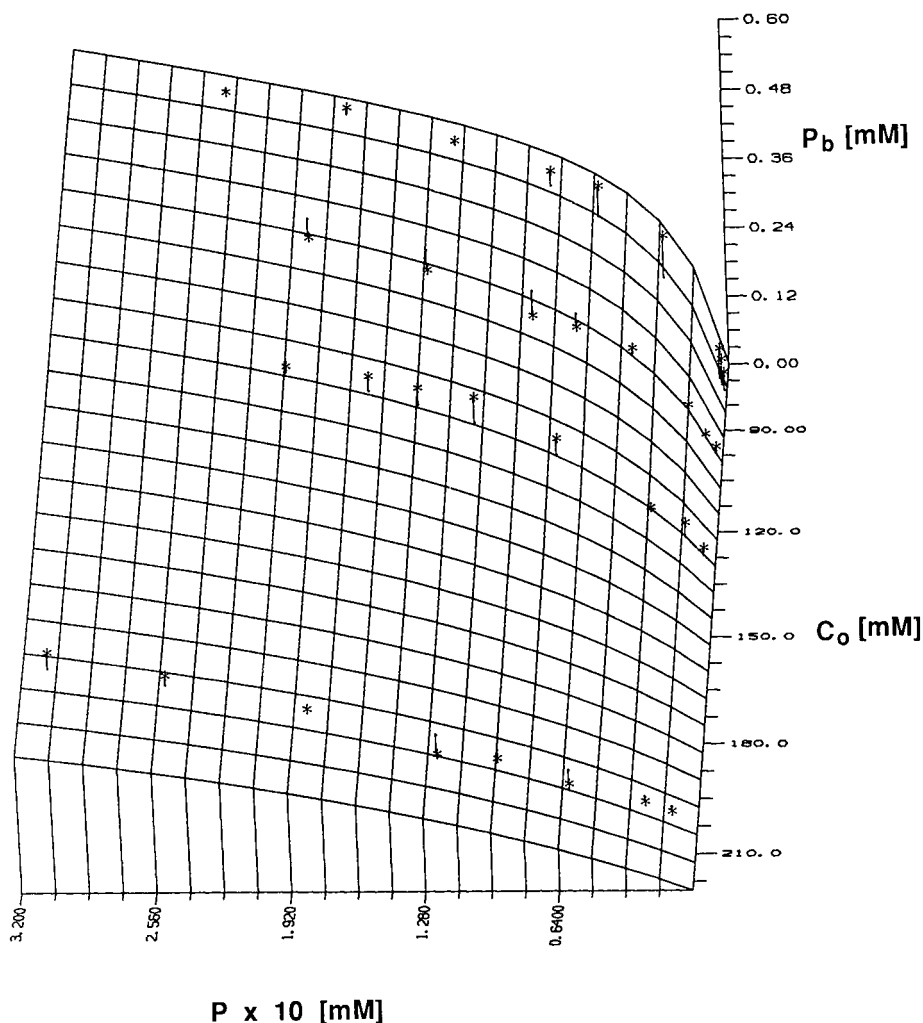


Fig. 9. Comparison of mass action model with batch data for BSA/DS/8. The vertical lines represent difference between model and data values of P_b .

a much lower value for saturation is justified. In general, with P_m fitted from the data, the curvature in the model isotherm still cannot match that of the data isotherm for the range of salt levels investigated. For all three C_0 levels in Fig. 10, the slope at the beginning and end of the isotherms is underestimated and overestimated, respectively. This behavior can be described by an initially high value of Z , decreasing as surface coverage increases. This inverse dependence of Z on protein concentration concurs with the behavior of basic amino acids reported by Wang *et al.*¹⁰ They studied cation exchange of basic amino acids which had two types of binding: monovalent binding at the side-chain amino group and divalent binding at both the α and side-chain amino groups. At a given pH and salt concentration, the average Z decreased from 2 to 1 with

increase in amino acid concentration. As proteins have numerous binding groups and potentially heterogeneous binding, similar behavior may be expected. This trait will be examined in more detail later.

Table IV shows the Z , K and P_m values for the sets of experiments. Confidence intervals for this model were determined by ignoring the correlation between the parameters beyond the first derivative (linear approximation). The approximate 95% confidence intervals are included in Table IV. The wide confidence interval for K indicates that there may be some correlation between the parameters. Correlations can be readily visualized by a contour analysis of the model. For the best parameter estimates, the SSE between the model and the data is minimized. Taking slightly perturbed values of any two parameters (keeping all others constant) will increase the SSE in a smooth manner. Connecting all points of equal SSE around the minimum will generate a series of contours. The shape of the contours and their orientation relative to the axes indicates the degrees of scaling problems and correlation, respectively. Perfectly circular contours indicate no correlation and perfect scaling. If the contours are elongated, correlation is indicated by orientation of the oval away from alignment with the axes. Scaling problems make the parameter search more difficult, but correlation between parameters renders individual confidence intervals less useful (one should consider combined confidence surfaces)¹¹.

The confidence level for the parameter estimates is related to the constant SSE curves by the F statistic¹¹:

$$S_c = S_{\min} + s_{\text{pool}}^2 n F_{0.05}(n, m) \quad (13)$$

where S_c is the sum of squares for the contour, $S_{\min}$ is the minimum sum of squares (for the best-fit of parameters), s_{pool}^2 is the pooled variance of the data sets, n is the number of parameters being estimated, F is the F statistic and m is the degrees of freedom (number of points minus n). All of the contour plots for K versus Z perturbations, including the test data set, showed ovals greatly elongated parallel to the K axis and oriented slightly clockwise (Fig. 11). Hence K could be scaled better, and the degree of correlation between K and Z is significant.

Ion-exchange capacity and the geometric model

The Langmuir model can provide a check on the values of P_m . The ratio of the Langmuir coefficients, a/b , is a measure of the effective exchange capacity for a given protein. These ratios at the lowest counter-ion concentrations approach the capacity of the exchanger for the protein and are compared with the P_m estimates in Table V. The two methods give reasonably good agreement considering the width of the confidence intervals. One may suspect that the large protein molecules are only adsorbing on the surface of the exchanger particles. However, through a series of void fraction experiments, Wachter¹² showed that the proteins in our system can access 57–72% of the interior of an exchanger particle, whereas NO_3^- ions can access 95%.

The geometric interaction of a charged protein surface with a charged exchanger surface has an overriding effect on Z . If the charge groups cannot orient themselves to be near each other, they cannot interact. To quantify this interaction, we have developed a geometric model of the interaction of protein charge groups with exchanger charge groups. For this model we need the average charge separation

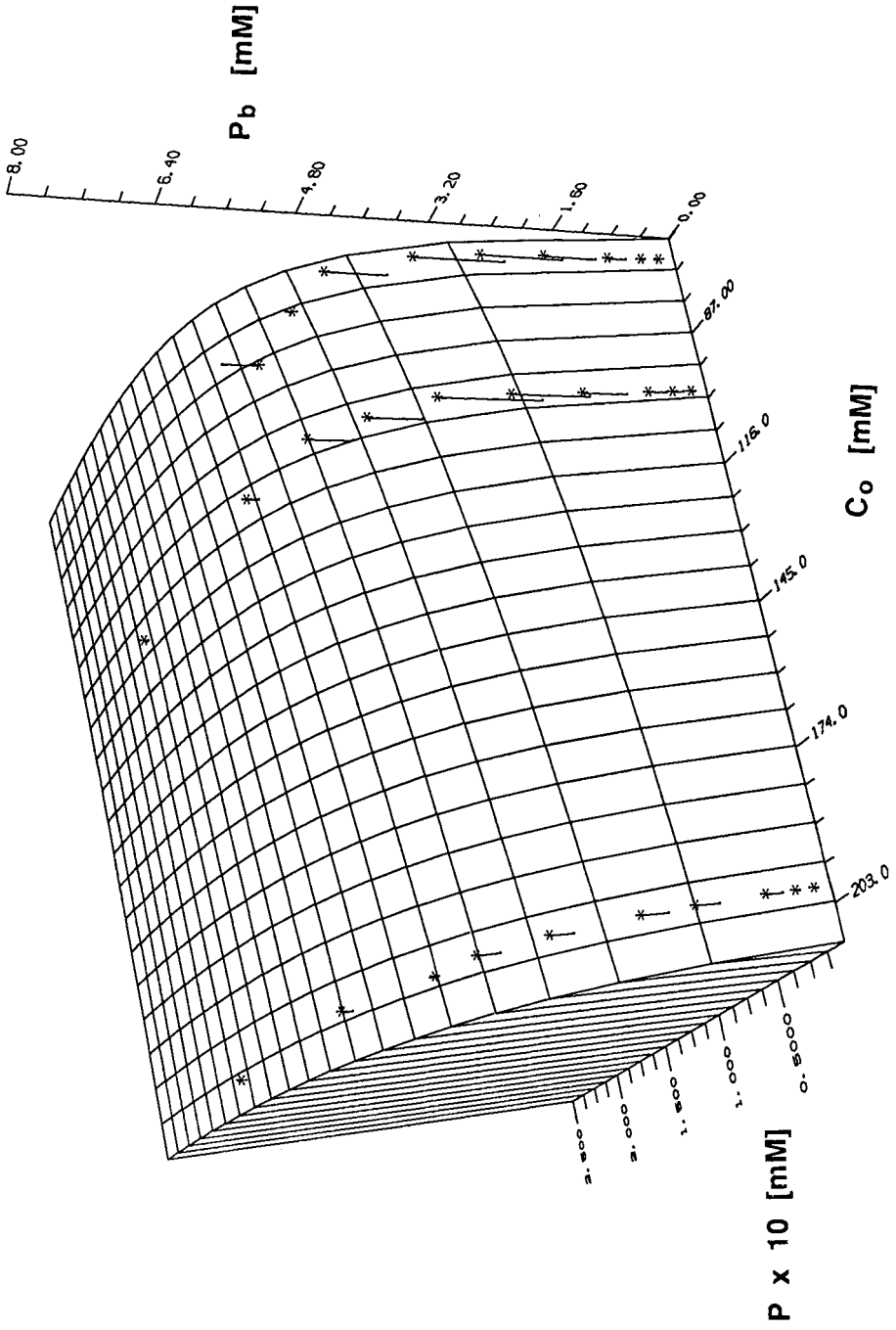


Fig. 10. Comparison of mass action model with batch data for MYO/CM/5. The vertical lines represent difference between model and data values of P_b .

TABLE IV
COMPLETE ISOTHERMS AND MODEL PARAMETERS

Conditions	SD (mM)	No. of points	Mass action			Slope method	
			$K/1000$	$P_m(\text{mM})$	Z	Z	$\text{Log } K$
MYO/CM/4	1.336	26	8.92 ± 21.76	7.98 ± 0.22	1.36 ± 0.69	0.6 ± 0.082	3.8 ± 0.087
MYO/CM/5	0.560	29	2.97 ± 2.89	5.54 ± 0.10	0.82 ± 0.20	1.3 ± 1.9	4.3 ± 2.5
MYO/SS/5	0.527	36	8.50 ± 8.29	7.42 ± 0.15	1.07 ± 0.22	1.15 ± 1.3	4.0 ± 1.6
LYO/CM/4	1.111	32	$27.9 \pm 3.68\text{e6}$	8.87 ± 0.91	1.82 ± 0.27	-0.15 ± 2.1	3.4 ± 2.2
LYO/CM/5	0.480	35	17400 ± 34721	8.76 ± 0.28	3.59 ± 0.49	3.8 ± 9.2	7.3 ± 10.3
LYO/SS/5	0.868	35	1818 ± 3100	10.25 ± 0.62	2.52 ± 0.39	2.1 ± 7.4	5.8 ± 7.8
BSA/DS/8	0.030	33	209.8 ± 380.0	1.01 ± 0.12	2.23 ± 0.29	3.0 ± 1.6	7.0 ± 3.4
BSA/QS/8	0.079	28	30005 ± 137979	1.01 ± 0.05	2.71 ± 0.74	3.1 ± 10.4	8.2 ± 21.6

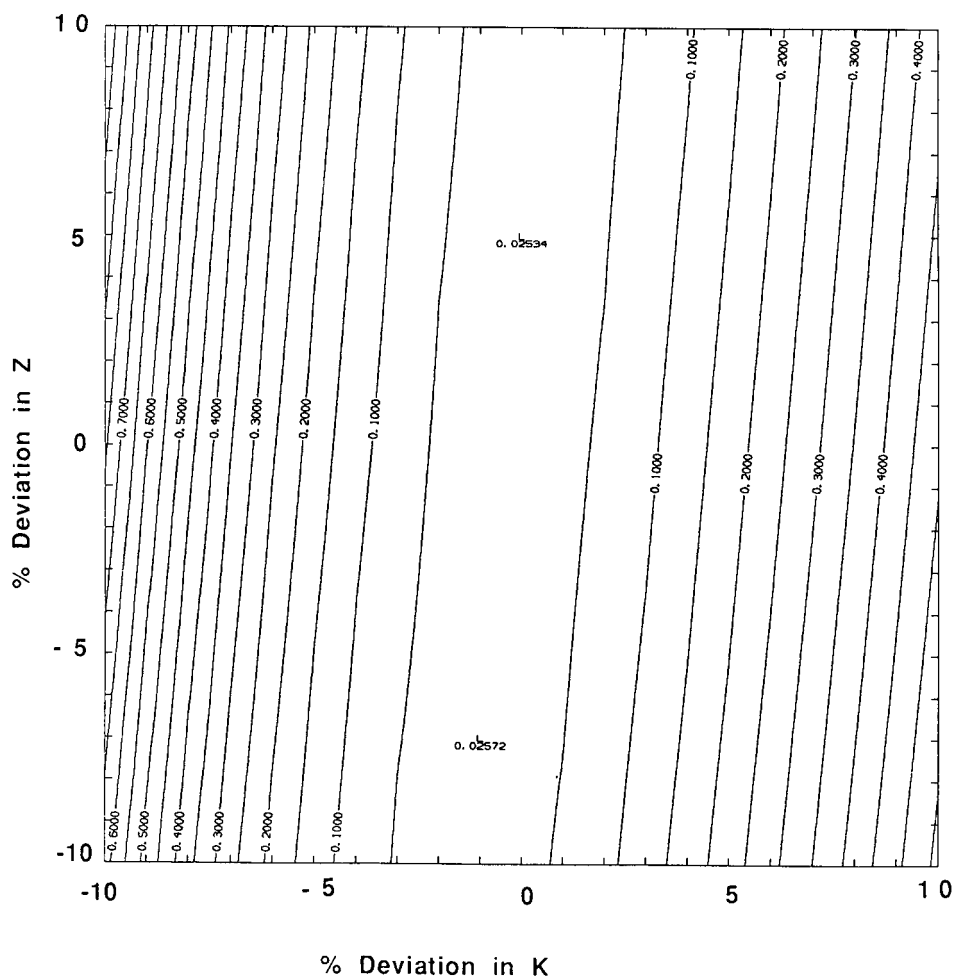
Fig. 11. SSE contours for the BSA/DS/8 isotherms with $Z = 2.23$, $K = 0.210 \cdot 10^6$ and $A = 1.01 \text{ mM}$. Span is $\pm 10\%$ of the parameter value.

TABLE V
COMPARISON OF PROTEIN CAPACITIES

Conditions	P_m (mM)	a/b (mM) ^a	ZP_m/q_m
MYO/CM/4	8.0	7.2	0.198
MYO/CM/5	5.5	5.1	0.046
MYO/SS/5	7.4	7.9	0.038
LYO/CM/4	8.9	7.3	0.295
LYO/CM/5	8.8	6.6	0.319
LYO/SS/5	10.3	9.2	0.124
BSA/DS/8	1.2	0.58	0.027
BSA/QS/8	1.0	0.75	0.013

^a Ratio for low salt isotherm.

distances (CSDs) for the exchangers and the overall dimensions for the proteins. Knowing the number of amino acids on the protein surface that are ionized, we made the following assumptions: (1) protein shape may be represented as a prolate spheroid and (2) excluding the void volume of 95%, the ion exchanger is a homogeneous phase in which the charges are uniformly distributed. For a large number of beads, the homogeneity will be realized. The last assumption slightly overestimates the CSD; it assumes the charges are in the 5% solid volume, but they are actually on the surface of the strands (they were determined by ion exchange with small ions). However, this error is minimal for small strands. Details of the calculations were given by Wachter¹², and the resulting numbers are shown in Table VI. The gel exchangers present an open matrix which allows even large proteins access to the interior of the beads. The "surface" seen by the protein molecules is a maze of wavy strands of varying diameters¹³. On average, the net effect is assumed to be equivalent to a flat exchanger surface with a uniform charge distribution. For the protein, we approximate the shape as an elliptical projection on to the exchanger surface.

With those approximations in place, we calculate the number of exchanger sites covered by the projection of the protein. As MYO and LYO have three different dimensions, there are two elliptical projections which represent the minimum and maximum area that the protein may cover. The corresponding numbers of sites covered for these projections are called SC_1 and SC_2 , respectively. The true number of

TABLE VI
ION EXCHANGER CSDs (Å)

pH	CM-Sepharose	S-Sepharose	DEAE-Sepharose	Q-Sepharose
4.0	10.1	6.3		
5.0	8.2	6.3		
6.0			7.8	6.3
8.0			9.0	6.3

exchanger sites covered should be anywhere between the two projections. The number of sites covered by one protein is compared with the number of moles of sites covered by 1 mole of protein molecules ($f_a q_m / P_m$), where f_a is the ratio of the particle void for a protein to that for NO_3^- . The values of f_a are 0.60 (BSA), 0.73 (MYO) and 0.76 (LYO). The results of this comparison are given in Table VII. In most instances the ratio $f_a q_m / P_m$ falls near SC_1 (within experimental error). This indicates that at high loading the protein orients itself with respect to the "planar" exchanger surface such that there is a minimum of contact. Even though many exchanger sites are covered by a protein, very few are actually involved in binding with the protein. Hence the charge densities of the exchangers studied appear to be in excess of that needed to reach the average Z . This may also be seen by the lack of correlation between Z (Table IV) and the average CSD on the exchanger (Table VI) for a given protein.

Apparent binding number

The associated plots for eqn. 12 are shown in Figs. 12–14. The slopes of these lines are the limiting values of Z for low concentration (Table IV); we arrived at the estimates by linear regression. The confidence intervals on the slope and intercept, Z and K , respectively, were calculated at a 95% confidence level. There are few data points for each of these lines, as only three or four C_0 levels were covered. Consequently, the confidence intervals are fairly wide, but the fitted values of Z show reasonably good agreement with the mass action estimates of Z .

Wada and Nakamura¹⁴ showed that most charges on a protein surface are surrounded by charges of opposite sign at neutral pH. Perutz¹⁵ noted that most of these charges are involved in salt bridging with neighboring charges of opposite sign. The bridging reduces the tendency of the protein charge groups to bind with the charge groups on the exchanger. This could explain the low number of binding sites reported here. Similar results were obtained with amino acids by Wang *et al.*¹⁰. They found that

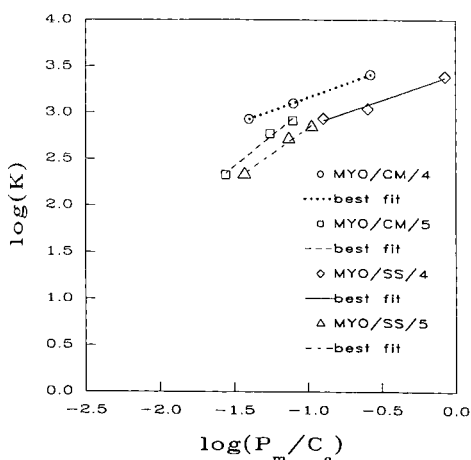


Fig. 12. Curves for slope method analysis of MYO isotherms.

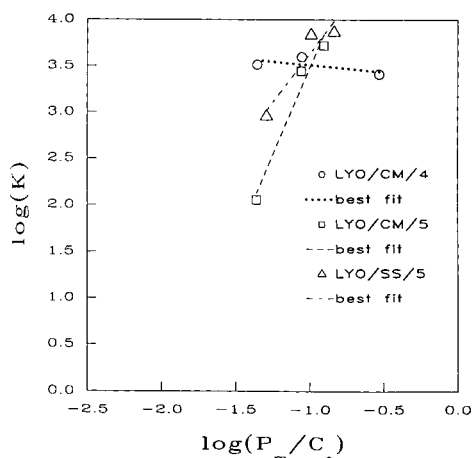


Fig. 13. Curves for slope method analysis of LYO isotherms.

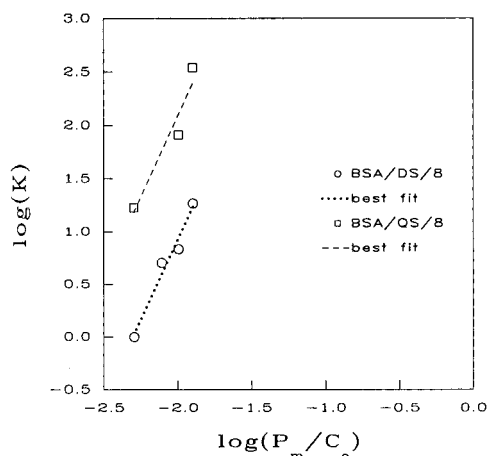


Fig. 14. Curves for slope method analysis of BSA isotherms.

when both the α -amino and α -COOH groups were ionized, bridging seemed to occur between these two nearby groups, reducing the chance of either group binding to an exchanger surface.

Since protein surface charges are due to weakly ionizable groups, the protein may carry a fractional average charge, resulting in a net fractional value for Z . Additionally, any given protein molecule may be oriented in several ways with respect to the exchanger, thus presenting different numbers of sites for ion exchange. Both of these factors contribute to the observed value of Z , which is a population-weighted average. Although the confidence intervals on the Z values in Table IV are wide enough to cover an integral number, several values of Z fall mid-way between two whole numbers. A Scatchard plot may be used to show that these fractional Z values result from two or more different binding forms of a protein in competition. Ruaan *et al.*¹⁶ used this graphing technique for pyruvate kinase adsorbing onto a gel containing both carboxylic acid groups and Cibacron Blue F3G-A. Writing for our system and assuming one type of interaction, we have



where P is the free protein, L is the exchanger site consisting of any fixed number of charge sites. The resulting Scatchard equation is

$$\frac{[P-L]}{[P]} = \frac{[L_{\max}]}{K_d} - \frac{[P-L]}{K_d} \quad (15)$$

where K_d is the dissociation equilibrium constant. If this relationship holds true, a plot of $[P-L]/[P]$ versus $[P-L]$ will be linear.

First, the test data set for the model was plotted in this manner. The resulting curve is concave-up, indicating negative cooperativity for the test set of parameters

($Z=4$). The Langmuir fit (which implies $Z=1$) of the test data set yielded a straight line. However, when the experimental isotherms were plotted in Scatchard form, almost all of them show a nearly exponential decay with increasing P_b (Fig. 15). This concave-up character is the result of heterogeneous binding forms in competition. The higher affinity systems showed a Gaussian-like peak (Fig. 16), which implies positive cooperative binding at low exchanger loading¹⁷. As the concentration increases, there is an inflection point, after which the concave-up pattern for heterogeneous binding is seen. The Langmuir model does not allow for either positive or negative cooperativity. The mass action model with $Z > 1$ can account for negative-cooperative binding, but it does not allow Z to change with concentration. Hence both models give a curve that represents some average due to competing forms.

As mentioned while discussing the three-dimensional nature of the model, the protein data, in addition to previously reported data for basic amino acids¹⁰, show that Z decreases with increasing concentration of protein or amino acid. The amino acid results and a study of cytochrome *c* by Boardman and Partridge¹ also showed that Z decreases with increasing salt concentration. In the latter work column elution volume was compared with sodium concentration. They overlaid curves predicted for various integral values of Z . At low sodium concentration (10 mM), the data followed the $Z=5$ curve, crossed over to the $Z=4$ curve as sodium concentration increased and finally approached the $Z=3$ curve at the end of their experimental conditions (Na^+ concentration = 18 mM).

Knowing that the apparent binding number is a population-weighted average of the two or more binding forms present, the task is to determine the relationship of Z to system variables. Over the course of a given separation, change in absorbed protein concentration usually has a direct effect on Z . There are two basic curve shapes proposed for this relationship: an exponential decay¹⁰ and a decreasing sigmoidal curve².

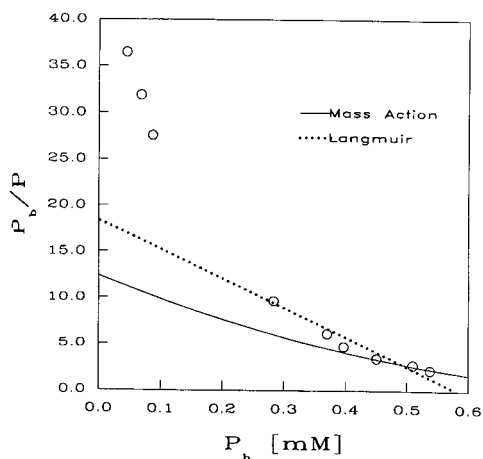


Fig. 15. Scatchard plot of BSA/DS/8 for $C_0=80$ mM, shown with best fits of Langmuir and mass action models.

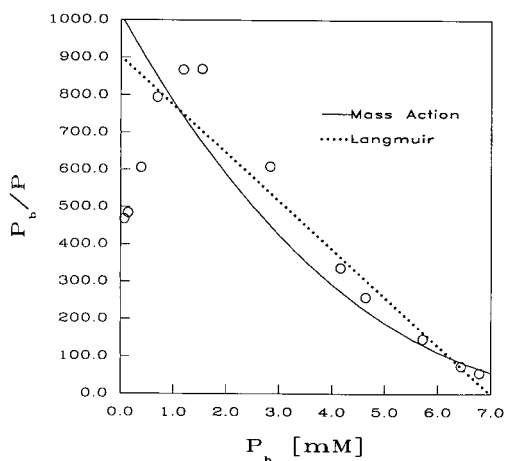


Fig. 16. Scatchard plot of LYO/SS/5 for $C_0=200$ mM, shown with best fits of Langmuir and mass action models.

TABLE VII
GEOMETRIC MODEL

Conditions	SC_1	SC_2	$f_a q_m / P_m$
MYO/CM/4	9.43	12.1	^a
MYO/CM/5	14.3	18.4	13.0
MYO/SS/5	24.2	31.2	20.7
LYO/CM/4	6.93	10.4	4.70
LYO/CM/5	10.5	15.8	8.59
LYO/SS/5	17.8	26.7	15.6
BSA/DS/8	57.4	57.4	49.7
BSA/QS/8	117.2	117.2	124.8

^a Insufficient data at high concentration.

The sigmoidal curve results in a total of five adjustable parameters for the proposed mass action expression. The model was applied to the set of almost linear isotherms for immunoglobulin G (IgG) on a strong anion exchanger which were reported by Mazsaroff *et al.*². The counter-ion concentration ranged from 100 to 108 mM. Fitted values for $Z_{\min}$ and $Z_{\max}$ were 2.36 and 3.61, respectively. We obtained a value of 2.53 ± 0.20 for the average Z . The other two parameters, $K = 671.2 \pm 141.4$ and $P_m = 9.30 \pm 7.27$ mg, are not directly comparable to our system as the IgG bound values were in units of mass and as a different ion exchanger was used. The standard deviation for bound mass was 0.00071 mg, indicating that a three-parameter model is sufficient to describe the IgG system at the low surface coverage reported.

CONCLUSIONS

At a given pH and ionic strength, isotherms of BSA, MYO and LYO can be well described by the Langmuir equation. At a given pH and varying counter-ion concentrations, isotherms of the proteins studied can be described by mass action equilibria. The accuracy of the P_m estimation has been confirmed by comparison with the Langmuir model and with the geometric model developed in this paper. The geometric model accurately predicts a range for the molar protein capacity of an exchanger based only on exchanger CSD and overall protein dimensions. This predicted capacity agrees with mass action and Langmuir estimates. For BSA, the largest protein studied, we observed partial size exclusion from some of the interior cavities of the exchangers.

The values of Z from mass action agree with those from a slope method. These small values are supported by other work which indicates that many of the potential binding groups on a protein surface are tied up in salt bridges and that steric hindrance prevents binding of many sites. The width of the Z confidence intervals is due to Z actually being an average value over the counter-ion and protein concentration ranges studied. The mass action model describes well the equilibrium data for IgG, and the average Z determined for these data falls mid-way between the maximum and minimum Z determined by a model allowing for variation in Z (ref. 2). This indicates that what our model shows is indeed the average Z over the concentration region of interest.

In addition, plotting our isotherm data as Scatchard plot supports the claim of competition between two or more binding forms of the proteins studied. For the high-affinity systems, the Scatchard plots also showed cooperative binding at low exchanger loading. Even though the Langmuir and mass action models give close fits of the isotherm data, the Scatchard plots clearly show that they really represent the average behavior.

ACKNOWLEDGEMENTS

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SYMBOLS AND ABBREVIATIONS

a, b	Langmuir coefficients
BSA	bovine serum albumin
C	salt counter-ion concentration in the bulk phase
C_b	salt counter-ion concentration in the adsorbed phase
C_0	initial salt concentration in the bulk phase
CSD	charge separation distance ($\text{\AA}$)
$F_{0.05}(n,m)$	F statistic, n parameters, m degrees of freedom
f_a	ratio of particle void for a protein over particle void for NO_3^-
k_d	dissociation equilibrium constant
K	mass action constant
K_b	binding constant
K_f	fractional accessibility coefficient
L	exchanger binding site (any number of charge sites)
$L_{\max}$	maximum number of exchanger binding sites
LYO	lysozyme
MYO	myoglobin
P	protein concentration in the bulk phase (mM)
P_b	protein concentration adsorbed on the exchanger [mmol protein per liter of exchanger (packed bed volume)]
$\hat{P}_b$	model prediction of P_b
P_m	protein capacity of the ion exchanger [mmol protein per liter of exchanger (packed bed volume)]
P-L	protein-exchanger binding complex concentration
q_m	small ion capacity of an exchanger [mequiv. ion per liter of exchanger (packed bed volume)]
S_c	sum of squares error for a contour
$S_{\min}$	minimum sum of squares from least-squares regression
SD	standard deviation
s_{pool}	pooled standard deviation (mM)
SSE	sum of squares of error
Z	average number of binding sites per molecule

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CHROM. 21 129

LINEAR VELOCITY CONTROL IN CAPILLARY SUPERCRITICAL FLUID CHROMATOGRAPHY BY RESTRICTOR TEMPERATURE PROGRAMMING

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SUMMARY

Increasing restrictor temperature decreases mass flow through capillary columns in a predictable way. Changes in mass flow can be related to changes in column linear velocity. Restrictor temperature programming, therefore, offers the potential for independent control of column efficiency while pump pressure, at constant oven temperature, controls fluid density. Experimental measurements of mass flow through restrictors at various temperatures and pressures confirm model predictions.

INTRODUCTION

The need to control the mobile phase linear velocity through the column is one of the most fundamental concepts of chromatography, but surprisingly little attention has been paid to linear velocity control in capillary supercritical fluid chromatography (SFC). This lack of attention may have arisen, in part, because there has not been, until recently¹, any accurate mathematical model for predicting mass flow through the system. In addition, no devices exist which can accurately adjust the extremely small mass flow-rates necessary for capillary SFC.

Capillary SFC almost invariably involves pressure programming from low to higher densities to consecutively solvate and elute a range of solutes in a single run^{2–4}. Most capillary SFC systems use a syringe pump which is run in a pressure-control mode. Fixed restrictors are used as mass-flow controllers at system pressures dictated by the pump. All fixed restrictors produce increasing mass flow with pressure. This increased mass flow can result in an increase in mobile phase linear velocity in the column, by as much as 10–15 times during a pressure-programmed run^{5–7}.

Changes in the geometry of restrictors can make the flow through them either laminar or turbulent¹. As pressure increases, laminar-flow restrictors produce more exaggerated increases in linear velocity than turbulent-flow devices. The worst case is a very-laminar-flow restrictor operated at a high temperature with moderate (*i.e.*, 40–60°C) column temperatures. Under these conditions column linear velocity can increase more than 10 times during a pressure-programmed run (from 80 to 400 atm).

Chromatographic efficiency is dictated by the ratio of the solute binary diffusion coefficient (D_{12}) in the mobile phase, to the linear velocity (u) of the mobile phase

through the column. Diffusion coefficients decrease in rough proportion to increasing density^{8,9} and viscosity^{10,11} of the mobile phase and with the square root of molecular weight¹² of the solute. To maintain constant efficiency and resolution, linear velocity should decrease 2–5 times during a pressure-programmed run to compensate for increasing density and viscosity. However, since late eluters are typically heavier (higher molecular weight), linear velocity should decrease more than to just offset the effects of density and viscosity. A further decrease of around 3.2 times would be needed to compensate for an order of magnitude increase in molecular weight. The combination of increasing density, viscosity and molecular weight suggests column linear velocity may need to drop as much as 15 times during a pressure-programmed run if constant column efficiency is to be maintained. It was pointed out above that most capillary chromatographs produce increasing column linear velocity during pressure-programmed runs, instead of the substantial decreases required.

No variable restrictors capable of controlling the very small mass flows of interest in capillary SFC have been reported. Many workers^{13–15} program oven temperature along with system pressure to change column density. In the extreme (*i.e.*, constant pressure) this isolates linear velocity changes from restrictor performance since mass flow out of the restrictor will remain constant if both the inlet pressure and temperature are fixed. Increasing column density while maintaining constant system mass flow will result in decreasing column linear velocity^{13,16} (provided the program rate is low enough to prevent significant contributions from charging effects^{7,17}). This, however, is not as complete a solution as it appears to be. Diffusion coefficients change more dramatically with temperature than with density. Diffusion coefficients are proportional to temperature to the 1.5–1.75 power¹², but are more nearly inversely proportional to density. Therefore, during inverse-temperature programs, diffusion coefficients drop faster than linear velocity. Inverse-temperature programming will lower the absolute value of linear velocity but it will not maintain constant reduced velocity of the mobile phase through the column or maintain constant column efficiency and resolution. In addition, chromatographic retention is a complex function of both fluid density and temperature. Semonian and Rogers¹⁸ point out that chromatograms obtained at the same density but different temperatures, or through inverse-temperature programming compared to increasing-pressure programming, will probably be different.

While inverse-temperature programming of the column has some advantages over pressure programming at fixed oven temperatures, it is preferable to have independent control over both column density and mobile phase linear velocity. The present work proposes the use of restrictor temperature programming to control mass flow and column linear velocity semi-independent of column density. The temperature programs required can be predicted mathematically for most types of commonly used restrictors. The approach is to program the restrictor temperature, not to change its internal diameter, but to vary the physical properties of the mobile phase.

THEORY

A previous report⁷ showed that restrictors can be modeled accurately, and that restrictors useful for capillary SFC range from those exhibiting very laminar to those with nearly turbulent flow. The degree of turbulence was shown to strongly impact

mass flow and the resulting column linear velocity vs. pressure profiles. The more laminar the device, the worse is the increase in linear velocity with pressure, corresponding to the worse loss in chromatographic resolution from start to finish of a pressure-programmed run. Two models are required to completely describe both very laminar and turbulent restrictors.

Laminar-flow restrictors

Laminar-flow restrictors tend to perform very poorly chromatographically. The loss in efficiency with increasing pressure was discussed above. In addition, since virtually all system pressure is dissipated inside the restrictor, part of the restrictor operates at very low density where solutes can drop out of solution and be lost to the restrictor walls or agglomerates and produce detector spikes. Most workers have, therefore, abandoned laminar-flow restrictor designs. However, some frequently used restrictors still produce laminar flow.

Modified Poiseuille equation

Restrictors that can be described by the modified Poiseuille equation (eqn. 1) exhibit laminar flow, and large increases in mass flow vs. pressure. Typical calculations using this equation indicate that mass flow decreases with increasing temperature. This is due to the large decreases in the ratio of density to viscosity with increasing temperature¹.

$$F_{\text{rest}} = \frac{\pi r^4}{8 L} \frac{g_c}{\eta_{\text{av}}} \frac{P_{\text{in}} \rho_{\text{av}}}{\eta_{\text{av}}} \quad (1)$$

where F_{rest} = restrictor mass flow in g/s, r = radius in cm, $g_c = 1.0136 \cdot 10^6$ g/cm s² atm, L = length in cm, P_{in} = restrictor inlet pressure in atm (assumes entire pressure drop occurs inside restrictor), and ρ_{av} and η_{av} = average density in g/cm³ and average viscosity in g/cm s, respectively.

Column linear velocity

Column linear velocity can be related to restrictor mass flow using eqns. 2 and 3. Under steady-state conditions, mass flow through all parts of the system must be equal, so

$$F_{\text{col}} = F_{\text{rest}} \quad (2)$$

where F_{col} = column mass flow in g/s. Also

$$u_{\text{col}} = F_{\text{col}} / (\rho_{\text{col}} \times A) \quad (3)$$

where u_{col} = velocity of mobile phase in the column, ρ_{col} = fluid density in the column, and A = column cross-sectional area.

Substituting eqns. 2 and 3 into eqn. 1 produces eqn. 4 which relates flow velocity in the column to restrictor and column parameters. Constants can be grouped in one term and the variables pressure, density, and viscosity, in another.

$$u_{\text{col}} = \frac{\pi r^4 g_c}{8 L A} \frac{P_{\text{in}} \rho_{\text{av}}}{\rho_{\text{col}} \eta_{\text{av}}} \quad (4)$$

Plotting the values of the right-hand term at different pressures and temperatures produces curves as shown in Fig. 1 showing the relative magnitude of changes in linear velocity independent of restrictor dimensions. Conditions producing constant column linear velocity independent of pressure would produce horizontal lines in Fig. 1. These results indicate that increasing restrictor temperature can be used to offset the normal increase in column linear velocity caused by increasing head pressure.

Temperature program rates

The restrictor temperature ramp rate required, to just offset increasing linear velocity caused by pressure ramps, can be predicted, as shown in Fig. 2. For the listed conditions, a temperature increase of 2°C is required to offset a pressure increase of 1 atm, and approximately linear temperature ramps compensate for linear pressure ramps. Constant linear velocity vs. pressure, however, was shown to be not enough to produce constant column efficiency over a run. Examining Fig. 1 shows that at least over narrow ranges of pressure, lines with negative slopes can be drawn, allowing some decrease in linear velocity with increasing pressure.

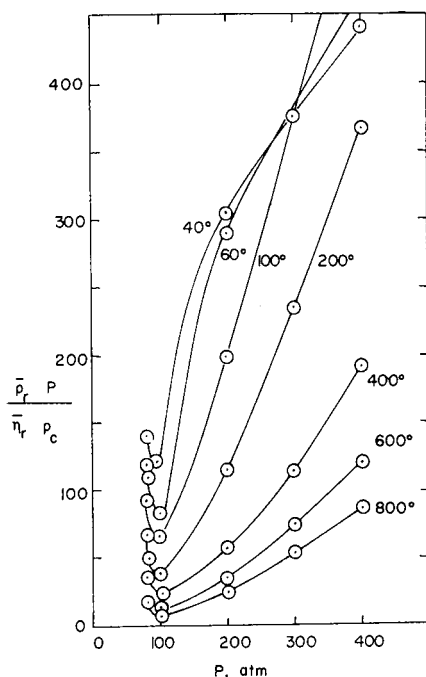


Fig. 1. Predictions of column linear velocity using the modifier Poiseuille equation. Term on left axis is proportional to column linear velocity. Numbers next to curves indicate restrictor temperature. Column temperature assumed to be 40°C. $\bar{\rho}_r$ and $\bar{\eta}_r$ are average density and viscosity in the restrictor, ρ_c is column density and P is restrictor inlet pressure.

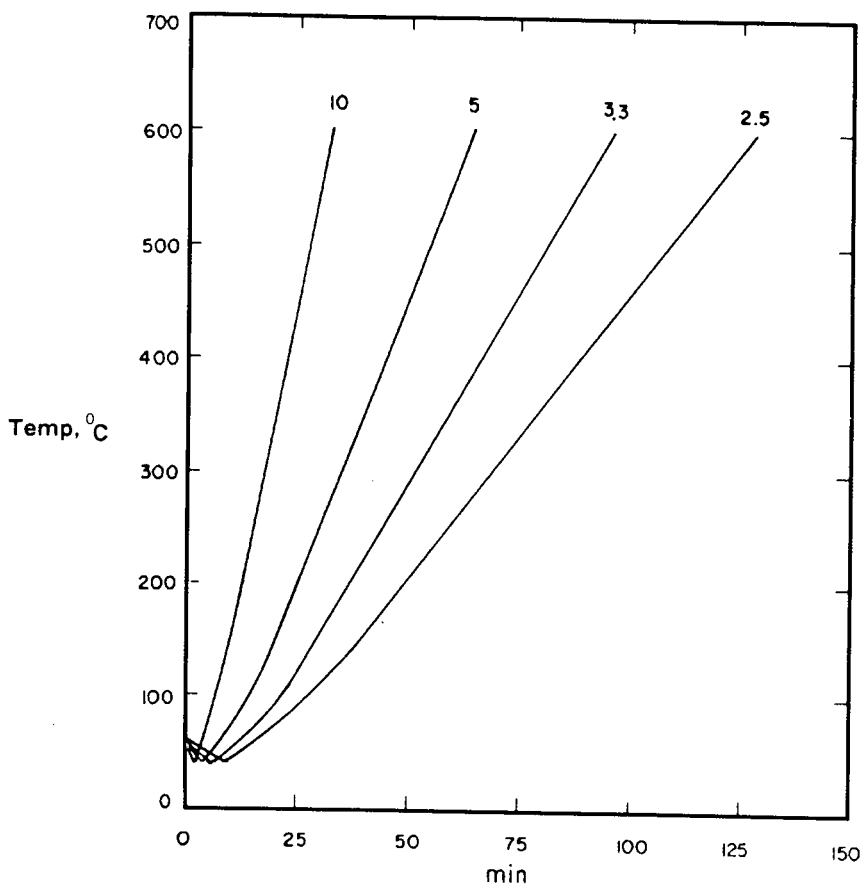


Fig. 2. Temperature (T) vs. time profile to produce constant column linear velocity during pressure programming using a linear (laminar flow) restrictor. Numbers next to curves are pressure program rates in atm/min starting from 80 atm and a column temperature and initial restrictor temperature of 40°C.

Turbulent-flow restrictors

It has been shown that turbulent-flow devices produce smaller increases in linear velocity vs. pressure than laminar-flow devices⁷. Mass flow still decreases with increasing restrictor temperature as in laminar-flow devices. This combination suggests turbulent-flow restrictors might give a wider range of linear velocity control than laminar-flow devices. Unfortunately, at the mass flows and pressures employed in capillary SFC, no restrictor type produces true turbulent flow. Integral restrictors¹⁹ which have steep internal tapers (decreasing from 50 μm to less than 1 μm I.D. within less than 1 mm) leading to a pinhole opening and other pinhole types, however, produce nearly turbulent flow. Turbulent-flow calculations using Lapple's method as well as a detailed discussion of turbulent-flow restrictor modeling have been previously described¹.

Calculations of column linear velocity resulting from turbulent restrictor mass flow vs. pressure for an arbitrarily chosen restrictor size of $2 \times 100 \mu\text{m}$ are presented in Fig. 3. The general shape of the curves are similar to those for the laminar-flow

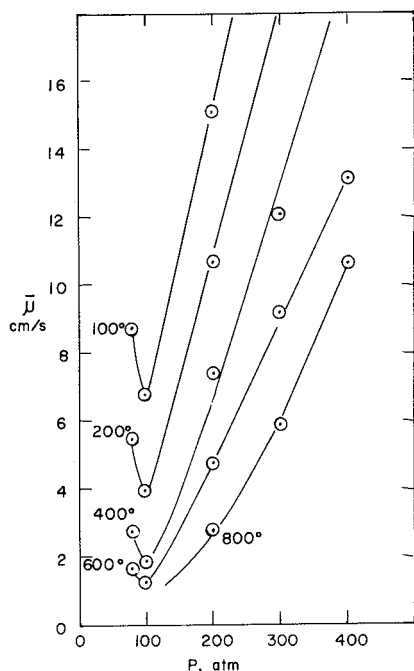


Fig. 3. Predicted column linear velocity using Lapple's method. Assumed column temperature is 40°C. Numbers next to curves are restrictor temperatures.

restrictor in Fig. 1 except the curves tend to exhibit less increase in linear velocity with increasing pressure, as expected. Constant linear velocity conditions could be represented with horizontal lines.

Heated-zone design

Very short restrictors are usually assumed to operate adiabatically. However, at low pressures and flow-rates, heat transfer may be adequate to maintain isothermal conditions. As pressure and mass flow increase, heat is more rapidly carried away by the expanding fluid and the device may operate more nearly adiabatically. The heated zones in most standard gas chromatographic detectors are not designed for use with such restrictors. There is typically a very large heated-zone I.D. compared to restrictor O.D., which tends to severely limit heat transfer. This is inadvertently compensated for by the heated-zone length being much too long. In very short restrictors the fluid upstream of the actual restriction must not be heated excessively compared to the column since this will drop fluid density and solute solubility in the restrictor below that in the column. For volatile solutes this is no problem since increased temperature will prevent such compounds from being lost. Some compounds, however, are thermally labile and their breakdown products are not volatile. In this case, tailing or loss of solutes can result.

EXPERIMENTAL

Instrumentation and chemicals

A Suprex Model 200 syringe pump was used to control the pressure of carbon dioxide supplied to the restrictors. Mass flows were measured with Sierra mass flow meters, and checked with bubble flow meters. The linear velocities reported in Figs. 4 and 5 are calculated values based on the measured mass flow-rates through integral (steep tapered) restrictors mounted in the heated zones described below. These mass flows together with the chosen column geometry, and column density vs. pressure and temperature^{20,21} relationships allows direct calculation of linear velocity. These values of column linear velocity have been randomly checked chromatographically. A Hewlett-Pachard Model 5890 gas chromatograph with two standard flame ionization detectors was used as the chromatographic oven and detector. Hexane was injected through an injection valve and "tee" arrangement. The "tee" was located in the oven, and the injection valve was a Valco Model 2WI4W.06 mounted on the gas chromatograph. The column was a methyl silicone 10 m $\times$ 50 μ m I.D. column from J.&W. Scientific. Constant-pressure chromatograms were collected with a Hewlett-Packard Model 3396 recording integrator. Hexane solvent peaks were assumed to be unretained and therefore a direct measure of the column linear velocity. This chromatographic set-up was not used throughout the experiment because the heated zone located at the base of the flame ionization detector was of conventional design, had inappropriate geometry, and a maximum temperature of 400°C.

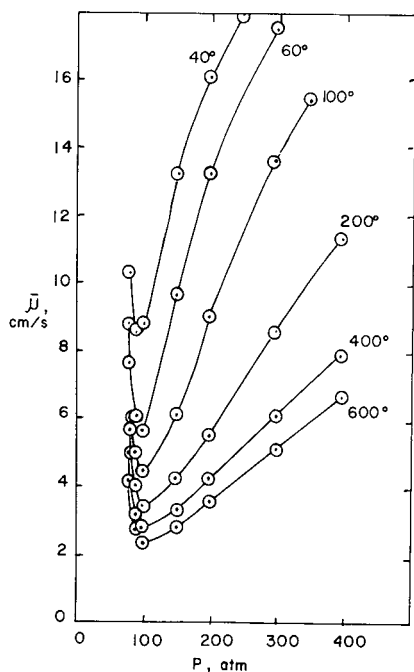


Fig. 4. Calculated column linear velocity from measured mass flows at different restrictor temperatures. Assumed column temperature is 40°C. Numbers next to curves are restrictor temperatures.

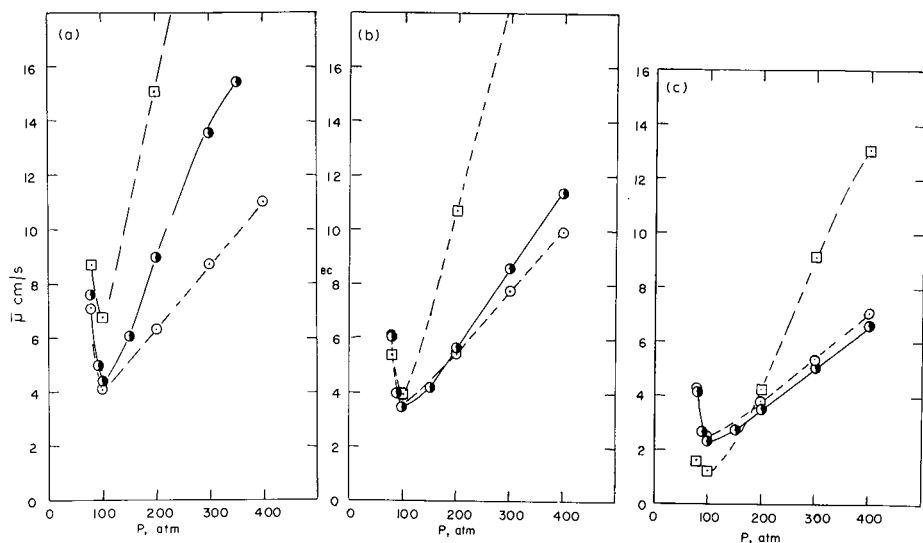


Fig. 5. Comparison of measured to predicted linear velocities. Column temperature is 40°C. $\square$ = Predicted results from Poiseuille model; $\circ$ = predicted results from Lapple's method; $\bullet$ = results from measured mass flows. Restrictor temperature: (a) 100°C; (b) 200°C; and (c) 600°C.

Carbon dioxide was SFC grade supplied in aluminum cylinders from Scott Specialty Gasses. "Integral" (steep tapered) restrictors were purchased from J.&W. Scientific. It was recognized that decreasing wall thickness would minimize heat transfer problems but problems in fabrication and the fragility of thin-wall restrictors with short taper lengths made the thick-wall material more attractive. Linear restrictors were pieces of small I.D. tubing (5 and 10 μm) purchased from SGE.

Heated zones were constructed of 316 stainless steel. Zones consisted of up to three stackable units, each approximately 1 cm thick and equipped with its own heater and temperature sensor. Units could be run simultaneously or individually to determine the minimum length of heated zone required for adequate heat transfer. Several versions of the inner diameter of the heated zones have been tried. All produce intimate contact between the restrictor outer diameter and the zone block, without an air gap.

RESULTS AND DISCUSSION

Heated-zone lengths

All heated-zone lengths gave the same mass flow at the same temperature, even at 600°C. This suggests that zones can be made less than 1 cm long and still allow adequate heat transfer while not losing solutes to the wall of the restrictor.

Integral restrictor mass flows

The mass flow of carbon dioxide through an integral restrictor (steep tapered) was measured as a function of both pressure and temperature, and the results are presented in Table I. This information was then used to calculate mobile phase linear

TABLE I

MASS FLOW ($\cdot 10^5$ g/s) THROUGH AN INTEGRAL RESTRICTOR AS A FUNCTION OF PRESSURE AND TEMPERATURE

T ($^{\circ}\text{C}$)	P (atm)						
	80	90	100	150	200	300	400
40	5.70	8.13	10.74	20.57	26.57	31.24 ^a	—
60	4.85	5.67	6.73	14.95	21.84	31.33	—
80	4.52	5.16	5.92	11.41	17.65	27.76	—
100	4.22	4.79	5.40	9.50	14.74	24.27	28.57 ^b
200	3.37	3.79	4.25	6.61	9.40	15.35	21.54
300	2.70	3.00	3.34	5.16	6.98	10.86	14.86
400	2.31	2.55	2.82	4.31	5.85	9.10	12.53

^a Collected at 250 $^{\circ}\text{C}$.

^b Collected at 350 $^{\circ}\text{C}$.

velocity expected at different column pressures and temperatures, an example of which is shown in Fig. 4.

These empirical results were compared to calculated values for both very laminar- (Fig. 1) and nearly turbulent-flow (Fig. 3) restrictors. The results appear remarkably like the calculated values in general shape and trends but more closely resemble the turbulent-flow calculations. Comparisons, as in Fig. 5a-c, indicate the degree of fit between the calculated and measured results at different restrictor

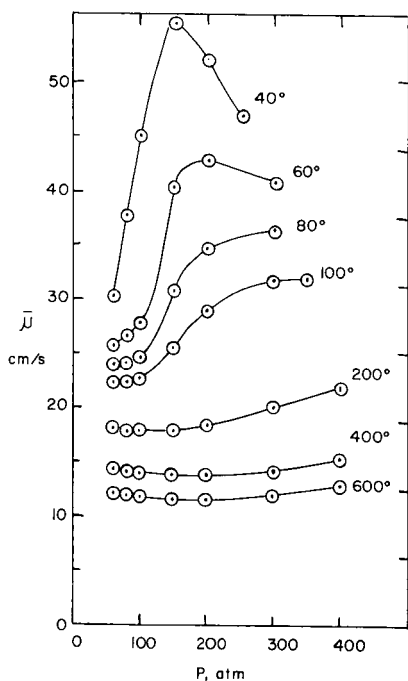


Fig. 6. Mass flow results of Fig. 4 replotted as linear velocities with column temperature increased to 200 $^{\circ}\text{C}$.

temperatures. The real restrictor acted less like a turbulent-flow device than expected. The discrepancies could arise from several sources. The restrictor could have a larger aspect ratio than estimated for the calculations or heat transfer may not be adequate to keep the restrictor at the set temperature (although the latter seems unlikely since both short and long heated zones gave the same result). We were somewhat surprised to find a more gradual than expected internal diameter taper in the commercially available integral restrictors when viewed under a microscope. This suggested that the discrepancies may be due to use of too small a restrictor aspect ratio in the calculations.

Real capillary columns are seldom operated at 40°C. The same mass-flow measurements used to calculate the linear velocities in Fig. 4, for a column temperature of 40°C, would yield the linear velocities in Fig. 6, if the column temperature were 200°C. This temperature is actually rather high for capillary SFC and represents an opposite extreme of the performance envelope. The results in Fig. 6 indicate that contrary to normal operation, where linear velocity increases from modest to large amounts during pressure-programmed runs, linear velocity can be decreased by at least a factor of three over the full pressure range and actually by larger amounts over narrower pressure ranges if restrictor temperature is programmed to counter the effects of increasing column pressure. Most chromatograms are collected using column conditions between the extremes represented by Figs. 4 and 6.

CONCLUSIONS

Restrictor flow can be accurately modeled and predictably controlled by changing restrictor temperature. The control concept employs changes in the fluid properties to throttle flow through a fixed restrictor. Since control is accomplished independent of column conditions it can be viewed as a means to independently control solute solubility through column density changes, and column efficiency through linear velocity control.

Work is progressing in interfacing a short, high-temperature heated zone into the base of a flame ionization detector with a programmable temperature controller so that chromatograms may be collected to determine the extent of expected efficiency improvements during pressure programming.

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COLUMN PRESSURE DROP IN CAPILLARY SUPERCRITICAL FLUID CHROMATOGRAPHY

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SUMMARY

Some relationships have been derived describing the pressure profile along a capillary column in supercritical fluid chromatography (SFC) in terms of experimental parameters. The calculated results for carbon dioxide as mobile phase suggest that, under usual operating conditions in capillary SFC, the pressure profile is nearly linear. The importance of the secondary effects detracting from applicability of the Hagen–Poiseuille equation is also discussed and shown to be negligible in most practical situations.

INTRODUCTION

In certain applications of capillary-column supercritical fluid chromatography (SFC) an accurate knowledge of the pressure drop across the column is necessary. For instance, when the diffusion coefficients of various solutes in dense fluids are measured with the chromatographic band-broadening technique^{1–5} the pressure drop is required to assess the portion of band broadening that results from expansion of the mobile phase along the column. In analytical SFC, the pressure drop is needed when the effects are investigated of the experimental parameters on the column efficiency and resolving power⁶. Moreover, a theoretical interpretation of SFC retention data is greatly simplified if the pressure drop across the column can safely be neglected^{7,8}.

Theoretical studies of the column pressure drop in SFC have focussed on packed columns^{9,10}. However, as pointed out by Schoenmakers *et al.*⁹, the pressure drops across typical packed and capillary SFC columns may be comparable under the usual experimental conditions. In packed-column SFC the pressure drop may actually become an operating parameter since both the column inlet and outlet pressures can be measured and controlled. In capillary-column SFC, however, flame-based detectors are frequently used. With these detectors the column effluent must be decompressed prior to detection. A pressure sensor between the column and the detector would inevitably degrade the system's efficiency by causing a large extra-column contribution to band broadening. For these reasons, a brief theoretical analysis of the pressure profile along a capillary column appears worthwhile.

In the following sections, relationships are derived for the pressure profile along

the SFC capillary column in terms of experimental parameters. A few examples are shown of the calculated pressure drops in capillary-column SFC with carbon dioxide as the mobile phase. Finally, the significance of various effects detracting from the applicability of the Hagen–Poiseuille equation is also discussed.

THEORETICAL

Starting from the Hagen–Poiseuille equation, the mass flow-rate of the mobile phase through the column cross-section positioned at a distance z from the column inlet may be written as:

$$\dot{m} = -[\pi r^4 \rho / (8\eta)] (dP/dz) \quad (1)$$

The meaning of the symbols is explained at the end of this article. The density and the dynamic viscosity of the mobile phase are those at the local pressure. The flow is assumed to be isothermal. The minus sign in eqn. 1 takes account of the fact that the pressure decreases in the direction of flow. Unlike the volumetric flow-rate or the mean linear flow velocity, the mass flow-rate in eqn. 1 is invariant with respect to temperature and pressure. This fact may be useful when there is no suitable substance to mark the dead retention time¹¹.

It follows from eqn. 1 that the local pressure, P , at a distance z from the column inlet satisfies the relationship:

$$\int_P^{P_i} v^{-1} dP = 8\dot{m}z/(\pi r^4) \quad (2)$$

To evaluate the integral in eqn. 2, the pressure dependence of v^{-1} must be known. It should be noted that in SFC both η and ρ vary with pressure, whereas in gas chromatography the pressure dependence of η may be neglected¹². Generally, v^{-1} may be expanded into a Taylor series with respect to pressure. As the pressure drop in capillary SFC is usually small compared to P_i , the series may be truncated after the second term:

$$v^{-1} = v_i^{-1} + (P - P_i)\kappa \quad (3)$$

Combination of eqns. 2 and 3 yields:

$$v_i^{-1}(P_i - P) - \kappa (P_i - P)^2/2 = 8\dot{m}z/(\pi r^4) \quad (4)$$

Eqn. 4 clearly shows the effect of κ upon the overall appearance of the pressure profile along the column. If $\kappa > 0$, the dependence of P on z is convex in the upward direction, *i.e.*, the pressure at half the column length is greater than the arithmetic mean of the inlet and outlet pressures. This is the case in gas chromatography. If $\kappa < 0$, the dependence is concave, and when $\kappa = 0$ a linear pressure profile results. Eqn. 4 may be solved to give:

$$P = P_i - \kappa^{-1} v_i^{-1} + \kappa^{-1} [v_i^{-2} - 16\kappa \dot{m}z/(\pi r^4)]^{\frac{1}{2}} \quad (5)$$

Finally, the mean column pressure may be obtained by averaging eqn. 5 over the column length, L :

$$\bar{P} = P_i - \kappa^{-1} v_i^{-1} + \frac{\pi r^4}{24\kappa^2 \dot{m}L} \left[v_i^{-3} - \left(v_i^{-2} - \frac{16\kappa \dot{m}L}{\pi r^4} \right)^{3/2} \right] \quad (6)$$

The column-outlet pressure, P_0 , may be calculated by substituting L for z in eqn. 5. Although eqns. 5 and 6 fail for strictly linear pressure profiles ($\kappa = 0$), an application of the l'Hôpital rule shows that they converge to their correct counterparts in the limit of $\kappa \rightarrow 0$ [$P = P_i - 8\dot{m}Lv_i/(\pi r^4)$ and $\bar{P} = P_i - 4\dot{m}Lv_i/(\pi r^4)$, respectively].

It follows from the above consideration that the pressure dependence of v^{-1} plays a key rôle in determining the pressure profile along the column. Unfortunately, no explicit equation can be given to express v^{-1} as a function of pressure throughout the temperature and pressure range pertinent to SFC. This is because, over such a broad range of fluid states, neither density nor dynamic viscosity can be related to temperature and pressure by a single explicit expression with an accuracy sufficient for the present purpose. For this reason, the assessment of the pressure dependence of v^{-1} should be based upon the experimental data for a given fluid, at least when η is concerned.

As an example, Fig. 1 shows v^{-1} as a function of pressure for carbon dioxide at three different temperatures. The dynamic viscosity values used were those recommended by Stephan and Lucas¹³. Their tabulated data were smoothed graphically with the Reichenberg correlation¹⁴ serving as an approximate guide. The densities were calculated from the corresponding-states correlation of Lee and Kesler¹⁵. The isotherms shown in Fig. 1 were drawn through the data points calculated at a pressure step of 5 bar. At any particular pressure, the respective value of κ to be used in eqns. 5 and 6 was approximated by the slope of the straight line drawn to the nearest neighbouring point on the lower-pressure side. The 315-K isotherm in Fig. 1 displays an enhancement of v^{-1} at pressures somewhat above the critical. The enhancement probably becomes more apparent as the critical temperature is approached; however, a verification of this conjecture seems to be precluded by the uncertainties regarding the dynamic viscosity of carbon dioxide in the critical region¹³. The uncertainty of the data plotted in Fig. 1 may be estimated to be $\pm 8\%$; this figure comes from the combined uncertainties of η and ρ , ± 5 and $\pm 3\%$, respectively.

For certain ranges of temperature and pressure, higher-order terms of the Taylor series should be included in eqn. 3 to account for the curvature of the isotherm showing v^{-1} as a function of pressure (*cf.*, the 315-K isotherm in Fig. 1 at pressures close to 100 bar). In such an instance, higher powers of the difference ($P_i - P$) would appear in eqn. 4, and eqn. 4 would have to be solved numerically to yield the pressure profile along the column. For such a procedure, however, the respective higher-order derivatives of v^{-1} with respect to pressure would be required. At present, the derivatives cannot be evaluated because of limited precision of the viscosity data.

It is apparent from Fig. 1 that κ may acquire both positive and negative values

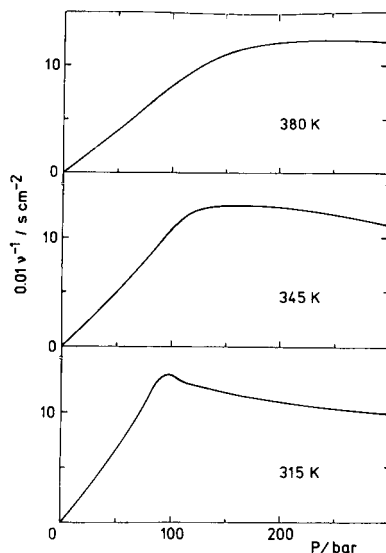


Fig. 1. Reciprocal kinematic viscosity of carbon dioxide as a function of pressure and temperature.

within the temperature and pressure ranges pertinent to SFC. Hence, in principle, both positive and negative departures from a linear pressure profile may occur in capillary-column SFC with carbon dioxide as the mobile phase, although the deviations are very small (see below). This situation differs markedly from that in low-pressure gas chromatography where only positive deviations from linear pressure profiles are possible.

RESULTS AND DISCUSSION

In Table I a few examples are listed of the calculated pressure drops for three capillary columns within the temperature and pressure ranges pertinent to SFC with carbon dioxide as the mobile phase. It follows from the $\Delta \bar{P}$ entries in Table I that, under operating conditions typical of isothermal SFC with carbon dioxide as the mobile phase, the pressure profile along a capillary column may be considered to be linear.

The considerations of the preceding section assumed that the flow is isothermal, and that the hydrodynamic behaviour of a capillary SFC column conforms strictly to the Hagen-Poiseuille equation. In practice, however, there are several effects that may detract from the validity of these assumptions for a real capillary column, *viz.*, (i) departures from the isothermal flow regime, (ii) departures from the laminar flow regime, (iii) column-end effects and (iv) distortion of the parabolic velocity profile within the column by coiling-induced secondary flow. Below, the significance is discussed of each of these effects in capillary-column SFC with carbon dioxide as the mobile phase. The treatment is far from being exact. Rather, the simple numerical examples given below are intended to illustrate the *maximum* impact of the particular effect.

TABLE I

CALCULATED PRESSURE DROPS AND THERMAL EFFECTS IN SEVERAL CAPILLARY SFC COLUMNS WITH CARBON DIOXIDE AS THE MOBILE PHASE

$T(K)$	$P_i(\text{bar})$	$\bar{u}_i(\text{cm/s})$	$\Delta P(\text{bar})$	$10^3 \Delta \bar{P}(\text{bar})$	$\dot{q}_d/\dot{m}(\text{J/g})$	$\dot{q}_e/\dot{m}(\text{J/g})$	
Column 20 m $\times$ 50 μm I.D.							
315	75	2	1.11	1.9	0.48	-2.94	
		5	2.81	12.3	1.20	-7.45	
	150	2	3.32	-1.7	0.43	-0.95	
		5	8.27	-9.9	1.08	-2.36	
	345	75	2	1.03	1.2	0.65	-1.16
			5	2.59	8.0	1.63	-2.92
	150	2	1.90	0	0.40	-1.84	
		5	4.76	0.2	0.99	-4.61	
	240	2	2.99	-1.0	0.42	-0.67	
		5	7.45	-5.5	1.05	-1.68	
	380	75	2	1.07	1.3	0.84	-0.78
			5	2.69	8.6	2.11	-1.96
150		2	1.43	0.8	0.46	-1.11	
		5	3.58	3.9	1.15	-2.79	
240		2	2.19	0	0.41	-0.84	
		5	5.47	0	1.03	-2.09	
Column 10 m $\times$ 25 μm I.D.							
315	150	2	6.63	-6.4	0.86	-1.89	
		5	16.43	-38.7	2.16	-4.70	
380	75	2	2.15	5.4	1.68	-1.57	
		5	5.50	36.6	4.21	-4.01	
Column 1 m $\times$ 10 μm I.D.							
315	150	2	4.15	-2.5	0.54	-1.19	
		5	10.32	-15.3	1.35	-2.95	
380	75	2	1.33	2.1	1.05	-0.97	
		5	3.38	13.7	2.63	-2.47	

(i) Thermal effects

When a fluid moves down a capillary tube two effects tend to change the temperature of the fluid. The first is the dissipation of the mechanical energy due to the viscosity of the fluid. To a first approximation, the heat formed by viscous dissipation per unit time under laminar flow conditions may be expressed by:

$$\dot{q}_d = 8v_i L \bar{u}_i \dot{m} / r^2 \quad (7)$$

In the sixth column of Table I, several values are listed of the ratio $\dot{q}_d/\dot{m}$ in capillary-column SFC with carbon dioxide as the mobile phase. The second thermal effect is the temperature change due to expansion of the fluid along the column. In the extreme case of a thermally insulated column, the expansion would be isenthalpic, and the rate of the internal energy change due to expansion could be approximated as:

$$\dot{q}_e = -\mu C_p \Delta P \dot{m} / M \quad (8)$$

The minus sign is to indicate that the fluid cools down when μ is positive, which is the case in supercritical fluids. The values of the ratio $\dot{q}_e/\dot{m}$ are shown in the seventh column of Table I. The values of the product μC_p were derived from the Lee and Kesler correlation¹⁵. An inspection of the $\dot{q}_d$ and $\dot{q}_e$ entries in Table I reveals that the two thermal effects tend to offset each other but that the cooling due to expansion prevails in most cases. Accordingly, in capillary-column SFC with carbon dioxide as the mobile phase, heat has to be supplied to the column to keep it at a constant temperature. It may be shown that, for a given fluid at a given temperature and pressure, the ratio $\dot{q}_e/\dot{q}_d$ is nearly independent of the column geometry and the mean linear flow velocity, at least under laminar flow conditions. However, the absolute amounts of heat involved are minute since very small mass flow-rates are used in capillary-column SFC (typically, 10^{-6} to 10^{-4} g/s for carbon dioxide as the mobile phase). Hence, in capillary-column SFC with carbon dioxide as the mobile phase, thermal effects appear to be insignificant.

(ii) *Departures from the laminar flow regime*

The Reynolds number for the flow in a capillary SFC column may be written as:

$$\text{Re} = ru_{\max}v^{-1} \quad (9)$$

Setting $r = 100 \mu\text{m}$, $u_{\max} = 10 \text{ cm/s}$ and $v^{-1} = 1500 \text{ s/cm}^2$ (cf., Fig. 1), the maximum value of the Reynolds number in capillary-column SFC with carbon dioxide as the mobile phase is 150. This is still well below the critical value ($\text{Re} \approx 2200$) at which the onset of turbulence starts. Hence, the departures from laminar flow appear to be negligible in capillary-column SFC. In packed-column SFC the effects of turbulence are more significant¹⁶.

(iii) *Column-end effects*

In the derivation of the Hagen–Poiseuille equation the parabolic velocity profile is supposed to be developed throughout the length of the capillary. At the inlet of any real capillary column, however, there is a transient region within which the fluid is accelerated and the velocity pattern developed. At the outlet end of the column the opposite occurs. According to the treatment quoted by Dushman and Lafferty¹⁷ for an incompressible fluid, the column pressure drop corrected for the end effects may be written as:

$$\Delta P = 8\eta L\bar{u}_i/r^2 + 1.14\rho\bar{u}_i^2 \quad (10)$$

Assuming that $\rho = 0.8 \text{ g/cm}^3$ and $\bar{u}_i = 5 \text{ cm/s}$, the second term on the right-hand side of eqn. 10 amounts to $2.3 \cdot 10^{-5} \text{ bar}$, which is negligible compared to ΔP in Table I.

(iv) *Effect of column coiling*

The Hagen–Poiseuille equation applies to a straight tube. Since a capillary column must obviously be coiled, the impact of coiling on the applicability of the equation should be assessed. In a coiled column, centrifugal forces cause the streamline of maximum velocity to be pushed out in the direction of increasing distance from the coil centre. Thus, a double helical secondary flow pattern is developed in the column,

TABLE II

EFFECT OF COLUMN COILING IN CAPILLARY SFC WITH CARBON DIOXIDE AS THE MOBILE PHASE

 $u_{\max} = 10 \text{ cm/s}$, $v^{-1} = 1500 \text{ s/cm}^2$, $R = 7.5 \text{ cm}$.

$r \text{ (}\mu\text{m)}$	λ	δ	α	β
25	3000	$1.7 \cdot 10^{-4}$	$1.7 \cdot 10^{-4}$	1
100	750	$1.9 \cdot 10^{-2}$	$2.8 \cdot 10^{-3}$	0.9996
160	469	$8.0 \cdot 10^{-2}$	$7.1 \cdot 10^{-3}$	0.9943

and, consequently, the parabolic velocity profile is distorted. Under the flow conditions typical of capillary-column SFC, the velocity profile in the coiled column may be described by the expressions of Dean and Topakoglu^{18,19}. These complex expressions will not be repeated here. Instead, the resulting typical values of several quantities characterizing the secondary flow in capillary-column SFC are listed in Table II. It is seen that, under the usual conditions in capillary-column SFC with carbon dioxide as the mobile phase, the effect of coiling upon the applicability of the Hagen–Poiseuille equation is not significant. However, this conclusion no longer applies when the radius ratio, λ , is minimized intentionally to produce a gain in column efficiency²⁰.

CONCLUSION

The model developed in this work makes it possible to calculate the pressure profile along a capillary column in SFC. It appears that, under operating conditions typical of isothermal SFC with carbon dioxide as the mobile phase, the deviations of the pressure profile from linearity are negligible. Simple calculations suggest that, under the same conditions, possible secondary effects (i)–(iv) do not introduce a significant error into the column pressure drop calculated from the Hagen–Poiseuille equation.

SYMBOLS

- C_p Isobaric molar heat capacity of the mobile phase at the column-oven temperature and the column-inlet pressure
 L Column length
 $\dot{m}$ Mass flow-rate of the mobile phase through the column
 M Molar mass of the mobile phase
 P Pressure
 P_i Column-inlet pressure
 P_o Column-outlet pressure
 ΔP Pressure drop across the column, $P_i - P_o$
 $\bar{P}$ Mean pressure in the column
 $\Delta \bar{P}$ Deviation of the mean pressure in the column from the arithmetic mean of the inlet and outlet pressures, $\bar{P} - (P_i + P_o)/2$

$\dot{q}_d$	Rate of heat formation due to viscous dissipation of the mechanical energy of the mobile phase in the column
$\dot{q}_e$	Rate of the internal energy change due to expansion of the mobile phase along the column
r	Column radius
R	Radius of the column coil
Re	Reynolds number
$u_{\max}$	Maximum linear velocity of the mobile phase at a given column cross-section
$\bar{u}_i$	Mean cross-sectional linear velocity of the mobile phase at the column inlet
z	Distance from the column inlet
α	Ratio of the maximum radial velocity to the maximum axial velocity of the mobile phase in the column
β	Ratio of the mass flow-rate through the coiled column to the mass flow-rate through the corresponding straight column with the same pressure drop
δ	Maximum relative deviation of the axial velocity from that corresponding to the parabolic velocity profile
η	Dynamic viscosity of the mobile phase
κ	Pressure derivative of v^{-1} at the column-oven temperature and the column-inlet pressure
λ	Aspect ratio, R/r
μ	Joule-Thomson coefficient of the mobile phase at the column-oven temperature and the column-inlet pressure
ν	Kinematic viscosity of the mobile phase
ν_i	Kinematic viscosity of the mobile phase at the column inlet
ρ	Density of the mobile phase

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SOLVENT STRENGTH AND SELECTIVITY PROPERTIES OF SUPERCRITICAL CARBON DIOXIDE RELATIVE TO LIQUID HEXANE

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SUMMARY

Non-polar, non-polar chromatography (chromatography with non-polar solvents and a non-polar stationary phase) was used to study the solvent properties of supercritical carbon dioxide. Six substituted benzenes were chosen as chromatographic test probes on C₁₈ reversed-phase columns to examine selectivity differences between hexane and supercritical carbon dioxide mobile phases. We have found that Lewis base properties of supercritical carbon dioxide give it selectivity properties not observed in hexane. Addition of 3.0% methanol modifier has only minor effects on the solvent strength and selectivity of supercritical carbon dioxide. Lewis acid–base interaction appears to be the primary mechanism for separation and elution order. By understanding Lewis acid–base pairing, hydrogen bonding and induced dipole interactions observed in non-polar, non-polar chromatography we can improve our understanding of chromatographic separations and industrial extractions using supercritical carbon dioxide.

INTRODUCTION

Supercritical carbon dioxide has unique properties as a non-toxic yet effective chromatographic mobile phase and extraction fluid. Applications in which supercritical carbon dioxide has been used include, the extraction of petroleum products, synthetic oils, foods, flavors, fragrances, drugs, biologically active compounds and the removal of toxic organics from environmental matrices^{1–4}. Analytical separations utilizing supercritical carbon dioxide include packed, capillary and preparative-scale chromatography^{5–7}. The addition of “modifiers” or “entrainers” to supercritical carbon dioxide has been used to improve its solubility and selectivity properties⁸. Although supercritical carbon dioxide has been used for a number of decades in the separation sciences, its selectivity properties and the mechanism of modifier action are not well understood. The field of supercritical fluid chromatography (SFC) is expanding rapidly and the increasing interest in supercritical fluid extraction (SFE) is

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out-pacing our understanding of supercritical fluid solubility and selectivity properties. This lack of knowledge prevents the accurate prediction of the optimal supercritical solvent systems for industrial and analytical applications.

The better our understanding is of the system we are studying the better our ability to predict the outcome of a given extraction or separation. By knowing the chemical composition of the matrix (stationary phase) and the chemical structure of the products (analytes) the appropriate supercritical solvent system may be chosen. In many cases carbon dioxide with appropriate modifiers can be used to achieve the desired separation. Pure supercritical carbon dioxide is a poor extractor of caffeine from coffee beans or nicotine from tobacco unless a small percentage of a polar modifier such as water or methanol is present. On the other hand the extraction of fatty acids from fish tissue, which is composed primarily of collagen, occurs rapidly with pure supercritical carbon dioxide.

Several researchers have compared the selectivity and solubility properties of supercritical carbon dioxide to hexane. Randall⁹ used an experimental design similar to that described by Snyder to characterize the solvent properties of supercritical carbon dioxide. Randall⁹ concluded that the solvent power of supercritical carbon dioxide was similar to hexane with some strong dipole selectivity tendencies. Mourier *et al.*⁷ examined four different stationary phases using supercritical carbon dioxide with various polar modifiers. Mourier *et al.*⁷ concluded that supercritical carbon dioxide was a non-polar solvent similar to hexane but its selectivity could be changed by modifier addition. Levy and Ritchey¹² investigated modifier effects on carbon dioxide when eluting polyaromatic hydrocarbons from C₈ and diol packed columns. The modifiers studied were methanol, 2-methoxyethanol, 1-propanol, tetrahydrofuran, dimethylsulfoxide, acetonitrile, sulfurhexafluoride and fluorotrichloromethane. They concluded that polar modifiers improved both the solvent strength and selectivity of supercritical carbon dioxide, however any selectivity changes observed were subtle¹⁰. Wright *et al.*¹³ investigated the solvent strength and selectivity of supercritical carbon dioxide with and without 2.5% methanol modifier on a 5% phenyl-poly(methylphenylsiloxane) capillary column. A polarity test mix was used to follow changes. Wright *et al.*¹³ saw little effect on the solvent power or selectivity of the supercritical carbon dioxide with the addition of modifier. Deviation from previously mentioned packed column work was attributed to the low number of active sites on the capillary column. Recently, Morin *et al.*¹⁴ reported both non-polar-non-polar partitioning and π - π interactions when comparing the separations of several substituted benzenes on a PRP-1 column. This paper will undertake a systematic investigation of the chromatographic separation of substituted benzenes on silica- and polystyrene-divinylbenzene (PSDVB)-based C₁₈ columns. The elution order and chromatographic peak shape observed when using analytes with various functional groups will be used to improve our understanding of supercritical carbon dioxide solubility and selectivity properties relative to liquid hexane.

EXPERIMENTAL

Apparatus

The high-performance liquid chromatographic (HPLC) system consisted of two Waters 6000 dual piston pumps, a Wisp 7108 autosampler, a Waters 440 vari-

able, dual-wavelength UV absorbance detector and a Waters system controller/data module. One pump was used to supply hexane and the other supplied methanol. The chromatographic conditions are found in Table I.

The SFC system is a Hewlett Packard 1084 liquid chromatograph which has been modified at the factory for supercritical-fluid operation. The modifications included heat exchangers on the pump heads to maintain the carbon dioxide as a liquid for proper compression, a high-pressure UV cell, a thermostatted backpressure regulator, an inlet pressure gauge and an exit pressure gauge. Two single-piston diaphragm pumps are at the heart of the system and a variable, dual-wavelength UV detector is placed prior to the back pressure regulator. One pump is used to pump carbon dioxide from a diptube cylinder and the second is used to pump liquid methanol. During 100% carbon dioxide operation both pumps are used to pump the carbon dioxide to prevent leakage of trace amounts of methanol into the system. The chromatographic conditions can be found in Table I.

Chromatographic columns

Separate columns from the same lot were used for carbon dioxide and for hexane to eliminate any confusion which may arise due to permanent phase modification by either solvent. The silica-based, high-coverage C₁₈ columns were purchased from Analytical Sciences, (Santa Clara, CA, U.S.A.). The PSDVB-based ACT-1 C₁₈ columns were purchased from Interaction Chemicals (Mountain View, CA, U.S.A.).

Chemicals and reagents

Dip tube, high purity (99.998%) carbon dioxide was purchased from Matheson (Seacaucus, NJ, U.S.A.). Hexane, methanol and benzene were all distilled-in-glass

TABLE I
CHROMATOGRAPHIC CONDITIONS

	HPLC	SFC
Mobile phase	Hexane	Supercritical CO ₂
Modifier	Methanol	Methanol
Modifier concentration	3.0% (v/v)	3.0% (v/v)
Pressure	1000–2000 p.s.i.g.	2400 p.s.i.g.
Temperature	24°C	45°C
Flow-rate	1.5 ml/min	1.5 ml/min
Column A	ASI C ₁₈	ASI C ₁₈
Material	Silica	Silica
Dimensions	30 cm × 4.6 mm I.D.	30 cm × 4.6 mm I.D.
Particle size	5 μm d _p	5 μm d _p
Plates	7900	7850
Column B	ACT-1 C ₁₈	ACT-1 C ₁₈
Material	PSDVB	PSDVB
Dimensions	17.5 cm × 4.6 mm I.D.	17.5 cm × 4.6 mm I.D.
Particle size	10 μm	10 μm
Plates	6800	6700
Wavelength	254 nm	254 nm

high-purity-grade solvents purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). The substituted benzenes were supplied by Aldrich (Milwaukee, WI, U.S.A.).

Experimental design

A set of eight experiments was chosen to limit the number of variables and to provide a solid base on which to build our understanding of supercritical carbon dioxide solvent properties. All researchers studying supercritical carbon dioxide have found it to be quite comparable to hexane. Since the solvent properties of hexane are well understood, hexane was used as a benchmark. Most matrices which are of interest for supercritical extraction are hydrocarbon based, therefore a C_{18} stationary phase was chosen for the test chromatographic column. Since C_{18} , hexane and supercritical carbon dioxide are all non-polar, subtle changes in solvent strength and selectivity will be more readily noticed. A PSDVB-based C_{18} column was also used to help distinguish the effects of partitioning from adsorption on silica-based columns.

Substituted benzenes were chosen as analyte test probes such that they would differ from each other only by their functional group. The following analytes were selected; toluene (non-polar hydrocarbon), aniline (base), benzoic acid (acid), phenol (hydrogen bonding), nitrobenzene (strong dipole), chlorobenzene (electrophilic) and benzene (non-substituted benchmark). To avoid solute interaction each analyte was run separately. Methanol was selected as the polar modifier, since its use has been referred to extensively in the literature^{9,12}. Other workers have shown that the most dramatic changes in solvent power occur due to coverage of active sites on the chromatographic column at modifier concentrations up to 1%⁸. A modifier concentration of 3% was chosen to ensure that the coverage of active sites was complete and not a variable in these studies. Each test probe would be analyzed under eight different conditions, hexane mobile phase on a silica-based C_{18} column, hexane on PSDVB C_{18} , supercritical carbon dioxide on silica C_{18} , supercritical carbon dioxide on PSDVB C_{18} , hexane-3% methanol on silica C_{18} , hexane-3% methanol on PSDVB C_{18} , supercritical carbon dioxide-3% methanol on silica C_{18} , and supercritical carbon dioxide-3% methanol on PSDVB C_{18} .

Procedure

Both the SFC and the HPLC conditions were as identical as possible with the exception of the mobile phase being used (Table 7). Injections of each analyte were made in triplicate to determine accurate retention times. After retention times for all components had been determined for the single solvent systems, 3.0% methanol modifier was added to each mobile phase and the experiments repeated. This procedure was carried out on both the silica- and polymer-based C_{18} columns.

For each set of chromatographic conditions, peak shape was noted with respect to tailing, fronting and sharpness. Retention times to the closest hundredths-of-a-minute were recorded but were rounded down to tenths-of-a-minute after averaging the three runs. Once the average retention times were compiled for each component, capacity factors were calculated according to $k' = (t_R - t_0)/t_0$ where k' is the capacity factor, t_R the retention time of the component and t_0 the retention time of an unretained component.

The t_0 for HPLC was calculated by dividing the column volume by the mobile

phase flow-rate. For packed-column SFC, this calculation does not hold since the mobile phase density and therefore volume changes with temperature and pressure. Therefore, the t_0 was determined experimentally by injecting C₄, C₃, C₂ and C₁ hydrocarbons. The packed-column SFC system was fitted with a flame ionization detector for this set of experiments. The retention times of the C₁–C₄ hydrocarbons were then plotted against carbon number and the C₀ retention time was used as t_0 .

RESULTS AND DISCUSSION

The t_0 capacity factor and peak shape results are summarized numerically in Table II. Peak shape and relative peak retention times are summarized graphically in Figs. 1 and 2. The figures are graphic representations of the peak shape of each component positioned on a time axis relative to the retention time of benzene. By plotting the retention time of substituted benzenes relative to benzene, the effect of each substituent can be clearly visualized. The retention of all analytes can be explained by Lewis acid–base pairing, hydrogen bonding, π electron interaction and induced dipoles. Table III lists the major forces affecting the retention of analytes in the chromatographic systems studies. The predominance of a particular force varies depending upon the chromatographic conditions. Lewis acid–base pairing can occur between solute and solvent, solute and modifier, solute and stationary phase, solvent and modifier, solute and stationary phase and modifier and stationary phase. The Lewis acid–base pair strength is primarily dependent on the electrostatic and covalent interactions between the acid and the base^{15,16}.

Solvent strength

The strength of a given solvent is related to its overall “polarity”, P' , which is often referred to as its chromatographic strength, solvent strength or solvating power. Snyder¹⁰ has characterized the three major types of solvent–solute interactions in liquid chromatography as proton acceptor, proton donor and strong dipole. The overall polarity, P' , of a solvent is approximately equal to the sum of these interactions and the proportion of these interactions determined the selectivity of the solvent:

$$P' = p' \text{ (proton acceptor)} + p' \text{ (proton donor)} + p' \text{ (strong dipole)}$$

The proton acceptor and proton donor interactions can be described more universally as electron pair donors and electron pair acceptors (Lewis bases and Lewis acids). Dipole interactions in non-polar, non-polar chromatography range in strength from weak induced dipoles and π electron interaction to stronger hydrogen bonding.

A way of comparing the relative solvent strength of two mobile phases is by comparing capacity factors (Table II). The greater the solvent strength of the mobile phase the smaller k' will be. When carbon dioxide is used as the mobile phase smaller k' values are obtained for most analytes. Thus carbon dioxide at 2400 p.s.i.g. and 45°C density ($d = 0.77$), has slightly greater solvent strength than liquid hexane. Unlike hexane, the solvent strength of supercritical carbon dioxide increases with density. This change is most dramatic near the critical point and is a parabolic function which passes through a maximum¹⁷.

TABLE II
ANALYTE CAPACITY FACTORS (k') UNDER VARIOUS CONDITIONS
T = Tail; t = slight tail; B = broad; S = sharp.

Compound	Silica C_{18}			PSDVB C_{18}				
	CO_2 ($t_0 = 1.3$ min)	CO_2 + methanol ($t_0 = 1.3$ min)	Hexane ($t_0 = 1.2$ min)	Hexane + methanol ($t_0 = 1.2$ min)	CO_2 ($t_0 = 1.2$ min)	CO_2 + methanol ($t_0 = 1.2$ min)	Hexane ($t_0 = 1.1$ min)	Hexane + methanol ($t_0 = 1.1$ min)
Aniline	17.85(T)	3.62(T)	3.83(t)	0.83(S)	2.92(S)	2.50(S)	3.55(S)	1.00(S)
Benzene	0.31(S)	0.69(S)	0.67(S)	1.33(S)	0.58(S)	0.50(S)	1.00(S)	2.27(S)
Benzoic acid	> 29.77	3.46(t)	> 32.33	2.00(T)	4.58(t)	4.17(S)	> 35.36	3.91(T)
Chlorobenzene	0.38(S)	0.77(S)	0.67(S)	0.75(S)	1.42(S)	1.17(S)	1.18(S)	2.82(B)
Nitrobenzene	0.62(S)	0.92(S)	1.00(S)	0.75(S)	2.08(S)	2.08(S)	2.64(S)	3.64(S)
Phenol	6.15(S)	1.62(t)	9.50(BT)	0.75(S)	3.42(t)	3.25(S)	10.45(t)	3.18(S)
Toluene	0.38(S)	0.69(S)	0.58(S)	0.67(S)	1.10(S)	0.75(S)	0.91(S)	0.82(S)

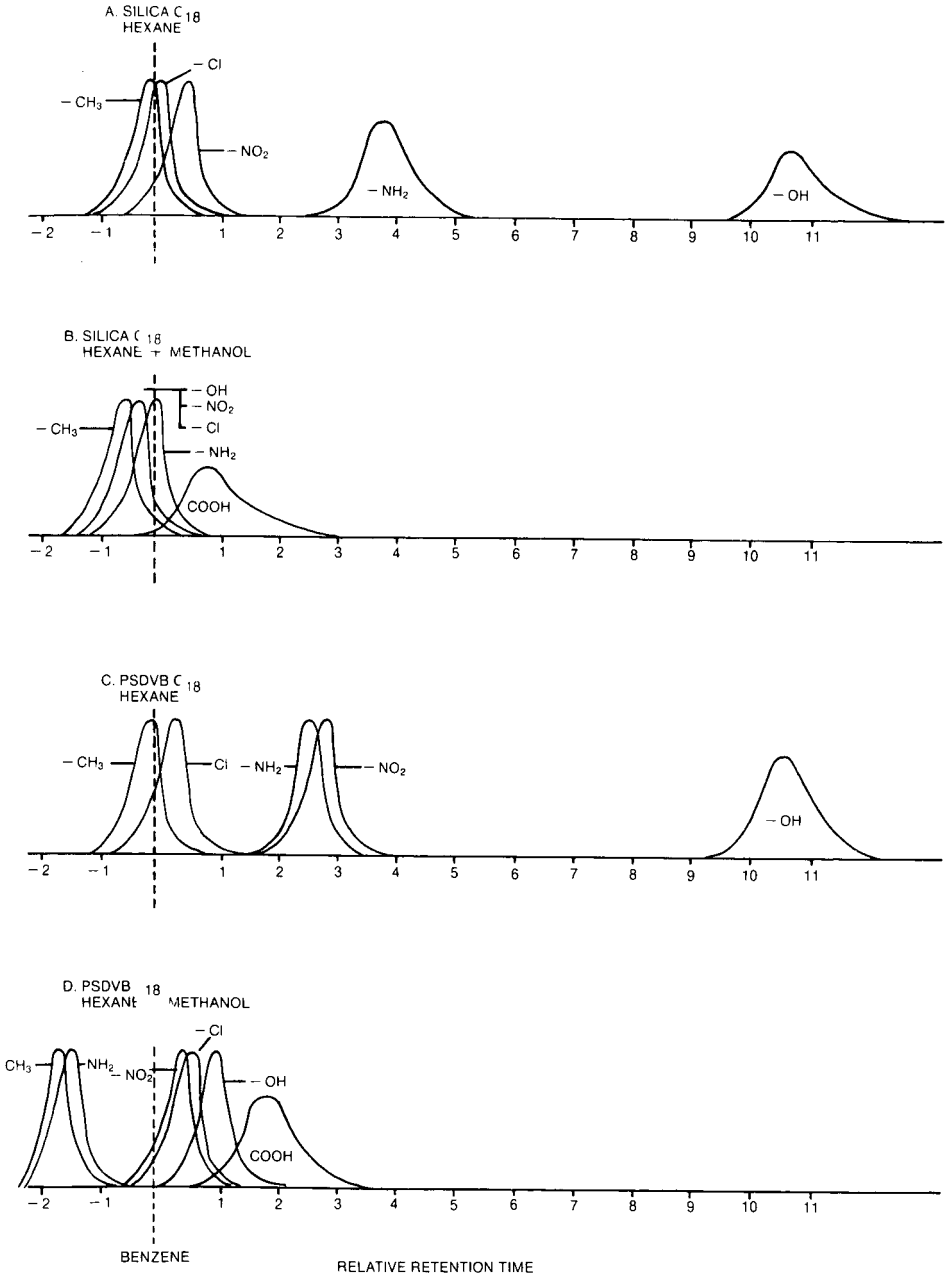


Fig. 1. Simulated chromatographic separation on various columns with a hexane-based mobile phase.

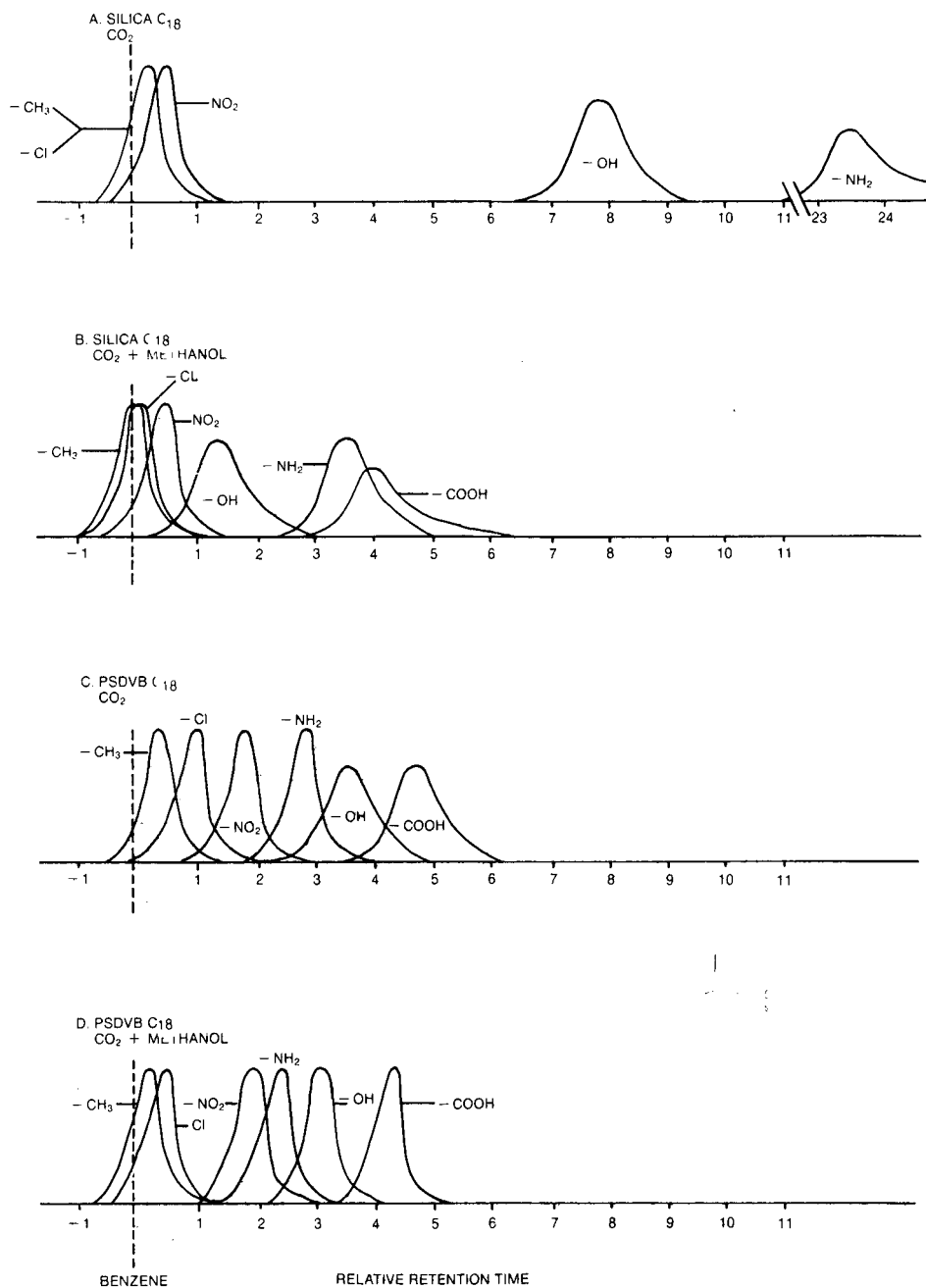


Fig. 2. Simulated chromatographic separation on various columns with a supercritical carbon dioxide-based mobile phase.

The addition of the polar methanol modifier increases the mobile phase solvent strength as can be seen by the decrease in k' values on the PSDVB-based column. The modifier effect is most pronounced with hexane and the effect of methanol modifier on supercritical carbon dioxide solvent strength appears to be minor. These results are in agreement with Wrights findings¹³.

Unique properties of supercritical carbon dioxide

Carbon dioxide has been shown to have a solvent strength similar to hexane, with minor dipole selectivity properties⁹. The analyte with the strongest dipole is nitrobenzene. Out of all the experimental conditions, we would expect dipole-dipole interaction to be most noticeable between nitrobenzene and PSDVB. We find that nitrobenzene elutes slightly faster in carbon dioxide than hexane on the PSDVB-based column indicating that carbon dioxide has slightly greater dipole selectivity than hexane (Figs. 1C and 2C).

TABLE III

SUGGESTED INTERMOLECULAR FORCES AFFECTING SOLUTE RETENTION IN ORDER OF OBSERVED PREDOMINANCE

<i>Species involved</i>	<i>Force involved</i>
<i>All mobile phases</i>	
Benzoic acid + PSDVB	Lewis acid-base, π - π electron
Benzoic acid + silica	Lewis acid-base, hydrogen bonding
Benzoic acid + methanol	Lewis acid-base, hydrogen bonding
Phenol + PSDVB	Lewis acid-base, hydrogen bonding and π - π electron interaction
<i>Supercritical CO₂ mobile phases</i>	
Benzoic acid + CO ₂	Lewis acid-base, hydrogen bonding
Phenol + CO ₂	Lewis acid-base, hydrogen bonding
Silica + aniline	Lewis acid-base, hydrogen bonding
Phenol + silica	Lewis acid-base, hydrogen bonding
Methanol + CO ₂	Lewis acid-base, hydrogen bonding
Methanol + silica	Lewis acid-base, hydrogen bonding
Methanol + PSDVB	Lewis acid-base
Phenol + methanol	Hydrogen bonding
Nitrobenzene + PSDVB	Induced dipole, π - π electron
Chlorobenzene + PSDVB	Induced dipole, π - π electron
Toluene + PSDVB	Induced dipole, π - π electron
Benzene + PSDVB	Induced dipole, π - π electron
<i>Hexane mobile phases</i>	
Methanol + aniline	Lewis acid-base, hydrogen bonding
Phenol + methanol	Lewis acid-base, hydrogen bonding
Phenol + silica	Lewis acid-base, hydrogen bonding
Methanol + silica	Lewis acid-base, hydrogen bonding
Methanol + nitrobenzene	Lewis acid-base, hydrogen bonding
Phenol + PSDVB	Lewis acid-base, π - π electron
Methanol + PSDVB	Lewis acid-base, π - π electron
Nitrobenzene + PSDVB	Induced dipole, π - π electron
Aniline + PSDVB	Induced dipole, π - π electron
Chlorobenzene + PSDVB	Induced dipole

Although supercritical carbon dioxide is similar to hexane in respect to its polarity, it is significantly different from hexane in its ability to Lewis acid–base pair and hydrogen bond¹⁸. It is interesting to note that phenol tails quite badly on the silica column in hexane, but not in carbon dioxide (Fig. 2A). Since carbon dioxide is a Lewis base, it readily pairs with the Lewis acid phenol so that it is unavailable for hydrogen bonding with the silicon dioxide groups. On the other hand carbon dioxide does not pair with the aniline, except for some hydrogen bonding, therefore aniline is free to interact with the silica. Under every chromatographic condition studied, benzoic acid is the last compound to elute due to Lewis acid–base pairing, hydrogen bonding and π electron interactions with the various supports. Benzoic acid tails significantly in hexane but not in supercritical carbon dioxide. The decrease tailing in carbon dioxide is most likely due to Lewis acid–base pairing and hydrogen bonding between benzoic acid and carbon dioxide.

PSDVB-based C₁₈ column

With the PSDVB-based C₁₈ column, the presence of silicon dioxide and silicon hydroxide sites are eliminated and compounds are no longer retained, via the mechanism of hydrogen bonding. Although tailing is significantly reduced on the PSDVB column, species capable of hydrogen bonding on the silica-based column are also retained on PSDVB (Fig. 1C). This can be explained by the π electrons in the extensive aromatic ring structure of PSDVB. These electrons are easily polarizable causing induced dipoles and π electron interaction with solute electron orbitals¹⁹. We would expect the PSDVB-based column to retain all analytes which are not capable of hydrogen bonding longer than the silica-based column due to chemical similarity. The NH₂ functionality in aniline is a ring activator which increases the electron density of the ring making it a better candidate for π – π electron interactions with the PSDVB. Nitrobenzene, the strongest dipole, is retained somewhat by the PSDVB due to induced dipole interactions. The benzene rings in PSDVB with their high electron density can act as weak Lewis bases²⁰. Supercritical carbon dioxide however is a slightly stronger Lewis base than PSDVB and successfully competes with the PSDVB for the Lewis acid analytes phenol and benzoic acid.

Modifier effects

It has been reported that small amounts of methanol modifier (less than 1.0%) added to non-polar solvents have a large effect on solute retention, especially when species capable of hydrogen bonding are involved^{7,8,21}. Once the active sites have been covered, additional amounts of methanol modifier do not have the same dramatic effect on the retention time of these species. This dramatic change in retention for hydrogen bonding species was attributed primarily to the coverage of active sites on the silica support. We observed the same phenomenon for hydrogen bonding species on the silica-based C₁₈ column with hexane as the mobile phase (Fig. 1A). Hydrogen bonding accounts for the increased retention of aniline, phenol and benzoic acid²¹. When methanol modifier is added, it acts as a Lewis acid, readily pairing with aniline, silicon dioxide groups and the PSDVB support. Lewis acid–base pairing and hydrogen bonding with methanol allow aniline, phenol and benzoic acid to elute from the column more rapidly (Fig. 18).

Methanol, like water, can act as either a Lewis acid or base and has hydrogen

bonding capabilities. As a Lewis acid, methanol readily pairs with aniline. The aniline-methanol complex is no longer a ring activator and therefore the aniline elutes close to toluene due to its similar partitioning characteristics. Since methanol is a slightly stronger Lewis base than benzene, it successfully competes with the PSDVB for phenol and benzoic acid²². Nitrobenzene, which is not retained on the PSDVB by a Lewis acid-base mechanism is not affected by the addition of methanol (Fig. 2).

A number of mechanisms have been proposed to account for the effects of polar modifiers in SFC: (1) interaction of polar modifiers with free silane active sites; (2) solute-modifier interaction forming a stable species which favors the mobile phase; (3) short-range clusters are formed between the modifier and the mobile phase; (4) the polar modifier acts as a surfactant causing a decrease in interfacial tension between the mobile phase and the stationary phase; (5) enhanced solubility and selectivity of certain analytes is caused by the formation of Lewis acid-base pairs between the modifier and the solute. At the 3.0% modifier concentration we cannot rule out mechanism 1. The coverage of active sites dramatically affects the retention of polar species in both hexane and supercritical carbon dioxide. Proposed mechanisms 2 and 5 are essentially the same. Although hydrogen bonding and induced dipole interactions should not be overlooked, Lewis acid-base pairing appears to be the most important force involved in solute-modifier interaction. Interaction between the modifier and the solute is only one type of interaction that can occur in chromatographic systems. Mechanism 3 focuses on another type of interaction. Short-range cluster formation may be very important, especially in the case of supercritical carbon dioxide with a Lewis acid modifier such as methanol. Mechanism 4 does not dominate because we do not observe a proportional decrease in the retention of all solutes with the addition of modifier.

CONCLUSION

Supercritical carbon dioxide is a Lewis base with proton acceptor selectivity properties. This selectivity may not have shown up in earlier studies due to dominant active-site interactions. Supercritical carbon dioxide has been shown to have hydrogen bonding capabilities and some dipole selectivity. The large effects caused by methanol on solute retention are due to the coverage of active sites on the stationary phase. The addition of methanol appears to give supercritical carbon dioxide slightly greater solvent strength but without changing its selectivity as far as solvent-solute interactions are concerned. We are only able to separate solvent-solute interactions from solvent-matrix and solute-matrix interactions in controlled experiments. Fortunately, the understanding of supercritical carbon dioxide solvent properties gained through such experiments can help us explain and predict its solvating power.

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INJECTION OF ELUITES IN SOLVENTS STRONGER THAN THE MOBILE PHASE IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

A study was made of the distortion and multiplication of peaks that occur when an elute is injected dissolved in a solvent that is significantly stronger than the mobile phase in reversed-phase liquid chromatography. The solvent strength and volume injected affected peak shape. Column length and diameter, particle size and type of reversed phase did not affect general peak shapes. A qualitative interpretation of the phenomenon is presented.

INTRODUCTION

Chromatographers have long practiced using the mobile phase as the injection solvent for the elute in liquid chromatography. Many workers have observed abnormal chromatograms when solvents other than the mobile phase were used. Commonly multiple peaks appeared in the chromatogram of a single elute.

Wu and Wittick¹ observed broad and tailing peaks for vitamins D₂ and B₁₂ when the injection solvent was methanol and the mobile phase contained water in reversed-phase chromatography. Using the mobile phase for injection sharpened the peaks and reduced tailing. Tseng and Rogers² found with a C₁₈ column *o*-, *m*- and *p*-dihydroxybenzenes gave single symmetric peaks when methanol was the injection solvent and the mobile phase. If water was used for injection, each isomer produced a split (double) peak. When water was the injection solvent and the mobile phase, single peaks were produced. When the injection solvent was methanol, the peak from each isomer had a leading shoulder. Kirschbaum *et al.*³ found that, with an aqueous mobile phase containing methanol, hydrochlorothiazide gave a doublet peak when injected in methanol. However, it gave a single sharp peak using the mobile phase for injection. Tsimidou and Macrae⁴ were able to eliminate a number of peak shoulders by reducing the injection volume of chloroform or changing to acetone for injection in triglyceride analysis. Yi *et al.*⁵ observed multiplet peaks for purine compounds. Peak multiplicity was altered by altering column temperature and mobile phase methanol concentration. Low *et al.*⁶ found peak splitting in the reversed-phase ion pair chromatography of sympathomimetic drugs. They suggested peak splitting was the

result of competitive ion pairing and *in situ* ion exchange. Ng and Ng⁸ simulated peak splitting with a microcomputer program based on a model of changing retention ratio as the injection solvent moved through the column leaving a memory effect in the stationary phase. Perlman and Kirschbaum⁹ correlated peak shape with internal hydrogen bonding of the eluite, but Chan and Yeung¹⁰ disagreed with this interpretation. Khachik *et al.*¹¹ found multiple peak formation in the reversed-phase chromatography of carotenoids. They believed the multiplicity to be dependent on the solubility of the carotenoid in the injection solvent and the mobile phase and on the interaction of the solvent and mobile phase as the injection solvent entered the column.

We wish to report a systematic study of injecting eluites dissolved in strong solvents in reversed-phase high-performance liquid chromatography (HPLC). We studied the peak shape that resulted when weak mobile phases were used. We investigated: (1) amino acids, whose charge was pH controlled; (2) electrically neutral eluites, phenylalkanols differing in the number of methylene groups; and (3) cimetidine, a compound that initially appeared insensitive to injection solvent effects. Column length and diameter, stationary phase type and particle size, injection solvent strength and volume were varied.

EXPERIMENTAL

The HPLC systems consisted of Waters M-6000 pumps; a Waters U6K, a Rheodyne 7125 or 7135 injector or a Waters WISP autoinjector; a Kratos 773 or Perkin-Elmer LC-55 variable-wavelength absorbance detector; and a Fisher Recordall 500, Houston 4511 recorder, or Waters 730 data module. A presaturation column packed with 50- μ m silica was inserted between the pump and injector in some of the work. For some of the work, a guard column of 37- μ m pellicular C₁₈ packing was located between the injector and analytical column.

Columns were obtained from Alltech (Deerfield, IL, U.S.A.), Hamilton (Reno, NV, U.S.A.), Phenomenex (Rancho Palos Verdes, CA, U.S.A.) or packed with a Micromeritics slurry packer.

L-Phenylalanine, L-tryptophan, cimetidine, benzyl alcohol, 2-phenylethanol, 3-phenyl-1-propanol, 4-phenyl-1-butanol and 1-propanol were purchased from Aldrich (Milwaukee, WI, U.S.A.). Acetonitrile and methanol were from Burdick & Jackson or Chempure. Buffers were 5 mM sodium hydrogenphosphate or sodium dihydrogenphosphate and the pH was adjusted with 85% orthophosphoric acid or 50% sodium hydroxide.

Eluite stock solutions were stored at room temperature. The amino acids disappeared very slowly, but no decomposition products' peaks appeared in their chromatograms. A maximum disappearance of 26% occurred with tryptophan. Other eluites were stable in solution. In studying the effect of injection volume of phenylalkanols, injection solution concentrations were prepared to give a constant 50 ng injected. Except where otherwise noted, the amino acids and cimetidine were injected in 20- μ l volumes, and the amount of amino acid was 0.91 μ g and the amount of cimetidine was 1.0 μ g. Composition of the injection solution and mobile phase are expressed in volume ratios. The detector wavelength used with phenylalkanols was 254 nm, and the wavelength used with amino acids and cimetidine was 210 nm. The flow-rate was 1.0 ml/min.

RESULTS AND DISCUSSION

In general, what was observed with a weak mobile phase when sufficient volume of a strong injection solvent was used for an eluite, was a range of peak distortions from a leading peak to a multiplicity of peaks. The peak obtained using the mobile phase for injection (referred to hereafter as peak a) was always present in the chromatogram as indicated by retention time, and any other peaks appeared at shorter retention times. Commonly one peak (peak b) appeared immediately preceeding peak a and another at the retention time of the injection solvent (peak c). Occasionally more than two peaks appeared prior to peak a.

Solvent strength

Fig. 1. illustrates the development of a double peak when benzyl alcohol was injected into a mobile phase of methanol–water (20:80) and the injection solvent was changed from water to acetonitrile–water solutions. The peak with a retention time (t_R) of 21.3 ± 0.1 min and a capacity factor (k') of 6.3, was present in all chromatograms. When the injection solvent composition reached 67% acetonitrile, significant leading was apparent. The leading region developed into a second peak with an injection solvent of neat acetonitrile.

Fig. 2 shows the changes in the peak of positively charged phenylalanine when it was injected into an acetonitrile–buffer (8:92) mobile phase from injection solvents of varying strength. Phenylalanine had a significantly smaller capacity factor than the benzyl alcohol previously discussed. A strong peak c developed as the injection solvent strength increased but a distinct peak b was not formed. With another more efficient column that gave a little larger k' , however, a distinct peak b was resolved.

Figs. 1 and 2 illustrate the qualitative differences found in the chromatograms obtained when using injection solvents of strength greater than that of the mobile phase. Because peak a was always present in these chromatograms, its height was used as a quantitative measure of changes in the chromatograms.

Table I compares the heights of the phenylalanine and tryptophan peaks. Tryptophan gave leading peaks as the acetonitrile content of the injection solvent increased but no peak splitting. The phenylalanine peak, $k' = 0.9$, was more readily

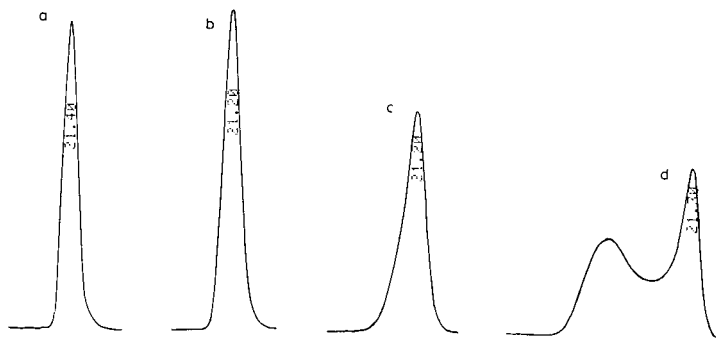


Fig. 1. Deterioration of the benzyl alcohol peak in a mobile phase of methanol–water (20:80). Injection solvent's acetonitrile percent in water: a = 0, b = 50, c = 67, d = 100. Injection volume: 70 μ l. Benzyl alcohol concentration: varied.

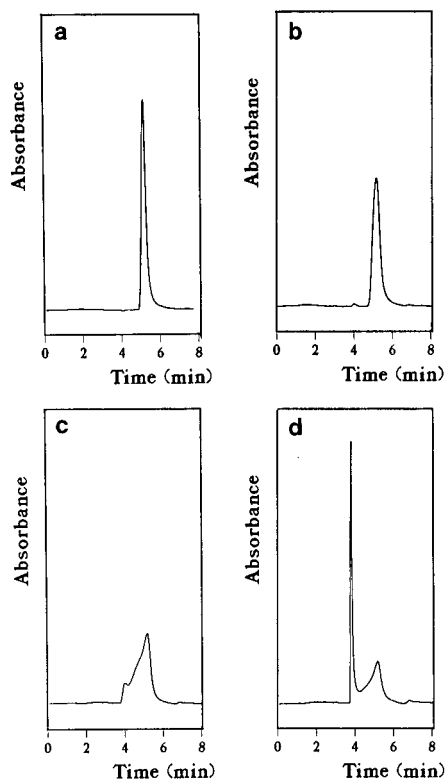


Fig. 2. Deterioration of the phenylalanine peak in a mobile phase of acetonitrile–buffer, pH = 3.5 (8:92). Injection solvent's acetonitrile percent in water: a = 0, b = 30, c = 50, d = 70. Injection volume: 20 μ l. Phenylalanine concentration: 46 ng/ μ l.

TABLE I

HEIGHTS OF PEAK a FOR PHENYLALANINE AND TRYPTOPHAN

Mobile phase, acetonitrile–water (8:92); pH 3.5; column, 25 $\times$ 0.46 cm I.D. (10 μ m, ODS).

Injection solvent % acetonitrile	Peak height (h_p) (cm)	
	Phenylalanine	Tryptophan
0		11.5
10	7.2	
20	7.0	11.7
30	6.2	
40	4.5	10.6
50	3.3	
60	1.2	9.7
70	1.0	
80	0.9	7.8
90	1.0	
100	0.6	4.9

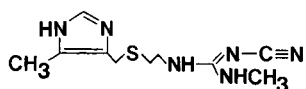


Fig. 3. Structure of cimetidine.

distorted than that of tryptophan, $k' = 2.7$. Cimetidine (Fig. 3), $k' = 6.5$, in the same volume of the same injection solvents as used for phenylalanine and tryptophan gave no peak distortion. Benzyl alcohol with a column and mobile phase that gave a k' of 1.1 as compared to 6.3 for the study shown in Fig. 1 gave multiple peaks when the injection solvent contained only 50% acetonitrile. Thus, the greater k' the less the tendency for the elute peak to be distorted when the injection solvent was stronger than the mobile phase.

Table II presents peak height changes for phenylalanine and tryptophan when the organic component of the injection solvent was changed to 1-propanol. The tryptophan results were expected. The 1-propanol being a stronger solvent than acetonitrile caused a drop in peak height of over 30% when present at 40% in the injection solvent. By comparison (Table I) acetonitrile at that volume % level in the injection solvent caused less than a 20% drop in peak height. Furthermore, the tryptophan peak was split into a peak b and a. The splitting did not occur with acetonitrile.

The results with phenylalanine, however, were not expected. First, not shown in Table II, the phenylalanine peak never showed any signs of splitting, not even a shoulder. Second, the retention time of the peak decreased as the 1-propanol content increased. Third, the peak height rose initially as the 1-propanol content increased, and this rise was markedly higher than the drop in k' dictated. The results show that, with respect to sensitivity, injecting in 60% 1-propanol is more optimal than injecting

TABLE II

INJECTION OF PHENYLALANINE AND TRYPTOPHAN IN 1-PROPANOL-WATER SOLUTION

Mobile phase and column as in Table I

Injection solvent % 1-propanol	Phenylalanine ^a		Tryptophan	
	t_R (min)	h_p (cm)	t_R (min)	h_p (cm)
0	7.6	7.1		
10	7.2	7.4	15.7	10.6
20	7.1	8.2	15.7	9.9
30	7.0	9.0	15.7	8.6
40	7.0	10.1	15.7	7.0
50	6.9	11.6	15.5	6.3
60	6.8	11.7	15.7	4.6
70	6.7	11.3	15.6	4.0
80	6.5	11.1	15.8	3.5
90	6.4	9.9	15.7	3.2
100	6.4	9.5		

^a $t_R = 6.4$, $h_p = 9.5$ for injection solvent of the mobile phase.

TABLE III

INJECTION OF NEAT STRONG SOLVENT AFTER TRYPTOPHAN INJECTION

All injections 20 μ l. Mobile phase and column as in Table I.

Injection solvent % acetonitrile	Initial injection		Tryptophan injection then 1-propanol ^a		Tryptophan injection then acetonitrile ^a	
	t_R (min)	h_p (cm)	t_R (min)	h_p (min)	t_R (min)	h_p (cm)
0	11.9	14.4	10.4	7.7	11.4	11.4
20	12.2	14.8	10.6	7.1	11.4	10.7
50	11.9	11.8	10.5	7.2	11.6	10.0
70	11.7	8.6	10.0	6.5	11.6	8.3
100	11.0	5.4	9.7	4.5	11.2	4.3

^a Injection of neat organic solvent followed the tryptophan injection 2 min later.

phenylalanine in the mobile phase. The pattern of peak height decrease with increasing solvent strength did not appear until levels of 1-propanol greater than 60% were reached. In all of our studies, these results with phenylalanine and 1-propanol were the only results that did not fit the pattern of peak deterioration with increasing solvent strength.

The purpose of the study shown in Table III was to determine if peak distortion would occur if neat organic solvent was injected after tryptophan had been injected into the column and had moved about 20% of the column length. Tryptophan injection in solvents of varying strength was followed 2 min later by an injection of neat acetonitrile or 1-propanol. The neat solvent injection caused peak leading, and this was indicated by a loss of peak height with both acetonitrile and 1-propanol. The neat solvent caused further peak leading even when the peak was already distorted because the tryptophan had been injected in a strong solvent. The effect of 1-propanol was greater than the acetonitrile effect in causing leading. Most significant in the results was that the leading caused by delayed injection of neat solvent was always smaller than the leading caused by injection in the neat solvent, *i.e.*, peak height (h_p) was always > 5.4 . We interpret this to mean the neat solvent band was diluted by the mobile phase before it reached the eluite band, and its effect was, therefore, diminished. This interpretation suggests that the cause of peak distortion when injecting in strong solvents acted very early in the column before the injection plug became too dilute.

To answer the question of whether the peak leading and the extra peaks formed might be the result of the appearance of new elutes, the sum of peak areas was measured in each chromatogram. With both phenylalanine and tryptophan, the total area remained constant when the amount of eluite injected was constant regardless of the shape of the peak(s). These results are consistent with those of others who showed by mass spectroscopy that the eluite was producing all of the split peaks⁶. Thus, new elutes were not being introduced by chemical or physical means, and then these producing asymmetry and multiple peaks.

Injection volume

Fig. 4 shows the increase of peak height as the injection volume of benzyl alcohol solutions increased. When the injection solvent was weak, 33% acetonitrile,

the peak height was proportional to injection volume. However, when the solvent contained 67% acetonitrile, the peaks were shorter than they should have been at injection volumes greater than 30 μl . When the injection solvent was neat acetonitrile, linearity, if it was present at all, ended at very low injection volumes. The peaks were shorter than they were with an injection solvent of 67% acetonitrile.

Cimetidine was injected in neat methanol with no peak distortion in a method for its determination in blood and plasma¹². In our initial studies, cimetidine was unusual because it showed no peak distortion in going from a weak to a strong injection solvent using acetonitrile or 1-propanol. However, this initial work used injections of 20 μl or less. Fig. 5, in which cimetidine's peak height is shown as a function of injection volume shows that cimetidine's peak height was significantly lower when it was injected in a strong solvent provided the injection volume was large enough.

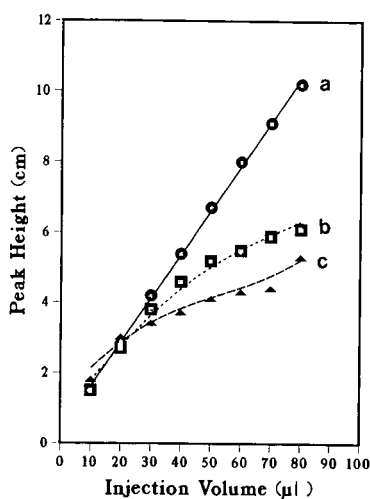


Fig. 4. Relationship between injection volume and peak height of benzyl alcohol injected into a mobile phase of methanol-water (20:80). Injection solvent's acetonitrile percent in water: a=33, b=67, c=100.

In a more systematic study, the loss of peak height of a series of phenylalkanols was investigated. The loss of peak height was accompanied by peak leading as injection volume was increased and eventually the appearance of multiple peaks preceding peak a. Table IV summarizes the results of phenylalkanol injection in acetonitrile-water (50:50) and Table V summarizes the results when the injection solvent was neat acetonitrile. In all cases peak height loss increased with increasing injection volume, and the losses were always greater when neat acetonitrile was the injection solvent. When either injection solvent was used, there appeared to be a slight tendency for the rate of peak height loss with injection volume to be greater, with lower molecular weight phenylalkanols.

Stationary phase

Tables VI and VII show the results of injecting 4-phenyl-1-butanol into a variety of columns using, respectively, 50% and neat acetonitrile. In all cases, peak leading

TABLE IV

INJECTION OF $C_6H_5(CH_2)_nOH$ IN VARYING VOLUMES OF 50% ACETONITRILEMobile phase, methanol-water (30:70); column, 8×0.46 cm I.D. ($5 \mu m$, methyl)

Injection volume (μl)	h_p loss (%)			
	$n = 1$	$n = 2$	$n = 3$	$n = 4$
2.0	0	0	0	0
4.0	8	11	8	9
8.0	12	17	17	14
16.0	33	36	31	26
32.0	62	61	58	53 ^a
64.0		78	68	

^a 73% loss with a 96- μl injection.

occurred as the volume increased and peak multiplicity appeared at the higher volumes. The loss of peak height was very similar from column to column even though the stationary phase changed. The linear velocity of the mobile phase also changed, despite the use of the same flow-rate (1.0 ml/min) throughout. Similar results between short and long columns suggest again that peak changes occurred at the beginning of the column. Consistent with other results, the loss of peak height was greater when the injection solvent was neat acetonitrile as compared to 50% acetonitrile. Despite the differences in columns, solvophobic sorption was common to all the chromatography indicated in Tables VI and VII. This similarity suggests that it is in the sorption process that peak distortion occurs.

Mobile phase

Provided sufficient injection volume was used, peak distortion occurred to the extent that there was a difference between the mobile phase strength and that of the injection solvent. Thus, when tryptophan dissolved in neat acetonitrile was injected into a mobile phase of acetonitrile-water (15:85) ($k' = 2.1$), the peak height loss was

TABLE V

INJECTION OF $C_6H_5(CH_2)_nOH$ IN VARYING VOLUMES OF NEAT ACETONITRILE

Same chromatographic conditions as in Table IV

Injection volume (μl)	h_p loss (%)			
	$n = 1$	$n = 2$	$n = 3$	$n = 4$
2.0	0	0	0	0
4.0	20	21	20	21
8.0	49	48	47	43
16.0	64	70	68	66
32.0	86	84	82	81
64.0		93	93	

TABLE VI

INJECTION OF 4-PHENYL-1-BUTANOL INTO DIFFERENT COLUMNS IN 50% ACETONITRILE

Column	h_p loss (%)				
	2.0 μ l	4.0 μ l	8.0 μ l	16.0 μ l	32.0 μ l
10 $\times$ 0.46 cm I.D. 10 μ m, SDVB ^a	0	9	17	29	59
15 $\times$ 0.42 cm I.D. 10 μ m, SDVB	0	8	19	38	47
25 $\times$ 0.46 cm I.D. 10 μ m, ODS	0	12	21	32	61
10 $\times$ 0.46 cm I.D. 5 μ m, octyl	0	7	19	35	51
8 $\times$ 0.70 cm I.D. 3 μ m, ODS	0	8	17	28	54
8 $\times$ 0.46 cm I.D. 5 μ m, methyl	0	9	14	26	53

^a Styrene-divinylbenzene copolymer or PRP-1.

48%. But, when the same injection was made into acetonitrile-water (35:65) ($k' = 0.4$) the loss was 12%.

In Table VIII is shown the effect of pH on the peak height of phenylalanine. At the pH values used, phenylalanine was electrically positive, net neutral, and negative. Regardless of charge, the peak underwent the changes observed with other elutes when the injection solvent strength was changed. At all three pH values, the magnitude of the peak height change was similar at a given solvent composition and the appearance of peak b occurred at the same solvent composition.

TABLE VII

INJECTION OF 4-PHENYL-1-BUTANOL INTO DIFFERENT COLUMNS IN NEAT ACETONITRILE

Column	h_p loss (%)				
	2.0 μ l	4.0 μ l	8.0 μ l	16.0 μ l	32.0 μ l
10 $\times$ 0.46 cm I.D. 10 μ m, SDVB	0	18	36	58	80
15 $\times$ 0.42 cm I.D. 10 μ m, SDVB	0	24	40	64	73
25 $\times$ 0.46 cm I.D. 10 μ m, ODS	0	28	31	66	88
10 $\times$ 0.46 cm I.D. 5 μ m, octyl	0	19	38	53	79
8 $\times$ 0.70 cm I.D. 3 μ m, ODS	0	18	29	54	71
8 $\times$ 0.46 cm I.D. 5 μ m, methyl	0	21	43	66	81

TABLE VIII

EFFECT OF MOBILE PHASE pH ON PEAK HEIGHT OF PHENYLALANINE AT DIFFERING SOLVENT STRENGTHS

 t_R (min): 5.8–6.1 (pH 3.5), 6.4–6.6 (pH 5.5), 5.9–6.1 (pH 7.6).

Injection solvent % acetonitrile	h_p (cm)		
	pH 3.5	pH 5.5	pH 7.6
0		8.0	
10	7.2	7.4	7.2
20	7.0	6.6	
30	6.2	6.2	4.8
50 ^a	3.3	2.5	2.7
60	1.2	2.0	
70	1.0	1.8	
80	0.9	1.4	1.7
90	1.0	1.3	
100	0.6	1.0	1.3

^a At each pH peak b appeared at 50% and higher levels of acetonitrile.

Interpretation

When an elute is injected dissolved in a solvent of mobile phase composition, it is distributed initially at the top of the column in diminishing concentration because the head of the injection plug is depleted of elute through sorption by the stationary phase as the plug moves into the column. But a symmetric peak quickly develops as the mobile phase moves the elute along¹³. The initial concentration gradient is steeper for larger capacity factors. As the strength of the injection solvent is increased, the elute concentration left at the very top of the column as the plug moves along is less but enough to produce a peak with a consistent retention time (peak a). The greater

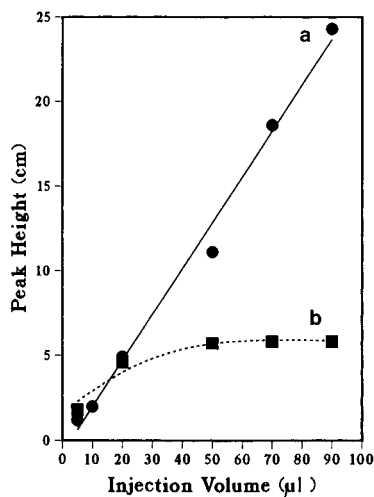


Fig. 5. Relationship between injection volume and peak height of cimetidine injected into a mobile phase of acetonitrile–buffer (15:85). Injection solvent: a = water, b = acetonitrile.

k' , the higher the amount left at the top of the column. The remaining amount is carried further into the column by the solvent plug. This extension of elute sorption produces a leading peak when the solvent is not too strong, but increasing the solvent strength extends the elute sorption even further and a peak (b) is produced. With larger capacity factors, the amount sorbed at the column top is larger so that the solvent strength required to produce peak b must be greater. As the solvent strength becomes very great, the plug carries some of the elute with it to the end of the column (peak c). The capacity factor must be small enough in this case to permit the plug to retain enough elute to produce a peak. One could imagine a hypothetical case in which the solvent is so strong that the elute is not retained and all of the elute is found in peak c.

The injection volume effect is the result of rapid dilution of the solvent as it moves through the column. When the volume is small, rapid dilution of the injection solvent with the mobile phase puts the elute in the same condition it would have been in had the injection solvent been mobile phase. As the volume for injection is increased, there is less dilution of the center of the plug. Thus, the solvent can carry the elute away from peak a to produce peaks b and c as discussed above.

This view of the production of leading and multiple peaks requires no "memory" in the stationary phase⁸. Nor does it consider the hydrocarbon stationary phase modified significantly by the injection solvent.

Occasionally more than one peak appeared between peaks a and c. This may be the result of plug dilution in the pre-column hardware such as the injector. Or it may be that injection in strong solvents accentuates the effects of poor packing on peak shape, and the appearance of more than one peak between peaks a and c was the result of defective packing.

Using this view of the production of peak distortion and multiplicity, we are presently investigating this phenomenon by computer simulation.

We have not developed an interpretation of the peak height and plate number improvement through increasing the ratio of 1-propanol to water in the injection solvent for phenylalanine. The effect is reproducible, and the peak as described in Table II is taller when neat 1-propanol is used than when neat water is used. We believe the phenomenon is related to the relative rates of band movement of phenylalanine and 1-propanol.

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INACCURACIES DUE TO SAMPLE-SOLVENT INTERACTIONS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Inaccuracies in quantitation by high-performance liquid chromatography (HPLC) due to sample-solvent interactions are described for two compounds, vancomycin and vinblastine. Erroneously high peak areas were obtained when water was used as the sample solvent without matching the ionic strength of the comparator solutions to the ionic strength of the sample solutions. The presence and magnitude of these errors appear to be instrument related with the sample injection system identified as a key variable. These interactions demonstrate that special care is needed to match sample and standard solution matrices when HPLC is used for precise quantitative determinations.

INTRODUCTION

A series of recent papers described the effects of sample solvent on high-performance liquid chromatography (HPLC) peak quantitation with UV detection and postulated explanations for the apparently anomalous observations^{1–3}. The extent and very existence of these effects were called into question and the results were explained by well-known chromatographic behavior⁴. In this report, the authors present their observations of anomalous HPLC peak quantitation that are not readily explainable. While experimental efforts are continuing, extensive characterization of these effects demonstrate that: (1) subtle sample solvent effects can significantly influence peak quantitation; (2) these effects may be due to differences in solution ionic strengths; and (3) these effects can be traced to specific instrumental characteristics, namely the sample injection system.

Perlman and Kirschbaum¹ described quantitation differences associated with sample-solvent interactions in HPLC and concluded that only compounds which form intramolecular hydrogen bonds exhibit this effect. Their observations were made on a series of 18 pharmaceutical compounds dissolved in water, methanol or ethanol. These compounds were eluted with solvents of various polarity and detected by their UV response (214–270 nm). Their observations are similar to the classical qualitative changes observed in HPLC peak profiles when certain organic solvents are

used to aid sample solubility, the "injected-solvent effect"⁵⁻⁷. The original observations by Perlman and Kirschbaum could not be reproduced by Berridge who suggested that only peak shape was affected, possibly by an instrumental artifact². In a second report by Perlman and Kirschbaum³, an additional compound (aztreonam) prepared in either mobile phase or water is described. In this case, changes in peak areas were noted without any apparent corresponding change in peak shape. More recently, Kirschbaum^{8,9} summarized the factors contributing to interlaboratory differences in quantitation including detector linearity, injection volume, and sensitivity of the analyte molar absorptivity to slight changes in environment. Finally, Chan and Yeung⁴ suggested that the original observations could be readily explained by well-known behaviors in HPLC such as the injected-solvent effect and by changes in the analyte molar absorptivity with slight changes in solvent composition. However, independent observations made in the present authors' laboratories for two additional compound types clearly indicate that inaccuracies due to sample-solvent interactions occur for a number of analytes and that they are not explained by classical injected-solvent effects or by changes in the molar absorptivity of the analyte.

Refs. 2 and 4-7 focus on changes due to the organic composition of the sample solvent, *e.g.*, chromatographic peak profiles are altered by band broadening, the appearance of ghost peaks, or peak fronting. In contrast, the two examples described herein exhibit differences in peak quantitation (peak area) as a function of sample solvent composition without qualitative changes in the peak profiles. A similar behavior is observed in Fig. 1 of ref. 3. Experiments designed to characterize this phenomenon and our conclusions are described in this report.

In this work, all sample solvents were primarily aqueous and the solvent strengths were less than or equal to that of the initial mobile phase solvent. Thus, classical injected-solvent effects should not be present and were not observed. Quantitative changes were traced to differences in the ionic strength of the sample solvent and to instrumental factors. Our experiments identified conditions which can be used to estimate the "true" peak area. Our results suggest that sample-solvent interactions are more widespread than commonly believed and that inaccurate results due to this phenomenon often go undetected.

EXPERIMENTAL

Apparatus

Vancomycin. The separations for vancomycin were performed on a Varian 5560 gradient HPLC apparatus with an integrated UV-200 variable-wavelength detector (Varian, Walnut Creek, CA, U.S.A.), and a Varian 8085 autosampler with a Valco (Valco, Houston, TX, U.S.A.) sampling valve, or a Perkin-Elmer ISS-100 autosampler (Perkin-Elmer, Norwalk, CT, U.S.A.) with an attached Valco valve and temperature control capabilities. Data collection and reduction were performed on a central laboratory chromatography computer system with data storage, manipulation, and graphics capabilities. The separation was performed on a Beckman UltrasphereTM ODS 25 cm $\times$ 4.6 mm I.D. stainless-steel column with 5- μ m packing (Beckman, San Ramon, CA, U.S.A.). Sample loops of 20 μ l nominal volume were used for all injections. Ultraviolet absorbance data were obtained on a Sargent-Welch Model 6-550 spectrophotometer (Sargent-Welch, Skokie, IL, U.S.A.).

Vinblastine. The assays for vinblastine were performed on two modular HPLC instruments. Components for the first instrument included a Waters M6000A pump (Waters Assoc., Milford, MA, U.S.A.), a Micromeritics autosampler (Micromeritics, Norcross, GA, U.S.A.), a Rheodyne 7126 sampling valve (Rheodyne, Cotati, CA, U.S.A.), and a Dupont variable-wavelength UV detector (Dupont, Wilmington, DE, U.S.A.). The second system consisted of a Varian 5000 pumping system, a Varian 8000 autosampler, a Valco valve, and a Dupont variable-wavelength UV detector. A Rheodyne 7125 manual valve was also used for one set of experiments. Data collection and reduction were performed on the same centralized laboratory chromatography computer data system. The separation was performed on an IBM C₁₈ 15 cm × 4.6 mm I.D. stainless steel column with 5- μ m packing (IBM, Danbury, CT, U.S.A.). Sample loops of 20 μ l nominal volume were used. Ultraviolet absorbance data were obtained on a Sargent-Welch Model 6-550 spectrophotometer.

Reagents

HPLC-grade acetonitrile and methanol were purchased from Mallinckrodt (Mallinckrodt, Paris, KY, U.S.A.). Purified water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, U.S.A.). All other reagents were of analytical reagent grade. All vancomycin and vinblastine samples were prepared at Eli Lilly and Company. The solution of 0.1 M phosphate buffer was prepared as a solution of sodium dihydrogen phosphate with the pH adjusted to 3.0 with phosphoric acid.

Methods

Vancomycin. The mobile phases were prepared from a stock buffer solution and acetonitrile. The buffer solution was prepared by adding 4 ml triethylamine to 2 l water and adjusting the pH to 3.2 with phosphoric acid. Mobile phase A was made by adding 50 ml acetonitrile to 950 ml buffer solution. Mobile phase B was prepared by adding 400 ml acetonitrile to 600 ml buffer solution. Each mobile phase was thoroughly mixed, degassed, and covered to reduce evaporation.

The gradient program was performed as a linear ramp from 5 to 65% mobile phase B over 40 min, corresponding to a 1.5% mobile phase B change per minute. The final conditions were held for five minutes and then ramped back to initial conditions over the next minute. Equilibration was reestablished within 60 min from injection. A constant flow-rate of 0.9 ml/min was used. A detector wavelength of 254 nm was used with a setting of 0.05 a.u.f.s.

Vinblastine. The aqueous portion of the mobile phase was prepared by adding 14 ml diethylamine to 1 l of purified water and adjusting the pH to 7.5 with phosphoric acid. The mobile phase was a mixture of 62% of methanol-acetonitrile (4:1, v/v) with the buffer solution. It was mixed thoroughly, filtered, and degassed prior to use. The separation was performed in an isocratic mode. The flow-rate was 1.5 ml/min and the detector wavelength was 262 nm at 0.5 a.u.f.s.

RESULTS AND DISCUSSION

Vancomycin

The effect of sample solvent on peak quantitation for vancomycin was observed during routine HPLC characterization on the Varian HPLC with the Varian auto-

TABLE I

VANCOMYCIN HYDROCHLORIDE AND BASE SAMPLE CHARACTERIZATION PROFILES

	<i>Hydrochloride</i>	<i>Base</i>
<i>Sample composition (%)</i>		
Water content	2.0	6.8
HCl content	2.5	—
Vancomycin base content	89.2	87.3
Related substances	6.3	5.9
<i>Sample solutions</i>		
Sample weight (mg)	126.2	126.0
Stock solution concentration as:		
mg sample/ml	2.524	2.520
mg vancomycin base/ml	2.251	2.200
Diluted solution concentration as:		
mg sample/ml	0.1010	0.1008
mg vancomycin base/ml	0.0901	0.0880
<i>HPLC peak areas</i>		
Main peak area (arbitrary units)	47 208	36 196
Normalized peak area (per mg/ml base)	$5.24 \cdot 10^5$	$4.11 \cdot 10^5$

sampler. Peak area differences were obtained for samples of vancomycin base and vancomycin hydrochloride at equal sample concentrations. The samples characterized in Table I illustrate typical peak area differences. The sample of vancomycin hydrochloride at a concentration of 0.1010 mg/ml (0.0900 mg vancomycin base/ml) produced a peak area count of about 47 200 (arbitrary units), whereas a sample of vancomycin base of similar relative purity at a concentration of 0.1008 mg/ml (0.0880 mg vancomycin base/ml) yielded a peak area count of about 36 200. Note that these sample concentrations are converted to vancomycin base content (*i.e.*, corrected for water, hydrochloride salt and related substance content) so that the normalized peak area responses should be equivalent whereas about a 30% difference is observed. In contrast, chromatograms of 2.5 mg/ml-solutions appeared nearly identical with no apparent differences in peak areas between the hydrochloride and base samples. These observations were repeatable within and across days. Sample preparation and instrumental parameters were studied to determine their roles in this anomalous behavior.

Sample solvent differences were investigated first. Routinely, a sample stock solution of 2.5 mg/ml vancomycin hydrochloride was prepared by dissolution of the salt in water followed by a dilution to 0.1 mg/ml with water. The pH of these solutions are about 3.5 and 4, respectively. Vancomycin base, however, has limited solubility above a pH of 4.5. To prepare the stock solution of 2.5 mg/ml of the base, concentrated phosphoric acid was added dropwise until complete dissolution of the sample was achieved. Subsequent dilutions to 0.1 mg/ml were made with water.

The effect of sample solvent was investigated further by chromatographic and spectroscopic evaluation of vancomycin solutions in numerous solvents. Stock solutions of vancomycin hydrochloride and base were prepared as described previously (data in Table I). Five different sample solvents were used for further dilution from

TABLE II

VANCOMYCIN HYDROCHLORIDE PEAK AREA AND ABSORBANCE AS A FUNCTION OF SAMPLE SOLVENT

See Table I for the sample composition.

<i>Sample solvent</i>	<i>Main peak area (relative units)</i>	<i>Absorbance (a.u., 254 nm)</i>	<i>Absorbance (a.u., 280 nm)</i>
Water	47 208	0.322	0.447
0.001 <i>M</i> HCl	35 678	0.323	0.448
0.1 <i>M</i> Phosphate buffer	35 191	0.324	0.450
Mobile phase A	35 602	0.317	0.447
Mobile phase B	4678	0.322	0.448

the stock solutions. The UV absorbances of these solutions were measured at 254 nm (wavelength used for HPLC detection) and at 280 nm (absorption maximum for vancomycin). Data from the solutions prepared from the vancomycin hydrochloride sample and from the solutions prepared from the vancomycin base sample are given in Tables II and III, respectively. Previous work indicates that vancomycin-related impurities have similar spectral characteristics and will contribute proportionately to the UV absorbance measurements¹⁰. That is, the UV absorbance will be proportional to the sum of the vancomycin base content and the related substance content whereas the HPLC peak area will include only the vancomycin base contribution.

The vancomycin hydrochloride solutions yielded equivalent absorbances for all five solvents. The five vancomycin base solutions also yielded equivalent absorbances. The vancomycin HPLC peak areas for these solutions are included in Tables II and III. The hydrochloride sample peak areas are approximately equivalent for the solutions with sample solvents of hydrochloric acid, phosphate buffer, and mobile phase A. However, the peak area for the water solution is about 33% greater, equivalent to the differences previously observed. Fig. 1 shows that no qualitative differences are observed for these chromatograms. In contrast, the peak area is reduced for the solution in mobile phase B since an injected-solvent effect is produced by a sample solvent which is stronger than the initial mobile phase conditions. This qualitative

TABLE III

VANCOMYCIN BASE PEAK AREA AND ABSORBANCE AS A FUNCTION OF SAMPLE SOLVENT

See Table I for the sample composition.

<i>Sample solvent</i>	<i>Main peak area (relative units)</i>	<i>Absorbance (a.u., 254 nm)</i>	<i>Absorbance (a.u., 280 nm)</i>
Water	36 196	0.311	0.434
0.001 <i>M</i> HCl	34 736	0.313	0.437
0.1 <i>M</i> Phosphate buffer	34 920	0.314	0.438
Mobile phase A	34 812	0.306	0.432
Mobile phase B	4022	0.308	0.432

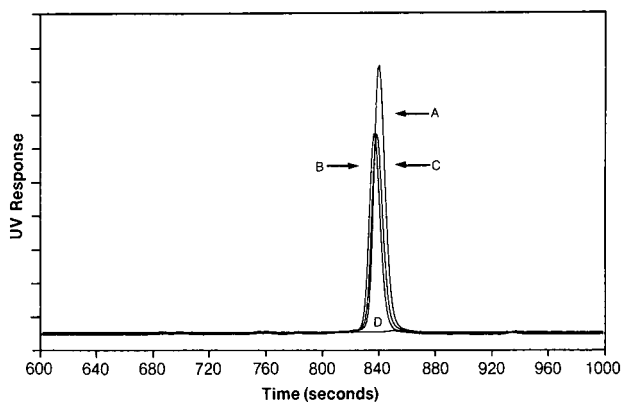


Fig. 1. Chromatograms from 0.1 mg/ml vancomycin hydrochloride diluted with (A) water, (B) 0.001 *M* hydrochloric acid and (C) mobile phase A. D was obtained from a water blank.

difference is shown in the chromatogram in Fig. 2. Note that no baseline artifacts appear in the retention window for vancomycin when a water blank was injected.

The vancomycin base sample peak areas were also approximately equivalent for the solutions with sample solvents of hydrochloric acid, phosphate buffer and mobile phase A. The peak area for the water solution was greater than the others, similar to the hydrochloride samples except that the increase was now only about 4%. The peak area for the sample in mobile phase B is again reduced by an injected-solvent effect. Again, the chromatogram from the water blank shows no baseline artifacts in the vancomycin retention window. Thus, the qualitative differences for these chromatograms (Fig. 3) mirror those of the hydrochloride sample. However, there are no differences in molar absorptivity which could explain these HPLC peak area differences for either the hydrochloride or base samples.

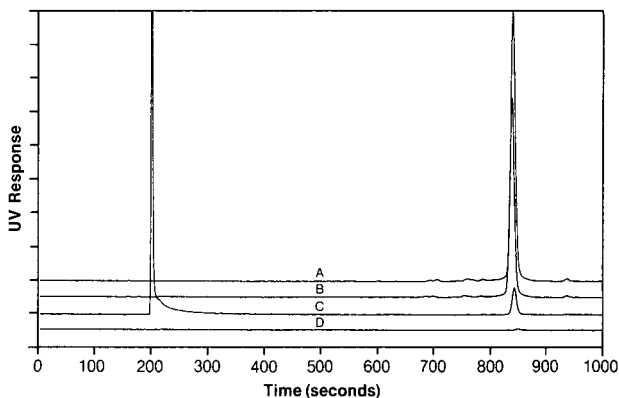


Fig. 2. Chromatograms from 0.1 mg/ml vancomycin hydrochloride diluted with (A) water, (B) 0.001 *M* hydrochloric acid and (C) mobile phase B which demonstrates the qualitative and quantitative differences observed for these sample solvents. D was obtained from a water blank.

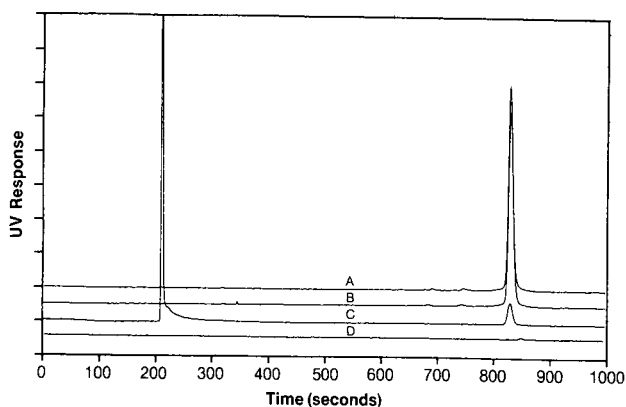


Fig. 3. Chromatograms from 0.1 mg/ml vancomycin base diluted with (A) water, (B) 0.001 *M* hydrochloric acid and (C) mobile phase B which demonstrate the qualitative and quantitative differences observed for these sample solvents. D was obtained from a water blank.

Data from either base or hydrochloride samples in the three sample solvents (0.001 *M* HCl, 0.1 *M* phosphate buffer of pH 3.0, and mobile phase A) indicate that peak areas are consistent when a minimum ionic strength is maintained for the sample solvent. In the absence of added buffer, the HPLC peak area increased without a corresponding increase in molar absorptivity. The smaller increase in peak area for the base sample in water is probably due to the increased ionic strength from the phosphoric acid required for dissolution of the original sample.

If similar peak areas were observed for the stock solutions while differences were observed for dilute solutions, the calibration curves for water and buffered sample diluents must diverge. Calibration curves for water and mobile phase A sample diluents were generated and are plotted in Figs. 4 and 5 (expanded scale). The deviation from zero of the *y*-intercept for the aqueous standards suggests that the areas for the water solutions were erroneously high and those for the mobile phase A

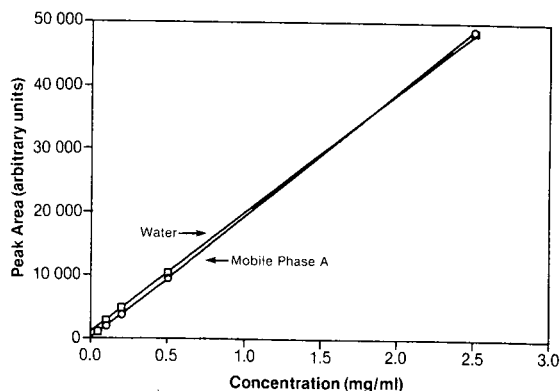


Fig. 4. Calibration curves from vancomycin hydrochloride diluted with water and mobile phase A.

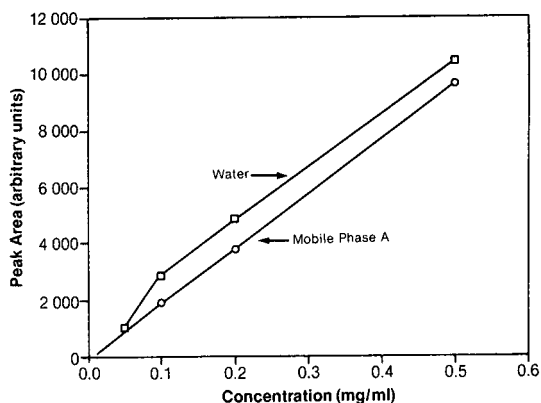


Fig. 5. An expanded view of the calibration curves from Fig. 4 which demonstrate the significant differences in y -intercepts.

solutions provide greater accuracy. Note that non-linearity is observed in the curve obtained from the water solutions below 0.1 mg/ml. The overlap of the two curves at 0.05 mg/ml supports the conclusion that the areas observed from water solutions are high.

Erroneously high peak areas are difficult to explain. While low peak areas are often attributable to precipitation or adsorption, high peak areas can occur for only a few reasons, *e.g.* by changes in the molar absorptivity of the analyte, a cause which has been eliminated in this example. To further characterize these observations obtained on the Varian autosampler, a second autosampler was evaluated on the same chromatographic system. The Perkin-Elmer ISS-100 autosampler yielded equivalent peak areas for hydrochloride samples dissolved in water or mobile phase A at both 5°C and ambient temperatures. These areas were approximately equivalent to the areas obtained from the Varian autosampler using mobile phase A as the sample diluent. It was noted that separate injection valves were used. Thus, the observed effect appears to be autosampler specific.

Vinblastine

The effect of sample solvent on HPLC peak quantitation for vinblastine was observed during an interlaboratory collaborative study. The collaborative study was undertaken prior to transferring assay methodology for vinblastine raw material and formulations. During the course of the study in which multiple days, instruments, and analysts were evaluated, low results (lower than expected peak areas) were sometimes obtained for the vinblastine ready-to-use formulation. The formulation contains 1 mg/ml vinblastine in the excipient matrix; samples were diluted to 0.4 mg/ml with water prior to performing the assay. The vinblastine ready-to-use matrix is 100 mg/ml mannitol, 1.3 mg/ml methylparaben, 0.2 mg/ml propylparaben, 1.5 mg/ml sodium acetate (anhydrous), and 4.7 ml/l glacial acetic acid adjusted to pH 5. The chromatographic behavior (peak shapes and retention times) was unchanged except for reduced peak areas (Fig. 6). Note that baseline artifacts were not apparent in a water blank. The low results, on the order of 10% below theory, correlated with an unac-

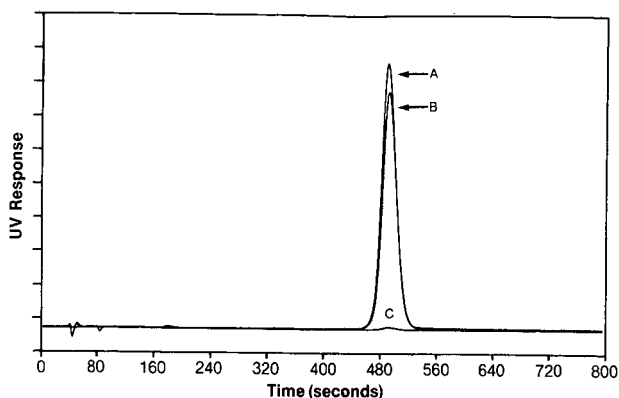


Fig. 6. Chromatograms from vinblastine samples diluted with (A) water and (B) formulation matrix. C was obtained from a water blank.

ceptably high normalized y -intercept-to-slope ratio for the respective standard curve. Our laboratory uses the normalized intercept-to-slope ratio as one of the parameters to evaluate standard curves. The normalized intercept-to-slope ratio (ISR) is defined as

$$\text{ISR} = (b/m) \cdot [2/(x_{\max} + x_{\min})]$$

where b is the y -intercept, m is the slope of the linear regression line fitted to the data, and $x_{\max}$ and $x_{\min}$ are the purity-corrected standard weights for the highest and lowest points on the standard curve. The ratio is deemed acceptable if it is less than 0.1, that is, the y -intercept is no more than 10% of the response of the midpoint of the curve.

The high intercept-to-slope ratios were determined to be instrument dependent. This led to the classification of instruments as acceptable and unacceptable for use in this assay. Arbitrarily, instruments yielding intercept-to-slope ratios of 0.1 or greater were designated as unacceptable. However, no changes in peak shapes or retention times were observed between instruments. Fig. 7 includes two standard curves obtained from an acceptable instrument and two curves obtained from an unacceptable instrument. The aqueous curve (standards prepared in water) and the matrix curve (standards prepared in the ready-to-use matrix) from the acceptable instrument both have near-zero intercepts. In contrast, the aqueous standard curve obtained on an unacceptable instrument has a very high intercept and is approximately parallel to, but offset from, its corresponding matrix curve. Using the observed response for a sample in the ready-to-use matrix, the error in the sample concentration that results from calculations *versus* the aqueous standard curve (true *vs.* apparent concentration) is approximately 14% for the unacceptable instrument.

The response on the unacceptable instrument was explored in greater detail by extending the range of the calibration curve. As expected, the curves begin to converge at low concentrations (<0.1 mg/ml). Thus, the standard curves generated in the sample matrix are correct whereas those generated for standards diluted in water are

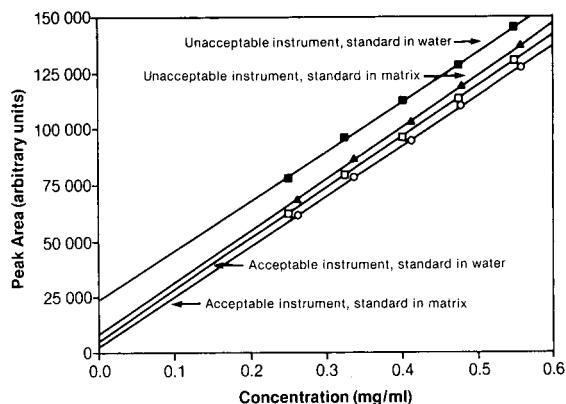


Fig. 7. Calibration curves from vinblastine standards prepared in water and in formulation matrix. Results are shown for an instrument which yields acceptable results and for an instrument which yields unacceptable results.

erroneously high. In addition to the matrix effect, there is a definite instrumental contribution to the error for aqueous vinblastine standard solutions.

Correction of the assay bias was a relatively simple matter. Calculations of the ready-to-use vinblastine formulation samples using the standard curve generated in the ready-to-use matrix eliminated the negative bias in the results. However, the underlying cause of the bias was still unknown. That is, what could produce an offset of the standard curve for the aqueous solutions?

Method conditions and the general state of the instruments involved were studied in order to eliminate sources of bias. Detector wavelength settings, sample carryover, lack of response linearity and differences in mobile phase makeup or columns were eliminated as factors contributing to bias. Absorbances of vinblastine in water, mobile phase, and acetate buffer were checked; the complete ready-to-use matrix cannot be used due to the UV absorbance of methylparaben and propylparaben. The

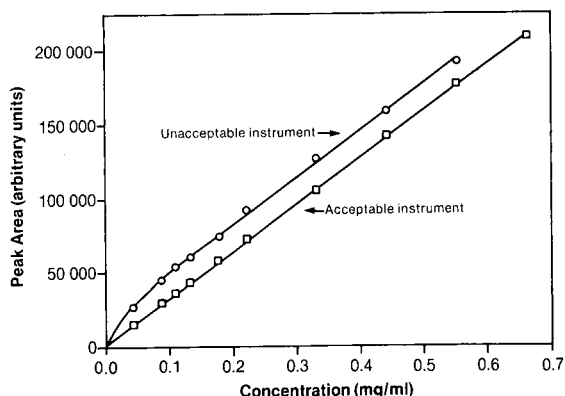


Fig. 8. Calibration curves from vinblastine standards prepared in water and run on an acceptable instrument and an unacceptable instrument.

absorptivities for vinblastine in water, pH 4 sodium acetate solution or HPLC mobile phase were equivalent whereas the absorptivity in the aqueous pH 7.5 diethylamine phosphate buffer was about 2% lower than any of the above solutions, probably because this solution is on the base side of the vinblastine pK_a . These minor absorptivity differences are thought to be masked in the HPLC method since all samples are eluted in mobile phase regardless of the initial sample solvent. Therefore, differences in UV absorptivity can be eliminated as the cause of the observed effect. Finally, fluorescence spectra of the mobile phase with and without vinblastine eliminated fluorescence as a factor contributing to the observed phenomena. Thus, further experiments focused on the matrix composition and instrument configuration.

The effect of mannitol concentration on vinblastine recovery was investigated on one acceptable instrument and one unacceptable instrument (Table IV). Vinblastine spiked with mannitol, ranging from 10 to 300 mg/ml, had comparable peak areas on both instruments. Thus, mannitol was eliminated as a source of the bias even though mannitol is the principal excipient and these solutions cover a wide range of viscosity.

TABLE IV
EFFECT OF MANNITOL CONCENTRATION ON VINBLASTINE RECOVERY

Mannitol concentration (mg/ml)	Vinblastine recovery (%)	
	Acceptable instrument	Unacceptable instrument
10	101	101
20	100	101
40	100	100
80	100	99
160	99	98
300	99	97

To determine the effect of the acetate, vinblastine samples at the normal acetate level were assayed *versus* aqueous standards. The acceptable instrument indicated 98% vinblastine recovery, while recovery on the unacceptable instrument was only 90%. Thus, acetate is a critical component in the matrix. However, the instrument dependence of this effect was still unknown.

The contribution of the various instrument components was investigated by sequentially interchanging the components of the acceptable instrument and the unacceptable instrument. The intercept-to-slope ratio of standard curves was used as the diagnostic parameter to detect changes in system performance. Table V is a compilation of the normalized intercept-to-slope ratios obtained for aqueous and matrix standards in this series of experiments. Changes in detectors produced no effect. However, the high intercept-to-slope ratio follows the sample injection system of the Varian instrument. Furthermore, when a Rheodyne 7125 manual injection valve was substituted, a normal intercept-to-slope ratio was obtained whereas manual injection using the Valco valve of that instrument produced a high intercept-to-slope ratio.

TABLE V

INTERCEPT-TO-SLOPE RATIOS FOR VINBLASTINE CALIBRATION CURVES AS A FUNCTION OF INSTRUMENT CONFIGURATION AND SAMPLE SOLVENT^a

Instrument configuration				Sample solvent	
Pump	Autosampler	Injection valve	Detector	Aqueous	Matrix
Varian 5000	Varian 8000	Valco	Dupont (1)	+ 0.18	+0.05
Varian 5000	Varian 8000	Valco	Dupont (2)	+ 0.18	+0.05
Varian 5000	—	Rheodyne 7125 ^b	Dupont (2)	+0.00	+0.00
Waters M6000A	Micromeritics	Rheodyne 7126	Dupont (2)	+0.04	+0.02
Waters M6000A	Micromeritics	Rheodyne 7126	Dupont (1)	+0.01	+0.03
Waters M6000A	—	Rheodyne 7125 ^b	Dupont (1)	-0.02	-0.00
Varian 5000	—	Rheodyne 7125 ^b	Dupont (1)	+0.02	+0.02
Waters M6000A	—	Rheodyne 7125 ^b	Dupont (2)	-0.01	+0.00
Varian 5000	Varian 8000	Valco	Dupont (1)	+ 0.21	+0.06
Waters M6000A	Micromeritics	Rheodyne 7126	Dupont (2)	+0.02	+0.07
Waters M6000A	Varian 8000	Valco	Dupont (2)	+ 0.22	—
Varian 5000	Varian 8000	Valco	Dupont (1)	+ 0.28	—
Varian 5000	—	Valco ^c	Dupont (1)	+ 0.16	—

^a Experiments reported in approximate chronological order for data obtained on two days. The original configurations were repeated at intervals to insure that the original behavior was still present. **Bold** entries denote standard curves with high intercept-to-slope ratios; +0 and -0 denote values between +0.005 and 0 or between -0.005 and 0, respectively.

^b The Rheodyne 7125 is a manual injection valve.

^c Denotes valve used in a manual mode.

Thus, the injection valve from this unacceptable instrument appears to be the primary source of high intercept-to-slope ratios.

The final experiment was designed to confirm the interaction between acetate level and instrument configuration. Vinblastine solutions were injected at various acetate levels using the Valco valve in an automatic and then in a manual mode on the acceptable instrument. The relative peak areas are summarized in Table VI. The decreased response of vinblastine as the acetate levels increased correlates with the use of this particular valve in both modes.

It should be noted that although the detailed experiments designed to isolate

TABLE VI

EFFECT OF ACETATE CONCENTRATION ON VINBLASTINE PEAK AREAS^a

Solution	Relative peak area (%)	
	Automatic injection	Manual injection
No acetate	100.0	100.0
1% Formulation acetate	97.6	97.4
10% Formulation acetate	92.7	95.2
100% Formulation acetate	90.5	92.7

^a Instrument is Varian 5000 pump, Dupont detector (1) and Valco valve operated in an automatic mode (Varian 8000 autosampler) or manual mode.

the cause of the high intercept-to-slope problem focused on permutations of components from two specific instruments, the effect has been observed with other instruments. However, there is no commonality in the configuration of the unacceptable instruments. Thus, no one component of the sample injection system can be assigned as the cause of this effect.

CONCLUSIONS

HPLC quantitation of the two compounds vancomycin and vinblastine appears to be highly dependent upon the sample solvent composition with some instruments. A constant true value for the peak area from vancomycin depends on the presence of a minimum ionic strength of the sample solvent, while the molar absorptivity was unchanged for all solvents studied. It was concluded that stock sample solutions diluted with water resulted in peak areas that were erroneously high. The presence and magnitude of the error appear to be instrument dependent. Errors in quantitation of vinblastine were detected by unacceptably high values for the normalized intercept-to-slope ratios of the calibration curves. Again, standards dissolved in water produced erroneously high peak areas on some instruments, correctable by the addition of acetate to the standard solvent. The instrument dependence of this phenomenon was systematically evaluated and a correlation to the sample injection system was found.

The observed sample-solvent interactions for these two compounds appear to be quite similar in their characteristics and their cause. However, the errors in quantitation produced by these effects were not discovered until considerable experimental evaluation was performed. Full explanations for the described observations have not been discovered, but experimental work is continuing towards that end. These observations may be similar to those described by Perlman and Kirschbaum^{3,8,9}. Certainly these results reinforce the belief that changes in HPLC peak areas due to sample-solvent interactions unrelated to classical injected-solvent effects are not uncommon.

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ELECTRON-CAPTURE DETECTOR WITH AN ION SIGNAL

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SUMMARY

A design of an electron capture detector equipped with two anodes is introduced. The conditions of the detector supply which result in a signal at the first anode of electron nature and a signal at the second anode of ion nature were defined. This construction enables one to design an electronic system which limits the influence of fluctuations in temperature and pressure on the value of the basic current of the detector.

INTRODUCTION

The detection of electronegative compounds in gas chromatography is based on the utilization of the electron capture phenomenon in the ionization chamber, *i.e.*, in the electron capture detector. The process of electron capture leads both to a decrease in the number of free electrons and an increase in the number of negative ions in the volume of the detector. The quantitative detection of the electronegative component reacting with electrons in the process of capture can be carried out by means of measurements of the current of negative ions, the current of electrons or the sum of both these currents.

At present, the second method of the detection is generally applied because it has the highest sensitivity. The duration of the voltage pulse creating an electric field between the detector electrodes is chosen such that only electrons reach the anode.

A dependence of the detector ionization current on the duration of the voltage pulse, t_p , supplying the detector shows whether we are dealing with measurements of the electron current only or of the electron and ion currents. If the ionization current is constant above a certain pulse width, t_p , then only electrons are collected by the anode in this region. If this characteristic has a certain inclination, then the measured detector ionization current has both electron and ion components. The first case can easily be fulfilled in a detector supplied with an argon–methane mixture. Due to the high mobility of electrons, the voltage pulse of duration from 0.1 to 1 μ s helps to collect the

electrons in the detector and the characteristic described above has a range of saturation. In the case of a detector supplied with nitrogen in which electrons have a decisively lower mobility, the curve describing the dependence of the ionization current on the pulse duration, t_p , does not have a distinct refraction that would indicate a flow of two kinds of electric charges having different mobilities, *i.e.*, electrons and negative ions to the anode. This fact is not of great significance as regards the detection sensitivity which is still high even when the voltage pulses supplying the detector are between 1 and 100 μ s. The improbability of accurate determination of the detector supply conditions where only the electron current is measured makes difficult an interpretation of the measurements especially when using mathematical models of the detector as a chemical reactor.

In this paper a new constructional type of electron-capture detector and its supply with a pulse voltage helping to measure the electron and ion components of the detector ionization current are presented. The possibility of the measurement of both components of the detector ionization current is of assistance when investigating the participation of the ion current in the measured detector signal, and the observation of changes in the electron and negative ion charges caused by the presence of molecules of the sample in the detector. It has been observed that changes in the pressure of the carrier gas and detector temperature cause opposite changes in the ion and electron currents, which has enabled the construction of a system of improved stability.

DESCRIPTION OF THE DETECTOR AND SUPPLY SYSTEM

Fig. 1 shows the construction of the electron-capture detector. The detector consists of a body (4) in which a radioactive source (3) (22 mm $\times$ 10 mm I.D., activity of ^{63}Ni up to 5 mCi) is situated. Two anodes (1 and 2) connected with generators of rectangular pulses by the capacitors are placed concentrically inside the radioactive source.

Fig. 2a presents the system of supplying the detector with pulse voltages and the method of measurement of the ionization current of both anodes. Fig. 2b shows the shape and time course of the voltage pulses from the pulse generators (1 and 2). The pulse amplitude is 50 V while the duration of the pulse supplying anode 1 is t_1 and that

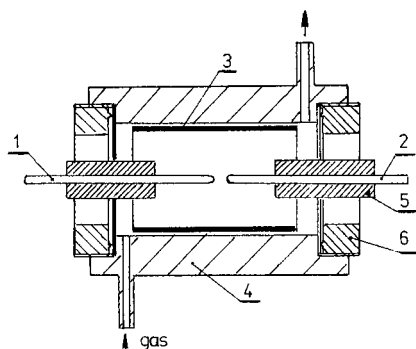


Fig. 1. The design of the electron-capture detector with two anodes (1 and 2), radioactive source (3), detector body (4), ceramic insulator (5) and nut (6).

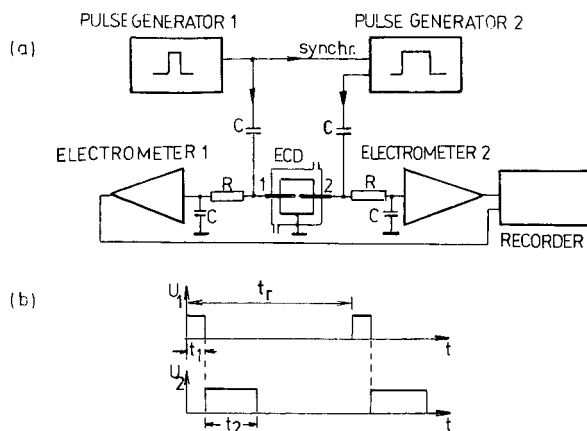


Fig. 2. (a) System of supply and measurement of the detector signal. (b) Time courses of the voltage pulses supplying anode 1, of amplitude U_1 and duration t_1 , and supplying anode 2, of amplitude U_2 and duration t_2 . ECD = Electron-capture detector; C = capacitor; R = resistor.

of anode 2 is t_2 . These durations can be regulated independently within the range from 1 to several hundred μ s. The generators of pulses 1 and 2 are synchronized such that the trailing edge of the pulse t_1 initiates the pulse t_2 supplying electrode 2. Generator 1 operates as described¹ and helps to measure automatically the dependence of the ionization current of anode 1 on the repetition time of the pulse, t_r . Since generator 2 is synchronized with generator 1, the measurement of the ionization current of anode 2 can also be made automatic as a function of the repetition time of the pulses supplying this electrode.

In the course of measurements the detector was supplied with nitrogen as the carrier gas (contamination level of *ca.* 1 ppm). The detector signal for strongly negative halogenated hydrocarbons separated on a chromatographic column of length 1 m packed with 10% DC-200 on 100–200 mesh Chromosorb W has been investigated.

RESULTS

The measurement of the electron component of the detector ionization current by one anode and the ion component by the other anode was the objective of the construction of the detector with two anodes supplied with voltage pulses of different durations. It was assumed then that the first pulse will collect a rapidly moving electron charge. The other pulse ought to collect the slower negative ions that were not collected during the short first pulse. It seems that all the electrons will not be collected by the first pulse, but their participation in the current of the second electrode will be less than that of the anions.

The collection properties of the two electrodes was compared in such a way that both electrodes were supplied with a pulse voltage of the same duration, *i.e.*, $t_1 = t_2$ and with subsequent pulses of duration $2t_1$, or they were combined in parallel and treated in this way as one electrode. Fig. 3 presents current characteristics corresponding to different variations of supplying the detector with pulses of duration $t_1 =$

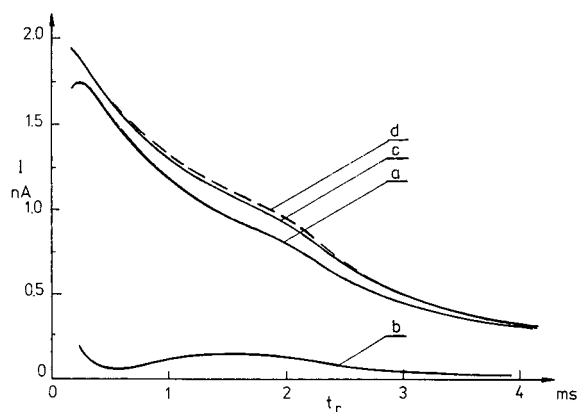


Fig. 3. The dependence of the ionization current of the detector on the pulse repetition for different ways of detector supply. See the text.

$t_2 = 20 \mu\text{s}$. Curve a represents the detector current characteristic of anode 1 which is supplied with the first voltage pulse and curve b is the ionization current of anode 2 supplied with a second pulse immediately after the collection of charge by anode 1. It is seen that a decisively greater charge is collected by anode 1. Anode 2 collects a considerably smaller charge which is evidence of the charge exhaustion, *i.e.*, of the nature of the source from which it arises. The detector is completely symmetrical, *i.e.*, inversion of the sequence of supplying the anodes does not influence the courses of the current characteristics a and b in Fig. 3. The fact that the sum of the currents measured by both anodes supplied successively by pulses of duration $20 \mu\text{s}$ equals the current collected by one anode supplied with a voltage pulse of duration $40 \mu\text{s}$ by (see curve c in Fig. 3) is evidence for the equivalence of the performances of the two anodes. When the two electrodes were connected together and supplied with voltage pulses of $40 \mu\text{s}$, the ionization current collected was as shown by curve d in Fig. 3.

From the characteristics presented in Fig. 3 it follows that electrodes 1 and 2 supplied successively with voltage pulses of $20 \mu\text{s}$ collect practically the whole of the available electric charge in the detector.

The influence of the pulse duration on the value of the charge collected is presented in Fig. 4. During the measurements both detector anodes were connected together. The pulses supplied were repeated every $320 \mu\text{s}$ (Fig. 4b) or $4000 \mu\text{s}$ (Fig. 4a) at the temperatures shown. An increase in the detector temperature resulted in an increase in the charge collected. This can be accounted for by the process of detachment of electrons from molecules of oxygen which is a contaminant of the carrier gas². When the repetition time, t_r , is $320 \mu\text{s}$ the charge collected reaches saturation for pulse durations greater than $20 \mu\text{s}$. For long repetitions (Fig. 4a) the saturation state is not obtained within the range of durations of the pulses, t_1 , applied. This makes the determination of t_1 at least difficult. From Fig. 4a it follows that the collection of charges occurs more slowly for long repetition times, what can be accounted for by the origin of the plasma and by a decrease in the electron mobility^{3,4} in the long voltageless state in the detector.

The influence of different times t_1 at a constant value of t_2 on the course of the

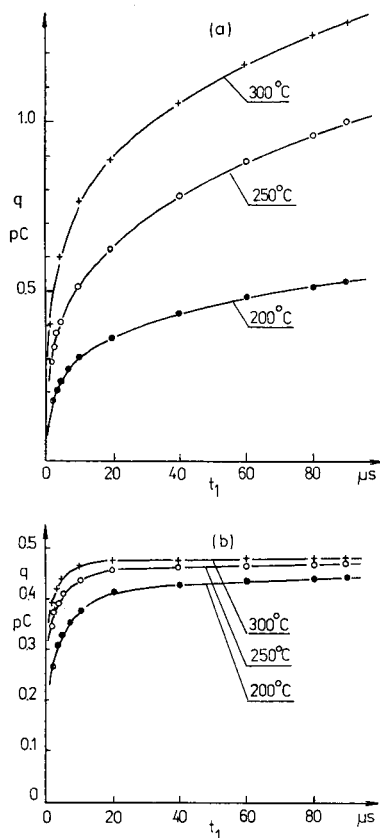


Fig. 4. The dependence of the charge collected by two detector electrodes joined together on the duration of the pulses supplied. Repetition times, t_r : 4000 (a) and 320 μs (b). The detector temperatures are given.

detector current characteristics is presented in Fig. 5. The characteristics I_1 and I_2 are the ionization currents measured by anodes 1 and 2 respectively. The dashed curves represent the ratio I_2/I_1 . Analyzing the course of the characteristics in Fig. 5, the following effect can be observed. A pulse of duration $t_1 = 3 \mu s$ collects only the electron component. On the other hand, a pulse applied to the other anode and lasting 120 μs collects negative ions and electrons, and the maximum current occurs for a repetition time of about 1200 μs (Fig. 5a). An increase in t_2 up to 9 μs results in an increase in the current collected by the first anode and the second anode collects the remaining electrons and negative ions, and the maximum current occurs at a repetition time of 1000 μs . A pulse of duration 90 μs at the first electrode collects practically all the electric charges in the detector, while the curve I_2 in Fig. 5c represents, most probably, the falling parts of the curves I_2 of Fig. 5a and b. The ratio I_2/I_1 is almost constant for repetition times over 2000 μs . For shorter repetition times this ratio is not uniquely determined because of the complex character of the factors influencing the values of the currents collected by both anodes.

The measurements the results of which are presented in Figs. 4 and 5 do not

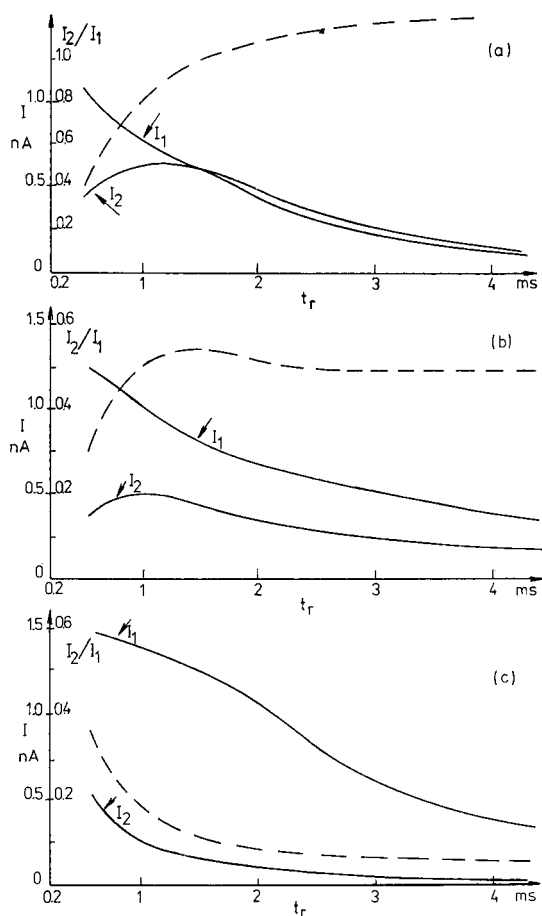


Fig. 5. Dependence of the ionization currents at anodes 1 and 2 on the repetition time, $t_1 = 3$ (a), 9 (b) and 90 μ s (c); $t_2 = 120$ μ s. ---, I_2/I_1 .

allow an unique determination of the parameters of the pulse supplying anodes 1 and 2 which would collect the electron and ion components of the detector ionization current. We tried to determine these conditions by measuring the polarity of the observed signal in the measurement circuit of anodes 1 and 2 caused by the sample introduced in the detector. If anode 1 collects electrons, then the sample introduced in the detector ought to cause a decrease in the current in its measuring circuit, *i.e.*, a signal of negative polarity. On the other hand, the signal ought to be of positive polarity in the measurement circuit of the second anode if it collects the anions. A small concentration of oxygen was introduced in the detector. It caused several percentage decrease in the ionization current. The signals of both anodes for different pulse durations, t_1 and t_2 , were observed. The charges collected in the measurement configurations of both anodes are denoted by Δq_1 and Δq_2 respectively. Exemplary results are shown in Fig. 6. It was observed that the signal Δq_1 is always of negative polarity. The polarity of the signal Δq_2 depends on the configuration of the parameters

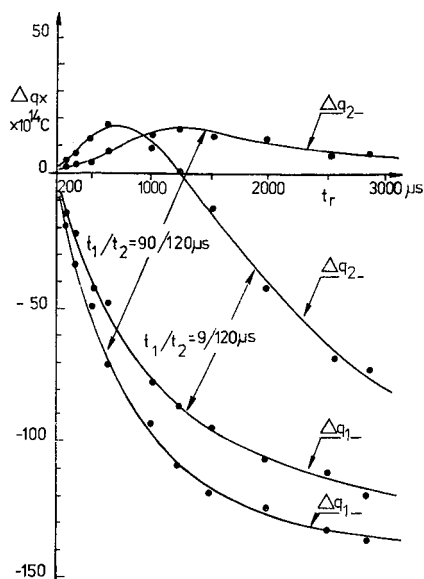


Fig. 6. The dependence of the signal at anode 1, Δq_1 , and at anode 2, Δq_2 , for the given pulse durations t_1 and t_2 on the pulse repetition for a constant oxygen concentration in the detector.

t_1 and t_2 and the repetition time, t_r . For $t_1/t_2 = 9/120 \mu s$, Δq_2 is of positive polarity for repetition periods from 200 to 1200 μs , i.e., in the period for which the current I_2 of Fig. 5b has a bulge. For $t_1/t_2 = 90/120 \mu s$ and the whole range of repetition time (from 200 to 3000 μs), Δq_2 has a positive polarity. Thus the curve I_2 in Fig. 5c refers to the ion current.

In order to determine optimum detection conditions, the times t_1 and t_2 were changed so that the signals of both anodes were of the opposite polarity and their ratio, $p = |\Delta q_2/\Delta q_1|$, was maximized. Fig. 7 shows the dependence of the absolute values of

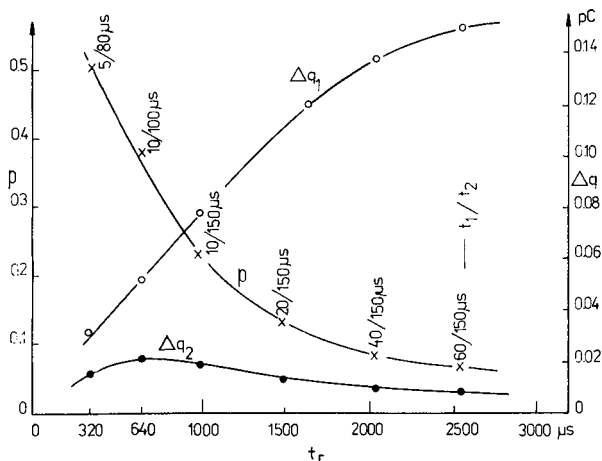


Fig. 7. The dependence of the electron signal, Δq_1 , and of the ion signal, Δq_2 , and the ratio, $P = |\Delta q_2/\Delta q_1|$ on the pulse repetition time for an oxygen sample. The values of t_1 and t_2 for which p was defined are marked on curve p .

Δq_1 and Δq_2 as well as their ratio, p , on the repetition time, t_r . The values t_1 and t_2 for which p was defined are marked on the curve p . It is seen that for short repetition times, t_r , the ion signal may reach 50% of the electron signal, and as t_r increases this proportion diminishes in favour of the electron signal. It was stated that for $t_1 < 3 \mu\text{s}$ the signal Δq_2 is of negative polarity, which means that anode 2 collects a lot of electrons and their loss connected with the presence of the sample molecules in the detector is bigger than the increase in the negative ions collected by this anode. It can also be stated that the proportion of the signals of both anodes decreases simultaneously with the filling factor of the pulsed voltage, defined as $k = (t_1 + t_2)/t_r$.

DISCUSSION

The results of the measurements presented in Fig. 7 prompt the question: can we treat the signals measured by anodes 1 and 2 as the electron and ion signal. Is it possible to measure the negative ion current in the detector supplied by a pulsed voltage if these ions move at least 1000 times more slowly than the electrons in the electric field of the detector. Maybe both signals are of electron character and the change of the signal polarity at anode 2 is connected with the change in the electron drift velocity, caused by the space charge that occurs in the detector.

The problem of the collection of anions was studied by Wells⁵ in order to explain the limitation of the linearity range in the constant-current system of the detector. He stated that the high concentration of the sample tested that accompanied the high frequency of pulses at the end of the measurement range caused the collection, by the anode, also of the negative ions.

The attempt to estimate the probability of the collection of the anions by the detector electrodes introduced below will allow us to conclude that the signal observed in the measurement system at anode 2 is the ion signal.

The conversion of the electron in the capture process into anions means the loss of the free charge observed in the detector where only the electrons would constitute the measured current. The pulsed electric field that occurs in cycles between the detector electrodes will cause a step drift of the ion in the direction of the anode and if recombination does not take place then the amount of electrons collected will not change in spite of the capture process that occurs in the detector.

It can be assumed that at the time $t = 0$ a certain number of anions, A_0 , occurs in the detector which has a plate-parallel geometry. Moreover, it can be assumed that for the correspondingly low anion concentration, there will be no change in the positive ion concentration, p . If we disregard the ion diffusion in the time between the pulses of uniform electric field, the number of ion jumps necessary to reach the anode, if at time $t = 0$ the distance between this ion and the anode is x , will be

$$n(x) = xL/U\mu_j t_p \quad (1)$$

where U is the amplitude of the voltage pulse in V, μ_j is the mobility of the negative ion in $\text{m}^2/\text{V} \cdot \text{s}$, t_p is the duration of the voltage pulse in s and L is the distance between the anode and cathode in m. The total residence time of the anion in the detector equals $n(x)t_r$, where t_r is the repetition time of the pulses supplied. The rate of loss of the anion in the ion-ion recombination process is represented by

$$da/dt = -\alpha_c a p \quad (2)$$

where α_e is the rate constant for the recombination of negative ions with positive ones in m^3/s , a and p are the concentrations of negative and positive ions respectively, in ions/m^3 . After integrating the dependence 2 in the range from $t = 0$ to $t = n(x)t_r$ we obtain the anion concentration in the layer of thickness dx drifting from the distance x to the anode

$$a_x = A_0/V \cdot \exp[-\alpha_e p n(x)t_r] \quad (3)$$

where V is the detector volume.

The layer considered contained initially $dA_0 = A_0 dx/L$ of the anions, of which

$$dA_x = (A_0/L) \cdot \exp[-\alpha_e p n(x)t_r] dx \quad (4)$$

reach the anode. From the whole detector volume the anode will be reached by

$$A_F = \int_0^L dA_x dx \text{ anions, and after integration of eqn. 4 the fraction of their initial}$$

amount in the detector is:

$$\frac{A_F}{A_0} = \frac{U\mu_j t_p}{\alpha_e p L^2 t_r} \left[1 - \exp(-\alpha_e t_r L^2 / U\mu_j t_p) \right] \quad (5)$$

A similar treatment carried out for a detector of cylindrical electrode geometry, where the anode and cathode have radii a and b respectively, leads to the following dependence defining the number of the negative ion jumps from the location r to the anode

$$n(r) = \frac{(r^2 - a^2)}{2U\mu_j t_p} \ln(b/a) \quad (6)$$

and the relative number of negative ions collected is

$$\frac{A_F}{A_0} = \frac{2\{1 - \exp[-z(b - a)^2] \cdot \exp(za^2)\}}{z(b^2 - a^2)} \quad (7)$$

where:

$$z = \frac{\alpha_e p t_r \cdot \ln(b/a)}{2U\mu_j t_p}$$

Calculations of A_F/A_0 were done for the following values of the parameters that occur in eqns. 5 and 7: $\mu_i = 3.3 \cdot 10^{-4} \text{ m}^2/\text{V} \cdot \text{s}$, $\alpha_e = 3 \cdot 10^{-12} \text{ m}^3/\text{s}$; ref. 6, $t_r = 1.5 \cdot 10^{-3} \text{ s}$, $t_p = t_1 + t_2 = 20 \mu\text{s} + 150 \mu\text{s} = 1.7 \cdot 10^{-4} \text{ s}$ (which gives the filling factor $k = t_p/t_r = 0.11$); $U = 50 \text{ V}$. For plane geometry, $L = 4.5 \cdot 10^{-3} \text{ m}$; for cylindrical geometry, $a = 0.5 \cdot 10^{-3} \text{ m}$, $b = 5 \cdot 10^{-3} \text{ m}$. For the detector saturation current $I_0 = 3 \cdot 10^{-9} \text{ A}$, the rate of generation of positive ions equals $1.78 \cdot 10^{10}/\text{s}$ and

their concentration in the detector volume, $V = 10^{-6} \text{ m}^3$, is $p = 0.78 \cdot 10^{14}/\text{m}^3$. The proportion A_F/A_0 calculated is 0.37 for plane geometry and 0.56 for cylindrical geometry, and is a measure of the probability of collection of the anions formed in the electron capture process. This probability depends to a great extent on the residence time of the ion in the detector which depends in turn on the filling factor of the pulsed voltage and the drift length. Both for the plane and cylindrical geometries the calculated probability of the negative ion collection is estimated to be large.

The initial number of anions, A_0 , equals the decrease in the number of free electrons in the detector as a result of their capture by the sample molecules. It can be assumed that the specifically defined ratio A_F/A_0 represents the ratio of the signals of both electrodes if one of them collects the electrons only and the other the anions.

The experimental values of the ratio of the electrode signals are lower than the ones calculated as above. For example, for the repetition time, $t_r = 1500 \mu\text{s}$ the value $p = 0.13$ while the calculated ratio $A_F/A_0 = 0.56$. This discrepancy may result from the longer distance and by the same token greater number of jumps required in the direction of the collecting electrodes under the influence of the electric field effected by the pulse applied to both anodes. In consequence, the ion's path is not along the detector radius but is more complex. The field of the space charge entails deceleration of the ion in the direction of the anodes.

Influence of the pressure and temperature changes on the signals of both electrodes

Fig. 8 represents an example of the analysis of halogenated hydrocarbons. The chromatograms designated S1 and S2 were obtained in the measuring circuit of anodes 1 and 2, respectively. Fig. 8a presents the chromatograms of the liquid sample of halogenated hydrocarbons into which 2 cm^3 of air were injected in order to obtain the

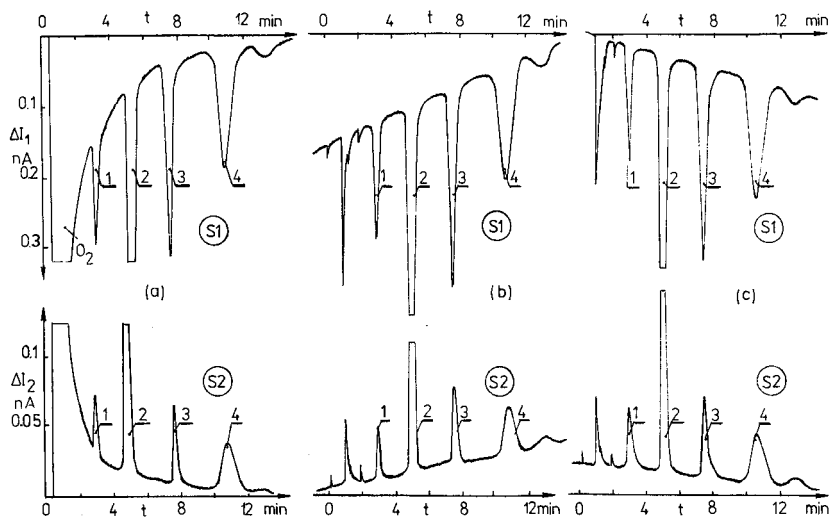


Fig. 8. Chromatograms obtained in the measurement of the signal of electrode 1, S1, and electrode 2, S2. Peaks: 1 = CHCl_3 ; 2 = CH_3CCl_3 ; 3 = CCl_4 ; 4 = C_2HCl_3 . The drift of zero line was caused by: (a) contamination of the carrier gas by oxygen; (b) change in carrier gas pressure in the detector; (c) change in the detector temperature.

drift of the zero line. It is seen that at both anodes the contamination of the signal of the carrier gas by oxygen decreases with time. Fig. 8b presents chromatograms where the zero line drift is caused by a change in gas pressure in the detector obtained by connecting its outlet to a closed vessel. It is seen that an increase in gas pressure results in a change in the ionization current at both anodes in the same directions. Fig. 8c presents chromatograms where the drift of the zero line is caused by a change in the temperature of the detector obtained by switching on the thermostat. In this case the changes in the current at both electrodes are of opposite sign.

The influence of the temperature and pressure on the direction of the changes in the ionization currents at anodes 1 and 2 introduced above may be used for constructing a system that demonstrates the stability of the detector operation. Fig. 9 presents an example of the operation of such a system. In Fig. 9a the signal of anode 1 for a vestigial quantity of CCl_4 and C_2HCl_3 is shown, while Fig. 9b represents the signal at anode 2 for the same sample. Fig. 9c shows the signal at anode 2 when it is five times amplified, while Fig. 9d represents the difference between the signals in 9a and c. In consequence, a readable and stable detector signal was obtained.

The utilization of the two signals of the detector equipped with two collecting electrodes stabilizing its operation is possible for pulse parameters such that the signal polarities of the electrodes have opposite signs.

The chromatograms in Fig. 8 are very similar to bipolar chromatograms presented by McMahon and Aue⁷. The possibility of obtaining a positive signal from an electron-capture detector was suggested by Aue *et al.*⁸. These authors used a three-chamber detector with a bifurcated current flow to two anodes. One anode was exposed to the gas chromatographic effluent and gave a negative signal. The second anode, not exposed to the effluent, produced a positive signal. Signals of opposite polarity from the two electrodes were generated owing to the space charge effect.

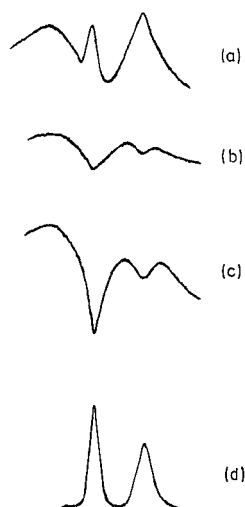


Fig. 9. Illustration of the stability of the basic current of the detector. (a) Electron signal of electrode 1 for vestigial concentration of CCl_4 and C_2HCl_3 . (b) Ion signal of electrode 2 for the same sample. (c) Ion signal amplified. (d) Difference between signals a and c.

Our chromatograms were obtained in a different way to the method described above. We used an one-chamber detector in which both anodes were identically exposed to the column effluent and placed close to each other. Signals of opposite polarity are generated owing to a properly selected sequence and width of the pulses. Theoretical relationships for the detector signal are based on the assumed homogeneity of the detector (reactor model). We expect that charge effects described in many works, e.g. refs. 8–11, may influence the signals. A quantitative description of space charge effects is difficult owing to the lack of exact spatial solutions of the Poisson equation for a detector cell working in the pulse mode.

CONCLUSIONS

Providing an electron-capture detector with two anodes and the application of a suitable system for supplying these anodes with pulsed voltages enables us to obtain two signals, one having the character of an electron signal, the other of an ion signal.

In spite of its research purpose, the detector described makes possible the construction of a system eliminating the influence of the pressure fluctuation on the detector basic current, and by the same token the improvement of the stability of its operation.

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HIGH-SENSITIVITY MICRO ULTRAVIOLET ABSORPTION DETECTOR FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A UV absorption detector with a 0.6- μ l flow cell for high-performance liquid chromatography (HPLC) was developed. In order to improve the signal-to-noise ratio when the cell volume is reduced, the flow cell has a reflective layer on the internal wall of the optical path. The cell transmittance is hardly affected by changes in solvent conditions, based on a pulse flow from a pump. The flow cell can enhance sensitivity in the low-volume UV detector by making it possible to reduce the baseline shift and noise which are caused by variations of solvent conditions. Extra-column dispersion in the detector flow cell is reduced at high flow-rates of over 0.5 ml/min. The detector is especially effective for fast and micro-scale HPLC.

INTRODUCTION

Recently, high-performance liquid chromatographic (HPLC) methods with packing particles smaller than 5 μ m and microbore columns have been developed for rapid analysis. However, it is necessary to decrease the extra-column dispersion that occurs outside the column when these smaller packings and microbore columns are used. A UV detector with a small flow cell and a new flow cell structure developed for this purpose was described in previous papers^{1–5}. Our aim is the effective use of columns of length 10–50 mm and I.D. 2–3 mm, packed with 1–3- μ m particles. Theoretically, a detector with a cell volume of less than 1 μ l is required for such small packings⁶.

In the design of the UV detector cell, it is necessary to minimize extra-column dispersion while maintaining sensitivity. It is difficult to minimize the cell volume in a simple structure, as the sensitivity of the UV detector depends on the Lambert–Beer law. In order to maintain sensitivity, the cell diameter should be reduced while keeping a long optical path. However, reducing the cell diameter makes the light flux smaller and results in noisier signals. In fact, our preliminary experiments showed that the signal-to-noise ratio was decreased on reducing the cell diameter and a baseline shift appeared based on changes in light energy due to refractive index (RI) phenomena of the solvent. The RI change occurs as the result of poor flow cell design and changes with the solvent flow, temperature, etc. and it causes light rays to bend in

the cell. In general, the cell wall is opaque to UV light and therefore part of the incident light to the cell is lost by absorption and scattering on the internal wall. The changes in cell transmittance are caused by variations in the loss on the internal wall due to changes in RI; hence the development of a low-volume UV detector cannot be achieved by simply miniaturizing current commercial instruments, but rather reduction of the light absorption and scattering in the cell must be realized.

In a current commercial instrument, the flow cell has a reflective surface, which is a polished surface of a bright metal⁷. However, the reflectance of the polished surface is worse than that of an evaporated surface⁸. In this paper, we describe a new micro flow cell with a reflective layer formed by an evaporation method to reduce the influence on RI changes.

EXPERIMENTAL

Detector flow cell

Three types of flow cells were designed for the UV detector, which had an I.D. of 0.5 mm for a 3-mm path length. The total volume of each flow cell was 0.6 μ l.

The first cell was made by drilling out a quartz block to make the necessary flow channels and fitting quartz windows in the ends of the block. The second was made in the same way, except that the internal wall was polished. The third had a reflective layer on the internal wall of the cell optical path. More specifically, this cell was composed of two plate-shaped flow cell bodies provided with a light path having a reflective layer, inlet and outlet passages and a pair of quartz windows on the side of the cell bodies. The reflective layer along the light path was formed by thermal evaporation or electron beam evaporation before the cell bodies were joined. A schematic diagram of the third cell is shown in Fig. 1.

The performances of the flow cells were compared with those of conventional semimicro flow cells of 1.0 or 1.1 mm I.D. for a 3-mm path length (cell volumes 2.4 or 3.0 μ l, respectively) with a slit width of 0.5 mm I.D.

Apparatus

A Hitachi 655A-12 liquid chromatograph was used. The mobile phase was passed by the pump to a sample injector (Tokyorika 5001) and through a column (50 mm $\times$ 4 mm I.D.) packed with 2- μ m glass beads. The sample injector was connected directly to a modified Hitachi 655-51 UV monitor without a column. The output from the UV detector was fed to an analysing recorder (YEW Model 3655).

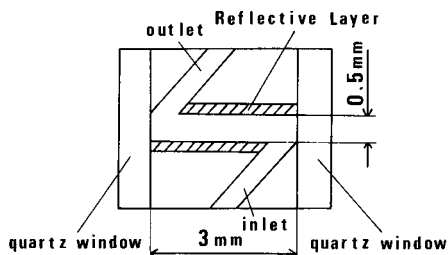


Fig. 1. Schematic diagram of flow cell.

The sensitivity of a UV monitor is dependent on the magnitude of the change in the cell transmittance due to changes in the RI of the solvent in the flow cell. Therefore, we measured the absorbance change that appeared as a baseline shift according to the stepwise change in the flow-rate. The sensitivity of the UV detector was evaluated from the magnitude of the baseline shift due to the change in flow-rate.

Methanol was used as the eluent, into which 0.1 μl of 0.5% (v/v) benzene was injected. Practical measurements of peak dispersion were carried out without a separation column. The problem in the experiment was that the band profiles to be determined were very narrow. As most of the measurements have to be carried out in less than 1 s, we used a system that had an overall time constant of 10 ms for the detector electronics and a sampling rate of 1 ms with a 1-kHz low-pass filter for the recorder. The dispersion was determined by the standard deviation of a concentration profile, taking the width at 60.7% of the maximum height.

RESULTS AND DISCUSSION

The first aim of the study was to obtain a detector cell with a volume of 0.6 μl . We wanted a structure of the micro flow cell for the UV detector that would be suitable for fast or micro HPLC. In order to identify the problems that occur on reducing the cell volume, the first micro flow cell was made by drilling out a quartz block according to the conventional method. This first cell was a Z-type of the same shape as a conventional Hitachi semimicro flow cell (3.0 μl). The magnitude of the baseline shift was measured quantitatively for flow cells of 0.5 and 1.0 mm I.D. diameter by the stepwise changes in the flow-rate. The results are shown in Fig. 2. The slope of the baseline shift for the 0.5-mm I.D. flow cell was about three times that for the 1.0-mm I.D. flow cell. Reduction in the cell diameter resulted in an increase in the ratio of the absorbed and scattered light on the internal wall to the incident light. Therefore, the baseline shift for the smaller diameter flow cell might appear even as a result of a slight change in the solvent conditions. This led to greater noise and drift, resulting in low sensitivity and instability in the analysis.

We then tried to reduce the baseline shift by setting a slit in front of the cell. It was expected that the slit might be effective in preventing the incident light from striking the internal wall, even when RI changes of the solvent would cause the light to bend. The effect of the slit was evaluated first with the 1.0-mm I.D. flow cell with a

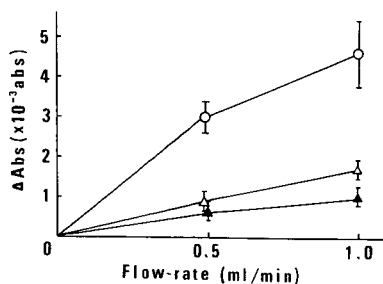


Fig. 2. Effect of flow-rate change on baseline shift. ○, 0.6 μl (0.5-mm I.D., 3-mm path length); Δ, 2.4 μl (1.0-mm I.D., 3-mm path length), ▲, 2.4 μl (1.0-mm I.D., 3-mm path length, with 0.5-mm I.D. slit).

0.5-mm I.D. slit. The magnitude of the baseline shift for this flow cell decreased to about 60% of that without the slit. However, reducing the cell diameter to 0.5 from 1.0 mm I.D. caused a larger baseline shift. Therefore, application of the slit was inadequate for a flow cell of less than 0.5 mm I.D. In addition, it became increasingly difficult to make straight, narrow-bore holes by drilling into quartz glass or even into plastics when the path length was greater than ten times the diameter. Fabrication by drilling may have limitations with regard to usable dimensions when a parallel-type flow cell is designed. Therefore, it is difficult to make a high-sensitivity flow cell smaller than 0.1 μl by using a conventional manufacturing method.

It is necessary to decrease the baseline shift to maintain the sensitivity when the cell volume is reduced by minimizing the diameter. In general, the cell wall is opaque to UV light and therefore the light strikes the cell wall due to RI changes and is absorbed or scattered. The loss in the cell must be decreased to maintain sensitivity, and consequently a new flow cell was needed with a reflective layer to reduce the changes in cell transmittance. In a current commercial instrument, a flow cell with a polished surface of a highly reflective material is applied. However, the reflectance of the evaporated surface used in this study is higher than that of a polished or electroplated surface in the same material⁸. We used an aluminium or rhodium layer as the reflective layer because of their high reflectance of UV light⁹. When using the aluminium layer, a silicon dioxide layer was formed on the reflective layer by electron beam evaporation to prevent corrosion on the reflective surface. When the flow cell was used under the usual conditions of reversed-phase chromatography, no reflectance change was observed.

The magnitude of the baseline shift with stepwise flow-rate changes is shown in Fig. 3 for different the flow cells. The baseline shift for the flow cell having a polished internal cell wall was about 50% of that of the untreated cell. This meant that the polished cell wall could enhance the sensitivity in the micro flow cell. Roughness of the internal wall seemed to produce a significant enhancement of the effects of RI changes of the solvent, even with only a slight change in flow-rate. The baseline shift with the flow cell having an aluminium or rhodium reflective layer decreased to about 80% of that with the untreated flow cell. In the flow cell having the reflective layer, it seemed that part of the incident light that did not pass straight along the light-path

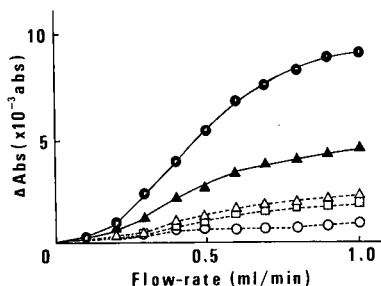


Fig. 3. Effect of flow-rate change on baseline shift. ●, 0.6 μl (0.5-mm I.D., 3-mm path length, untreated); ▲, 0.6 μl (0.5-mm I.D., 3-mm path length, polished); △, 0.6 μl (0.5-mm I.D., 3-mm path length, Rh-coated), □, 0.6 μl (0.5-mm I.D., 3-mm path length, Al-coated); ○, 3.0 μl (1.1-mm I.D., 3-mm path length, with 0.5-mm I.D. slit)

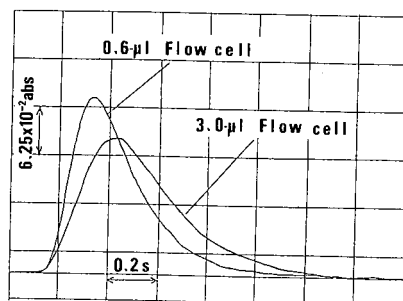
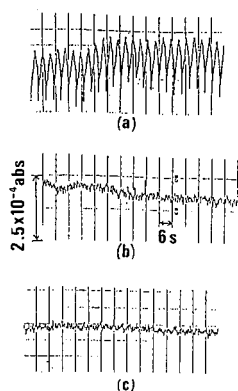


Fig. 4. Noise on chromatograms. (a) 0.6 μ l (0.5-mm I.D., 3-mm path length, untreated); (b) 0.6 μ l (0.5-mm I.D., 3-mm path length, Al-coated); (c) 3.0 μ l (1.1-mm I.D., 3-mm path length, with 0.5-mm I.D. slit). Flow-rate, 1.0 ml/min; eluent, methanol.

Fig. 5. Variance contribution by UV detector cells. Sample volume, 0.1 μ l; flow-rate, 1.0 ml/min; solute, benzene; eluent, methanol.

axis was reflected by the internal wall. Therefore, the ratio of the loss by absorption and scattering on the internal wall to the incident light was hardly changed, resulting in a very small baseline shift. Consequently, lower noise and high sensitivity and stability could be obtained with the flow cell having the reflective layer.

The noise levels on some chromatograms are shown in Fig. 4. The noise level on the chromatogram obtained with the flow cell having an aluminium or rhodium layer was as low as that with the conventional semimicro 3.0- μ l (1.1 mm I.D. for a 3.0-mm path length) flow cell with a 0.5-mm I.D. slit. Therefore, the new micro flow cell seemed to be effective when applied with micro-bore columns or small particle columns.

The effect of reducing the cell volume on the band dispersion is shown in Fig. 5. The concentration profiles were obtained by detecting benzene eluted at a flow-rate of 1 ml/min. The reduction in cell volume had an effect on the band width. The relationship between band dispersion and flow-rate is shown in Fig. 6. The effect of the reduction in cell volume was clearly apparent at flow-rates above 0.5 ml/min. The qualitative dependence of dispersion on various parameters, especially diffusion coef-

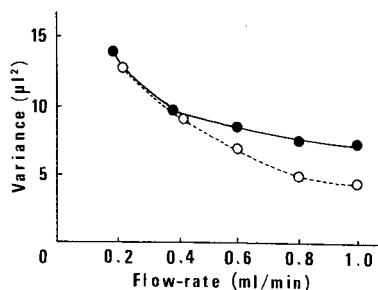


Fig. 6. Effect of flow-rate on variance. ●, 3.0- μ l flow cell; ○, 0.6- μ l flow cell.

ficient and flow-rate, is given by the Golay equation¹⁰. However, the present results showed that the dispersion decreased with increasing flow-rate, contrary to the theoretical prediction. The present method did not take into account the band broadening in the connectors and the injector. The theory suggested that such band broadening might be negligible, being much smaller than that in the detector cell. Hupe *et al.*¹¹ reported the same dispersion behaviour as ours and the major source of the band broadening might be the injection procedure. The shape of the curves in Fig. 6 suggested that a laminar flow profile existed at low flow-rates and might be destroyed by a secondary flow at higher flow-rates. If the flow profile in a cell is similar to that in an open tube, the flow profile in the Z-type flow cell in the main channel should be of the Poiseuille type, and including secondary flow or turbulence in the channel connections. However, there is no theoretical explanation regarding dispersion in a flow cell or even a zig-zag tube, because the relevant Navier–Stokes differential equations for complex geometrical forms have not been solved.

CONCLUSION

The UV detector with a 0.6- μ l flow cell having a metallic reflective layer shows high sensitivity and stability. The results showed that a baseline shift was almost completely absent with variations in the solvent conditions and flow-rate. Therefore, the new flow cell should be useful for fast and micro liquid chromatography using small particle packings or narrow-bore columns.

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CALCULATION OF THE CHARACTERISTICS OF THE BONDED LAYERS OF REVERSED STATIONARY PHASES

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SUMMARY

A new stationary phase parameter, R , the percentage of rigid structure, was introduced. It will be determined by the structural characteristics of the initial support and the modifier employed. This parameter will permit the characterization of any reversed stationary phase more completely and its relation to other types of bonded layer structure. Reproducibility is expected only for stationary phases with similar structures of the bonded layer. The carbon content, bonding density and R were calculated for different stationary phases from data on the pore structure of the initial support and the modifier structure.

INTRODUCTION

Reversed stationary phases with bonded long chain hydrocarbon groups are the most widely used stationary phases for the separation of a large variety of mixtures by high-performance liquid chromatography (HPLC). More than half of all HPLC analyses are carried out in this way. The main drawback of commercially available stationary phases, is their poor reproducibility^{1,2}.

In spite of the apparent simplicity of the bonded layer structures of these stationary phases they are rather complicated systems. Therefore much attention has recently been paid to the structure of reversed stationary phases^{3–6}. The majority of suppliers give only the value of carbon content. Evidently, the description of stationary phases in this way is insufficient since this macrovalue enables only their capacity to be calculated. Stationary phases having similar values of the carbon contents may be quite different in their chromatographic characteristics.

The concept of bonding density (the number of bonded modifier groups per unit area) gives additional and essential information about reversed phases. The use of these parameters and structural data for the initial silicas (specific surface area, average pore diameter, pore size dispersion) allows any sample of modified silica to be characterized. In this context it could be of interest to introduce a new parameter which would include structural characteristics of both the initial support and of the bonded layer one. Such parameter can be obtained by structural-geometrical consid-

eration of a bonded layer of modified silica as described in our paper⁷. It was shown that there are three basic types of bonded layer structures of reversed stationary phases, namely: flexible, rigid and intermediate, the last consisting of both flexible and rigid structures in different ratios. Also that the bonded molecules in flexible and rigid structures have different conformational mobilities. In a rigid structure the conformation of the bonded hydrocarbon chains is practically independent of the external conditions, and under chromatographic conditions the adsorption retention mechanism is dominant. The conformation of the bonded chains in a flexible structure is determined by the temperature and solvent composition. The probe molecules can penetrate the bonded layer and a distribution retention mechanism is dominant.

We have introduced a parameter R (ref. 7), the percentage of rigid structure as the characteristics of a bonded layer which will be determined by the structural characteristics of initial support and modifier employed. We believe that this additional parameter will permit the characterization of any reversed stationary phase more completely and its relation to other types of bonded layer structure. Reproducibility should be expected only for stationary phases with similar structures of the bonded layer.

This paper deals with the calculation of the carbon content, bonding density, p and R from data on the pore structure of the initial support and the modifier structure.

RESULTS AND DISCUSSION

In the previous paper⁷ we offered a new calculation method for the characteristics of a bonded layer from data on the pore structure of the initial support (average pore diameter and pore size distribution) and the modifier structure (size of anchor group, the number of carbon atoms in the case of alkyl modifiers).

The following assumptions have been taken as a basis for the calculation of the characteristics of the modified surface:

- (i) support pores have a cylindrical form;
- (ii) bonded modifier molecules are evenly distributed throughout the surface;
- (iii) bonded chains adopt such a conformation which provides the maximum bonding density;
- (iv) pore diameters are distributed according to the normal law.

In the framework of this model, simple equations have been obtained which contain integrals easily calculable on a computer. The calculation data have been shown⁷ to be in good agreement with experimental data.

Effect of pore diameter and pore size distribution on carbon content for C_8 and C_{18} reversed stationary phases

The results of the calculations are given in Figs. 1 and 2. The carbon content remains constant for a number of silicas with the same pore volume when the average pore diameter is less than 10 nm for C_{18} bonded phases and 4 nm for C_8 bonded phases, but decrease linearly with increasing average pore diameter. Thus, we believe, it is wrong to use a narrow pore support to increase the carbon content owing to the increase in specific surface area, S , of the initial support. Indeed S increases with decreasing average pore diameter, simultaneously to the decrease in bonding density, that is why the carbon content remains unchanged and, hence, the maximum pos-

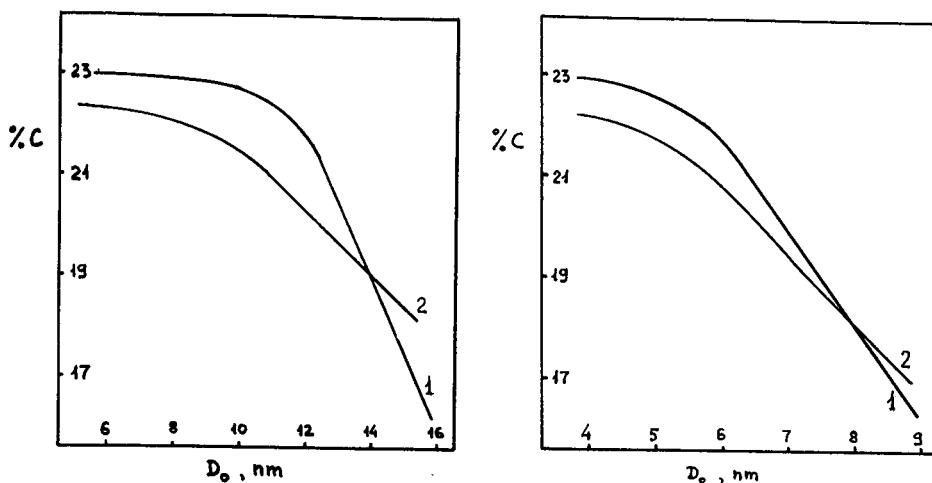


Fig. 1. Effect of the average pore diameter and dispersion of the pore size distribution, σ , on the carbon content for C_{18} reversed stationary phases. Pore volume = 0.75 ml/g. 1, $\sigma = 1$ nm; 2, $\sigma = 3$ nm.

Fig. 2. Effect of the average pore diameter and dispersion of the pore size distribution, σ , on the carbon content for C_8 reversed stationary phases. Other details as in Fig. 1.

sible. For instance, for C_{18} bonded phases with pore volume 0.75 ml/g, the maximum carbon content is 23%.

The carbon content also depends upon the width of the pore size distribution. However, the calculations show that this dependence is fairly slight and one can conclude that the carbon content is practically independent of σ , the dispersion of the pore size distribution (Figs. 1 and 2).

Effect of chain length on carbon content at fixed pore diameter

The effect of the chain length of a bonded n -alkyl modifier for a support with an average pore diameter of 10 nm (the most frequently used support in HPLC) on the carbon content is presented in Fig. 3. Increasing the chain length to 18 carbon atoms (C_{18} stationary phase) leads to a linear increase in carbon content. It is interesting that the application of a longer chain modifier (C_{20} , C_{22} , etc.) does not result in further increases in the carbon content.

Bonding density: effect of average pore diameter and dispersion of pore size distribution

The results of the calculations are given in Fig. 4. In the case of alkyltrimethyl- and alkyltrichlorosilanes, the bonding density for C_{18} stationary phases with an average pore diameter of more than 10 nm remains constant and equal to 2.3 groups per nm^2 . However, when employing stationary phases with an average pore diameter less than 10 nm, the bonding density reduces linearly with decreasing average pore diameter. For instance, in the case of C_{18} stationary phases with average pore diameter 6 nm and dispersion $\sigma = 1$ nm, the maximum possible bonding density of alkyl chains is about 55% from 2.3 groups per nm^2 .

Only stationary phases with the same bonding density can be reproducible

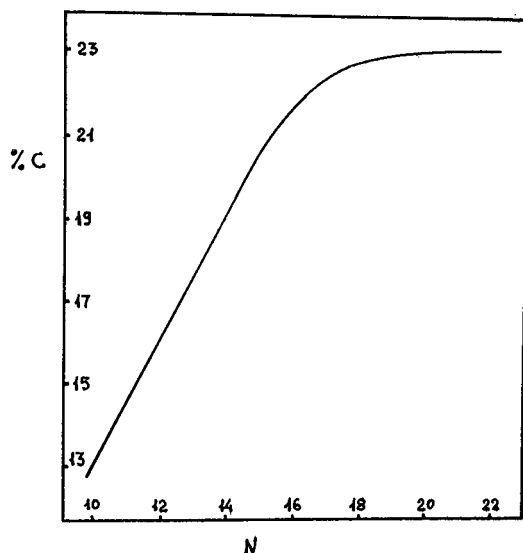


Fig. 3. Effect of the chain length of the modifier molecule on the carbon content. Average pore diameter = 10 nm; pore volume = 0.75 ml/g.

ones. It is preferred if $p = p_{\max} = 2.3$ groups per nm^2 , *i.e.*, remaining silanol groups on the surface are maximally shielded by modifier molecules. In the case of C_{18} stationary phases, these conditions will be realized for supports with $D_0 \geq 12$ nm, *i.e.*, within sufficiently wide pores. For such supports even considerable changes in D_0 from lot to lot do not result in large changes in p value (Fig. 4). Unfortunately, this is not true of commercially available C_{18} stationary phases, the majority of which have $D_0 = 8\text{--}12$ nm and $p < p_{\max}$ and depends on D_0 .

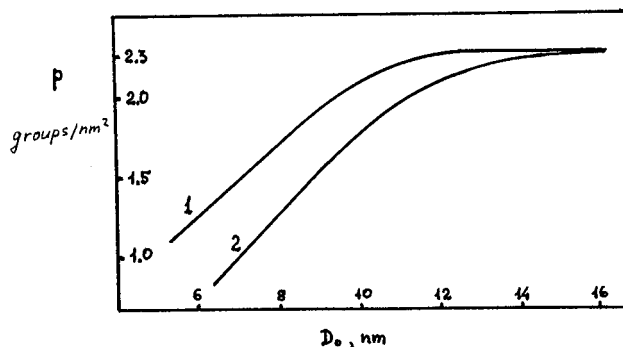


Fig. 4. Effect of the average pore diameter and dispersion of the pore size distribution, σ , on the bonding density for C_{18} reversed stationary phases. 1, $\sigma = 1$ nm; 2, $\sigma = 3$ nm.

Calculation of percentage of rigid structure: effect of D_0 and σ for C_{18} stationary phases

The results of the calculations show (Fig. 5) that in the case of the support with a narrow pore size distribution ($\sigma = 1$ nm), the rigid structure is transformed into a flexible one within a narrow interval of D_0 . Indeed, when $D_0 = 8$ nm, R is 100% and if $D_0 = 14$ nm, $R = 0$. For wide pore size distribution this interval is extended considerably (Fig. 5).

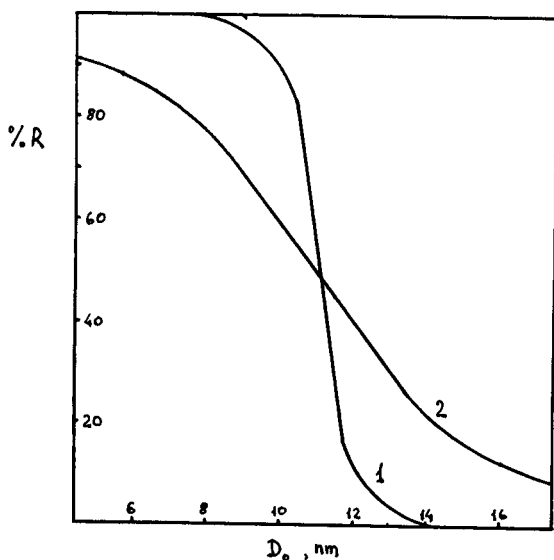


Fig. 5. Effect of the average pore diameter and dispersion of the pore size distribution, σ , on the percentage of rigid structure for C_{18} reversed stationary phases. Pore volume = 0.75 ml/g. 1, $\sigma = 1$ nm; 2, $\sigma = 3$ nm.

Reproducibility of chromatographic characteristics is expected where R is 0 or 100%. Then a negligible change in the average pore diameter of the initial support will not lead to a considerable change in the percentage of rigid structure.

To modify the silica surface chemically, one should choose the support with a narrow and symmetric pore size distribution in order to reduce the interval of the rigid-flexible transformation.

As mentioned above, the majority of commercial C_{18} stationary phases are manufactured on the basis of supports with average pore diameters of 8–12 nm. Comparing two samples of C_{18} stationary phases with $D_0 = 11$ and 12 nm ($\sigma = 1$ nm in both cases), which differentiate insignificantly, both their carbon contents and bonding densities (Figs. 1 and 4), they can differ in four times by the percentage of rigid structure (R). The number of adsorption sites in the bonding layer is also different, hence, the adsorption and chromatographic characteristics will also be different.

Diagram of bonded layer state for reversed stationary phases

Finally we combined the results of the calculations for the bonded layer of reversed stationary phases into a state diagram (Fig. 6). This allows the kind of the

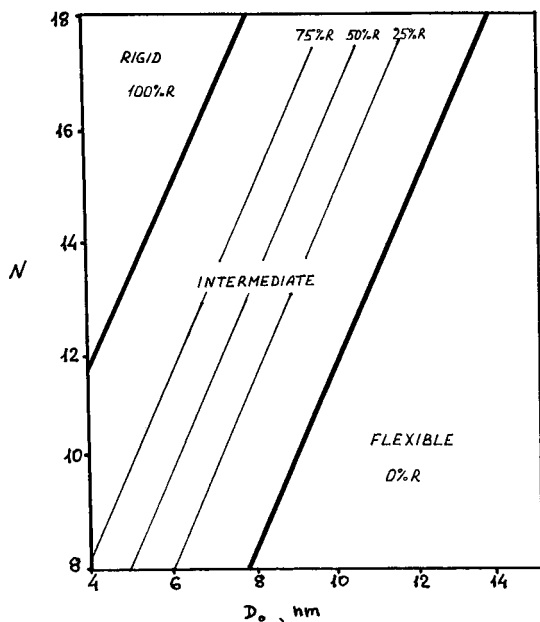


Fig. 6. Diagram of the bonded layer state for reversed stationary phases. Pore volume = 0.75 ml/g; $\sigma = 1$ nm.

bonded layer structure to be identified from the average pore diameter of the initial support and the number of carbon atoms in the bonded n -alkyl chain of the modifier.

The diagram contains three parts: rigid, intermediate and flexible structures. To employ the diagram it is necessary to raise a perpendicular from the value of the average pore diameter (D_0) on the abscissa to crossing with a perpendicular raised from the N value, the number of carbon atoms in the bonded n -alkyl chain of a modifier. The point of intersection will lie within the region, which demonstrates the state of the bonded layer, namely rigid, intermediate or flexible. For instance, supports with an average pore diameter of 10 nm with bonded octyl groups (C_8 stationary phases) have bonded layers with a flexible structure, and similar supports with bonded octadecyl groups (C_{18} stationary phases) have bonded layers with intermediate structure comprising 75% rigid structure.

It is interesting that stationary phases corresponding to the intersection points, lying in the upper left part of the diagram (100% rigid structure), are characterized by identical carbon contents. These results additionally confirm that carbon percentage does not depend on D_0 for small pores. For stationary phases with a pore volume 0.75 ml/g this value of the carbon content is 23%, for stationary phases with a pore volume of 0.6 ml/g it is 18.4% and it is impossible to reach higher carbon contents for supports with the stated pore volume neither by increasing the specific surface nor by decreasing D_0 .

The given diagram was constructed for narrow distributions of pore diameters (dispersion is 1 nm). In the case of wider distributions, the limits of the intermediate structure region will be essentially broadened (Fig. 7).

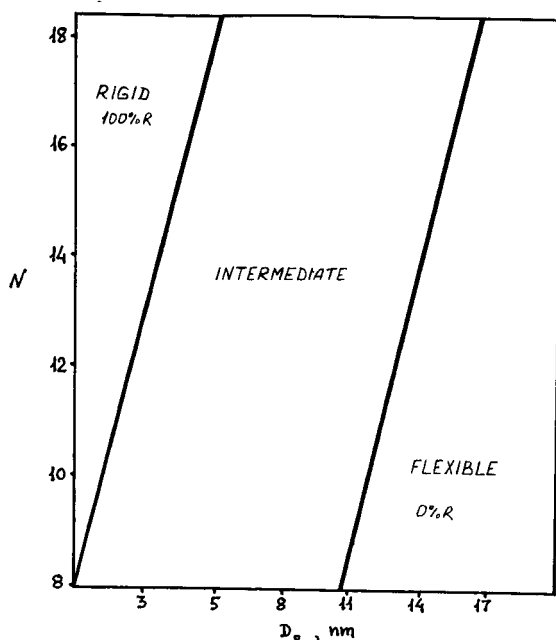


Fig. 7. Diagram of the bonded layer state for reversed stationary phases. Pore volume = 0.75 ml/g; $\sigma = 3$ nm.

CONCLUSIONS

The given results testify once more to the fact that to obtain reproducible reversed stationary phases one needs rigorous control of the porous structure of the initial support.

The introduction of a new parameter giving the percentage of rigid structure of a bonded layer permits the reversed stationary phases to be classified into three basic groups according to the structure of the bonded layer: rigid, flexible and intermediate. In our opinion, only stationary phases with rigid or flexible structures of the bonded layer can possess reproducible characteristics. Only the same lot of an initial support is likely to be able to reproduce the characteristics of a stationary phase with intermediate structure, since even negligible changes in the pore size distribution of the initial support can result in significant changes in the adsorption and chromatographic characteristics of the stationary phases. Unfortunately, the majority of commercially available stationary phases with bonded octadecyl groups are manufactured on the basis of silicas with average pore diameters ranging from 8 to 12 nm, which leads to bonded layers of intermediate structure. In our opinion, this is one of the main reasons why their characteristics are not reproducible.

The diagrams presented here reveal the trends in the basic parameters of the bonded layer depending on the porous structure of the initial support and the *n*-alkyl chain length of the modifier, as well as enable the choice of better support-modifier pair to make a stationary phase with the required characteristics.

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TOTALLY ORGANIC STATIONARY PHASE FROM PERFLUOROALKYL ACRYLATE FOR LIQUID CHROMATOGRAPHY

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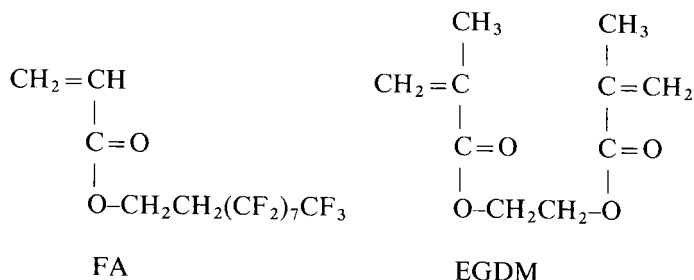
SUMMARY

Porous and fluorine-containing spherical beads for column packings in high-performance liquid chromatography were prepared by suspension copolymerization of heptadecafluorodecyl acrylate with divinyl monomers as a cross-linking agent. The fluorine content was easily adjustable by changing the monomer ratio. The packings show efficient separation for fluorine-containing compounds, *e.g.*, 5-fluorouracil and uracil, mixtures of fluorinated steroids, etc. In addition, they are highly acid- and alkali-resistant, show no abnormal adsorption for ionic compounds and no excessive retention for large hydrophobic compounds. Therefore, they are applicable also to the chromatographic separation of hydrocarbon substances. These favourable properties are related to the low surface energy based on the higher fluorine content.

INTRODUCTION

Recently, the synthesis of fluorinated stationary phases for liquid chromatography and their specific separation behaviours based on fluorine–fluorine interactions were reported. However, most fluorinated packings are of the silica-bonded type^{1–7}. Therefore, these packings have some defects due to the matrix materials.

We have succeeded in making totally organic packings by copolymerization of a perfluoroalkyl monomer (FA). Although modified poly(chlorotrifluoroethylene) is known^{8–15}, our packings have many advantages: (1) they are made by one-step copolymerization, (2) they are spherical and uniform in size because of suspension



polymerization and they become macroporous when a diluent is added in the process of polymerization and (3) the content of perfluoroalkyl group is easily adjustable by changing the molar ratio of FA to comonomer [for example, ethylene glycol dimethacrylate (EGDM) as a cross-linking agent].

In this communication we report the synthesis of perfluoroalkyl polymer packings and the successful separation of hydrophobic or fluorine-containing compounds.

EXPERIMENTAL

Synthesis

Heptadecafluorodecyl acrylate (FA, Nippon Oil & Fats), ethylene glycol dimethacrylate (EGDM) or divinylbenzene (DVB) as a cross-linking agent, diethylbenzene (DEB) as a diluent and benzoyl peroxide as an initiator were mixed at room temperature. The mixture was added to a 1–3% (w/w) solution of aqueous poly(vinyl alcohol) kept at 75°C as a suspension medium, and stirred vigorously at this temperature for 24 h. The spherical polymers produced were collected by filtration and washed with water, hot water and ethanol. By adjusting the stirring speed and sieving, porous spheres of average diameters 5–10 and 25–44 μm were obtained.

Chromatography

The spheres were packed into a stainless-steel column (10–30 cm $\times$ 0.4 cm I.D.) under pressure of 60–80 kg cm⁻². The chromatograph included a JASCO 880-PU pump, a spectrophotometer UVIDEK-100-IV and a Shodex refractometer SE-51. An Unisil Pack (Type 5C18-250A, 25 cm $\times$ 0.46 cm I.D., 12 500 theoretical plate numbers, pore size 250 Å; Gasukuro Kogyo) was used as a reference column. All chromatography was carried out in aqueous solutions at 25°C.

RESULTS AND DISCUSSION

Perfluoroalkyl polymer beads

Fluorine-containing spherical beads were prepared by suspension copolymerization under the conditions given in Table I. The beads obtained are spherical and unevenly sized as shown in Fig. 1. It was confirmed by elemental analysis and infrared

TABLE I

PREPARATIVE CONDITIONS AND ELEMENTAL ANALYSIS OF PERFLUOROALKYL POLYMERS

No.	Molar ratio FA:EGDM:DVB	DEB (%, v/v for monomer)	Elemental analysis (%)		
			C	H	F
FA-EGDM-1	70:30:—	100	35.9	2.2	53.5
FA-EGDM-2	50:50:—	100	39.8	3.2	45.1
FA-EGDM-3	30:70:—	100	47.4	4.7	32.9
FA-DVB-1	70:—:30	50	36.8	2.2	56.3
FA-DVB-2	50:—:50	50	44.2	2.9	49.8
FA-DVB-3	30:—:70	50	52.3	3.9	39.3

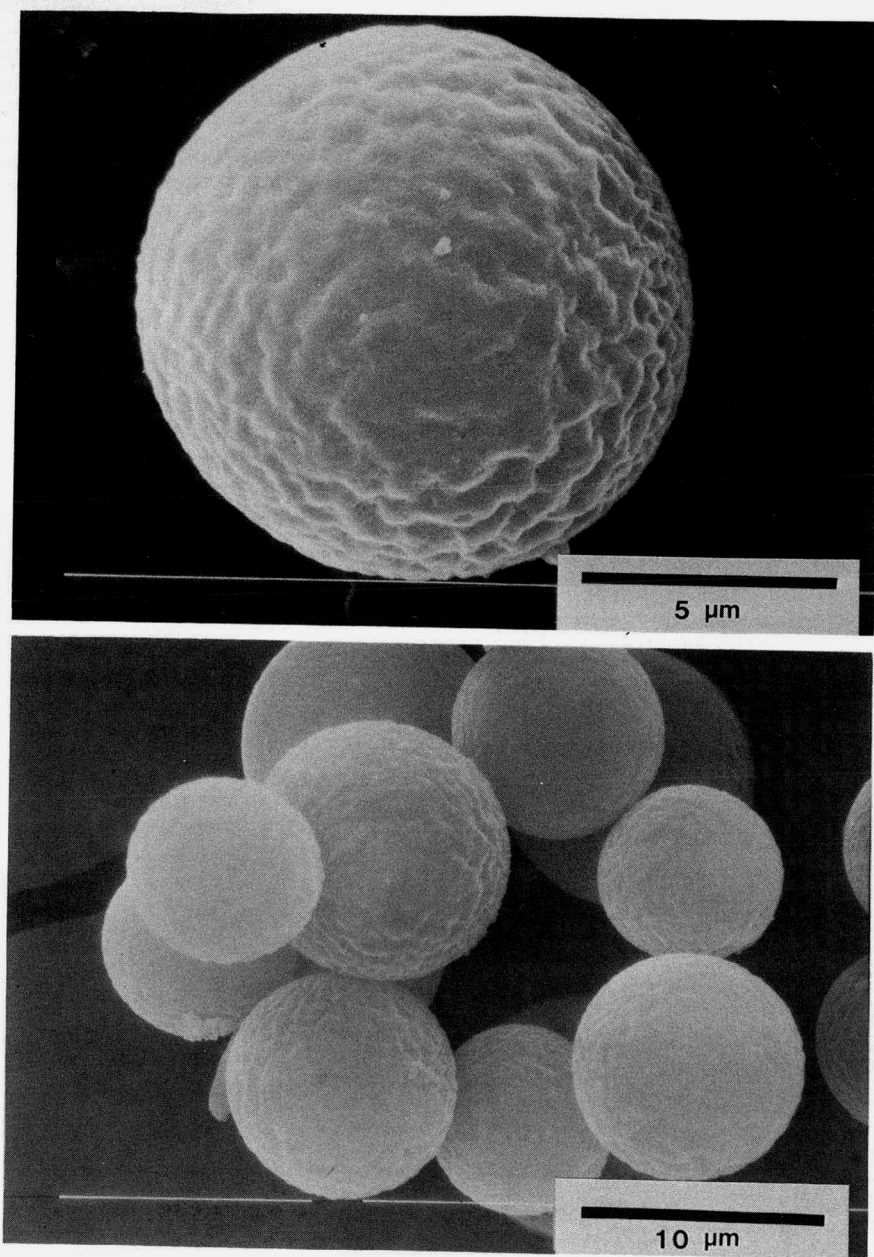


Fig. 1. Electron micrographs of perfluoroalkyl polymer beads prepared by suspension polymerization (FA-EGDM-3).

spectra ($1100\text{--}1250\text{ cm}^{-1}$) that the fluorine content of the beads increased with increasing monomer ratio of FA to the cross-linking agent: spherical beads with higher fluorine contents from 33 to 56% (w/w) are obtained with ease (21.1% was reported by Berendsen *et al.*² in the case of silica-based types).

The packings became porous by use of diethylbenzene as a diluent in the polymerization process. Organic solvents have little influence on the degree of swelling of FA-EGDM type beads. It was determined by gel permeation chromatography (GPC)¹⁶ in tetrahydrofuran that the porosity and the exclusion molecular weight of the FA-EGDM type beads were in the ranges of 40–60% and 100 000–300 000 (for polystyrene), respectively. However, the corresponding values were not determined for the FA-DVB type beads because of the large swelling in tetrahydrofuran, due to the hydrophobicity of the DVB moiety.

Chromatographic properties

Fig. 2 shows the relationship between the monomer ratio of FA and the capacity factor, k' , for benzene and hexafluorobenzene as a solute. It would be expected that an increase in the monomer ratio of FA would lead to an increase in the k' value of hexafluorobenzene due to the fluorine-fluorine interaction between packings and solutes. However, as shown in Fig. 2, k' was independent of the monomer ratio. This may be attributable to the fact that the packing density of FA groups increases with an increase in the monomer ratio of FA, thereby causing a stronger interaction among the fluorocarbon chains than between the fluorocarbon chains and the solutes. This is supported by our observation that the k' value increased with an increase in the monomer ratio of FA when the polymerization was carried out in the presence of a co-monomer, such as propyleneglycol methacrylate, indicating that the orientation of the FA groups is disordered by the dilution due to the presence of the co-monomer. In contrast, the value of k' for hexafluorobenzene is independent of the monomer ratio of FA, but the value for benzene decreased with increasing monomer ratio. This is attributable to a lower surface energy based on the orientation of the perfluoroalkyl groups. The lowering of the surface energy results in an "oilproof" property, which gives limited miscibility between hydrocarbon and fluorocarbon substances. However, this property is not disadvantageous for chromatographic separation of hydrocarbon substances.

The value of k' is always higher with DVB than with EGDM. This indicates that DVB as a cross-linking agent confers sufficient aromaticity upon the packings to result in π - π interactions^{17,18} between the packing and the solute.

Fig. 3 shows a comparison with the reversed-phase packing, octadecyl-bonded silica gel (ODS), using perfluorinated benzenes (a) and normal hydrocarbons (b) as the

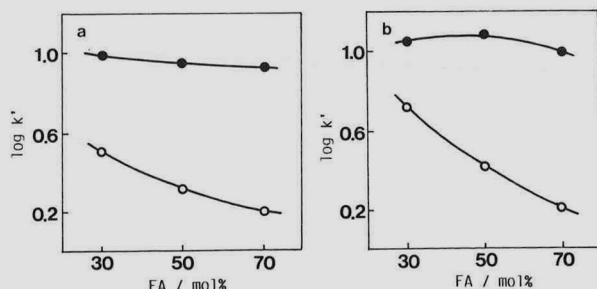


Fig. 2. Relationship between the monomer ratio, $\{[FA]/([FA] + [\text{cross-linking agent}])\} \cdot 100$, and the value of k' for benzene (○) and hexafluorobenzene (●) on the FA-EGDM column (a) and the FA-DVB column (b). Mobile phase: acetonitrile-water (50:50, v/v).

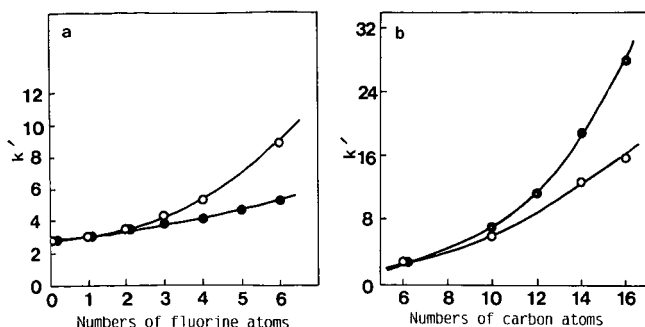


Fig. 3. Retention of fluorinated benzenes (a) and aromatic compounds (b) on FA-EGDM-3 (O) and ODS (●) columns. Mobile phases: acetonitrile–water (50:50, v/v) (a) and (90:10, v/v) (b). Solutes: (a) benzene, fluoro-, 1,2-difluoro-, 1,2,4-trifluoro-, 1,2,3,5-tetrafluoro-, pentafluoro- and hexafluorobenzene; (b) benzene, naphthalene, biphenyl, anthracene and pyrene.

solutes. It is clear that sufficiently large capacity factors are obtained for both types of solutes in the case of the FA-EGDM column. In the case of the ODS column, lower capacity factors are obtained for perfluorinated benzenes and increased retention is observed for hydrocarbons, especially long-chain alkyl compounds. The advantages of the FA-EGDM beads are attributable as follows: for fluorinated solutes, the fluorine–fluorine interaction between the stationary phase and the solutes plays a significant role in producing large capacity factors and the FA-EGDM beads contain a sufficient amount of fluorine atoms. For hydrocarbon solutes, the ODS packings show higher affinity than do the FA-EGDM packings. Therefore, the ODS column is unsuitable for isocratic separation of solutes with significantly different hydrophobicities and gradient elution should be applied in this case. On the contrary, FA-EGDM packings enable chromatographic separation in the isocratic mode even for such solutes because of the retention of large hydrophobic compounds is not excessive. This is also due to the “oilproof” property based on the lower surface energy of the perfluoroalkyl groups.

A similar limited miscibility between hydrocarbon and fluorocarbon substances is observed in micellar systems. Long-chain alkyl groups are difficult to incorporate into the micelles from a surfactant with an heptadecafluorodecyl chain, or are phase-separated in the micelles (Fig. 4)^{19,20}.

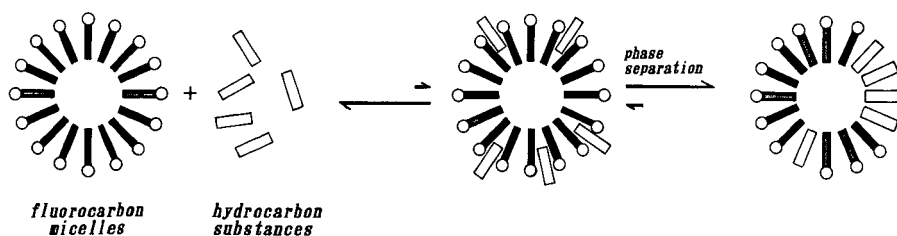


Fig. 4. Schematic illustration of the limited miscibility between fluorocarbon micelles and hydrocarbon substances.

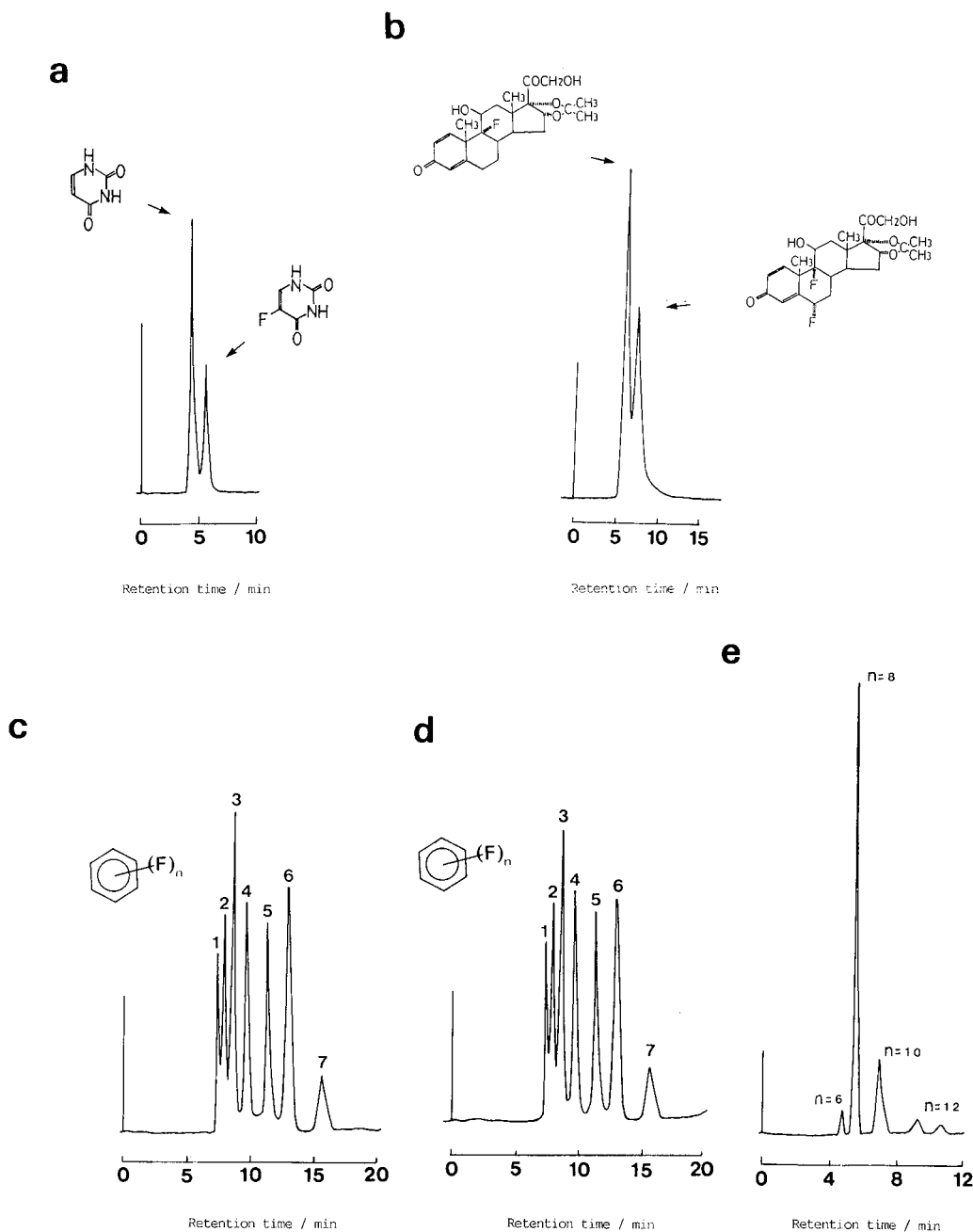


Fig. 5. Typical chromatograms using the FA-EGDM-3 column. Mobile phases and solutes: (a) water for uracil and 5-fluorouracil; (b) methanol-water (60:40, v/v) for steroids; (c) acetonitrile-water (50:50, v/v) for fluorinated benzene (1 = benzene, 2 = fluorobenzene, 3 = 1,2-difluorobenzene, 4 = 1,2,4-trifluorobenzene, 5 = 1,2,3,5-tetrafluorobenzene, 6 = pentafluorobenzene and 7 = hexafluorobenzene); (d) as (c) after treatment with 0.1 M NaOH for 1 day; (e) ethanol for perfluoroalkyl alcohols $\text{CF}_3(\text{CF}_2)_{n-1}\text{CH}_2\text{CH}_2\text{OH}$.

TABLE II
RESISTANCES TO ACID AND ALKALI OF FA-EGDM-3 PACKINGS

Time ^a (day)	0.1 M HCl (aq.)		0.1 M NaOH (aq.)	
	%C	%H	%C	%H
0	47.4	4.7	47.4	4.7
60	47.4	4.7	47.4	4.8
120	47.4	4.8	47.5	5.0

^a Time of standing at room temperature.

TABLE III
RETENTION BEHAVIOURS FOR BASIC SUBSTANCES

Mobile phases: acetonitrile–water (80:20, v/v).

Solute	<i>k'</i>	
	FA-EGDM-3	ODS
Pyridine	0.08	0.27
Quinoline	0.39	0.70
α-Naphthoquinoline	1.19	1.66
Benzylamine	0.18	Adsorption (not eluted)

As shown in Figs. 2 and 3a, the FA-EGDM column is highly efficient for the separation of fluorinated compounds. Fig. 5 shows typical chromatograms for mixtures of fluorine-containing compounds. 5-Fluorouracil, a carcinogenic substance, could not be separated from uracil on the ODS column under the same conditions.

Resistances to acid and alkali

The FA moiety in the packings is also a typical “waterproof” group. The FA-EGDM packings are remarkably acid- and alkali-resistant on the basis of this property. As shown in Table II, the packings can withstand use at pH 1 and 13. The chromatograms c and d in Fig. 5 show that the retention time does not change after treatment of the column with an alkaline solution.

In addition, the FA-EGDM packings are composed of a totally organic and non-ionic polymer. Abnormal adsorptions are often observed for ionic substances in the case of silica-bonded packings^{21,22}. This is due to undesirable silanol residues in the packing. Such adsorption behaviours are not detected with the FA-EGDM packings (Table III).

CONCLUSIONS

- (1) Totally organic and non-ionic packings were prepared by suspension copolymerization using perfluoroalkyl acrylate.
- (2) The packings are spherical and the size is adjustable with ease.

- (3) The fluorine content of the packings is adjustable by changing the monomer ratio.
- (4) The packings enable efficient separation of fluorine-containing substances.
- (5) The packings show no excessive retention for large hydrophobic hydrocarbons.
- (6) The packings are highly acid- and alkali-resistant and show no ionic adsorption for ionic substances.

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LIQUID CHROMATOGRAPHIC SEPARATION OF SEVERAL CLASSES OF ACIDIC AND BASIC AROMATIC COMPOUNDS ON SILICA-BONDED 8-QUINOLINOL STATIONARY PHASES

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SUMMARY

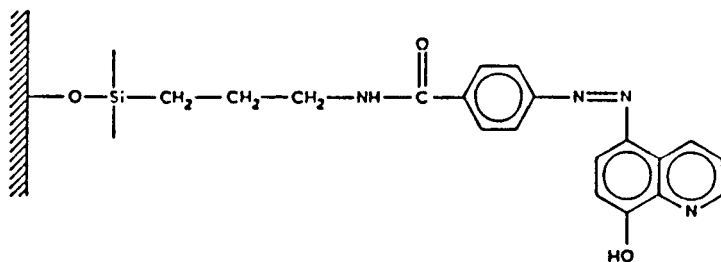
Silica-bound 8-quinolinol (QSG) phases were used for the liquid chromatographic separation of various substituted aromatic compounds including acids, anilines, sulfa drugs, nitrogen heterocyclics and positional isomers. Some iron(III)- and copper(II)-loaded QSG materials were used in addition to non-loaded phases. Aqueous acetate–organic eluents were employed. Two different synthetic routes were used to prepare the QSG phases, resulting in different chromatographic behavior. Those phases obtained via an initial aminopropylsilane surface modification favor acidic compounds, while QSG prepared by an initial nitrobenzamide silylation exhibits greater retention of basic analytes. Phases of relatively low 8-quinolinol content exhibited better resolution and, in some cases, greater retention than higher-capacity materials.

INTRODUCTION

For high-performance liquid chromatography (HPLC), today mostly nonpolar alkyl-chain stationary phases and polar water–organic mobile phases are employed, the so-called reversed-phase mode. Other stationary phases are commercially available, however, such as the nitrile, phenyl and alkylamine columns which permit additional interactions between the analyte and stationary phase such as dipole, π – π and hydrogen bonding. These columns can be employed in either the reversed-phase or normal-phase (non-polar eluent) mode.

Several selective stationary phases^{1,2}, as well as a chelating phase³, have been recently employed for the separation of organic analytes. In our laboratory we have been assessing the general utility of selective phases, especially silica-bound chelating-agent materials, for both metal-ion^{4,5} and organic analyte⁶ separations. Bonded chelating-agent materials differ from most commercially available phases and others commonly studied in that they are usually multi-site, multi-mode materials. The chelating phases discussed in this report employ azo-coupled 8-quinolinol attached to the silica surface by an organic backbone.

The selectivity of these phases can be enhanced by loading the chelating moiety



8-Quinololinol silica gel (QSG)

with an appropriate metal, and operating the column in the ligand-exchange mode. We have used iron(III)-loaded QSG as a ligand-exchange phase for the separation of phenols⁶, and other workers have successfully employed the secondary chemical equilibria of bound metal ions in the separation of various organic "ligands"⁷⁻⁹.

The present manuscript reports on the use of QSG phases prepared by two different synthetic methods, at both relatively high and low coverages, with and without loaded metal, for separation of several classes of organic species, including organic acids, phenols, aromatic amines, nitrogen heterocycles and sulfa drugs. The intent of this study was to demonstrate the versatility of these phases. Only minimal effort was made to optimize mobile phase composition and other chromatographic parameters. Indeed, one of the silica supports was a preparative-grade material from which, at best, only modest efficiency was to be expected. The drawback of multi-site phases, namely the efficiency loss which may occur, is recognized, but the intent is that, for samples not needing great resolving power, the added versatility will be beneficial in separating widely different analyte types.

EXPERIMENTAL

Apparatus

Most of the chromatographic studies were performed on a Spectra-Physics SP-8000 gradient liquid chromatograph with either a Beckman Model 153 or a Kratos Model 770 detector operated at 254 nm. Some runs employed a Waters Model 501 pump with a Gilson HM Holochrome detector, or an IBM LC-9533 system. A Haskel pneumatic pump (Model 26980-4) was used to slurry-pack the columns.

Spectroscopic studies employed a Hitachi 100-80 for UV-VIS or a Perkin-Elmer Model 272 for atomic absorption studies.

Reagents and solutions

The stationary phase support materials used were Adsorbosil-LC, a preparative-grade, 10- μ m diameter, 70- $\text{\AA}$ pore, 480-m²/g silica from Applied Science (now Alltech); and Polygosil 60-10, a 10- μ m diameter, 60- $\text{\AA}$ pore, 500-m²/g silica made by Machery-Nagel and supplied by Rainin. The reagents used in the syntheses, and suppliers, have been previously documented^{4-6,10}.

Water used for this work was deionized, passed through an activated-carbon column, a mixed-bed ion-exchange column (Barnstead) and then distilled from an

all-glass apparatus and stored in a plastic bottle for no more than a few days (to minimize degradation). Mobile phases were prepared using reagent-grade methanol. Acetate buffers were made with ultrapure acetic acid (Baker "Instra-Analyzed") and HPLC-grade sodium acetate (Baker). All mobile phases were filtered through 0.45- μm membrane filters. Samples were obtained from several suppliers and used as received. Sample concentrations were about $1 \cdot 10^{-4}$ M, in 100% methanol or methanol-water (50:50), and were also filtered before use.

Stationary phase synthesis

Two different approaches to the production of 8-quinolinol silica gels were employed in this study. Method I involves the silylation of silica with 3-aminopropyltriethoxysilane, subsequent amidization with *p*-nitrobenzoyl chloride, reduction of the nitro group with sodium dithionite, conversion to the diazonium salt in nitrous acid, and finally coupling to 8-quinolinol. Details of this procedure have been previously published¹¹.

Method I was used for the Adsorbosil supports, which were prepared originally as part of an earlier study⁶. Two preparations of method-I QSG were accomplished, each starting from a different batch of parent aminopropyl silica gel. The first used an excess of dissolved reactants over silica-bound groups for all reactions, resulting in a QSG stationary phase with about 180 $\mu\text{mol/g}$ of copper(II) uptake capacity⁶, and, by implication, 180 $\mu\text{mol/g}$ of 8-quinolinol groups¹². This batch of material had about 650 $\mu\text{mol/g}$ of aminopropyl groups on the surface at the initial silylation, as determined by copper(II) extraction.

The second batch of method-I QSG was intended to be of lower capacity. While the initial silylation produced about 500 $\mu\text{mol/g}$ of bound aminopropyl groups, the final 8-quinolinol content of this phase was limited by using a substoichiometric amount of sodium dithionite in the reduction of the *p*-nitrobenzamide silica gel (NBSG) to the aminobenzamide material (ABSG). Subsequent reactions employed excess reagents. The copper(II) uptake of this QSG was about 40 $\mu\text{mol/g}$. These materials will be referred to as QSGI-180 and QSGI-40, signifying their preparative approach and metal-uptake capacity.

Method II employs triethoxysilylpropyl-*p*-nitrobenzamide silylation, and eliminates the lengthy amidization step of method I¹⁰. After the initial silylation to produce the propyl-substituted NBSG, all subsequent reactions are identical to those of method I. The Polygosil was modified using this second procedure. The NBSG prepared by method II was shown by elemental nitrogen determination to have about 225 $\mu\text{mol/g}$ of nitrobenzamide groups bound to the surface. Two batches of QSG were prepared from this method-II NBSG, again a low- and relatively high-capacity material. The QSGII-110 was prepared with reagent excess at each step. The QSGII-56 was prepared by adding a limited amount (a few drops) of a dilute 8-quinolinol solution in 95% ethanol to the diazonium-salt silica gel slurried in 50 ml of ethanol.

The materials produced by both methods were deactivated by "end-capping" residual silanol groups with trimethylchlorosilane (5% solution in dry toluene, 5 h reflux) at the NBSG stage. The method-II NBSG phase was very hydrophobic after the capping reaction, and had to be "wet" with methanol before it could be slurried into aqueous dithionite solution for the reduction to ABSG¹⁰.

Capacity determination

The numbers of aminopropyl and 8-quinolinol groups were determined by batch copper(II) extraction¹¹, assuming 2-to-1 ligand-to-metal stoichiometry for the former and 1-to-1 for the latter¹². Atomic absorption spectroscopy (AAS) was used to quantitate the copper(II). Elemental analyses were performed by Desert Analytics (Tucson, AZ, U.S.A.).

Column packing

Column blanks were 25 cm long and either 4.6 mm I.D., stainless steel or 4.0 mm I.D., glass-lined stainless steel (Scientific Glass Engineering). All columns were packed at about 6000 p.s.i., by an upward slurry-packing technique¹³, with packing pressure maintained for about 30 min. Methanol was used as both the slurry and packing solvent.

Column metal loading

Packed QSG columns were first washed briefly with pH 1 nitric acid, rinsed with water, and equilibrated with pH 2.5 nitric acid. After filling the pump and tubing with the pH 2.5, 0.01 M metal-ion solution, the column was attached and the metal-ion solution was pumped through the column at 1 ml/min. Iron(III) breakthrough was determined visually by testing the effluent with potassium thiocyanate. Effluent from copper-loading runs was mixed (postcolumn) with $3 \cdot 10^{-5}$ M 4-(2-pyridylazo)resorcinol (PAR)^{4,5} and passed through a detector set at 510 nm. These breakthrough values gave a check on the batch copper(II) capacity determination. A further check was obtained by stripping the metal ion off the column with pH 1 nitric acid after use, and determining the amount of metal ion by AAS.

General chromatographic conditions

Mobile phases were helium-degassed for about 20 min. Columns were equilibrated for 30–45 min, longer if a pH change was involved. Equilibration was assumed to have been attained when effluent pH matched that of the mobile phase and when a steady baseline was observed. Flow-rates were from 1 to 2 ml/min. Sample volumes were either 55, 20 or 10 μ l, depending on which chromatographic system was in use. All injections were done at least twice, and all runs were replicated on different days to be sure retention data were consistent. Anomalous data were rerun several times over a period of days or weeks as a further check on reproducibility. Chromatographic runs were made at ambient temperature, and detectors were set at 254 nm.

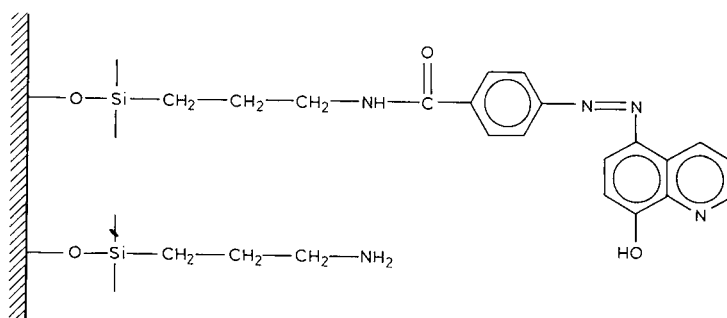
RESULTS AND DISCUSSION

An earlier study from this laboratory which employed a QSG stationary phase for the separation of phenols⁶ has been extended to several additional classes of organic analytes. Two different silica support materials and two different surface-modification chemistries were employed for stationary phases. The preparation and characteristics of these materials are treated below.

Stationary phase considerations

Two stationary phases prepared by method I and two by method II were used in

this study. The method-I materials had comparable initial silylation coverages, 500 and 650 $\mu\text{mol/g}$ of aminopropyl groups¹² for QSGI-40 and QSGI-180, respectively. All subsequent reactions of the higher-capacity method-I material were performed in an excess of reagents, but only about 180 $\mu\text{mol/g}$ of coupled 8-quinolinol groups resulted, presumably due to steric constraints at the amidization reaction with the bulky *p*-nitrobenzoyl chloride and/or the coupling reaction with the even larger 8-quinolinol^{10,11,14}. These observations suggest that unreacted aminopropyl groups may remain using the method-I approach, as shown below. Some of these may be present in an oxidized form unreactive to metal ions¹⁰⁻¹² and *p*-nitrobenzoyl chloride, but may provide a site which interacts with acidic analytes. The relatively low method-II NBSG coverage (see below), implies steric constraints also for the nitrobenzamide silylation reaction.



Both routes to QSG employed "end capping" with trimethylchlorosilane at the NBSG stage of the synthesis. Phases produced by method-II silylation probably contain a greater number of trimethylsilyl groups than those produced by method I, as the initial silylation yielded only about 225 $\mu\text{mol/g}$ surface coverage as compared to 500–700 $\mu\text{mol/g}$ for the aminopropyl silylation, leaving a greater number of unreacted silanols¹⁰. In any case, other studies have shown that only about half of the original silanols can be reacted, even with the smallest silane (*e.g.* ref. 15, p. 278), and coverage decreases as the size of the silane increases.

These QSG phases contain sites which make possible several types of retention mechanisms. Dispersion interactions may occur at the propyl chain and aromatic portions, as well as at the trimethylsilyl groups. The aromatic groups also permit π - π interactions, while the amide, azo and quinolinol portions of the bound species allow for polar and hydrogen-bonding interactions¹⁶. If the heterocyclic nitrogen atom is protonated, anion exchange interactions can also occur. Additionally, interactions at any unreacted aminopropyl, nitrophenyl and silanol, as well as diazonium salt decomposition sites are possible. The presence of all these types of interaction sites provides great versatility, but also may degrade column efficiency¹⁵, as is seen in the chromatographic data which follow.

TABLE I
EFFECT OF pH, IRON LOADING AND IONIC STRENGTH ON RETENTION OF ORGANIC ACIDS

Compound	pK_a	Capacity factor, k'		$pH\ 4.8^b$							
		$pH\ 3.1^a$		$pH\ 4.8^b$							
		$QSGI-180Fe$	$QSGI-180$	$QSGI-180$	$QSGII-110$	$QSGI-180Fe$	$QSGI-180$	$QSGII-110$	$QSGI-180Fe^c$		
<i>p</i> -Hydroxybenzoic acid	4.48	1.13	0.8	0.8	0.32	1.80	1.4	0.27	1.36		
<i>o</i> -Hydroxybenzoic acid	2.97	1.20	—	—	—	1.73	—	—	1.36		
Hydrocinnamic acid	4.69	1.6	1.0	1.0	0.35	3.01	2.5	0.36	1.73		
Vanillic acid	4.62	1.73	—	—	—	2.27	—	—	1.73		
Benzoic acid	4.19	1.93	—	—	—	2.48	—	—	1.82		
<i>m</i> -Coumaric acid	4.4	2.27	—	—	—	4.0	—	—	3.1		
<i>o</i> -Coumaric acid	4.61	2.27	—	—	—	5.1	—	—	3.3		
Gentisic acid	2.97	2.73	—	—	—	19.0	—	—	—		
<i>p</i> -Coumaric acid	4.72	2.9	1.6	1.6	0.53	3.9	—	0.45	2.73		
Cinnamic acid	4.45	3.5	2.4	2.4	0.55	5.2	5.2	0.45	4.0		
<i>p</i> -Methoxycinnamic acid	4.54	5.75	3.0	3.0	0.94	8.1	7.8	0.64	5.36		
<i>p</i> -Methylcinnamic acid	4.56	6.0	—	—	—	7.64	—	—	5.0		
2,4,5-Trimethoxycinnamic acid	—	7.0	3.8	3.8	1.8	10.4	9.0	1.3	7.0		
3-Furoic acid	4.5	1.3	1.4	1.4	0.33	1.7	2.8	0.04	1.0		
2-Furoic acid	3.6	6.0	9.0	9.0	0.41	2.7	6.4	0.09	1.1		

^a Mobile phase: water-methanol-1 *M* acetic acid (25:50:25) (apparent pH 3.1).

^b Mobile phase: water-methanol-0.1 *M* pH 4 acetate buffer (25:50:25) (apparent pH 4.8).

^c Same mobile phase as ^b except 0.075 *M* in NaClO₄.

TABLE II
EFFECT OF pH, IRON AND COPPER LOADING ON RETENTION OF SUBSTITUTED ANILINES

Compound	pK_b	Capacity factor, k'		Water-methanol-0.1 M pH 5 acetate buffer (45:45:10) (apparent pH 5.5)		
		Water-methanol (55:45) ^a				
		QSGII-110	QSGI-180	QSGI-180Fe	QSGI-180Cu	
<i>p</i> -Anisidine	8.70	6.81	0.48	1.32	0.19	0.7
<i>p</i> -Toluidine	8.92	4.0	0.29	1.65	0.17	0.85
<i>m</i> -Toluidine	9.30	1.05	0.36	1.65	0.27	—
Aniline	9.38	1.31	0.24	1.18	0.25	0.57
<i>m</i> -Anisidine	9.52	0.87	0.56	1.28	0.56	—
<i>o</i> -Anisidine	9.53	1.5	0.56	1.73	0.41	0.7
<i>o</i> -Toluidine	9.58	2.05	0.36	1.5	0.19	—
<i>p</i> -Chloroaniline	10.0	0.72	1.11	2.68	1.06	1.2
<i>m</i> -Chloroaniline	10.5	0.56	1.11	2.78	1.28	1.2
<i>m</i> -Nitroaniline	11.5	0.36	1.27	3.57	1.86	1.6
<i>p</i> -Nitroaniline	13.0	0.41	1.67	4.9	2.28	2.1
<i>o</i> -Nitroaniline	14.2	0.56	1.72	5.4	2.36	2.4

^a Apparent pH 4.2.

Organic acids

The QSGI-180 columns used for the phenol study⁶ were also employed in the present investigation for aromatic carboxylic acids, as were the method-II stationary phases. Results similar to those of the phenol study were obtained, in that the iron(III)-loaded QSGI-180 column exhibited greater retention than the non-loaded column for all the benzene-based acids at both pH 3.1 and 4.8 (Table I). A curious exception to this is that the furoic acids exhibited decreased retention on the iron-loaded column relative to the nonloaded.

Several acids were also run on the non-loaded QSGII-110 column at pH 3.1 and 4.8. Retention was much smaller on this method-II Polygosil phase than on the method-I Adsorbosil column. An increase in pH from 3.1 to 4.8 produced slightly lower k' values on the QSGII-110, compared to at least *doubled* retention on the QSGI-180 column. This behavior difference can be ascribed to the different surface-modification chemistries. The Adsorbosil-based method-I phase appears to display a relatively "basic" surface, possibly due to the unreacted aminopropyl groups suggested above. The Polygosil-based method-II material displays more typical "reversed-phase" character with reduced retention of acid analytes at higher pH values. As the pK_a values of most of the acids used as analytes were about the same (in the 4–5 range) no correlation of k' with acid strength emerged from this data. A typical separation of several organic acids on QSGII-110 at pH 2.9 with an acetic acid-methanol mobile phase is shown in Fig. 1.

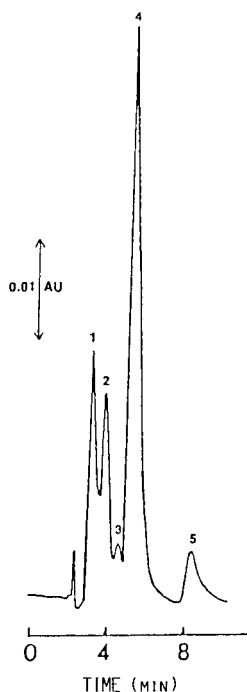


Fig. 1. Separation of cinnamic acids on a QSGII-110 column. Mobile phase: water-methanol-1 *M* acetic acid (25:40:35) (apparent pH 2.9); sample size 20 μ l; flow-rate 1.5 ml/min. Peaks: 1 = hydrocinnamic acid; 2 = *p*-coumaric acid; 3 = cinnamic acid; 4 = *p*-methoxycinnamic acid; 5 = 2,4,5-trimethoxycinnamic acid.

Nitrogen-containing analytes

Several substituted anilines were run on both metal-loaded and non-loaded method-I columns, with retention increasing with decreasing base strength of the analyte (Table II). The presence of iron increased retention from two- to five-fold over the non-loaded column, the copper only slightly, if at all. Retention was lower on the iron-loaded column in the acetate mobile phase than in water-methanol. The copper-loaded column exhibited the opposite behavior. This difference in behavior may be due to the greater competitive interaction of the acetate ion with iron(III) than with copper(II)⁶.

The anilines were also run on the QSGII-110 column in water-methanol (55:45), yielding striking differences compared to the QSGI-180 column (Table II). First, elution order is reversed on the Polygosil-based method-II column; and second, the range of k' values is much greater than for the Adsorbosil-based method-I phase. These results also imply that a different mechanism operates on the QSGI phases than on the QSGII.

Although peak shapes for the anilines on the copper-loaded and non-loaded columns were acceptable, the iron produced noticeable tailing. For example, asymmetry values in acetate mobile phase for *m*-chloroaniline were about 1.4 on both the copper-loaded and the non-loaded QSGI-180 column but 2.3 on the iron-loaded phase. Aniline itself had a value of 2.9 on the iron phase. Similar results were obtained for naphthalene, a neutral probe analyte. Retention was greater on the iron-loaded columns than on the non-loaded, but efficiency was badly degraded by the iron. For example, on the iron-loaded QSGI-180 column in 100% methanol ($k' = 0.3$) the asymmetry factor was 1.7, while in methanol-water (80:20) ($k' = 0.8$) the asymmetry was 4.0. These results imply competing mechanisms with different mass-transfer rate

TABLE III

EFFECT OF COPPER LOADING, pH AND BUFFER CONCENTRATION ON CAPACITY FACTORS OF SULFA DRUGS ON QSGI AND QSGI-Cu COLUMNS

NR = Not retained.

Compound	pK_a	Capacity factor, k'					
		QSGI-180		QSGI-180Cu-80		QSGI-180Cu-100	
		pH 6.35 ^a	Unbuffered ^b	pH 6.35 ^a	pH 6.35 ^c	pH 6.35 ^a	pH 5.0 ^d
Sulfathiazole	7.2	NR	1.4	1.93	3.86	7.57	3.57
Sulfamerazine	6.9	NR	1.14	1.28	2.14	4.7	2.43
Sulfadiazine	6.4	NR	1.0	1.28	2.28	4.7	2.14
Sulfapyridine	8.4	NR	1.14	0.86	1.21	2.14	1.28
Sulfisoxazole	4.9	NR	2.43	0.71	1.43	1.28	0.43
Sulfabenzamide	4.6	1.14	6.14	0.71	0.57	1.28	1.57
Sulfacetamide	5.4	NR	1.28	—	—	0.43	0.43
Sulfanilamide	10.4	NR	1.28	—	—	0.14	0.14

^a Methanol-0.1 M pH 5 acetate buffer (70:30) (apparent pH 6.35).

^b Methanol-water (45:55).

^c Methanol-0.1 M pH 5 acetate buffer-water (70:10:20) (apparent pH 6.35).

^d Methanol-0.1 M pH 4 acetate buffer (70:30) (apparent pH 5.0).

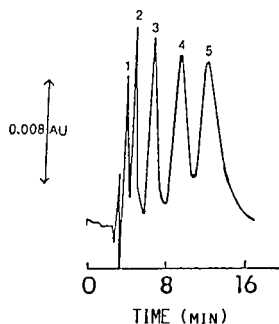


Fig. 2. Separation of sulfa drugs on QSGI-180Cu, about 80 $\mu\text{mol/g}$ copper(II) content. Mobile phase: water-acetonitrile-0.1 M pH 5 acetate buffer (50:40:10) (apparent pH 5.5); sample size 10 μl ; flow-rate 1 ml/min. Peaks: 1 = sulfanilamide; 2 = sulfacetamide; 3 = sulfapyridine; 4 = sulfamerazine; 5 = sulfathiazole.

constants, as found by Kunzru and Frei¹⁷ for amine separation on cadmium-impregnated silica gel.

The QSG phases also were employed for the separation of a number of sulfa drugs. Separation of these analytes is somewhat difficult because of their wide range of pK_a values, hydrophobicity, structures and functional groups¹⁸⁻²¹. Both QSGI-180 and QSGII-110, with and without copper(II), were used as stationary phases for these

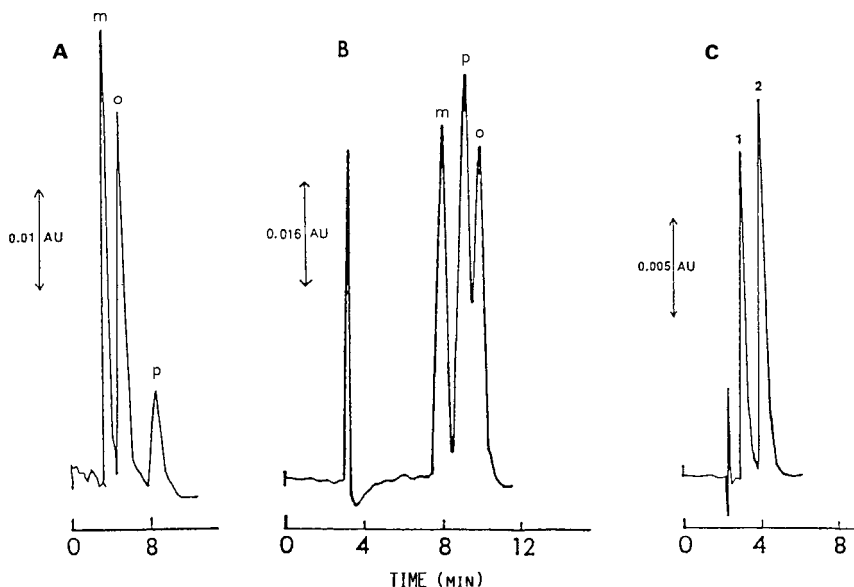


Fig. 3. Separation of positional isomers on QSG phases. (A) Toluidine isomers on a QSGII-110 column in water-methanol (50:50) mobile phase; 20 μl sample size; 1.8 ml/min flow-rate. (B) Nitroaniline isomers on a QSGI-40 column in water-methanol (55:45) mobile phase; 10 μl sample size; 1 ml/min flow-rate. (C) 3-furoic (1) and 2-furoic acid (2) on a QSGII-110 column in water-methanol-1 M acetic acid (25:50:25) (apparent pH 3.1) mobile phase; 10 μl sample size; 1.5 ml/min flow-rate.

analytes. Most of the sulfa drugs were unretained in acetate mobile phases on both columns in the absence of copper, while on the copper-loaded columns these solutes exhibited significant retention, with larger k' values at higher pH, lower buffer concentration, and greater copper loading (Table III). It appears that the greatest contribution to sulfa drug retention is interaction with chelated copper(II), as qualitatively similar behavior was observed on the copper-loaded method-I and method-II columns, rather than the opposite behavior found for the acids and anilines. A typical separation of several sulfa drugs is shown in Fig. 2.

Both the QSGI and QSGII phases were employed for the separation of positional isomers of anisidine, toluidine, nitroaniline, and furoic acid. The toluidine and anisidine isomers elute in different order on the two phases (Table II), again highlighting the apparently different character of the materials prepared by these two methods. Further, even though the elution order of the furoic acids is the same on the two phases, the very large k' value for the 2-furoic isomer on the QSGI phase is striking, and cannot be accounted for by differences in the amount of 8-quinolinol alone (Table I). Chromatograms of some isomers are shown in Fig. 3.

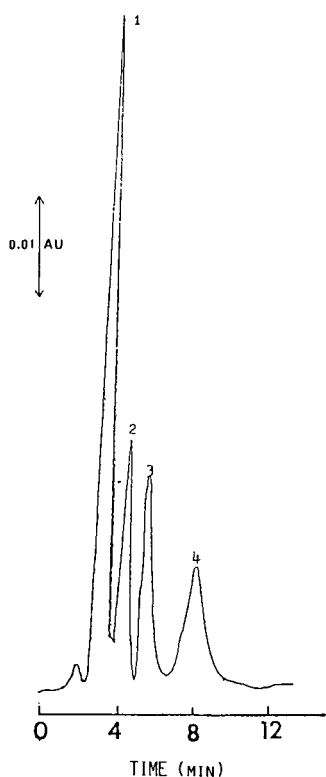


Fig. 4. Separation of some nitrogen-heterocyclic compounds on a QSGI-180Fe column. Mobile phase: water-methanol-0.1 *M* acetic acid (50:40:10) (apparent pH 4.0). Peaks: 1 = cytosine; 2 = uracil; 3 = thymine; 4 = purine.

A number of nitrogen-heterocyclic bases were run on the QSG and iron-loaded QSG phases, including various purine and pyrimidine bases and structurally related compounds. Separation on iron-loaded QSGI-180 of a mixture of uracil, thymine, cytosine and purine is illustrated in Fig. 4. Like the aniline-bases these analytes displayed opposite pH effects on the two classes of stationary phases.

Stationary phase capacity effects

Several classes of analytes were compared on the low- and high-capacity columns prepared by methods I and II. Significantly enhanced resolution was obtained on the QSGI-40 compared to the QSGI-180 column for phenols as well as anilines (Fig. 5). More surprising, however, is the fact that *larger* k' values were obtained on the *lower*-capacity material (Table IV). The effect is most pronounced for the carboxylic acids, less so for the phenols and aromatic amines.

Although the two method-II columns available for high- and low-capacity comparison did not have as large an 8-quinolinol difference as the method-I columns, some efficiency improvement was still found for the QSGII-56 column compared to the QSGII-110. However, retention was dramatically *larger* on the *lower*-capacity than the higher-capacity column for the aromatic amines (Table IV), some of the stronger bases being irreversibly retained in water-methanol (55:45).

Several factors may be at work here. With fewer 8-quinolinol groups present there is less steric crowding on the low-capacity phases allowing the bonded moieties to

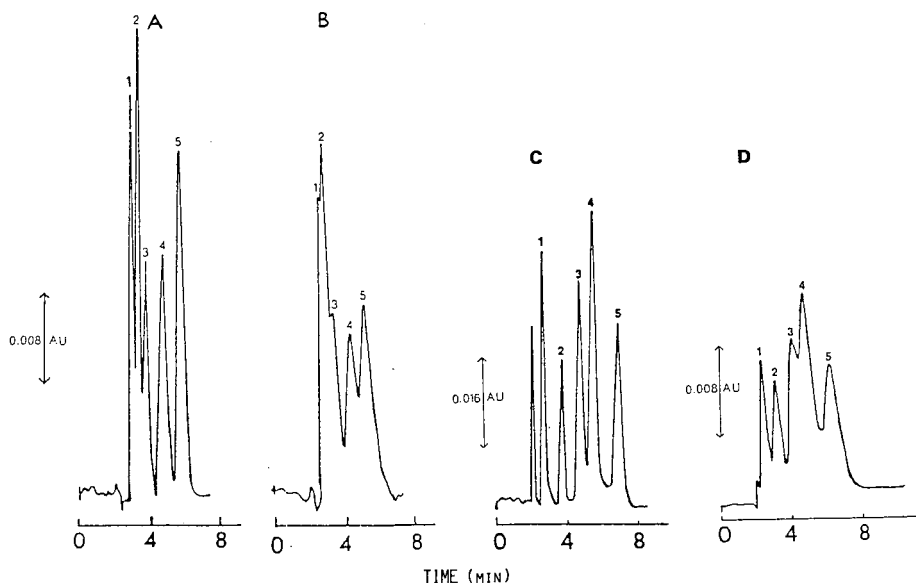


Fig. 5. Effect of stationary phase capacity on separation of organic analytes. Five phenols on QSGI-40 (A) and QSGI-180 (B) in a water-methanol-1 *M* acetic acid (25:50:25) mobile phase (apparent pH 3.1). Peaks: 1 = phenol; 2 = *p*-cresol; 3 = *o*-chlorophenol; 4 = *p*-nitrophenol; 5 = 2,4-dichlorophenol. Five anilines on QSGI-40 (C) and QSGI-180 (D) in a water-methanol-0.1 *M* pH 4.0 acetate (45:45:10) mobile phase (apparent pH 4.6). Peaks: 1 = *p*-anisidine; 2 = *o*-anisidine; 3 = *p*-chloroaniline; 4 = *m*-nitroaniline; 5 = *o*-nitroaniline.

TABLE IV

EFFECT OF STATIONARY PHASE CAPACITY ON RETENTION OF ORGANIC ANALYTES

NR = Not retained.

Compound	Capacity factor, k'			
	QSGI-40	QSGI-180	QSGII-56	QSGII-110
<i>Acids^a</i>				
Hydrocinnamic acid	2.6	1.0	0.2	0.35
Cinnamic acid	6.6	2.4	0.4	0.55
<i>p</i> -Methoxycinnamic acid	8.1	3.0	0.6	0.94
<i>p</i> -Hydroxybenzoic acid	1.8	0.8	—	—
2-Furoic acid	35.0	9.0	NR	0.41
3-Furoic acid	4.2	1.4	NR	0.33
<i>Phenols^a</i>				
Phenol	0.5	0.4	—	—
<i>p</i> -Cresol	0.7	0.5	—	—
<i>o</i> -Chlorophenol	1.0	0.8	—	—
<i>p</i> -Nitrophenol	1.8	1.0	—	—
2,4-Dichlorophenol	2.1	1.7	—	—
<i>Amines^b</i>				
<i>p</i> -Toluidine	0.37	0.4	> 12.0	4.0
<i>o</i> -Anisidine	0.87	0.4	14.2	1.5
<i>m</i> -Toluidine	0.5	0.5	> 15.0	1.05
<i>m</i> -Anisidine	0.5	0.3	> 24.0	0.87
<i>p</i> -Chloroaniline	0.9	0.8	4.8	0.72
<i>m</i> -Chloroaniline	1.0	0.8	1.8	0.56
<i>p</i> -Nitroaniline	1.5	1.3	0.8	0.41

^a Mobile phase: water-methanol-1 *M* acetic acid (25:50:25) (apparent pH 3.1).^b Mobile phases: QSGI columns: water-methanol-0.1 *M* pH 4 acetate buffer (40:50:10) (apparent pH 4.8); QSGII columns: water-methanol (55:45).

be more "mobile" and accessible, and more rapid phase transfer to occur. The efficiency-degrading effect of too much stationary phase liquid is well known in gas chromatography, and has also been demonstrated for a bonded phase in liquid chromatography²².

Once more, opposite behavior was observed for the method-I and method-II phases, with acids exhibiting the greatest k' enhancement on the low-capacity QSGI phase, bases on the low-capacity QSGII column. These facts again imply that there are basic sites underlying the backbone on the QSGI bonded material and that they are more accessible with fewer 8-quinolinol groups present. Steric effects do seem to be a factor on the higher-capacity materials, however, because the aniline bases are also more retained on the lower-capacity QSGI phase.

In general, then, greater affinity for acidic analytes was found on the method-I materials, and greater retention of basic species on the method-II phases, the reasons for which remain unclear. We plan to systematically prepare and study the materials which result from these two synthetic routes to better understand the nature of the modified surface and the mechanisms of interaction for the various classes of analytes.

Nevertheless, from the present and earlier results, it would seem that the multi-site QSG materials described in this study offer attractive versatility, as they can be used to separate metal ions⁴; "as is" for a variety of classes of organic analytes; and loaded with metal ions in the more selective ligand-exchange mode⁶. These phases do appear to be less efficient than single-mechanism materials¹⁶. Indeed, the efficiency of the separations in this study (5000–8000 plates per meter) is lower than typically found on non-polar alkyl phases. It may be that the complexity of the phases which yields the versatility precludes high efficiency, and that this trade-off "goes with the territory". However, as mentioned above, this was a preliminary study, even using preparative-grade silica for some columns. Efficiency optimization was not the goal of the work. We plan further study, especially of the more promising low-capacity phases, to see if plate counts can be increased to more acceptable levels. Nevertheless, these materials do appear to have some utility as multipurpose liquid chromatographic phases.

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CHROM. 21 160

DIRECT LIQUID CHROMATOGRAPHIC SEPARATION OF ENANTIOMERS ON IMMOBILIZED PROTEIN STATIONARY PHASES

VII. SORBENTS OBTAINED BY ENTRAPMENT OF CROSS-LINKED BOVINE SERUM ALBUMIN IN SILICA

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SUMMARY

Chiral sorbents have been prepared by physical immobilization of bovine serum albumin (BSA), cross-linked with glutaraldehyde, into silica pores. BSA is first loaded into the silica and then entrapped by the cross-linking reaction. Sorbents of this kind were prepared even from 60-Å silica without loss of BSA content due to limited access by size exclusion. The cross-linker contributes to a large extent to the increased hydrophobicity, leading to significantly larger capacity factors for hydrophobic solutes. Treatment with organic solvents leads only to a minor irreversible reduction of enantioselectivity.

INTRODUCTION

The successful use of biopolymers, such as proteins, as chiral stationary phases in liquid chromatography¹ has led to an interest in understanding how the performance of sorbents of this kind is influenced by the method used for immobilization. One of the most relevant questions has been whether or not it is necessary for the protein to reside in its native structure. Related to this problem is the possible denaturing effect that may be caused by the presence of large amounts of organic co-solvents in the mobile phase. Since the availability of the enantiomer-discriminating binding sites is of primary importance, one might assume that steric effects arising from inappropriate immobilization techniques would be reflected in decreased separation factors.

Two recent publications by Aubel and Rogers^{2,3} are of great interest in this respect. They found that a glutaraldehyde-bound bovine serum albumin (BSA)-silica sorbent may show increased performance after pretreatment with organic solvents followed by 4 *M* aqueous urea.

For the last couple of years, we have been using BSA-silica sorbents based exclusively upon physical immobilization of BSA within the silica pores by glutar-

aldehyde cross-linking. We therefore feel that a comparison of results is justified. The present paper describes our method of immobilization and the properties of the chiral sorbent, and also presents a discussion on the rôle of the silica matrix as well as the effects of the hydrophobicity of the material obtained.

EXPERIMENTAL

Chemicals

The amino acids were from Sigma (St. Louis, MO, U.S.A.). The N-benzoyl derivatives were prepared using benzoyl chloride by a standard method⁴. The preparation of the N-(2,4-dinitrophenyl) (DNP) amino acids was performed as described previously⁵. ($\pm$)-Benzoin was from Fluka (Buchs, Switzerland). The glutaraldehyde, 50% in water from Eastman Kodak (Rochester, NY, U.S.A.), was diluted in water to give a 5% solution before use.

BSA was a "pure" grade quality (Cat. No. 11930) from Serva (Heidelberg, F.R.G.). The silica used was from Macherey-Nagel (Düren, F.R.G.) and was either spherical (Nucleosil) or irregular (Polygosil). Different pore sizes and particle sizes were used in the experiments. Information concerning the pore diameters and particle sizes was obtained from the suppliers of the materials.

All other chemicals and solvents were of analytical grade.

Preparation of the BSA-silica sorbent

BSA (2 g) was added to 15 ml of an ammonium dihydrogenphosphate buffer (100 mM, pH 4.4) and placed in an ultrasonic bath for 3 min. Silica (4 g) was added to the BSA solution in small portions and then ultrasonicated for 10 min. The slurry was slowly shaken for 24 h at room temperature. Then, 250 μ l of 5% glutaraldehyde were added to the mixture and the shaking continued for 2 h.

The slurry was filtered on a Millipore membrane (RA, 1.2 μ m) and the silica washed twice with 20 ml of the buffer. It was then resuspended in 20 ml of the buffer and 600 μ l of the glutaraldehyde solution were added. The slurry was shaken at 37°C overnight and the silica sorbent isolated by filtration. It was washed with phosphate buffer (50 mM, pH 7.0) and thereafter with 40% 1-propanol in the same buffer.

In the case of larger batches, the filtration steps were replaced by centrifugation at 1600 g for 5 min.

The BSA content of the packing material was determined by elemental analysis of nitrogen and sulphur. The analyses were performed by Mikro Kemi (Uppsala, Sweden).

Column packing

The columns were packed by the ascending slurry packing method⁶. As slurry and packing medium a 50 mM phosphate buffer, pH 7.0, containing 40% 1-propanol, was used. A stainless-steel tube of volume *ca.* 18 ml was used as a slurry reservoir and the packing was performed using a pressure of 300 bar.

Liquid chromatography

The chromatographic system consisted of a Model 2150 pump (LKB, Bromma, Sweden), a 7125 sample injector (Rheodyne, Cotati, CA, U.S.A.) equipped with a

20- μ l loop and a Model V⁴ variable wavelength UV absorbance detector (ISCO, Lincoln, NE, U.S.A.). The high-performance liquid chromatographic (HPLC) columns were 150 mm $\times$ 4.6 mm or 200 mm $\times$ 2.6 mm packed with the BSA-silica prepared as described. For comparative experiments a 150 mm $\times$ 4.0 mm Resolvosil-BSA column from Macherey-Nagel was used.

The mobile phase was a phosphate buffer containing 0–10% of 1-propanol. All experiments were made under isocratic conditions. Retention times and peak areas were obtained by an electronic integrator, Model 740; (Waters, Milford, MA, U.S.A.) interfaced with the detector.

Conditioning with organic solvent

The packing material was generally used without any further conditioning after being washed with 40% 1-propanol solution. In two cases, however, a conditioning of the packing material with organic solvents was performed. The first case entailed rinsing with water, acetone and then hexane. In the second case the conditioning sequence described by Aubel and Rogers² was employed: water, methanol, ethanol, hexane, hexane-dichloromethane (80:20)

RESULTS AND DISCUSSION

Column performance

The performance of a column packed with physically immobilized, cross-linked BSA is shown in Fig. 1 for the resolution of ($\pm$)-benzoin. The capacity factors (k') obtained under the conditions used are 9.3 and 25.3, respectively, giving an α value of 2.7. The resolution factor, R_s , calculated is 7.9. This and many other chromatograms obtained with this type of sorbent show that the polymeric nature of the chiral stationary phase does not give rise to peak broadening due to slow exchange and, in the majority of cases, does not negatively affect the α values.

Ultrasonic treatment of the silica-BSA mixture during the preparation of the

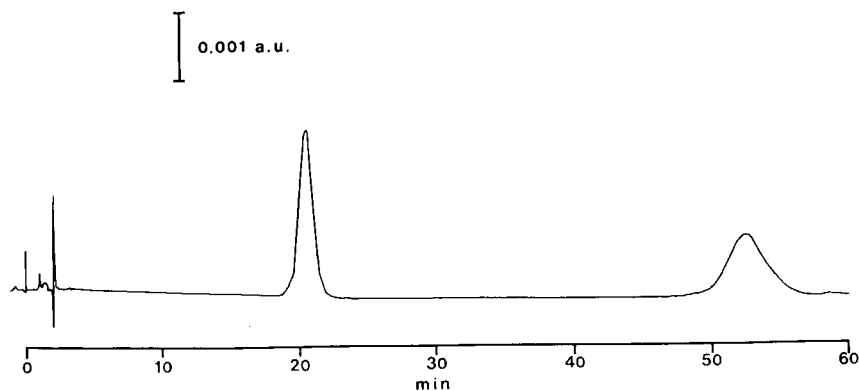


Fig. 1. Enantiomers of ($\pm$)-benzoin separated on entrapped, glutaraldehyde cross-linked BSA. The silica used was Nucleosil 100-7 which was packed in a 150 mm $\times$ 4.6 mm column. Phosphate buffer (50 mM, pH 8.0) + 2% 1-propanol. Flow-rate 1.0 ml/min UV detection at 247 nm. A 50 μ M solution was injected.

sorbents was used to degas the silica and hasten the solubilization of BSA. We have prepared sorbents both with and without ultrasonic treatment, and found no difference in column performance.

Effect of silica properties on BSA content

Sulphur and nitrogen analyses of the dried sorbent showed considerable variation in the amount of entrapped BSA, depending on the pore diameter of the silica material (Table I). The BSA molecule is an ellipsoid with axes of $140.9 \text{ \AA} \times 41.6 \text{ \AA}$ (ref. 7). The low amount of BSA in the 50- $\text{\AA}$ silica is thus probably caused by restricted diffusion. On the other hand, in the case of the 300- $\text{\AA}$ material, the poor entrapment may be caused by the rinsing step. The method described here is based on addition of the bifunctional glutaraldehyde reagent after the BSA; it is essential that the cross-linking reaction is carried out in two steps to avoid the formation of external polymer and agglomeration of the silica particles. Therefore, a washing step is included in order to eliminate BSA from the exterior of the silica. The second addition of glutaraldehyde will then yield an insoluble polymer structure, well anchored within the silica pores.

TABLE I

BSA CONTENT BASED ON NITROGEN AND SULPHUR ANALYSIS OF BSA-SILICA SORBENT MATERIAL (7 μm SILICA PARTICLES)

Pore diameter $\text{\AA}$	Amount of BSA (mg/g)
50	131
100	215
300	78

The column performance is a reflection of the amount of entrapped BSA. The k' values and the separation factors, α , for ($\pm$)-benzoin were found to be dependent on the amount of entrapped BSA (Table II). Both the resolution and the retention of the enantiomers on the columns increase with increasing amount of entrapped BSA. Also from the asymmetry factors, η , the 100- $\text{\AA}$ material (highest BSA content) has a higher column load threshold than those of the 50- and 300- $\text{\AA}$ materials.

A comparison between a sorbent prepared with the method described here and a commercially available Resolvosil-BSA column shows that the latter is significantly less hydrophobic and thus gives lower k' values. This is well illustrated by Fig. 2 which shows chromatograms of N-DNP-aspartic acid on the two types of columns under otherwise identical conditions. While the elution is more rapid, there is a certain decrease in resolution. A comparison of data is given in Table III. The Resolvosil-BSA columns are prepared by an immobilization technique other than the one described in this paper, and contain *ca.* 100 mg BSA per gram silica.

Effect of treatment with organic solvents

The washing procedure, using 40% 1-propanol in buffer, gave a stable packing material that showed no signs of leakage. A portion of this sorbent was washed with

TABLE II

COMPARISON OF THE RESOLUTION OF ($\pm$)-BENZOIN ON SILICA WITH DIFFERENT PORE DIAMETERSMobile phase: phosphate buffer (50 mM, pH 7.9) + 6% 1-propanol. UV detection at 247 nm. Solute: ($\pm$)-benzoin.

Injected quantity (nmol)	k'_1	k'_2	η_1	η_2	α	R_s
<i>Nucleosil: 50 Å, 7 μm</i>						
2	3.5	4.5	0.9	0.9	1.3	1.7
10	3.5	4.2	1.1	1.5	1.2	1.3
50	3.4	4.0	1.4	1.7	1.2	0.8
<i>Nucleosil: 100 Å, 7 μm</i>						
2	5.9	8.6	0.8	0.8	1.5	2.0
10	5.5	7.5	0.8	1.0	1.4	1.6
50	5.0	6.5	0.9	1.0	1.3	1.0
<i>Nucleosil: 300 Å, 7 μm</i>						
2	2.3	3.0	1.1	1.4	1.3	1.7
10	2.3	2.8	1.1	1.6	1.2	1.2
50	2.1	2.4	1.8	2.0	1.2	0.6

water, acetone and finally hexane. A column was then prepared using hexane as the liquid for packing. Another column that had been packed in the normal way (in aqueous buffer) was rinsed in turn with water, acetone and hexane. These columns were then tested with the use of 2–6% 1-propanol in hexane as the mobile phase. Although ($\pm$)-benzoin was retained on the columns, no separation of the enantio-

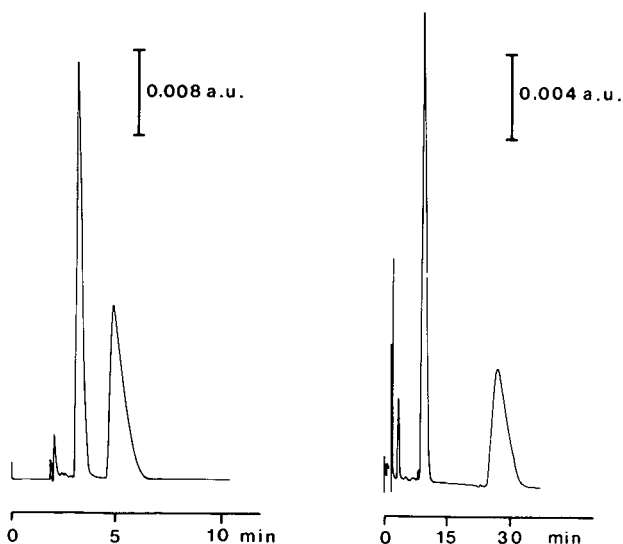


Fig. 2. Optical resolution of N-DNP-aspartic acid on Resovosil-BSA (left) and on entrapped, glutaraldehyde cross-linked BSA (right). Phosphate buffer (50 mM, pH 7.7), 3% 1-propanol. Flow-rate 1.0 ml/min UV detection at 340 nm. Solute concentration: (left) 100, (right) 200 μ M.

TABLE III

COMPARISON OF CHROMATOGRAPHIC DATA OBTAINED WITH THE TWO DIFFERENT BSA COLUMNS

(a) Solute: ($\pm$)-benzoin. Mobile phase: phosphate buffer (50 mM, pH 8.0) + 2% 1-propanol; 1.0 ml/min; UV 247 nm. (b) Solute: N-(2,4-dinitrophenyl)-D,L-aspartic acid. Mobile phase: phosphate buffer (50 mM, pH 7.7) + 3% 1-propanol; flow-rate 1.0 ml/min. UV detection at 240 nm.

Column		k'_1	k'_2	α	R_s
BSA-nucleosil 100-7	a	9.9	24.7	2.5	7.6
	b	4.0	14.0	3.5	4.2
Resolvosil-BSA	a	5.6	6.9	1.2	1.3
	b	0.7	1.7	2.4	1.9

mers was achieved. The columns were rinsed with acetone and water, equilibrated with the aqueous mobile phase and ($\pm$)-benzoin was again readily resolved. These data are shown in Table IV. However, the separation factor was found to be somewhat lower than that before the organic solvent treatment (Fig. 3).

Aubel and Rogers² tested two N-benzoylamino acids on their BSA-silica columns and found that organic conditioning of the column increased the separation factors for these compounds. Since this was contrary to our results with benzoin, one of our columns was conditioned according to their procedure and tested with the use of three N-benzoylamino acids. The results are given in Table V. While the organic conditioning decreases the R_s values for the least hydrophobic compounds, *i.e.*, the serine and alanine derivatives, an opposite, but small, effect was observed for the phenylalanine derivative. There seemed to be no general trend in the effect on the capacity factors, however.

On the other hand, a general effect caused by the organic conditioning of the column seems to be a significant improvement of peak symmetry (Table V). However, this effect was not associated with any gain in column efficiency in terms of plate number. The reason for this change in chromatographic behaviour is not yet known.

These results resemble those of Aubel and Rogers³ insofar as they indicate that changes created in the protein conformation by treatment with organic solvents can be almost completely restored after prolonged treatment with the initial buffer. The

TABLE IV

RESOLUTION OF ($\pm$)-BENZOIN BEFORE (a) AND AFTER (b) TREATMENT OF THE COLUMN WITH ORGANIC SOLVENTS

Column: 150 mm $\times$ 4.6 mm BSA-Polygosil 60-7. Mobile phase: 50 mM phosphate buffer (pH 7.0, 6% 1-propanol), 0.8 ml/min. UV detection at 254 nm.

	k'_1	k'_2	α	R_s
a	3.1	4.4	1.4	2.4
b	3.0	4.0	1.3	1.9

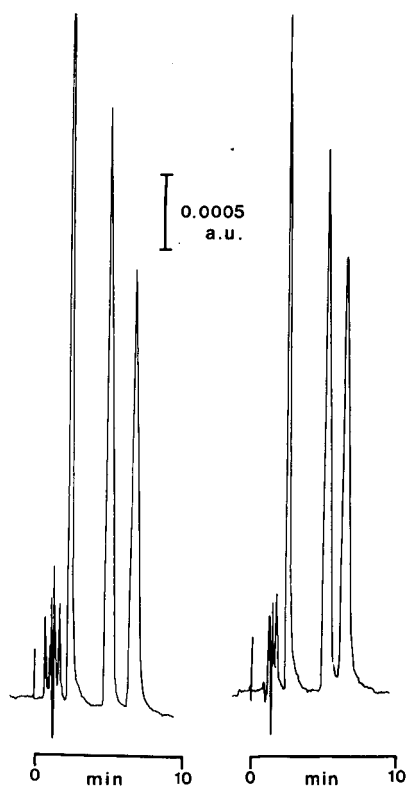


Fig. 3. Optical resolution of ($\pm$)-benzoin on a 200 mm $\times$ 2.6 mm BSA-Polygosil 60-7 column before and after treatment with organic solvents. Phosphate buffer (50 mM, pH 7.0) + 6% 1-propanol. Flow-rate 0.8 ml/min UV detection at 247 nm. A 25 μ M solution was injected.

immobilized protein therefore behaves quite differently from its free state in solution. The most likely reason for this is the decreased conformational mobility caused by the multipoint attachment to the solid phase.

TABLE V

CHROMATOGRAPHIC DATA OBTAINED WITH A GLUTARALDEHYDE CROSS-LINKED BSA SORBENT BEFORE (a) AND AFTER (b) EXPOSURE TO ORGANIC SOLVENTS

Column: 150 mm $\times$ 4.6 mm BSA-Nucleosil 100-7. Mobile phase: phosphate buffer (pH 6.5, 5% 1-propanol), flow-rate 1.0 ml/min. UV detection 225 nm.

Solute		k'_1	k'_2	η_1	η_2	α	R_s
N-Benzoyl-D,L-serine	a	0.33	0.91	—	—	2.8	2.1
	b	0.12	0.50	—	—	4.2	1.7
N-Benzoyl-D,L-alanine	a	1.8	3.7	2.8	2.1	2.0	3.8
	b	1.6	2.9	1.0	1.0	1.8	2.6
N-Benzoyl-D,L-phenylalanine	a	6.1	21.0	1.0	1.6	3.4	6.8
	b	6.1	23.1	1.0	1.2	3.8	7.1

ACKNOWLEDGEMENTS

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IDENTIFICATION OF TABUN IMPURITIES BY COMBINED CAPILLARY COLUMN GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A munitions-grade tabun sample containing a number of sample components was studied by combined capillary column gas chromatography–mass spectrometry under electron-impact and ammonia and deuterated ammonia chemical ionization conditions. Interpretation of the data resulted in the identification of nineteen impurities, including twelve monophosphorus and pyrophosphate compounds previously unreported in tabun. Ammonia and deuterated ammonia chemical ionization was particularly useful as this technique provided molecular ion information for all the identified sample components.

INTRODUCTION

The alleged use of the nerve agent ethyl dimethylphosphoramidocyanidate (tabun) in the Iran–Iraq war emphasizes the need for specific detection and identification procedures for this and other chemical warfare agents^{1–4}. Gas chromatographic–mass spectrometric (GC–MS) techniques have been used extensively for the analysis of these and other organophosphorus compounds. Electron-impact (EI) mass spectra generally provide excellent structural information⁵, but the presence of little or no molecular ion information often hinders the identification of unknown organophosphorus compounds. Chemical ionization mass spectrometry (CI–MS)⁶, a milder ionization technique, has been used with increasing frequency to provide the molecular ion information necessary for the identification of these organophosphorus compounds⁷.

CI–MS using methane, isobutane, ethylene or methanol has been applied to the analysis of organophosphorus chemical warfare agents^{8,9} and pesticides^{10–15}. More recently, the efficacy of ammonia CI–MS¹⁶ has been demonstrated for phosphorus oxyacids¹⁷, several organophosphorus pesticides^{10,14,18} and numerous chemical warfare agents, decomposition products and related impurities^{19–22}.

Previous GC–MS analysis of tabun samples in our laboratory¹⁹ and by others^{3,4} resulted in the detection of several impurities, including diethyl dimethylphosphoramidate, ethyl isopropyl dimethylphosphoramidate, triethyl phosphate, ethyl tetramethylphosphorodiamidate, tetramethylphosphorodiamidic cyanide and two pyrophosphates. Subsequent analysis of a munitions-grade tabun sample confirmed

the presence of these compounds and a number of other impurities. The identity of these unknown sample components would be valuable for future chemical event verification.

Tabun and nineteen related impurities were detected in the munitions sample during capillary column GC-MS analysis under EI and ammonia and deuterated ammonia CI conditions. Interpretation of the data resulted in the identification and characterization of twelve monophosphorus and pyrophosphate compounds previously unreported in munitions-grade tabun.

EXPERIMENTAL

Standards

All tabun samples were prepared in high-performance liquid chromatographic grade chloroform (Fisher Scientific, Edmonton, Canada) and stored in PTFE-lined screw-capped glass vials prior to analysis. The munitions-grade tabun, used for impurity identification, was taken from a sample which had been in a steel container for at least 30 years.

Synthetic samples of tabun, triisopropyl phosphate and bis(diethyl phosphoric) anhydride were prepared in our Organic Chemistry Laboratory. Anhydrous ammonia (99.99%; Liquid Carbonic, Scarborough, Canada) and deuterated ammonia (99%; MDS Isotopes, Dorval, Canada) were used as CI reagent gases.

Instruments

A Varian (Georgetown, Canada) Model 3700 gas chromatograph with flame ionization detection (FID) was used for all capillary column GC-FID analyses. Data were recorded on a Varian 4270 integrator and/or a Nelson Analytical 6000 data system. GC analyses were performed on 15 m $\times$ 0.32 mm I.D. capillary columns (J & W Scientific, Rancho Cordova, CA, U.S.A.) coated with DB-5 [(95%) methyl-(5%) diphenylpolysiloxane], DB-1701 [(86%) dimethyl-(14%) cyanopropylphenylpolysiloxane] and DBWAX (100% polyethylene glycol) films. An on-column injector of our own design was used for sample introduction²³. Injections were made at 50°C. This temperature was maintained for 2 min and followed by a 10°C/min temperature program. Maximum temperatures of 260, 280 and 230°C were maintained for 5 min for the DB-5, DB-1701 and DBWAX capillary columns, respectively. High-purity helium, at a linear velocity of 35 cm/s (methane injection at 50°C), was used as the carrier gas.

Capillary column GC-MS analyses were performed with a VG Micromass 70/70E double-focusing mass spectrometer (VG Analytical, Wythenshawe, U.K.) interfaced with a Varian 3700 gas chromatograph. The EI operating parameters were as follows: source pressure, $2 \cdot 10^{-6}$ Torr; source temperature, 200°C; electron energy, 70 eV; and electron emission, 100 μ A. The ammonia and deuterated ammonia CI operating conditions were as follows: source pressure, $5 \cdot 10^{-5}$ – $6 \cdot 10^{-5}$ Torr; electron energy, 50 eV; and electron emission, 500 μ A. The CI source temperature was initially 120°C and gradually increased to 140°C by the end of each GC-MS analysis owing to heating by the filament. Details of ammonia CI optimization have been reported²⁰. All EI and CI mass spectra were obtained at a resolution of 1000 (10% valley definition) and an accelerating voltage of 6 kV. Data were collected from 500 to 35 u (exponential down) at a scan rate of 1 s per decade.

RESULTS AND DISCUSSION

Capillary column GC-FID analysis of a munitions-grade tabun sample indicated that impurities accounted for 28% of the volatile organic content. Samples of the tabun were analysed by capillary column GC-MS under EI, ammonia CI ($\text{NH}_3\text{-CI}$) and deuterated ammonia CI ($\text{N}^2\text{H}_3\text{-CI}$) conditions in an effort to acquire the molecular and fragmentation ion information necessary for sample component identification. Comparison of acquired EI mass spectral data with previously published data confirmed the presence of several monophosphorus compounds including triethyl phosphate, diethyl dimethylphosphoramidate, ethyl isopropyl dimethylphosphoramidate, ethyl tetramethylphosphorodiamidate and tetramethylphosphorodiamidic cyanide¹⁹. Triisopropyl phosphate and the pyrophosphate bis-(diethyl phosphoric) anhydride were confirmed by comparison of acquired EI data with that obtained for synthesized standards.

The remaining monophosphorus and pyrophosphate compounds were identified on the basis of mass spectral interpretation. Characteristic fragmentation ions and in some instances molecular ions were obtained under EI ionization conditions. Pseudo-molecular $(\text{M} + \text{X})^+$ and/or $(\text{M} + \text{NX}_4)^+$ ions (where $\text{X} = \text{H}$ during $\text{NH}_3\text{-CI}$ and $\text{X} = {}^2\text{H}$ during $\text{N}^2\text{H}_3\text{-CI}$), together with a limited number of fragmentation ions, were observed for all sample components during CI operation.

Most in-house CI-MS analyses of organophosphorus compounds have been conducted with ammonia reagent gas, as this technique provides excellent molecular ion information at trace levels. Deuterated ammonia CI-MS was investigated as a complementary technique in this study because of the demonstrated usefulness of labelled reagent gases for structural studies (*e.g.*, determination of active hydrogen sites by isotope exchange) and for the elucidation of fragmentation pathways²⁴⁻²⁹. In addition, situations may arise where it may be possible to improve signal-to-noise ratios by monitoring $(\text{M} + {}^2\text{H})^+$ and/or $(\text{M} + \text{N}^2\text{H}_4)^+$ when $(\text{M} + \text{H})^+$ and/or $(\text{M} + \text{NH}_4)^+$ ions are interfered with by background chemical noise in complex environmental or biological samples.

Table I lists the identities of the monophosphorus and pyrophosphate compounds detected during capillary column GC-MS analysis of the munitions-grade tabun sample. Several different capillary columns were used to resolve the sample components, the best chromatographic separations being obtained with the more polar DB-1701 and DBWAX films. Fig. 1 illustrates a typical EI total ion current chromatogram for the tabun sample using the DBWAX film.

Monophosphorus compounds

The EI and $\text{NH}_3\text{-CI}$ mass spectra of tabun, diethyl dimethylphosphoramidate, ethyl isopropyl dimethylphosphoramidate, triethyl phosphate, ethyl tetramethylphosphorodiamidate and tetramethylphosphorodiamidic cyanide were the same as previously recorded¹⁹. $\text{N}^2\text{H}_3\text{-CI}$ mass spectra for these six compounds, listed in Table II, were dominated by $(\text{M} + {}^2\text{H})^+$ and/or $(\text{M} + \text{N}^2\text{H}_4)^+$ pseudo-molecular ions and exhibited pseudo-molecular relative ion intensities similar to those observed during $\text{NH}_3\text{-CI}$ operation.

Isotope-exchange during $\text{N}^2\text{H}_3\text{-CI}$ was observed for compounds with CI fragmentation ions containing active hydrogen sites. For example, the triethyl

TABLE I

COMPOUNDS IDENTIFIED (OR TENTATIVELY IDENTIFIED) IN MUNITIONS GRADE TABUN

Me = Methyl, Et = ethyl; *i*Pr = isopropyl.

<i>Compound No.^a</i>	<i>Mol. wt.</i>	<i>Compound</i>	<i>Structure</i>
1	209	Diisopropyl dimethylphosphoramidate ^b	$ \begin{array}{c} \text{O} \\ \parallel \\ i\text{-PrO}-\text{P}-\text{NMe}_2 \\ \\ \text{O}i\text{-Pr} \end{array} $
2	195	Ethyl isopropyl dimethylphosphoramidate ^c	$ \begin{array}{c} \text{O} \\ \parallel \\ i\text{-PrO}-\text{P}-\text{NMe}_2 \\ \\ \text{OEt} \end{array} $
3	224	Triisopropyl phosphate	$ \begin{array}{c} \text{O} \\ \parallel \\ i\text{-PrO}-\text{P}-\text{O}i\text{-Pr} \\ \\ \text{O}i\text{-Pr} \end{array} $
4	181	Diethyl dimethylphosphoramidate ^c	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{EtO}-\text{P}-\text{NMe}_2 \\ \\ \text{OEt} \end{array} $
5	210	Diisopropyl ethyl phosphate ^b	$ \begin{array}{c} \text{O} \\ \parallel \\ i\text{-PrO}-\text{P}-\text{OEt} \\ \\ \text{O}i\text{-Pr} \end{array} $
6	196	Diethyl isopropyl phosphate ^b	$ \begin{array}{c} \text{O} \\ \parallel \\ i\text{-PrO}-\text{P}-\text{OEt} \\ \\ \text{OEt} \end{array} $
7	182	Triethyl phosphate ^c	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{EtO}-\text{P}-\text{OEt} \\ \\ \text{OEt} \end{array} $

TABLE I (continued)

Compound No. ^a	Mol. wt.	Compound	Structure
8	194	Isopropyl tetramethylphosphoramidate ^b	$i\text{-PrO}-\overset{\text{O}}{\underset{\text{NMe}_2}{\parallel}}\text{P}-\text{NMe}_2$
9	180	Ethyl tetramethylphosphoramidate ^c	$\text{EtO}-\overset{\text{O}}{\underset{\text{NMe}_2}{\parallel}}\text{P}-\text{NMe}_2$
10	176	Isopropyl dimethylphosphoramidocyanidate ^b	$\text{CN}-\overset{\text{O}}{\underset{\text{O}i\text{-Pr}}{\parallel}}\text{P}-\text{NMe}_2$
11	162	Ethyl dimethylphosphoramidocyanidate (tabun)	$\text{CN}-\overset{\text{O}}{\underset{\text{OEt}}{\parallel}}\text{P}-\text{NMe}_2$
12	161	Tetramethylphosphorodiamidic cyanide ^c	$\text{CN}-\overset{\text{O}}{\underset{\text{NMe}_2}{\parallel}}\text{P}-\text{NMe}_2$
13	318	Diethyl phosphoric diisopropyl phosphoric anhydride ^b	$\text{EtO}-\overset{\text{O}}{\underset{\text{OEt}}{\parallel}}\text{P}-\text{O}-\overset{\text{O}}{\underset{\text{O}i\text{-Pr}}{\parallel}}\text{P}-\text{O}i\text{-Pr}$
14	317	Diisopropyl phosphoric ethyl dimethylphosphoramidic anhydride ^b	$\text{EtO}-\overset{\text{O}}{\underset{\text{NMe}_2}{\parallel}}\text{P}-\text{O}-\overset{\text{O}}{\underset{\text{O}i\text{-Pr}}{\parallel}}\text{P}-\text{O}i\text{-Pr}$

(Continued on p. 276)

TABLE I (continued)

Compound No. ^a	Mol. wt.	Compound	Structure
15	304	Diethyl phosphoric ethyl isopropyl phosphoric anhydride ^b	$\begin{array}{c} \text{O} \quad \quad \text{O} \\ \parallel \quad \parallel \\ \text{EtO}-\text{P}-\text{O}-\text{P}-\text{OEt} \\ \quad \quad \\ \text{OEt} \quad \text{O}i\text{-Pr} \end{array}$
16	303	Diethyl phosphoric isopropyl dimethylphosphoramidic anhydride ^b	$\begin{array}{c} \text{O} \quad \quad \text{O} \\ \parallel \quad \parallel \\ \text{EtO}-\text{P}-\text{O}-\text{P}-\text{NMe}_2 \\ \quad \quad \\ \text{OEt} \quad \text{O}i\text{-Pr} \end{array}$
17, 19	288	Bis(ethyl dimethylphosphoramidic) anhydride	$\begin{array}{c} \text{O} \quad \quad \text{O} \\ \parallel \quad \parallel \\ \text{Me}_2\text{N}-\text{P}-\text{O}-\text{P}-\text{NMe}_2 \\ \quad \quad \\ \text{OEt} \quad \text{OEt} \end{array}$
18	290	Bis(diethyl phosphoric) anhydride	$\begin{array}{c} \text{O} \quad \quad \text{O} \\ \parallel \quad \parallel \\ \text{EtO}-\text{P}-\text{O}-\text{P}-\text{OEt} \\ \quad \quad \\ \text{OEt} \quad \text{OEt} \end{array}$
20	289	Diethyl phosphoric ethyl dimethylphosphoramidic anhydride ^b	$\begin{array}{c} \text{O} \quad \quad \text{O} \\ \parallel \quad \parallel \\ \text{EtO}-\text{P}-\text{O}-\text{P}-\text{NMe}_2 \\ \quad \quad \\ \text{OEt} \quad \text{OEt} \end{array}$
21	287	Ethyl dimethylphosphoramidic tetramethylphosphorodiamidic anhydride	$\begin{array}{c} \text{O} \quad \quad \text{O} \\ \parallel \quad \parallel \\ \text{Me}_2\text{N}-\text{P}-\text{O}-\text{P}-\text{NMe}_2 \\ \quad \quad \\ \text{OEt} \quad \text{NMe}_2 \end{array}$

^a See Fig. 1.^b Tentative identification based on interpretation of acquired EI and CI mass spectral data.^c Compounds previously reported as tabun impurities.

phosphate $\text{N}^2\text{H}_3\text{-CI}$ fragmentation ion at m/z 157 may be rationalized by the neutral loss of C_2H_4 from the $(\text{M} + ^2\text{H})^+$ ion followed by hydrogen-deuterium exchange of the hydroxyl hydrogen. Under $\text{NH}_3\text{-CI}$ conditions an ion due to loss of C_2H_4 from the $(\text{M} + \text{H})^+$ ion with a relative intensity similar to m/z 157 was observed at m/z 155.

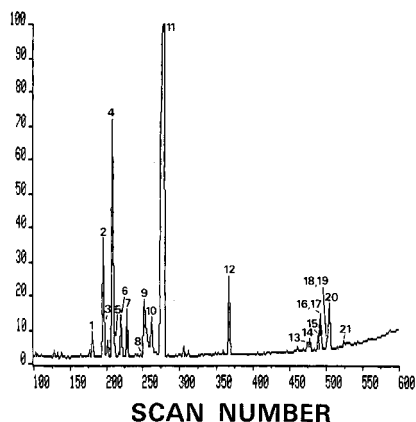


Fig. 1. Capillary column GC-MS total ion current (500–35 u) chromatogram of 1 μ g of munitions-grade tabun obtained under EI conditions (15 m $\times$ 0.32 mm I.D. DBWAX; one MS scan = 1.6 s). Numbered peaks are identified in Table I.

TABLE II

N²H₃-CI DATA FOR MONOPHOSPHORUS COMPOUNDS

Compound	<i>m/z</i> (relative intensity, %)						
	(<i>M</i> + ² <i>H</i>) ⁺	(<i>M</i> + <i>N</i> ² <i>H</i> ₄) ⁺	Other ions				
Diisopropyl dimethylphosphoramidate	211 (100)	231 (2)	213 (2)	212 (10)	210 (11)	170 (5)	169 (5)
Ethyl isopropyl dimethylphosphoramidate	197 (100)	217 (3)	198 (10)	196 (12)	195 (3)	156 (9)	155 (8)
Triisopropyl phosphate	226 (100)	246 (3)	227 (11)	225 (6)	205 (4)	185 (9)	164 (4)
Diethyl dimethylphosphoramidate	183 (100)	203 (3)	185 (2)	184 (8)	182 (13)	181 (2)	153 (2)
Diisopropyl ethyl phosphate	212 (100)	232 (7)	213 (12)	211 (6)	191 (3)	171 (11)	150 (7)
Diethyl isopropyl phosphate	198 (100)	218 (7)	200 (2)	199 (11)	197 (3)	177 (4)	157 (11)
Triethyl phosphate	184 (100)	204 (11)	186 (2)	185 (10)	183 (12)	157 (7)	130 (2)
Isopropyl tetramethylphosphorodiamidate	196 (100)	—	197 (11)	195 (8)	194 (6)	156 (5)	140 (6)
Ethyl tetramethylphosphorodiamidate	182 (100)	—	184 (2)	183 (9)	181 (11)	180 (5)	138 (3)
Isopropyl dimethylphosphoramidocyanide	178 (16)	198 (100)	199 (9)	197 (29)	196 (4)	179 (1)	157 (2)
Ethyl dimethylphosphoramidocyanide	164 (21)	184 (100)	185 (8)	183 (31)	182 (4)	165 (1)	163 (3)
Tetramethylphosphorodiamidic cyanide	163 (100)	183 (73)	184 (6)	182 (14)	164 (8)	162 (8)	135 (4)

Fragmentation ions for compounds containing isopropoxy substituents also exhibited isotopic exchange following the neutral loss of C_3H_6 . Exchanges of this nature within the ion source for these and other compounds are reasonable as a sample ion may suffer many collisions with neutral gas molecules prior to exiting the source. This CI

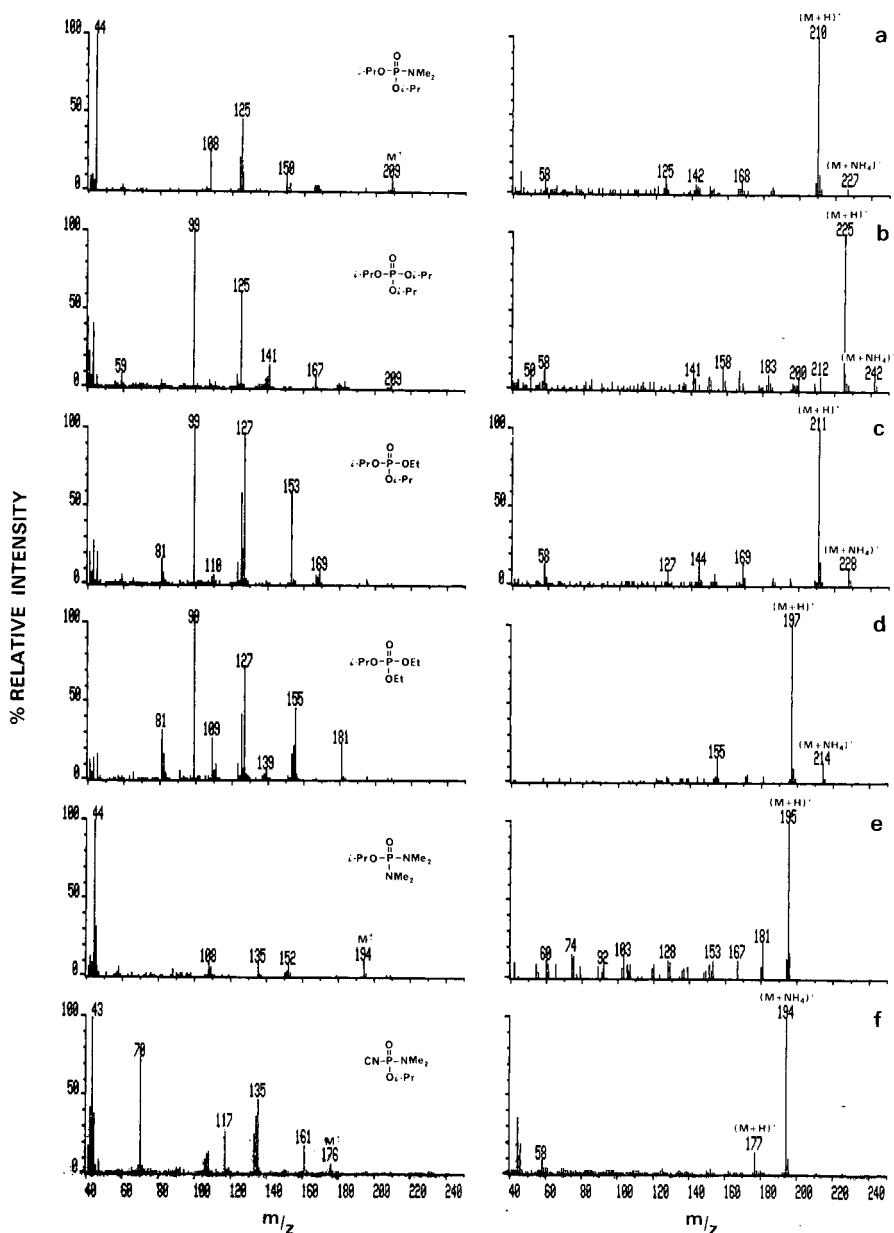


Fig. 2. EI and ammonia CI mass spectra of (a) diisopropyl dimethylphosphoramidate, (b) triisopropyl phosphate, (c) diisopropyl ethyl phosphate, (d) diethyl isopropyl phosphate, (e) isopropyl tetramethylphosphordiamidate and (f) isopropyl dimethylphosphoramidocyanidate.

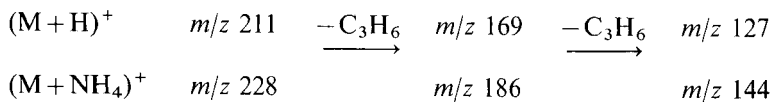
fragmentation ion information, together with the molecular ion data, was particularly useful for the identification of unknown isopropoxy-substituted monophosphorus compounds.

The EI mass spectra of diisopropyl dimethylphosphoramidate, triisopropyl phosphate, diisopropyl ethyl phosphate, diethyl isopropyl phosphate, isopropyl tetramethylphosphorodiamidate and the isopropyl analogue of tabun, isopropyl dimethylphosphoramidocyanide, illustrated in Fig. 2, were rich in EI fragmentation ion information but contained little or no molecular ion data. Ions due to losses of CH_3 , OC_3H_5 , OC_3H_7 , C_3H_5 , C_3H_6 and/or C_3H_7 from the molecular ion were observed for these isopropoxy-containing compounds. Ethoxy or cyano substitution was often indicated by a subsequent loss of C_2H_4 and HCN , respectively. In addition, an intense $[\text{P}(\text{OH})_4]^+$ ion at m/z 99 indicated the presence of a phosphate structure and the presence of an ion at m/z 44 or 43 suggested dimethylamine substitution.

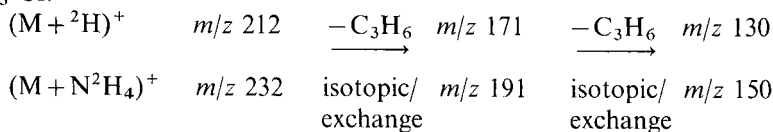
Triisopropyl phosphate was synthesized to support the identification of these isopropoxy compounds. EI fragmentation ions characteristic of only isopropoxy substitution at m/z 209, 183, 167, 141, 125 and 99, due to $(\text{M} - \text{CH}_3)^+$, $(\text{M} - \text{C}_3\text{H}_5)^+$, $(\text{M} - \text{OC}_3\text{H}_5)^+$, $(\text{M} - \text{C}_3\text{H}_5 - \text{C}_3\text{H}_6)^+$, $(\text{M} - \text{OC}_3\text{H}_5 - \text{C}_3\text{H}_6)^+$ and $[\text{P}(\text{OH})_4]^+$, respectively, were observed during GC-MS analysis.

$(\text{M} + \text{X})^+$ and/or $(\text{M} + \text{NX}_4)^+$ pseudo-molecular ions dominated the NH_3 -CI (Fig. 2) and N^2H_3 -CI (Table II) mass spectra of the isopropoxy compounds previously unreported in tabun. Neutral loss of C_3H_6 from the $(\text{M} + \text{X})^+$ and/or $(\text{M} + \text{NX}_4)^+$ pseudo-molecular ions was usually observed with isotopic exchange during N^2H_3 -CI analysis. For example, diisopropyl ethyl phosphate exhibited successive losses of C_3H_6 from both the $(\text{M} + \text{X})^+$ and $(\text{M} + \text{NX}_4)^+$ ions. The following scheme illustrates these losses and supports the presence of a structure with two isopropoxy substituents:

NH_3 -CI:



N^2H_3 -CI:



The acquired NH_3 -CI mass spectrum of triisopropyl phosphate was similar to published data¹⁷, with the major exception being less CI fragmentation and the observation of an $(\text{M} + \text{NH}_4)^+$ ion (and ions due to losses of C_3H_6 from this ion) during this study. Successive losses of C_3H_6 from the $(\text{M} + \text{H})^+$ and $(\text{M} + \text{NH}_4)^+$ pseudo-molecular ions at m/z 183 and 141 and at m/z 200 and 158, respectively, were observed. Differences in source design or ionization conditions could easily account for these subtle spectral differences.

Pyrophosphate compounds

A previous tabun analysis¹⁹ indicated the presence of two pyrophosphate

compounds. Compounds of this type, probably formed by "dimerization" of two monophosphorus compounds containing ethoxy and/or isopropoxy substituents, accounted for 3% of the volatile organic content. Eight compounds of this general structural type were identified during this capillary column GC-MS study.

Fig. 3 illustrates the EI mass spectra acquired for the pyrophosphates diethyl phosphoric diisopropyl phosphoric anhydride, diisopropyl phosphoric ethyl dimethylphosphoramidic anhydride, diethyl phosphoric ethyl isopropyl phosphoric anhydride, diethyl phosphoric isopropyl dimethylphosphoramidic anhydride, bis(ethyl dimethylphosphoramidic) anhydride, bis(diethyl phosphoric) anhydride, diethyl phosphoric ethyl dimethylphosphoramidic anhydride and ethyl dimethyl-

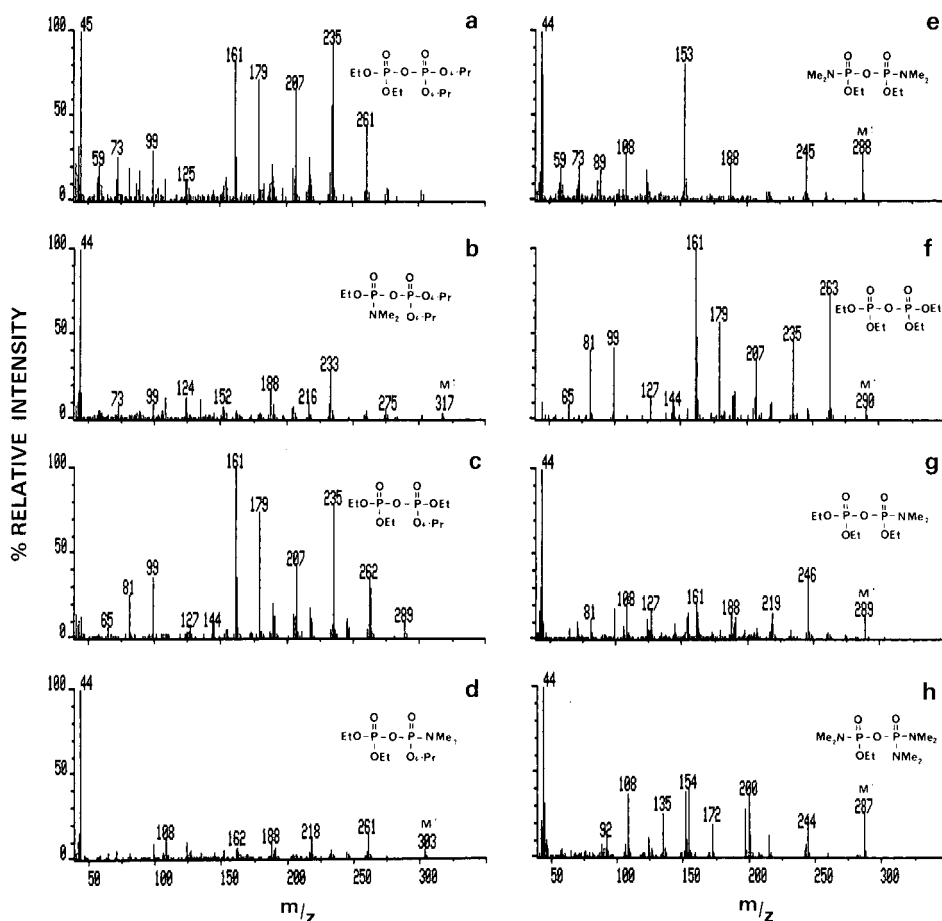


Fig. 3. EI mass spectra of (a) diethyl phosphoric diisopropyl phosphoric anhydride, (b) diisopropyl phosphoric ethyl dimethylphosphoramidic anhydride, (c) diethyl phosphoric ethyl isopropyl phosphoric anhydride, (d) diethyl phosphoric isopropyl dimethylphosphoramidic anhydride, (e) bis(ethyl dimethylphosphoramidic) anhydride, (f) bis(diethyl phosphoric) anhydride, (g) diethyl phosphoric ethyl dimethylphosphoramidic anhydride and (h) ethyl dimethylphosphoramidic tetramethylphosphorodiamidic anhydride.

phosphoramidic tetramethylphosphorodiamidic anhydride. Pyrophosphates containing only ethoxy and/or isopropoxy substitution exhibited characteristic EI fragmentation ions at m/z 99, 161, 179, 207 and 235 due to $[P(OH)_4]^+$, $[(HO)(O)P-O-P(O)(OH)_2]^+$, $[(HO)_3P-O-P(O)(OH)_2]^+$, $[(EtO)(OH)_2P-O-P(O)(OH)_2]^+$ and $[(EtO)_2(OH)P-O-P(O)(OH)_2]^+$ or $[(EtO)(OH)_2P-O-P(O)(OH)(OEt)]^+$, respectively. Those containing dimethylamine substitution in addition to ethoxy and/or isopropoxy groups all exhibited a base ion at m/z 44 and an ion at m/z 108 due to $[N(CH_3)_2]^+$ and $[N(CH_3)_2-P(O)(OH)]^+$, respectively. Higher mass EI fragmentation ions due to loss of OC_2H_3 , C_3H_6 , $(C_3H_6)_2$, $(OC_3H_7 + C_3H_6)$, $[OC_2H_3 + (CH_3)_2]$, $[N(CH_3)_2 + (C_2H_4)_3]$ or $[(N(CH_3)_2)_2 + C_2H_3]$ were also frequently observed.

Bis(ethyl dimethylphosphoramidic) anhydride contains two asymmetric phosphorus atoms, which resulted in the detection of two chromatographic peaks with similar peak areas (compounds 17 and 19), one due to an enantiometric pair and the other to the mesodiastereoisomer. The mass spectra of both diastereoisomers, illustrated in Fig. 3e, were identical and exhibited characteristic EI fragmentation ions due to $(M - OC_2H_3)^+$, $[M - N(CH_3)_2 - (C_2H_4)_2]^+$ and $[M - C_2H_4OP(O)N(CH_3)_2]^+$. Most of the other pyrophosphates (compounds 14, 15, 16, 20 and 21) probably possess only one asymmetric phosphorus atom and therefore elute as unresolved enantiomers on the non-chiral GC column. Compound 13 does not possess an asymmetric phosphorus atom and therefore exists as a single species.

The CI data were valuable for molecular ion determination as the mass spectra of all eight pyrophosphates were dominated by $(M + X)^+$ and/or $(M + NX_4)^+$ pseudo-molecular ions. Pseudo-molecular relative ion intensities, listed in Table III, were similar during NH_3 -CI and N^2H_3 -CI analysis. CI fragmentation was minimal above m/z 50 and usually due to neutral loss of C_3H_6 or C_2H_4 , with isotopic exchange being observed during N^2H_3 -CI operation.

TABLE III

 N^2H_3 -CI MOLECULAR ION DATA FOR PYROPHOSPHATE COMPOUNDS

Compound	m/z (relative intensity, %)			
	$(M + H)^+$	$(M + NH_4)^+$	$(M + ^2H)^+$	$(M + N^2H_4)^+$
Diethyl phosphoric diisopropyl phosphoric anhydride	319 (100)	236 (21)	320 (100)	340 (30)
Diisopropyl phosphoric ethyl dimethylphosphoramidic anhydride	318 (100)	335 (8)	319 (100)	339 (6)
Diethyl phosphoric ethyl isopropyl phosphoric anhydride	305 (100)	322 (23)	306 (100)	326 (22)
Diethyl phosphoric isopropyl dimethylphosphoramidic anhydride	304 (100)	321 (9)	305 (100)	325 (9)
Bis(ethyl dimethylphosphoramidic) anhydride	289 (100)	306 (7)	290 (100)	310 (5)
Bis(diethyl phosphoric) anhydride	291 (100)	308 (23)	292 (100)	312 (23)
Diethyl phosphoric ethyl dimethylphosphoramidic anhydride	290 (100)	307 (10)	291 (100)	311 (10)
Ethyl dimethylphosphoramidic tetramethylphosphorodiamidic anhydride	288 (100)	—	289 (100)	—

CONCLUSION

Tabun and a number of related organophosphorus impurities were identified during a GC-MS study of munitions-grade tabun. EI fragmentation ions provided valuable structural information for the sample components, but the presence of little or no molecular ion information for many components necessitated the use of CI techniques. Complementary molecular ion information in the form of $(M+X)^+$ and/or $(M+NX_4)^+$ pseudo-molecular ions were obtained for all sample components during CI operation. Interpretation of both the EI and CI mass spectral data acquired during this study led to the identification or tentative identification of nineteen tabun impurities, including twelve organophosphorus and pyrophosphate compounds previously unreported in tabun.

The mass spectral data provided are sufficient for the identification of the tabun and related impurities. Observation of these sample components may provide analysts with information on sample origin or synthetic procedures when dealing with samples contaminated with this chemical warfare agent.

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TRACE DETECTION OF MODIFIED DNA BASES VIA MOVING-BELT LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY USING ELECTROPHORIC DERIVATIZATION AND NEGATIVE CHEMICAL IONIZATION

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SUMMARY

Electrophoric derivatives of 5-methylcytosine and 5-hydroxymethyluracil nucleobases are determined using high-performance liquid chromatography–mass spectrometry coupled via a moving-belt interface. Standards as well as samples derived from DNA are analysed. As little as 9.9 pg (signal-to-noise ratio 5) and 180 fg (signal-to-noise ratio 10) of the respective nucleobases are detected in the electron-capture negative chemical ionization mode, and linear responses are observed over a moderate dynamic range. In a comparison study, liquid chromatography–electron-capture negative chemical ionization mass spectrometry is found to have a sensitivity comparable to gas chromatography–electron-capture negative chemical ionization mass spectrometry for 5-hydroxymethyluracil. A detection limit of 60 fg (signal-to-noise ratio 5) by gas chromatography–mass spectrometry is only three-fold better than the amount detected by liquid chromatography–mass spectrometry using the same mass spectrometer.

INTRODUCTION

The usefulness of mass spectrometry (MS) as a tool for structural elucidation of nucleic acid constituents has been well documented. Many modes of ionization have been employed including electron impact ionization^{1,2}, chemical ionization (CI)³, fast atom bombardment^{4,5}, field desorption⁶, and thermospray⁷. Some of these techniques, however, while providing important structural information, do not have the sensitivity necessary for the analysis of components derived from small amounts of human DNA.

Recently we reported on the application of gas chromatography (GC)–elec-

tron-capture detection (ECD) and GC–electron-capture negative CI-MS (ECNCI-MS) for the analysis of modified bases of DNA using electrophoric chemical derivatives^{8–11}. Picogram and sub-picogram amounts of 5-methylcytosine (5MC) and 5-hydroxymethyluracil (HMU), respectively, were detected. In this work we found that post-column derivatization cleanup of the sample by high-performance liquid chromatography (HPLC) was useful. This has led us to investigate the potential applicability of LC–ECNCI-MS to this problem. Not only would this potentially simplify the analysis, but it would eliminate the difficulty of concentrating picogram quantity samples down to the small volumes necessary for on-column injection onto a capillary GC column.

While there are a number of applications of LC–MS for quantitative analysis using a variety of interfaces^{12–15}, there is a paucity of work concerning the use of LC–MS for quantitative analysis at the subnanogram level^{16,17}, or describing the use of LC–ECNCI-MS¹⁸. In this paper we report the results of such studies using a moving-belt interface equipped with a spray device recently developed in our laboratory¹⁹.

The pentafluorobenzyl (PFB) derivatives of 5MC and HMU, which have good electron-capture properties, were examined, both as standards and derived from DNA. These electrophoric derivatives are well suited for work with the moving-belt LC–MS interface, being non-ionic and adequately volatile. Moreover, the HPLC mobile phases used for the separation of the analytes and the internal standards are compatible with the solvent requirements of the interface. Finally, our examination of the same samples by GC–ECNCI-MS provides a convenient and unique opportunity for a comparison of the GC–MS and LC–MS methods of analysis using the same mass spectrometer.

EXPERIMENTAL

Mass spectrometry

A Finnigan (San Jose, CA, U.S.A.) 4021B mass spectrometer equipped with a moving-belt HPLC–MS interface (Kapton[®] belts) and an HP-5890 gas chromatograph was used in all experiments.

The mass spectrometer was operated in the ECNCI mode (0.20 to 0.25 Torr methane) with a source temperature of 250°C. The reagent gas pressure was optimized during each experiment for maximum sensitivity. Quantitative data were acquired using selected ion monitoring (SIM), with dwell times of 0.5 s per selected unit mass. Full-scan spectra were recorded by scanning from 50–700 a.m.u. in 1 s.

The compounds were deposited on the belt by spraying the HPLC eluent via an electrically heated spray device¹⁹. To determine the transfer efficiency of the sprayer, we deposited a sample directly onto the belt with a hypodermic syringe, and compared the peak area with that obtained via flow injection of the same amount. The syringe was assumed to provide a 100% transfer of solute to the belt. The vaporizer temperature was set at 300°C for the HMU derivative and 385°C for the 5MC derivative. The cleanup heater of the interface was set at 330°C in both cases. At 300°C the belt speed was 3 cm/min, and at 380°C it was increased to 4 cm/min to avoid damage to the belt.

GC–MS analyses were carried out using an HP-5 cross-linked 5% phenyl-

methyl silicon capillary column, 25 m $\times$ 0.32 mm I.D., 0.25 μ m film thickness. The column was interfaced directly to the mass spectrometer. Scan rates and programs are the same as those described for the LC-MS experiments. The oven was programmed from 60–300°C at 30°C/min. Helium was used as a carrier gas. The head pressure was maintained at 15 p.s.i. Injections of 1.5 μ l or less were made in the on-column mode.

HPLC

LC was performed with a Waters (Milford, MA, U.S.A.) M 6000A solvent delivery system and a Rheodyne (Cotati, CA, U.S.A.) 7125 injection valve (20- μ l loop). A Kratos (Ramsey, NJ, U.S.A.) Spectroflow 773 UV detector was used to optimize the separations and to determine the retention time of a non-retained compound, t_0 .

The column was a Supelcosil LC-18-DB (150 $\times$ 4.6 mm, either 5- or 3- μ m particles), protected by a Supelcosil LC-18-DB guard column (20 $\times$ 4.6 mm, 5- μ m particles) from Supelco (Bellefonte, PA, U.S.A.). The flow-rate was maintained at 1 ml/min, and the elution volume of a non-retained compound (V_0) was determined to be 1.9 ml. In order to reduce adsorption of the derivatives, the columns were pretreated with a 10 mM acetic acid, adjusted to pH 4.5 with tributylamine and containing 100 mg/l each of adenine, guanosine, cytosine, thymine and 5MC.

Water and methanol were HPLC grade, purchased from Baker (Phillipsburg, NJ, U.S.A.).

All evaporations were carried out on a Speed Vap.

Adducts and derivatives

Internal standards were prepared by replacing one of the PFB groups with a tetrafluorobenzyl (TFB) group. The respective internal standards were added just prior to the final GC or HPLC analysis, and therefore do not serve as recovery standards. The TFB derivatives are only used to quantitate the final concentration of the analyte.

The work-up of calf thymus DNA for the analysis of HMU¹⁷ was accomplished in the following manner. The DNA was hydrolyzed by heating an appropriate amount in 3 M hydrochloric acid for 5 h at 95°C. Upon completion, each sample was cooled to room temperature and evaporated. The sample was reconstituted in 500 μ l of 10 mM potassium phosphate pH 4.6 and applied to silanized Pasteur pipettes packed with 500 mg of C₁₈ silica (prewashed with 3 ml each of methanol and water). The sample was eluted with methanol using vacuum, and the eluent collected and evaporated. To each dried sample was added a stirring bar and 250 μ l of a stirring potassium carbonate suspension (7.76 mg in 20 ml of acetonitrile). The sample was stirred at medium speed for 20 min, after which 250 μ l of a stock PFB bromide solution (28 μ mol in 20 ml of acetonitrile) was added, and the solution gently swirled for 1 h. The sample was then heated at 60°C with medium stirring for 3 h, cooled to room temperature and evaporated.

A stock solution of tetrabutylammonium sulphate (TBAS) was prepared (166.4 mg of TBAS in 20 ml of 1 M potassium hydroxide and 200 μ l were added to each sample. After stirring at medium speed for 20 min at room temperature, 200 μ l of a PFB bromide solution (700 μ mol in 20 ml of dichloromethane) were added and stirring was continued for 6 h at room temperature. The sample was then washed with

500 μl of dichloromethane and water. The aqueous phase was removed and washed with an additional 500 μl of dichloromethane. The dichloromethane layer was then combined and evaporated. The sample was further purified by reversed-phase (C_{18} ; acetonitrile–water, 70:30) and normal-phase (silica; isooctane–isopropanol, 99:1) HPLC respectively. Using retention data from standards, the appropriate 2-ml fraction was collected and evaporated. Prior to analysis each sample was reconstituted in warm toluene containing the internal standard. Additional details for the entire procedure are presented in ref. 17.

The work-up of calf thymus DNA for the analysis of 5MC¹¹ began with a hydrolysis of the DNA in 200 μl of formic acid at 150°C for 3 h. After evaporation, 100 μl of a PFB bromide solution (0.1 *M* in acetonitrile) and 1–2 mg of dry potassium carbonate was added. Each sample was heated at 60°C, with vortexing every 30 min. After 3 h, 500 μl of ethyl acetate–dichloromethane–acetonitrile (3:1:1) (solvent A) was added and the solution was applied to a silanized Pasteur pipette packed with 500 mg of end-capped propyl-cyano silica that had been prewashed with 2 ml of solvent A. The column was then washed with 2 ml of solvent A, and 4 $\times$ 500 μl of methanol. The final two methanol washes were collected and evaporated.

The dried residue was treated with 100 μl of pivalic anhydride (0.1 *M*) and 100 μl of dimethylaminopyridine (DMAP) (0.2 *mM*) in dry acetonitrile. After heating for 1 h at 60°C, 241 pg of the internal standard were added. The sample was diluted with 500 μl of acetonitrile–2-propanol (9:1) (solvent B) and applied to a silica extraction column. After washing with 2 ml of hexane–dichloromethane (3:1), the compounds were eluted with 4 $\times$ 500 μl of solvent B. The final two washes were collected and evaporated. The extract was further purified by reversed-phase HPLC (C_{18} , 20–70% acetonitrile in 10 min, then to 80% acetonitrile in 10 min) with blind collections of the appropriate 2 ml fractions. The samples were evaporated for storage. Prior to analysis the sample was reconstituted in warm toluene. Additional details for the entire procedure are presented in ref. 11.

Standard solutions of the 5MC derivative were prepared in concentrations ranging from 10 to 282.5 pg/ μl , containing 39 pg/ μl internal standard. The HMU derivatives were prepared from 0.190 to 11.9 pg/ μl with 6.2 pg/ μl internal standard. All weights refer to the base content of the derivatives. All standard solutions were made in methanol using acid-cleaned and gas-phase silanized glassware, pipettes, and syringes to avoid adsorption and cross-contamination. Injection volumes for HPLC were 3 μl for the standard solutions, and 10–20 μl for the DNA extracts.

RESULTS

This work focused on the analysis of two modified DNA bases, 5MC (**1**) and HMU (**2**). These compounds were converted to the corresponding pivalyl-N1-pentafluorobenzyl-5-methylcytosine, (**1a**) and 5-pentafluorobenzylloxymethyl-N1,N3-bis(pentafluorobenzyl)uracil (**2a**) derivatives. Internal standards were prepared by replacing one of the PFB groups in each of these compounds by a 2,3,5,6-tetrafluorobenzyl group. Thus the molecular weights of the analytes differ from their respective internal standards by 18 mass units, corresponding to the replacement of one fluorine by a hydrogen atom. All structures are shown in Fig. 1.

Mass spectral characteristics of the derivatives under ECNCI-MS conditions

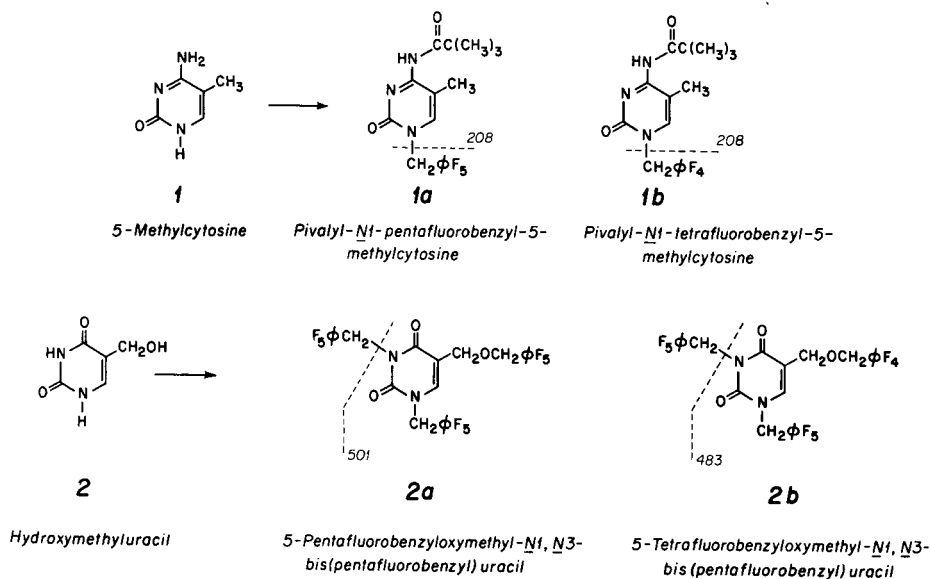


Fig. 1. Structures of 5-methylcytosine and 5-hydroxymethyluracil and their derivatives showing cleavages leading to diagnostic ions. Φ = Phenyl group.

were examined using the moving belt and by GC-MS. The spectra recorded off of the moving belt were identical with those recorded by GC-MS, and changes in the LC interface temperatures had no apparent effect on the fragmentation of the derivatives. No attempts were made to study the effect of lowering the ion source temperature, because at 250°C the spectra already show only one ion, and the higher temperature prolongs the operating lifetime of the source.

The derivatives fragment by loss of a fluorobenzyl group to yield essentially one ion. As shown in Fig. 1, this fragmentation leads to an ion of m/z 208 for both the 5MC derivative (**1a**) and its internal standard (**1b**). Chromatographic resolution of this pair of compounds is therefore necessary for LC-MS analysis using single ion monitoring. No such separation is necessary, however, for the analysis of the HMU derivative (**2a**) and its tetrafluorobenzyl internal standard (**2b**) since the fragmentation of these compounds leads to ions of different mass, m/z 501 and m/z 483, respectively.

As a first step in the development of an LC-MS assay for the compounds of interest we optimized the moving belt interface conditions. Subsequently, detection limits and calibration curves were obtained for the analytes using LC-MS. Finally, samples derived from DNA, which had been previously quantified by GC-ECD and GC-ECNCl-MS, were analyzed by LC-MS.

Better detection limits were obtained throughout this work for the HMU derivative as opposed to the 5MC derivative, consistent with the better GC behavior of the former compound observed in our laboratory.

Optimization of the HPLC-MS interface

Vaporizer temperature. An obvious requirement for LC-MS via a moving-belt interface is the ability to vaporize the analyte into the ion source. Consequently, the

determination of the optimum temperature for the flash vaporizer is an essential first step in this type of study. Accordingly, we measured the response of the detector to both model compounds by monitoring the single ion current of each derivative as a function of vaporizer temperature as they were spray-deposited onto the belt (Fig. 2). Significant differences are found in the behavior of the two compounds. Whereas the response of the 5MC derivatives **1a** increases in an exponential manner without reaching a maximum below 300°C, that of the HMU derivative **2a** rises very fast between 150 and 225°C, reaching a plateau above 250°C. We later tried a vaporizer temperature of 385°C for the 5MC derivative and observed a two-fold higher response than at 300°C. No experimental difficulties were encountered when the interface was kept at 385°C for several hours.

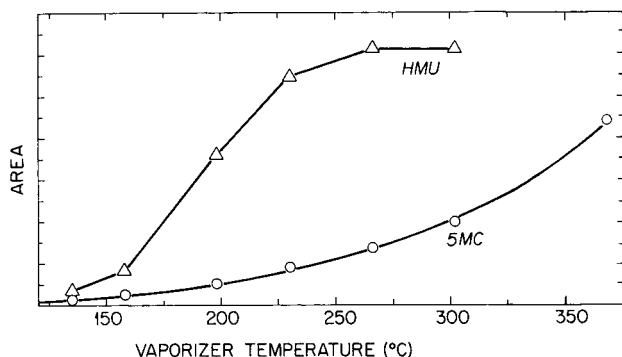


Fig. 2. Response of equimolar amounts of the 5MC and HMU derivatives *versus* vaporizer temperature. Technique: flow injection analysis with single ion monitoring; source temperature: 250°C; belt speed: 3 cm/min; methane CI pressure: 0.21 Torr.

Transfer efficiency. The transfer efficiency was established by injecting (rotary valve) 3- μ l volumes of sample through a capillary tube connected to the spray device (flow-rate 1.0 ml/min), and comparing the response against that obtained by direct deposition of 3 μ l of the same solution onto the belt with a syringe. The recovery was $74 \pm 5\%$ for 28 pg of 5MC and $72 \pm 4\%$ for 25 pg of HMU. It is notable that these recoveries are at the picogram level. Moreover, for the HMU derivative an essentially constant transfer efficiency is observed over three orders of magnitude in the range from 0.60 to 25 pg. These observations further support the utility of the moving belt interface for quantitative analysis.

HPLC-MS analysis

The 5MC derivative and its corresponding tetrafluorobenzyl internal standard both have the same diagnostic ion at m/z 208. Thus, in order to maintain reliable quantitation, it is necessary to achieve good chromatographic resolution of both compounds. Using methanol-water (90:10) as an eluent, the compounds are nearly baseline resolved with a capacity factor (k') of 0.4 for **1a**.

Since the HMU derivative and its internal standard, **2a** and **2b** respectively, have diagnostic ions with different masses, we had greater flexibility in developing the

chromatographic conditions. Nevertheless, as our long range goal is to develop a general method for the extraction, derivatization and analysis of as many modified bases as possible, we chose the same chromatographic conditions as those used for the analysis of the 5MC derivatives. The resolution of the HMU derivative and its internal standard was determined to be greater than 2.0 with a k' of 1.2 for **2a**. The separation of the four derivatives (two analytes and their corresponding internal standards) by LC-MS is shown in Fig. 3.

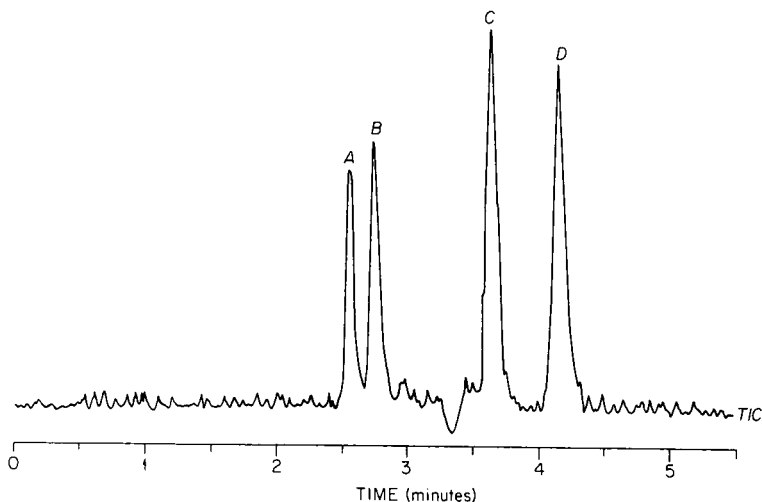


Fig. 3. Chromatogram of the 5MC and HMU derivatives and their respective internal standards. Column: Supelcosil LC-18-DB, 150 $\times$ 4.6 mm, 3 μ m particles; eluent: methanol-water (90:10); flow-rate: 1.0 ml/min; vaporizer temperature: 300°C; belt speed: 3 cm/min; methane CI pressure: 0.21 Torr; ions monitored: m/z 208, 483 and 501. Peaks: A = 80 pg internal standard 5MC; B = 80 pg 5 MC; C = 2.25 pg internal standard HMU; D = 1.62 pg HMU. TIC = Total ion current.

Calibration curves for **1a** and **2a** were obtained by triplicate 3- μ l injections at five different concentrations. For **1a** a linear response ($y = 0.002x + 0.037$) was obtained with a correlation coefficient $r^2 = 0.9921$ in the range corresponding to 10 to 280 pg of the base. Similarly, a linearity over the range from 0.56 to 36 pg of the free base with a value of $r^2 = 0.9978$ was obtained for the calibration curve of **2a** ($y = 0.923x + 0.060$). Response factors on a weight basis were determined to be 0.822 (S.D. = 4.6%) for 5MC and 1.04 (S.D. = 4.5%) for HMU.

The generally favorable behavior of these analytes encouraged us to establish the detection limits for these compounds by LC-MS. Chromatographic profiles of derivatized 5MC and HMU at or near the detection limits are shown in Fig. 4. Detection limits of 9.9 pg for 5MC (signal-to-noise ratio 5) and 180 fg for HMU (signal-to-noise ratio 10) are observed. These values significantly advance the sensitivity that has been achieved to date by LC-MS irrespective of the nature of the interface. Precision and accuracy data for concentrations within the working range of both analyses are shown in Table I.

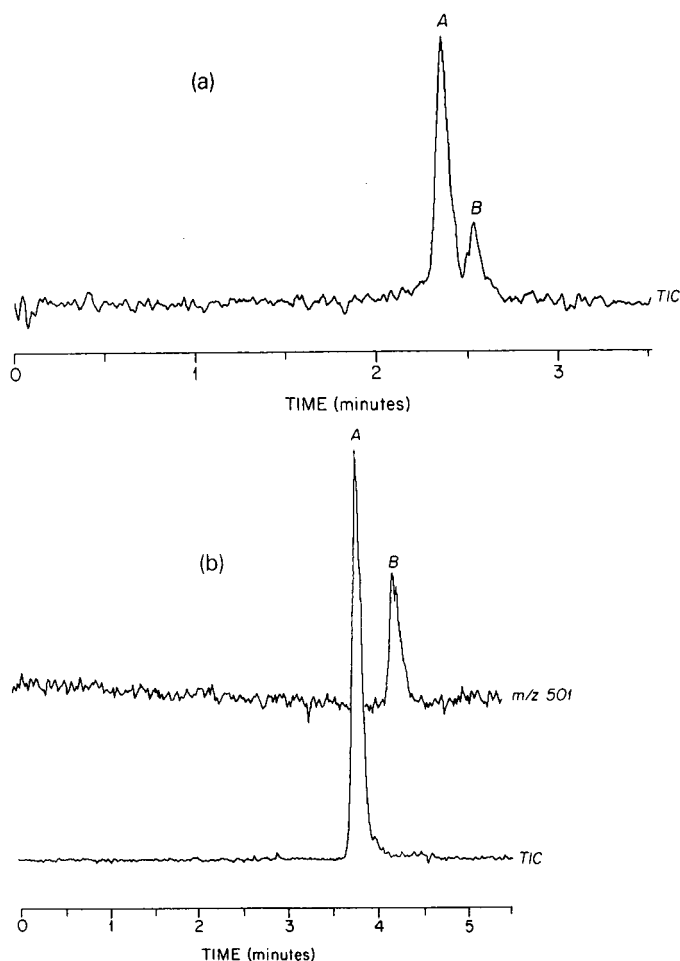


Fig. 4. (a) Demonstration of the detection limit for Piv-PFB-5-MC. Column: Supelcosil LC-18-DB, 150×4.6 mm, $3\text{-}\mu\text{m}$ particles; eluent: methanol-water (90:10); flow-rate: 1.0 ml/min; vaporizer temperature: 385°C ; belt speed: 4 cm/min; methane CI pressure: 0.21 Torr; ion monitored: m/z 208. Peaks: A = 118.3 pg internal standard; B = 9.9 pg 5MC. Demonstration of the detection limit for $(\text{PFB})_3\text{HMU}$. Column, eluent and flow-rate as in (a); vaporizer temperature: 300°C ; belt speed: 3 cm/min; methane CI pressure: 0.21 Torr; ions monitored: m/z 501 and 483. Peaks: A = 6.0 pg internal standard; B = 185 fg HMU.

TABLE I

ACCURACY AND PRECISION DATA FOR LC-MS ANALYSIS

Compound	Amount injected (pg)	Amount measured (pg)	Relative error (%)	Coefficient of variation (%)
SMC	28.0 ($n = 4$)	24.6 ± 3.5	-12.0	9.3
	55.0 ($n = 6$)	54.6 ± 5.7	-0.7	6.7
	282.8 ($n = 6$)	282.8 ± 14.2	+0.1	2.4
HMU	1.9 ($n = 6$)	1.7 ± 0.3	-8.9	11.9
	10.9 ($n = 6$)	11.6 ± 0.5	+6.4	3.5
	32.1 ($n = 6$)	32.4 ± 0.7	+0.9	4.7

Analysis of DNA extracts

Samples of DNA, previously analyzed by GC-ECD and GC-ECNCl-MS^{11,20}, were also examined. The LC-MS chromatogram of the 5MC derivative and its internal standard isolated from calf thymus DNA is shown in Fig. 5a. The data correspond to 410 pg of 5MC in 100 ng DNA, equivalent to a mole percentage of 1.0 for 5MC relative to the total base content of the DNA sample. The latter was determined by independent acid hydrolysis of the DNA to the bases and then subsequent quantitation of the bases by HPLC with UV detection¹¹. This result compares favorably with a value of 1.2 ± 0.1 mole% for the same analysis by GC-ECD¹¹, and the 1.2

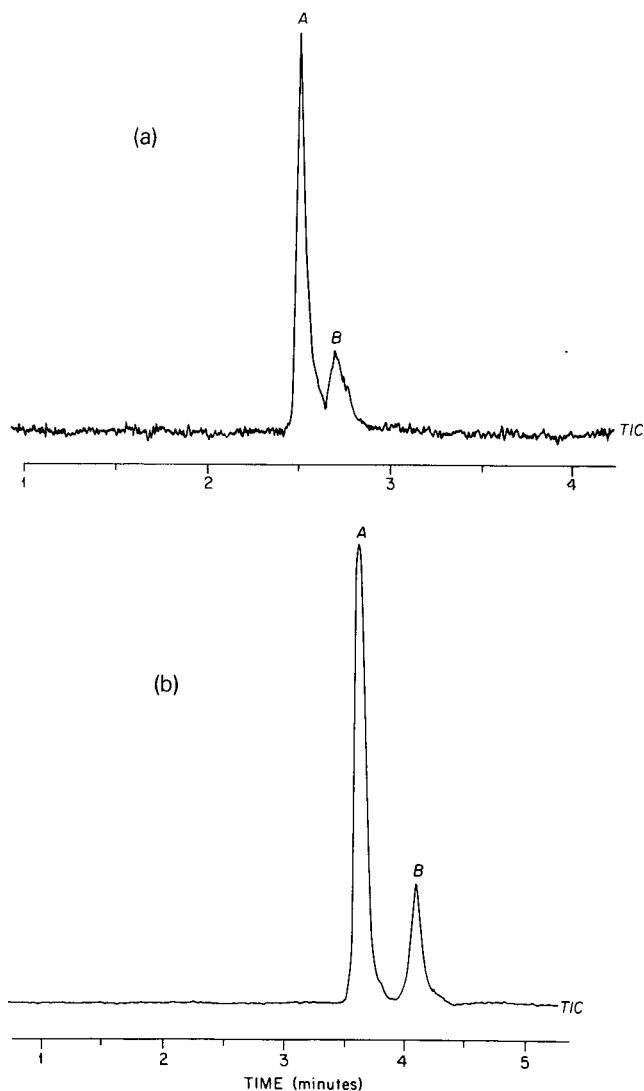


Fig. 5. (a) Determination of 5MC in 100 ng of calf thymus DNA. Conditions as in Fig. 4a. Peaks: A = 96.4 pg in internal standard; B = 5MC. (b) Determination of HMU in 7.8 mg of calf thymus DNA. Conditions as in Fig. 4b. Peaks: A = 33.2 pg internal standard; B = HMU.

mole% found by Crain and McCloskey²¹ using isotope dilution MS. Similarly, LC-MS analysis of a sample of human leucocyte DNA found 0.63 mole% of 5MC compared to $0.8 \pm 0.04\%$ by GC-ECD.

An HMU sample derived from 7.8 mg of calf thymus DNA was also analyzed by LC-MS. A representative chromatogram is shown in Fig. 5b. The data correspond to 0.7 ng of HMU in the hydrolyzed DNA sample. This is in good agreement with values of 0.7 ng by GC-ECNCI-MS and 1.0 ng by GC-ECD²⁰ for this hydrolyzed DNA preparation. Similarly, 4.4 pg of HMU base was measured in the sample obtained from 7.8 μ g of DNA compared to 3.0 pg by GC-ECD.

This value is artifactual since acid hydrolysis of thymidine, using the conditions described here, yields thymine contaminated with 0.001% HMU. The problem can be corrected by purifying the HMU deoxynucleotide derived from DNA by HPLC prior to acid hydrolysis. The discrepancy in the values of HMU found in the smaller *vs.* the larger DNA extracts can be attributed to the higher susceptibility of the smaller sample to undergo oxidation. It is not associated with the LC-MS instrumental method, which has been demonstrated to be highly reproducible, and therefore, does not detract from the validity of the LC-MS method to detect picogram quantities of damaged DNA bases.

DISCUSSION

The results presented here provide convincing evidence regarding the capability to conduct quantitative analysis at the trace level by LC-MS via the moving-belt interface. This is, in part, facilitated by the versatility of the interface which allows selection of the optimal ionization operating conditions of the mass spectrometer for a given type of analyte, in this case ECNCI-MS.

A comparison of Figs. 4 and 5 shows that the sensitivity achieved by the method for pure standards translates well to the analysis of complex matrices. The 5MC peak in the chromatogram of the DNA hydrolysate (Fig. 5a) represents 25.3 pg. The peak height and signal-to-noise ratio of this peak are consistent with the 5MC peak in Fig. 4b resulting from the injection of 9.9 pg of pure standard.

An examination of the HMU internal standard peaks in Figs. 4a and 5b again shows good correlation in terms of the signal-to-noise ratios for the standard and the sample. It is noteworthy that the HMU peak in the hydrolysate chromatogram (Fig. 5b) corresponds to 20 pg detected, and that this is $100\times$ the amount shown for the pure standard in Fig. 4a. The HMU peak in Fig. 4a is barely visible next to the internal standard in the bottom trace, which represents the TIC (m/z 501 + m/z 483).

In this work, we observed better performance for HMU compared to 5MC in two general respects. First, initial attempts to handle solutions containing trace amounts of these compounds in non-silanized glassware resulted in erratic results with 5MC while consistent measurements were obtained for HMU. No memory effects were observed, however, on the moving belt for either compound when operated at a constant temperature.

Secondly, under LC-MS conditions the response of the 5MC derivative dropped off precipitously as the water content of the mobile phase was increased. For example, at 70% aqueous methanol, a 5% increase in the water concentration resulted in a 50% decrease in the measurable peak area. The mobile phase used for the

LC-MS analysis of the 5MC derivative (methanol-water, 90:10) represents a compromise between minimizing this problem and selecting an optimum composition for the LC separation. This phenomenon was not observed under LC-UV conditions, nor was it encountered in the development of the HMU assay.

For both the 5MC and HMU derivatives we observed, using MS detection, that there was no difference, within experimental error, in the recovery of the analyte for manual spotting onto the belt *vs.* spray deposition after flow injection. On the other hand, under identical solvent composition and flow conditions, when spray deposition followed HPLC a significantly lower response was observed. This difference amounted to 40% less recovery for the 5MC derivative and 75% less recovery for the HMU derivative.

Related to these problems, and perhaps to other aspects of the system as well, the relative molar response of the HMU derivative to the 5MC derivative by LC-MS was about 100:1 under the conditions selected for most of the work (see Fig. 3). This is in contrast to a more comparable molar response of the two derivatives (3:1) under GC-MS conditions. Thus 180 fg of HMU could be detected by LC-MS compared to 10 pg of 5MC.

The better performance of the HMU derivative over the 5MC derivative in these respects is probably due, in part, to the active hydrogen on the latter compound, resulting in greater surface interaction. This points to the importance of optimizing the structure of the derivative in order to take full advantage of LC-MS for high sensitivity.

The HMU analysis of calf thymus DNA affords a rather unique opportunity to compare LC-ECNCI-MS to the vapor phase methods of GC-ECD and GC-ECN-CI-MS. The results are presented in Table II. Rather striking is the good correlation of the results and, in particular, the comparable detection limits (180 fg and 60 fg) attainable by LC-ECNCI-MS and GC-ECNCI-MS, respectively. This comparison of course does not take into account the differences in band widths associated with LC and GC. It is conceivable that the correspondence in the detection limits would be even closer if peaks of identical width could be attained. Clearly, such comparisons are also compound dependent. For example, it is unlikely that substances that are highly volatile or highly ionic or very polar in nature will behave in an identical

TABLE II
COMPARISON OF HPLC-MS AND GC-ECD RESULTS FOR HMU

<i>Sample</i>	<i>Method</i>	<i>Amount^a</i>
7.8 mg Calf thymus DNA	GC-ECD	1.0 ng / 7.8 mg
	GC-ECNCI-MS	0.7 ng / 7.8 mg
	LC-ECNCI-MS	0.7 ng / 7.8 mg
7.8 μ g Calf thymus DNA	GC-ECD	3.0 pg / 7.8 μ g
	LC-ECNCI-MS	4.4 pg / 7.8 μ g
Detection limits:	GC-ECNCI-MS:	60 fg (signal-to-noise ratio 5)
	LC-ECNCI-MS:	180 fg (signal-to-noise ratio 10)

^a As explained in the text, the amount of HMU is arbitrary since acid hydrolysis converts 0.001% of thymidine to HMU.

manner by LC-MS and GC-MS. However, these results indicate that, given suitable physical characteristics of an analyte, LC-MS via the moving-belt interface can compare favorably with GC-MS as a quantitative tool for trace analysis.

In summary, we have observed that LC-MS with a moving-belt interface can be nearly as sensitive as GC-ECNCl-MS for quantifying a derivatized DNA base as long as an appropriate derivative is selected. Furthermore, electrophore-based analytical methodology terminating in LC-MS shows great promise for the sensitive determination of alkyl and related chemical damage to DNA.

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ANALYSIS OF HEPARINS BY SIZE-EXCLUSION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH PHOTO-DIODE-ARRAY DETECTION

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SUMMARY

High-performance liquid chromatography (HPLC) of heparins was carried out with on-line photodiode-array detection. Average molecular weights and molecular weight distribution were calculated using computer data acquisition and handling; size-exclusion chromatography was performed using different selective microparticulate columns and narrow molecular weight distribution heparin standards for calibration; heparin peak purity was investigated using several methods for evaluation. Additional UV-absorbing compounds present in heparin preparations were characterized and quantitated by reversed-phase HPLC. These methods are useful for the analysis of the molecular weight distribution and peak purity of heparins and for the determination of additional drugs, additives or impurities.

INTRODUCTION

In recent years research on the chemical, biochemical and therapeutic properties of heparins has been carried out^{1–6}, particularly for low-molecular-weight (LMW) heparins^{7–9}; these are obtained generally by the fractionation or depolymerization of unfractionated (UF) heparins (molecular weight range 3000–40 000 daltons)¹. LMW heparins are claimed to possess antithrombotic and anticoagulant properties with reduced bleeding potential, greater bioavailability, longer biological half-lives and duration of therapeutic action compared with UF heparins^{7–9}. Heparins are polydisperse heterogeneous sulphated glycosylaminoglycans^{1,6} whose anticoagulant properties are related to their molecular weight distributions (MWD)⁷; therefore, the determination of the average molecular weights (MWs) and MWDs are important parameters for their characterization. Heparin solutions are administered parenterally^{10,11} and the pharmaceutical products^{12–14} may also contain other drugs, additives, preservatives and undeclared compounds; it is therefore necessary to analyse samples for these substances, which may be pharmacologically active or lead to unwanted reactions¹⁵.

We have previously reported¹⁶ the characterization of heparins using size-ex-

clusion chromatography (SEC) and the determination of their average MWs and MWDs and the different methods for the determination of the MW of heparins were discussed. Although the calculated MWs and MWDs determined by that method¹⁶ were in agreement with those reported by other workers, it had several limitations: the manual quantitative evaluation of SEC data was slow and tedious; the use of tritiated heparin derivatives as calibration standards required special precautions and had restricted LMW ranges; and UV detection at a single wavelength could not be applied to the determination of heparin peak purity or to the characterization of heparin peak purity or to the characterization of additional extraneous chromatographic peaks.

To overcome the above difficulties, we report here the SEC analysis of heparins using high-performance liquid chromatography (HPLC) with on-line photodiode-array detection (DAD), computer data acquisition and handling and narrow-MWD heparin standards for calibration. Reversed-phase HPLC was also used for the qualitative and quantitative analysis of other additional UV-absorbing compounds.

EXPERIMENTAL

Equipment

A Model 1090M liquid chromatograph with a DR5 ternary solvent delivery system, autoinjector, autosampler and a photodiode-array detector (range 190–600 nm, with 4.5- μ l cell volume and 6-mm path cell) was used (Hewlett-Packard, Waldbronn, F.R.G.). It was connected to a Model 79994A analytical workstation (Hewlett-Packard) for system control, data acquisition and evaluation and report generation; the installed software was HP 79995A (rev. 4.05) operating software, HP 79997A color view software and HP 79999A (rev. 4.05) GPC software (Hewlett-Packard).

Chromatographic conditions

SEC analysis. Ultropac TSK columns (300 $\times$ 7.5 mm I.D.) were used: G 2000 SW (2135-230) or G 3000 SW (2135-230) (LKB, Bromma, Sweden). The eluent was 0.1 M aqueous sodium chloride (isocratic), at 0.5 ml/min and the injection volume was 20 μ l.

Reversed-phase HPLC. A Hypersil ODS (5 μ m) column (100 $\times$ 2.1 mm I.D.) and a guard column (20 $\times$ 2.1 mm I.D.) (Hewlett-Packard) with the same filling were used. The eluent (flow-rate 0.5 ml/min) consisted of (A) phosphate buffer (0.01 M, pH 4.0) and (B) acetonitrile, with a solvent programme of 0–1 min 0% B and 1–10 min to 90% B with a run time of 13 min; the equilibration time was 2 min and the injection volume 2–10 μ l. Eluents were filtered and gassed with helium before use.

DAD parameters. The sample wavelengths were set at 206, 220, 240 and 280 nm (4 nm band width) and the reference wavelength at 550 nm (100 nm band width); spectrum storage (range 200–400 nm) was set in the all-acquisition or peak-controlled mode.

Chemicals

All chemicals were of analytical-reagent grade; solvents for HPLC were chromatographically pure. Pharmaceutical preparations of UF and LMW heparins were

commercially available. Heparin USP XXI (Lot 936275), LMW heparin and heparin calibration standards were gifts from Sandoz (Nürnberg, F.R.G.). Reference narrow-MWD heparin calibration standards with known MW were used for SEC calibration (MW 7500, 10 400, 14 500 and 19 000 daltons); their MWs had been determined previously using low-angle laser light scattering (LALLS).

Calculations

SEC analysis. Average MWs and MWDs were calculated from the calibration standards and sample data obtained at 206 nm. Using the GPC software¹⁷, the calibration graph was obtained, and average MWs [M_n , number-average MW; M_w , weight-average MW; M_z , z-average MW; M_{z+1} , ($z+1$)-average MW; M_{z+2} , ($z+2$)-average MW; D , polydispersity = M_w/M_n] and MWDs (differential and cumulative) were calculated¹⁷⁻¹⁹.

Peak purity. Heparin peak purity was obtained from the sample data and the operating software; comparison of peak spectra, extract chromatograms at different wavelengths and absorbance ratios between two wavelengths were calculated and displayed.

Additional peak analysis. Identification of signals from other peaks was made in a similar manner to that for heparin. Quantitative analysis was performed by integrating the absorbance signals, with internal or external calibration, both for SEC and reversed-phase HPLC.

RESULTS

SEC analysis

Stationary phases were selected according to the nature of the sample; some analyses were run with bimodal column coupling¹⁸; aqueous sodium chloride was used as the eluent. An LMW heparin was analysed with these columns and the chromatograms are shown in Fig. 1.

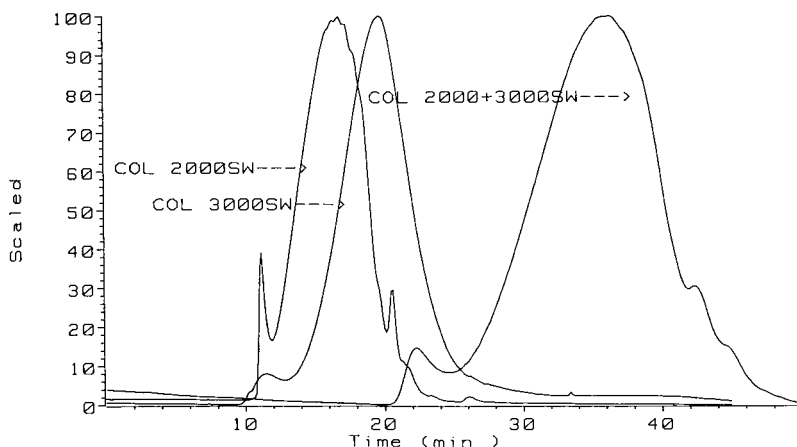


Fig. 1. SEC analysis of an LMW heparin with different columns and column coupling. Maximum absorbance (at 206 nm) scaled to 100.

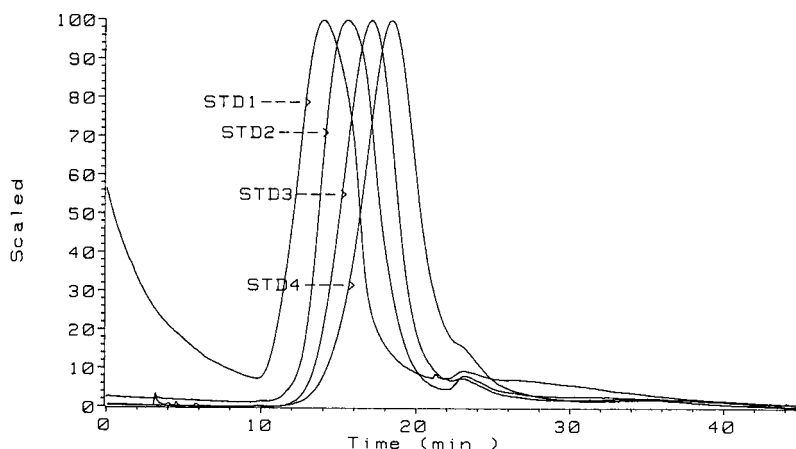


Fig. 2. SEC analysis of narrow-MWD heparin calibration standards (standards 1–4 with MW 19 000, 14 500, 10 400 and 7500, respectively); column, 3000 SW. Maximum absorbance (at 206 nm) scaled to 100.

MW averages and MWD. Narrow-MWD calibration heparin standards with known MW were analysed and the chromatograms are shown in Fig. 2. Data from the analysis of samples and the previously established calibration graph were processed using the GPC software^{17–19} to calculate MWDs (cumulative and differential), average MWs and polydispersity. The precision of the average MW determination was better than 1%.

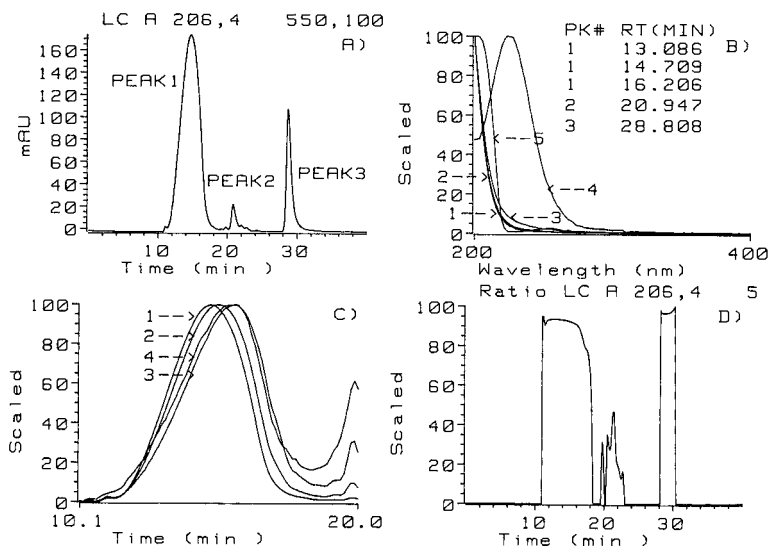


Fig. 3. Analysis of a commercial LMW heparin pharmaceutical product (column 2000SW). (A) Chromatogram at 206 nm; (B) UV spectra of heparin peak 1 (spectra 1, 2 and 3) and peaks 2 and 3 (spectra 4 and 5); (C) merged chromatograms from heparin peak (retention time 10–20 min) with multi-wavelength detection; (D) absorbance ratio 206/220 nm.

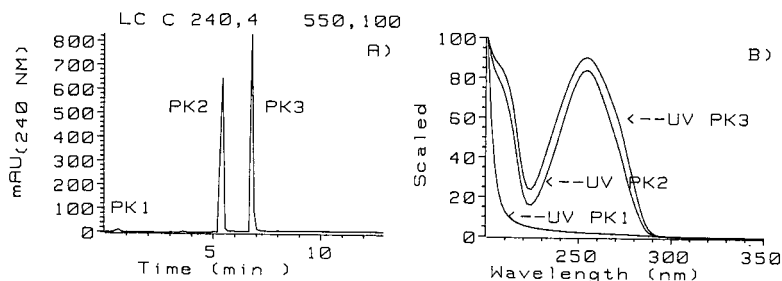


Fig. 4. Analysis of a heparin sample containing methyl (M) and propyl (P) 4-hydroxybenzoates. (A) Chromatogram with reversed-phase column, heparin (peak 1), M (peak 2) and P (peak 3); (B) corresponding UV spectra.

Heparin peak purity. A commercial heparin pharmaceutical product was analysed by SEC and the chromatogram is shown in Fig. 3A; supplementary peaks (peaks 2 and 3) appear apart from that of heparin (peak 1); several criteria for peak purity determination are exemplified in Fig. 3B–D: UV spectra on the ascending, apex and descending portions of the heparin peak (spectra 1, 2 and 3) and those for peaks 2 and 3 are displayed in Fig. 3B; Fig. 3C represents the merged chromatograms at different wavelengths during the heparin peak time interval; and the absorbance ratio between the signals at two wavelengths is shown in Fig. 3D for the same sample.

Reversed-phase HPLC analysis of heparin additives

Heparin samples were analysed by reversed-phase HPLC. Fig. 4 shows the analysis of a commercial heparin sample containing methyl and propyl 4-hydroxybenzoate as preservatives; external calibration allowed the quantitation of the components. Other additives mentioned by manufacturers were analysed in a similar way (retention times in minutes): dihydroergotamine mesylate (7.71); benzyl alcohol (4.97), *p*-cresol, (5.59), xylocaine (7.55) and L-ephedrine (4.54).

DISCUSSION

The use of different SEC columns, narrow-MWD calibration heparin standards, DAD, computer data acquisition and data handling induced us to reinvestigate our previous HPLC methodology for the analysis of heparins. We shall mention the most significant differences from our previous method¹⁶.

The advantages of DAD over conventional UV detection were discussed previously^{20–28}; improved analysis of heparins, for the determination of both average MWs and MWDs, peak purity and the analysis of other components result from its use.

SEC analysis

Gel permeation columns with modified microparticulate fillings were used for aqueous SEC analysis; however, compact columns (30 cm instead of 60 cm) were used with no loss of resolution; columns with different operating MW ranges were selected according to the heparin sample, to allow better differentiation of products from

several origins. Heparins were usually analysed with the 3000SW or 2000SW columns; for example, Fig. 1 shows that the 2000SW column is inadequate for the analysis of this heparin sample, as a peak at the exclusion volume is present. Heparins with a wide MWD were analysed with bimodal column coupling, but longer analysis times were needed (Fig. 1). Different salts, buffers and pH did not show advantages over the previously used aqueous sodium chloride¹⁶ as eluent.

Average MWs and MWDs. Compared with our previous technique¹⁶, the present method offers advantages for the determination of average MWs and MWDs as follows. (a) The calibration standards used in this work had the same chemical composition as the samples, narrow-MWD ranges and their MWs had been previously determined by LALLS (Figs. 2 and 3); these conditions and frequent column recalibrations^{18,19,29} are essential for the correct measurement of average MWs and MWDs of heparin samples. Previously a mixture of tritiated chemically modified LMW heparins was used for calibration¹⁶; apart from the restricted range in the LMW region which did not allow extrapolations to higher MW, and the differences in the structures of the standard and the sample, the use of radioactive material afforded special precautions. (b) A wavelength of 206 nm was used, as previously explained¹⁶, as this wavelength also allows the detection of other UV-absorbing components. The advantages of multi-wavelength and spectral scan analysis in this work are detailed below.

Heparin peak purity. Apart from the determination of average MWs and MWDs of heparins by SEC and DAD, the peak purity of heparins was additionally characterized. Fig. 3A shows the chromatogram of a commercial LMW heparin and the peak purity was investigated using several criteria, as follows. (1) The UV spectra at different retention times are displayed and compared in Fig. 3B, which shows the spectra on the ascending, apex and descending portions of the heparin peak (spectra 1, 2 and 3, respectively). Whereas the first two spectra are superimposable, spectrum 3 shows a significantly higher absorption, indicating an impurity; peaks 2 and 3 elute with higher retention times than heparin and indicate the presence of additional components (spectra 4 and 5, respectively); mathematical processing of spectral data such as derivatization or subtraction of spectra are additional ways of comparing spectra (not shown). The identification of an unknown peak merely from the retention time and spectral characteristics is difficult in the absence of data from known standards; if these are available they may be stored in a library for comparison with unknown compounds. (2) Fig. 3C shows the merged chromatograms obtained at different wavelengths during the elution of the heparin peak; a pure peak not containing foreign UV-absorbing substances should show four superimposable traces; this does not occur with this sample (Fig. 3C), indicating an impure heparin peak. (3) The ratios of the absorbances at two different wavelengths may be displayed, and pure peaks present a plateau. Absorbance ratios for the heparin sample are displayed in Fig. 3D; the descending part of the heparin peak contains a UV-absorbing impurity and the peak at 20.855 min is in fact a multi-component moiety; the peak at 28.743 min is pure.

These methods (and others such as three-dimensional plots of absorbance, wavelength and time) are useful for the additional evaluation of heparin SEC results.

Reversed-phase HPLC analysis of heparin additives

Additional extraneous LMW substances do not show sharp peaks or are not eluted by SEC; HPLC with reversed-phase columns is more suitable for their qualitative and quantitative analysis. Fig. 4 shows the chromatogram of a commercial heparin containing declared preservatives, which were readily identified and quantitated; optimal UV wavelengths may be selected according to the spectral characteristics of the compounds to be investigated. The use of microbore columns leads to fast analysis with good accuracy and precision.

SEC analysis using differential refractometers or on-line LALLS as detectors have been described recently^{29,30} for the determination of average MWs and MWDs of heparins; these methods, however, are insensitive for the analysis of traces of impurities or for the identification of additional compounds. The method described above with DAD is suitable for obtaining more information in this respect.

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PROFILING OF *ALTERNARIA* MYCOTOXINS IN FOODSTUFFS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIODE-ARRAY ULTRAVIOLET DETECTION

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SUMMARY

The potential of high-performance liquid chromatography (HPLC) with diode-array detection for the profiling of mycotoxins in food samples has been demonstrated. A gradient elution reversed-phase chromatographic method coupled with a suitable extraction procedure was devised for the separation and detection of major *Alternaria* mycotoxins in foodstuffs. Altenuene, alternariol, alternariol methyl ether (dibenzo- α -pyrone derivatives), altertoxin-I and altertoxin-II (perylene derivatives) were profiled in extracts of artificially infected maize, rice and tomato samples and naturally contaminated sunflower seeds. First evidence of the occurrence of a new dibenzo- α -pyrone derivative in *Alternaria* cultures is also reported.

INTRODUCTION

The genus *Alternaria* is one of the most common moulds contaminating foods and feeds, causing considerable economic losses to the food industry due to rotting of fruits and vegetables¹. *Alternaria* produces many metabolites of different chemical structure, and some exhibit mammalian toxicity. The dibenzo- α -pyrone and perylene derivatives shown in Fig. 1 are, together with tenuazonic acid, the *Alternaria* metabolites of major concern because of their toxicity and/or natural occurrence in a number of fungal-contaminated commodities.

Thin-layer chromatography is the most widely used technique for the detection of these mycotoxins (for a review, see ref. 2), although gas chromatography (with flame ionization³ or mass spectrometric detection⁴) and high-performance liquid chromatography (HPLC)⁵⁻¹¹ have also been used with varying degrees of success. According to Schade and King², HPLC may eventually be the procedure of choice for detecting *Alternaria* mycotoxins, but further developments are necessary to ensure the identification and quantitation of some toxins in foodstuff extracts.

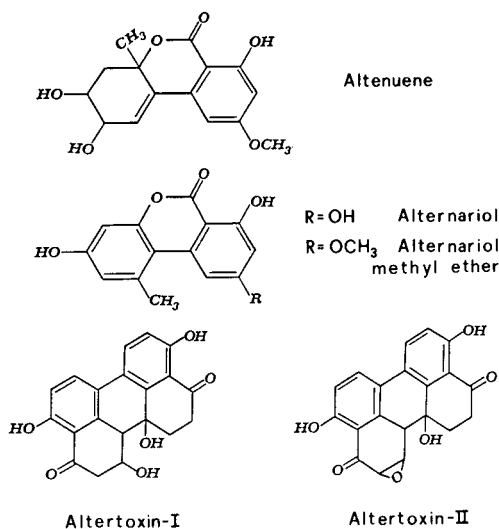


Fig. 1. Structures of representative dibenzo- α -pyrone and perylene derivatives.

Profiling of mycotoxins in fungal-contaminated foodstuffs by liquid chromatography coupled to detectors that are unable to give retention data corroborated by peak purity and peak identity tests may result in unreliability owing to the complexity of the matrix. The use of a blank extract (obtained from an uncontaminated sample) to check for potential interferents does not necessarily solve the problem. With such an approach interferences arising from fungal metabolites other than the selected toxins cannot be definitely excluded.

The advent of computer-controlled photodiode-array UV detectors in HPLC¹² has provided considerable help solving such a complex analytical problem by greatly improving peak identification, peak purity assessment and quantitation. The high spectral acquisition rate during elution provides a matrix of absorbance-wavelength-time data that can be treated by computer-aided techniques for their reduction, manipulation and presentation.

In this paper, the potential of diode-array detection in the HPLC analysis of *Alternaria* contaminated foodstuffs is demonstrated. A gradient elution reversed-phase chromatographic method was devised that simultaneously separated alternuene (ALT), alternariol (AOH), alternariol methyl ether (AME), altertoxin-I (ATX-I) and altertoxin-II (ATX-II). Tenuazonic acid, actually isolated in the extraction step, is determined separately using a procedure described elsewhere⁹ and is not considered here. An *Alternaria* metabolite with spectral characteristics very similar to those of alternuene (but separately eluting) has been found in the extract of *Alternaria alternata* (Fr.) Keiss. cultured on rice.

EXPERIMENTAL

Chemicals

Reference standards of AOH, AME, ALT, ATX-I and ATX-II were produced and isolated as described elsewhere⁹.

Water and methanol were of HPLC grade (Carlo Erba, Milan, Italy). All other chemicals were of analytical-reagent grade.

Apparatus

A Perkin-Elmer (Norwalk, CT, U.S.A.) Model 3B pump module equipped with a Rheodyne 7125 injection valve and a 125 × 4.6 mm I.D. reversed-phase ODS column (Bischoff, Leonberg, F.R.G.) with a Spherisorb ODS (5 µm) packing was used as the chromatographic system. A Bischoff guard column (30 × 4.6 mm I.D.) with Spherisorb ODS (5 µm) packing was used to protect the analytical column. The detector was an HP 1040A photodiode-array spectrophotometer (Hewlett-Packard, Palo Alto, CA, U.S.A.) interfaced to an HP 85 computer equipped with an HP 9121 dual disk drive and an HP 7470A plotter.

Sources of contaminated samples

Strains of *Alternaria alternata* were cultured on rice, maize and tomatoes in order to have artificially infected samples. For example, rice (200 g) was brought to about 50% moisture in a 500-ml erlenmeyer flask, autoclaved for 20 min at 120°C before fungal inoculation and then incubated in the dark for 3 weeks at room temperature (this is one of the best ways to simulate naturally contaminated foodstuffs).

Sunflower seeds naturally infected by *Alternaria alternata* were collected in the field in southern Italy and finely ground in a Buehler mill before toxin extraction.

Toxin extraction

Undried cultures or naturally contaminated samples (50 g) were extracted in a blender with 75 ml of methanol and filtered. A 40-ml portion of the filtrate was clarified by addition of 80 ml of 5% aqueous ammonium sulphate and filtered. A 90-ml volume of the filtrate (corresponding to 20 g of the original substrate) was extracted twice with 5 ml of methylene chloride. For oleaginous samples (sunflower seeds or corn kernels) a defatting step, with 30 ml of *n*-hexane, preceded the methylene chloride extraction. The combined extracts containing the dibenzo- α -pyrone and perylene derivatives were evaporated to dryness and reconstituted with 1 ml of methanol. The aqueous layer could be processed according to a procedure described elsewhere⁹ to determine tenuazonic acid.

For recovery experiments the uncontaminated substrate was spiked with the required amount of standard toxins, extracted as above and analysed by HPLC. Recovery was determined by calculating the peak-height ratio of samples and standards. Each experiment was done in triplicate.

Chromatographic conditions

A binary gradient system composed of methanol (solvent A) and H₃PO₄-acidified (pH 3) water was used. The elution programme was (1) from 50 to 70% solvent

A in 10 min, (2) from 70 to 85% in 0.1 min, then (3) isocratically for 8 min. The equilibration time between two successive runs was 8 min.

The injection volume was 2–5 μ l of the crude extract. Unless specified otherwise, spectra were acquired in the 210–400 nm range on the apex and on the ascending or descending part of each peak using a pilot signal at 257 nm, a reference signal at 550 nm (100 nm band width) and a threshold value of 1 mAU¹³. Up to seven chromatograms at different wavelengths could be retrieved and processed after the chromatographic run. Reference spectra were acquired during the elution of a standard mixture and used for peak identification purposes by comparison after spectral normalization.

Thin-layer chromatography was performed as described elsewhere⁹.

RESULTS AND DISCUSSION

A standard mixture of ALT, AOH, AME, ATX-I and ATX-II was injected in order to obtain retention time data and an archive of reference spectra under the operational experimental conditions for the samples. Linearity of the absorbance vs. concentration plot was observed for more than three decades of concentration and detection limits calculated from the calibration parameters were 5, 10, 3 and 10 ng on-column for ALT, ATX-I, AOH, and AME, respectively (no quantitative data were obtained for ATX-II because of the limited amount of standard available). Each of the values given above refers to the absorption maximum wavelength for each toxin (4 nm band width) and could be slightly enhanced by diode bunching¹⁴.

Recoveries from rice samples spiked with different amounts of toxin (from 0.2 to 10 μ g/g) ranged from 50 to 76%, as shown in Table I.

Fig. 2A shows the overall spectrochromatogram of a rice extract in which the spectra of alternariol and alternariol methyl ether are clearly identified. This furnishes an immediate example of the usefulness of three-dimensional plots in giving a first indication of the presence and identity of certain analytes possessing characteristic UV spectra. Obscured peaks were observed by rotating the viewing angle of the wavelength–time data plane and, eventually, zooming the zone of interest on the spectrochromatogram. Fig. 2B shows a typical example in which the examination of the sub-spectrochromatogram in the time window 11.6–13.0 min reveals the presence of ATX-II. Multi-signal chromatograms could be used to optimize, within a

TABLE I

RECOVERY OF SOME *ALTERNARIA* MYCOTOXINS FROM SPIKED RICE SAMPLES

Added (μ g)	Recovery (%) (mean $\pm$ S.D., n=3)			
	<i>Altenuene</i>	<i>Alternariol</i>	<i>Alternariol methyl ether</i>	<i>Altartoxin-I</i>
0.2	62.0 $\pm$ 6.7	72.0 $\pm$ 3.6	74.7 $\pm$ 3.8	—
1.0	51.6 $\pm$ 4.7	70.0 $\pm$ 3.2	76.0 $\pm$ 3.5	70.0 ^a
10.0	51.0 $\pm$ 4.1	50.0 $\pm$ 7.1	73.5 $\pm$ 4.0	—

^a Single determination.

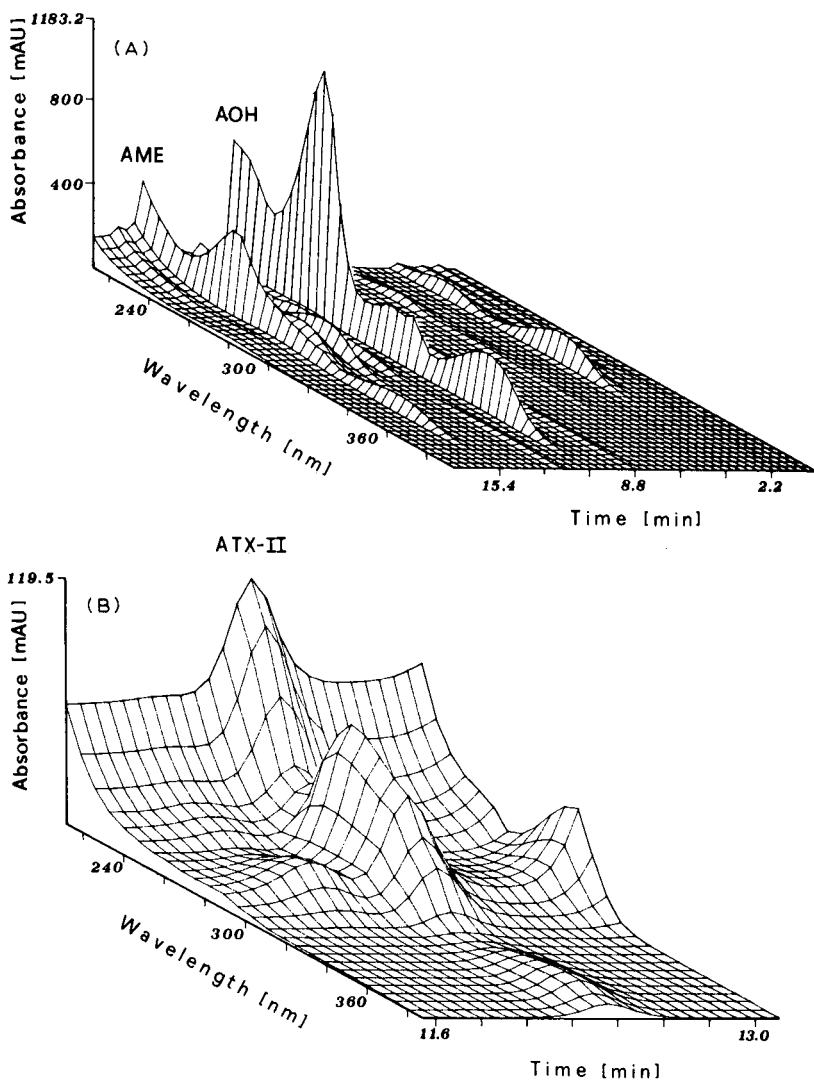


Fig. 2. (A) Overall spectrochromatogram for the extract of an artificially infected rice sample. (B) Sub-spectrochromatogram in the time region where altetroxin-II is eluted. Experimental conditions as described in the text except for spectral acquisition parameters; here 16 spectra/min were acquired.

single run, the sensitivity for the simultaneous detection of different toxins; this reduced considerably the analysis time compared with traditional methods^{4,5,9} requiring multiple sequential injections at different wavelengths selected on the absorption maxima of each toxin.

A multi-signal chromatogram relevant to a rice sample artificially infected with *Alternaria* is shown in Fig. 3. Trace A (240 nm, 4 nm band width) may be regarded as a non-selective "total-peak chromatogram", and provides the maximum sensitivity for ALT; trace B at 257 nm (4 nm band width) optimizes the sensitivity for AOH, AME, ATX-I and ATX-II; trace C at 340 nm (40 nm band width) may be regarded as

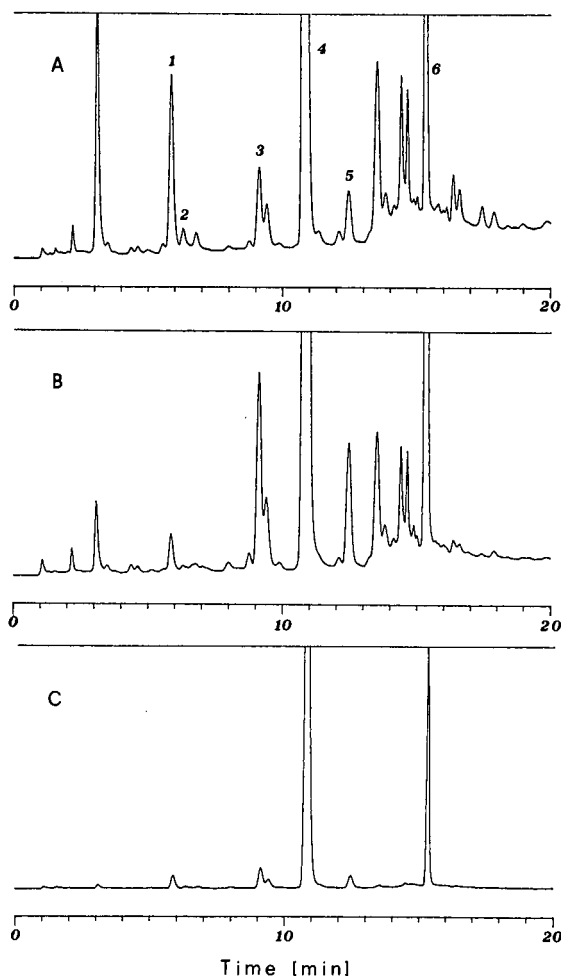


Fig. 3. Multi-signal plot of an extract of an artificially infected rice sample. Chromatographic conditions and spectral acquisition as specified under Experimental. Detection at 240 (trace A), 257 (trace B) and 340 nm (trace C). Spectral band width: 4 nm for traces A and B and 40 nm for trace C. Attenuation: 200 mAU full scale. Peaks: 1 = ALT (5 ppm); 2 = ALT isomer; 3 = ATX-I (3.7 ppm); 4 = AOH (200 ppm); 5 = ATX-II (2.3 ppm); 6 = AME (17.5 ppm).

a “toxin-selective” chromatogram because in this wavelength region the background contribution is minimized; it may be helpful for peak recognition with naturally contaminated samples.

For each peak a homogeneity test was performed by overlaying (after normalization) the spectra acquired on the apex and the ascending and descending parts of the peak. Provided no inhomogeneity was found, a peak identity test was performed by comparing the spectra of the unknown with the spectra of the standard (see Fig. 4 for an example).

Close inspection of spectrochromatograms of *Alternaria* culture extracts in the region around the elution times of altenuene revealed the presence of an additional

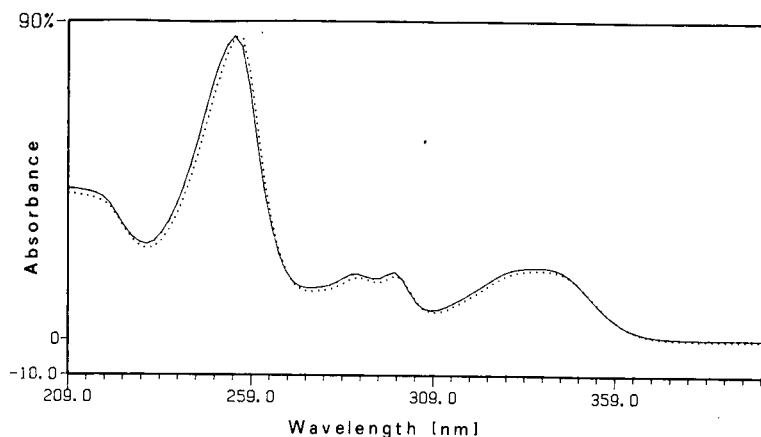


Fig. 4. Peak identity test for alternariol in a rice extract. Solid line, sample spectrum; dotted line, reference spectrum retrieved from a previously created archive.

compound with strictly similar UV spectral characteristics. We have isolated a suitable amount of the altenuene-like compound by preparative-scale HPLC from a culture of *Alternaria alternata* on rice, and identified it as an isomer of altenuene¹⁵. Note that a poor separation of the above compounds could have led to a failure of peak purity criteria, such as peak overlaying and ratio chromatograms, due to spectral similarity.

The method described here has been used successfully for profiling *Alternaria* mycotoxins in extracts of artificially infected rice, maize and tomato samples. For example, the chromatogram relevant to an extract of tomatoes containing 0.09 $\mu\text{g/g}$ of ATX-I, 35 $\mu\text{g/g}$ of AOH and 0.2 $\mu\text{g/g}$ of AME is shown in Fig. 5. Fig. 6 shows the spectrochromatogram, acquired in the elution region of AOH, relevant to the extract of a naturally contaminated sunflower seed sample; in this sample AOH and AME were both unambiguously detected and quantitated (by the standard additions method) at levels of 0.36 and 0.13 $\mu\text{g/g}$, respectively. The above findings were corroborated by thin-layer chromatographic analysis of the same extract and demonstrate the applicability of the proposed method to real fungal-contaminated samples containing *Alternaria* metabolites at sub-ppm levels.

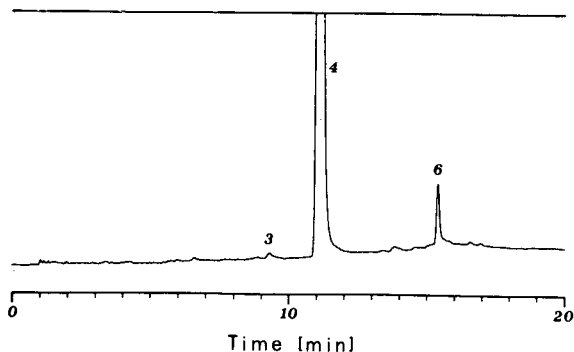


Fig. 5. Chromatogram relevant to the extract of an artificially infected tomato sample. Detection at 340 nm with a spectral band width of 40 nm. Attenuation, 10 mAU full scale. Peaks as in Fig. 3.

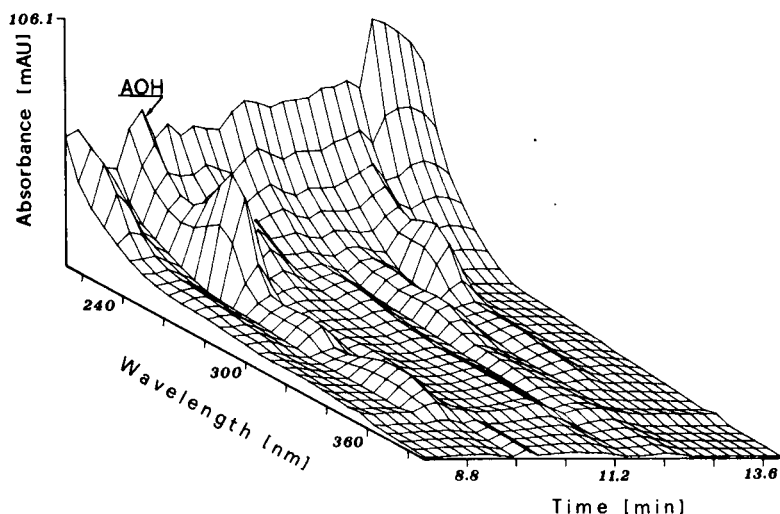


Fig. 6. Sub-spectrochromatogram (AOH elution region) relevant to the extract of a naturally contaminated sunflower seed sample.

CONCLUSIONS

The potential of diode-array detection for *Alternaria* mycotoxins profiling in food samples has been demonstrated. The multi-signal plotting capability may be an aid for a first identification (through the choice of selective wavelengths) and at the same time can optimize the detection sensitivity for compounds (or classes) having different absorption maxima. In addition, three-dimensional spectrochromatograms give UV spectra and the possibility of identification of those toxins having characteristic spectra. Spectra of unknown compounds can be compared with reference data previously stored and peak homogeneity can be checked.

Although UV spectra alone can rarely give an absolutely certain identification of a compound, a reasonable degree of confidence can be reached in most instances. Coupling of a diode-array UV detector to a functional group-specific detector, *e.g.*, electrochemical (in progress in our laboratory), should provide additional evidence (and sensitivity). This could avoid the need for sophisticated LC detection techniques such as mass spectrometry.

ACKNOWLEDGEMENTS

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AUTOMATED ZONE-ELECTROPHORETIC SAMPLE TREATMENT FOR THE ANALYSIS OF BIOLOGICAL SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The automation of zone-electrophoretic sample treatment for liquid chromatography is described. The procedure is completely controlled from a liquid chromatograph. The carry-over of proteins from human serum under different experimental conditions was studied. The influence of the presence of proteins in the sample is illustrated with the anionic compound salicylic acid and the increase in selectivity for cationic compounds is demonstrated with the determination of ephedrine, norephedrine and amphetamine in urine.

INTRODUCTION

Recently a new on-line sample treatment method for high-performance liquid chromatography (HPLC) using zone electrophoresis was introduced¹. This zone-electrophoretic sample treatment (ZEST) method is based on the different migration velocities of compounds in free solution in an applied electric field. The system, consisting of three interconnected pieces of capillary, is coupled on-line to an HPLC apparatus.

The ZEST procedure consists of four stages: (1) all capillaries are filled with electrophoresis buffer; (2) the first (sample) capillary is filled with sample solution; (3) an electric field is applied over the connected capillaries and migration of ionic compounds with the appropriate charge takes place from the sample capillary, through the second (transfer) piece of capillary towards the injection capillary; and (4) after a given time the electrophoresis is stopped. The compounds that have migrated into the

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injection capillary are flushed by the mobile phase towards the column and separation by HPLC can be achieved.

In preliminary studies with a manually controlled system it was shown that it is possible to isolate low-molecular-weight ionic compounds from biological fluids such as blood serum. A theoretical description was given by Kok² regarding optimization of the geometry and the electrophoretic conditions.

In this paper the automation and further evaluation of the method and applications to the analysis of serum and urine samples are described. Automation of the method is necessary, as the procedure consist in a sequence of consecutive steps and is sensitive to disturbances caused by manual manipulations. The introduction of an air bubble, for instance, in the capillary system during the injection of the buffer or the sample would interrupt the electrophoretic pathway, and exact timing is important for the reproducibility of the method. The influence of automation on the reliability and reproducibility has been studied.

Two aspects of this method were evaluated: the possibility of achieving the complete isolation of small ionic compounds from proteins in a biological matrix, and the increase in selectivity which is expected from this pretreatment method. For the first aspect, the carry-over of proteins from human serum to the injection capillary under different experimental conditions was studied. The influence of the presence of proteins in the sample was investigated in the determination of the anionic compound salicylic acid.

For the second aspect, the isolation of amphetamine and its derivatives ephedrine and norephedrine as cationic compounds from urine samples was investigated. Because of the relatively high absorption coefficient at low wavelength (about 40 times higher than at 254 nm), these compounds are preferably detected at 210 nm³. However, the selectivity of liquid chromatography alone for the determination of these compounds in a complex matrix such as urine is inadequate with detection at 210 nm⁴. Therefore, ZEST was evaluated as a method for increasing selectivity in this application.

EXPERIMENTAL

Chemicals and solutions

Sodium dihydrogenphosphate, disodium hydrogenphosphate, methanol and Folin-Ciocalteu phenol reagent were obtained from Merck (Darmstadt, F.R.G.). Salicylic acid, phthalic acid, copper sulphate, sodium *d*(+)-tartrate, sodium carbonate and ammonium sulphate were purchased from BDH (Poole, U.K.) and benzoic acid from Fluka (Buchs, Switzerland). Norephedrine was obtained from Janssen Chimica (Beerse, Belgium) and *d*-ephedrine from Aldrich (Milwaukee, WI, U.S.A.) The test compound solutions of salicylic acid, amphetamine, norephedrine and ephedrine were prepared daily from concentrated aqueous stock solutions. Pooled human serum was kept at -24°C and was used without any further treatment.

Electrophoresis was carried out in a 0.05 *M* phosphate buffer (pH 6.5) containing 5% methanol. The mobile phase for the determination of salicylic acid was an aqueous solution containing 60% (v/v) methanol and 0.05 *M* phosphate buffer (pH 2). For the determination of amphetamine, ephedrine and norephedrine the mobile phase contained 10% methanol, 0.2 *M* ammonium sulphate and 0.05 *M* phosphate buffer (pH 2).

Apparatus

The chromatographic equipment consisted of a Hewlett-Packard 1090 liquid chromatograph, an autoinjector with a 250- μ l syringe, an autosampler and a Model 1040 diode-array detector. The detection wavelength was 230 nm in the determination of salicylic acid and 210 nm in the determination of amphetamine and its derivatives. The column (150 $\times$ 4.6 mm I.D.) was packed with Zorbax C₈ (5 μ m). Electrophoresis was performed with an LKB 3371 power supply. The whole system was controlled by a Hewlett-Packard HP-85 computer.

The ZEST procedure itself was carried out in a set of three six-port valves connected by PTFE capillaries as described previously¹. The PTFE capillaries (1/16 in. O.D., 0.48 mm I.D.) were obtained from Chrompack (Middelburg, The Netherlands). The length of the sample, transfer and injection capillaries were 40, 40 and 70 mm, respectively. Taking into account the internal volumes of the valves used, the volume of the sample loaded was 8 μ l and 14 μ l of buffer solution were injected onto the column.

Conductivities of solutions were measured with a Wayne Kerr B642 autobalance universal bridge with a conductivity cell (cell constant = 1.35 cm⁻¹, Pt black electrodes, length 1 cm)

Protein determination

Protein concentrations were determined by the detection method described by Lowry *et al.*⁵, modified for small amounts. After electrophoresis the injection capillary was flushed with water, 250 μ l of effluent were collected in a tube and 2 ml of a solution containing 2% Na₂CO₃, 0.1 N NaOH, 0.01% CuSO₄ and 0.02% sodium tartrate in water were added. After 10 min 200 μ l of Folin-Ciocalteu reagent, diluted 1:1 with water, were added and mixed. After 30 min the absorption was measured at 750 nm. For calibration the same determination was carried out with dilute serum solutions pipetted directly into the reaction tubes.

For detection a Philips PU 8700 spectrophotometer was used.

RESULTS AND DISCUSSION

Automation and development

The automated set-up is shown in Fig. 1. The air-pressure-driven switching valves and the power supply, the injection syringe and the autosampler are controlled

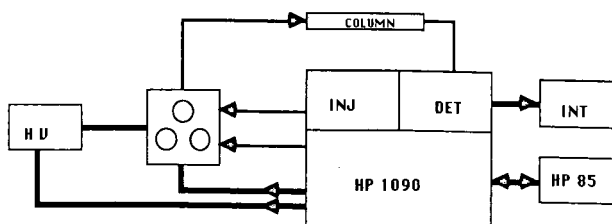


Fig. 1. Schematic diagram of set-up for automated zone electrophoretic sample treatment. INJ = injector; HV = high-voltage power supply; INT = integrator; DET = detector.

TABLE I

TIME SCHEDULE FOR ZONE ELECTROPHORETIC SAMPLE TREATMENT

Electrophoresis time, 16 min.

<i>Time (min)</i>	<i>Event</i>	<i>Valve</i>	<i>HV</i>
0.00	Program loading		
0.50	Flushing with buffer	PREP	
3.00	Sample injection	LOAD	
5.50	Start of electrophoresis	PHOR	On
6.00	Flushing with buffer		
21.50	End of electrophoresis		Off
21.55	Injection on column	ELUT	
22.00	End		

from the chromatograph. A typical timing schedule, a simplified representation of the program retrieved from disk, is shown in Table I.

The procedure starts by flushing of the capillaries with $2 \times 200 \mu\text{l}$ of buffer solution. It appeared to be necessary to use this large volume in order to avoid carry-over of previous samples. For safety reasons (high voltage) the electrophoretic system is contained in a separate compartment, so that the length of the tubing between the injection syringe and the switching valves has to be considerable. After changing the valve positions, the sample loop is filled by injecting $2 \times 200 \mu\text{l}$ of the sample solution. A decrease in the amount of sample resulted in irreproducible results. While electrophoresis is in progress, after another change in the positions of the valves, the connecting tube is flushed with buffer solution to wash out remaining sample solution. When the electrophoresis is terminated, the valves are switched to the injection position and compounds that have migrated to the injection capillary are separated on the column. During the separation the next ZEST procedure starts.

The electrical field strength in the capillaries was determined by measuring voltage-current relationships with different valve positions. With the maximum voltage of the power supply (1350 V) the electrical field strength is about 50 V cm^{-1} in the 0.48 mm I.D. capillaries. A large part of the voltage is lost in the tubing connecting the electrode vessels to the valve system. To examine the correctness of the connections of the capillaries to the valves, the measured currents were compared with calculated values. With 0.05 M phosphate buffer (pH 6.5) containing 5% methanol the current measured immediately after turning on the high-voltage supply at 1350 V was 0.37 mA. Calculation of the current, using a measured conductivity value of $5.1 \text{ ohm}^{-1} \text{ cm}^{-1}$ for this buffer, yields 0.36 mA. The fair agreement between the current outputs shows that there are no major disturbances to the electrophoretic pathway.

During the electrophoresis the current increased, until after about 10 min a steady-state level of 0.45 mA was reached using the buffer described above. The current increase is caused by the increase in the temperature in the capillaries, which can be calculated from these figures to be in the range 10–15°C.

The reproducibility of the automated system was evaluated using standard solutions of organic acids as model compounds. In Fig. 2 the results of recovery studies are shown. The recoveries were calculated by comparing peak areas obtained

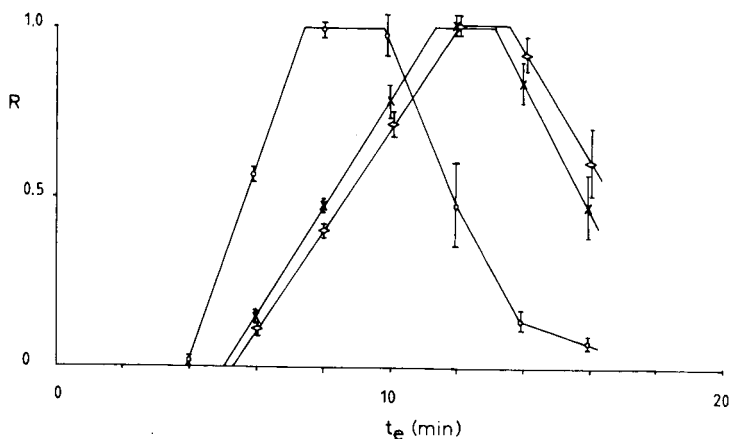


Fig. 2. Recovery (R) of (○) phthalic, (◇) benzoic and (×) salicylic acid from 0.05 M phosphate buffer (pH 6.5) versus time of electrophoresis (t_e). Each point represents the mean and standard deviation of three experiments. Mobile phase, 0.05 M phosphate buffer (pH 2)–methanol (60:40).

after ZEST with those after direct injection of the standard solutions. Fig. 2 shows that phthalic acid, which is doubly charged in the buffer used at pH 6.5, migrates faster than the monocarboxylic benzoic and salicylic acids. Partial separation can be achieved. As the injection capillary is longer than the sample capillary, a range of electrophoresis times is available for complete recovery of a compound. The reproducibility is considerably better on the plateau than on the slopes of the recovery curves. The relative standard deviation was 2% on this plateau, whereas it could be more than 20% on the slopes. Apparently, reproducibility of the migration speed is an important factor.

Protein migration

Experiments were carried out to investigate the carry-over of the proteins from the serum samples. The electrophoresis was performed in 0.05 M phosphate buffer and the serum sample was diluted with buffer and water (serum:buffer:water = 1:8:1) to avoid conductivity disturbances at the sample zone boundaries. The amount of proteins which reached the injection loop after electrophoresis was determined at different pH values and different directions of the electrical field. After electrophoresis the loop was purged with 250 μ l of an aqueous solution and collected in a tube directly after the injection loop and reagents were pipetted into the tubes. Recoveries in the ZEST procedure were obtained by comparing units of absorption. Detection of less than 1 μ g/ml of protein was possible.

Fig. 3 shows the results of the protein electrophoresis experiments. At pH 2 proteins began to enter the injection loop after electrophoresis for about 8 min when the electrophoresis was carried out in the direction of the negatively charged electrode. At pH 2 proteins are highly positively charged and migrate rapidly to the injection loop. Therefore, at this pH the isolation of small cations will be difficult. After 20 min virtually all proteins have migrated to the loop. Complete carry-over corresponds to 67 μ g of serum proteins injected (8 μ l of diluted serum).

Comparable results are obtained with a buffer of pH 11 with electrophoresis in

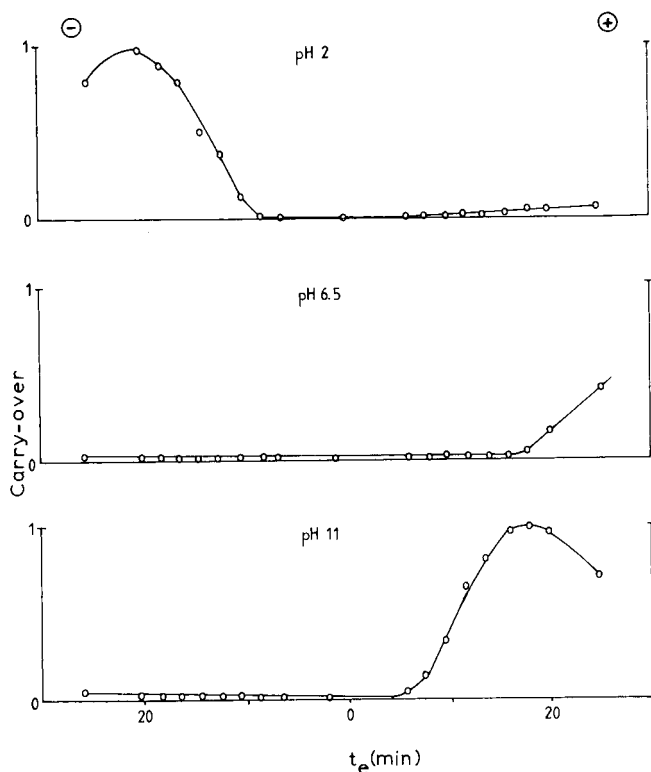


Fig. 3. Carry-over of blood serum proteins as a function of the electrophoresis time in the direction of the positive electrode (right-hand side) and the negative electrode (left-hand side), at different pH values of the buffer.

the direction of the positive electrode. Carry-over of proteins started after 5 min of electrophoresis, so that the isolation of small anions will be difficult.

At pH 6.5 electrophoresis can be carried out in both electrical field directions up to 18 min before the first proteins enter the injection loop.

Salicylic acid in human blood serum

As a result of the studies mentioned above and preliminary studies¹ phosphate buffer of pH 6.5 was chosen for ZEST of the anionic drug salicylic acid. Fig. 4 shows the results of recovery studies of salicylic acid from standard buffer solutions and from human blood serum. The recovery from serum samples is not complete, which indicates that salicylic acid undergoes a protein binding process. The maximum recovery from serum is 85% compared with standard solutions. However, it is not yet clear how this relates to the extent of protein binding of the drug.

The calibration graphs for salicylic acid in the range 10^{-5} – $5 \cdot 10^{-4}$ M (Fig. 5), the concentration range in serum found after a therapeutic dose, are linear (buffer and serum, $r = 0.999$). The reproducibility was better than 3% for standard solutions ($n = 7$) and 6% for serum samples ($n = 4$).

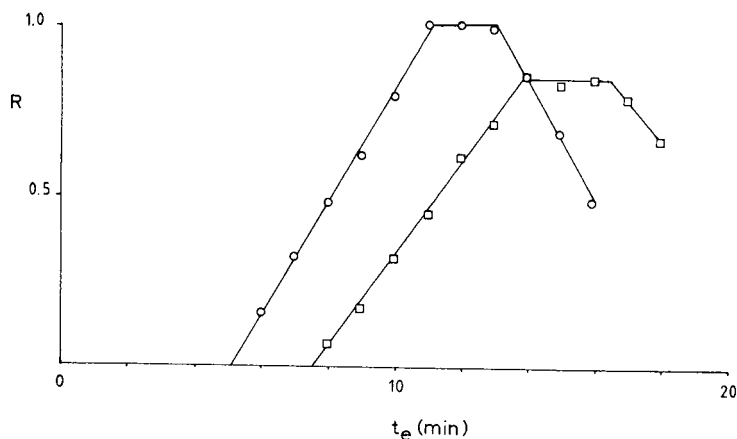


Fig. 4. Recovery of salicylic acid (10^{-4} M) from (O) 0.05 M phosphate buffer (pH 6.5) and (□) human blood serum versus electrophoresis time. Each point represents the mean of three experiments.

Amphetamines in urine

The applicability of ZEST to the prepurification of urine samples with cationic analytes is demonstrated with amphetamine and two of its major metabolites, ephedrine and norephedrine. The pK_a values of these compounds are 9.8, 9.7 and 9.4, respectively⁶.

Urine samples were diluted 1:1 with water to avoid high conductivity disturbances at the sample zone boundaries. Electrophoresis was performed in phosphate buffer of pH 6.5, resulting in migration of the positively charged molecules to the

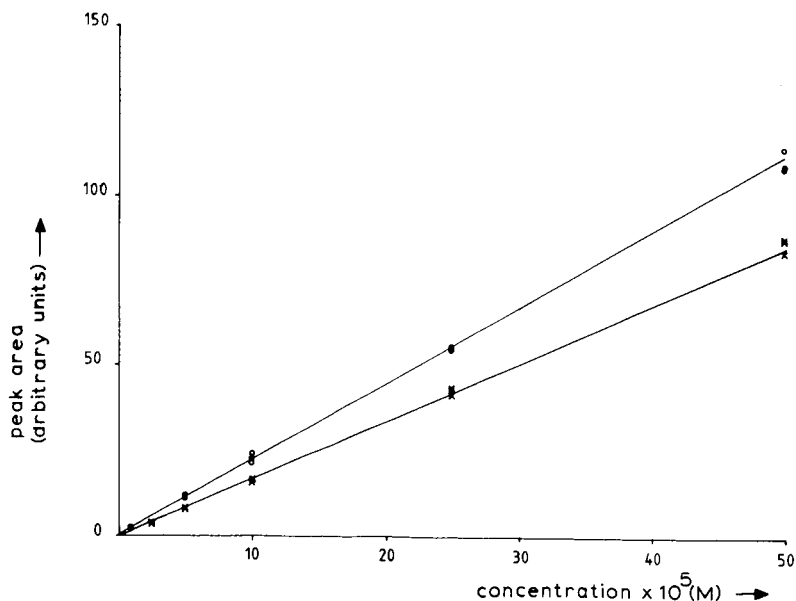


Fig. 5. Calibration graphs for salicylic acid in (O) 0.05 M phosphate buffer (pH 6.5) and (x) blood serum.

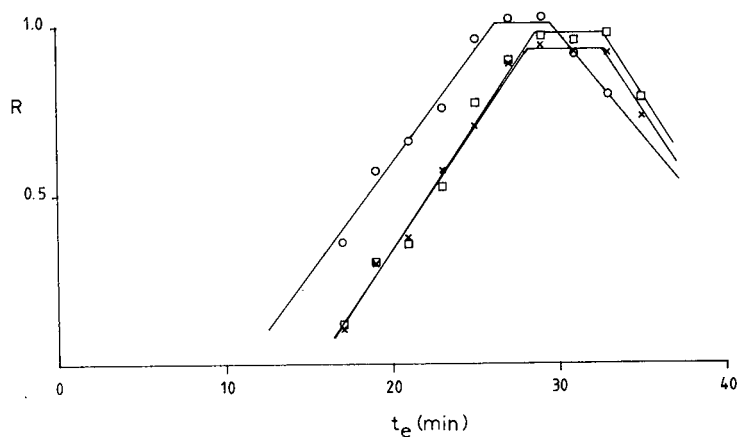


Fig. 6. Recovery of (○) amphetamine, (□) norephedrine and (×) ephedrine *versus* time of electrophoresis; standard solutions $5 \cdot 10^{-5}$ M in 0.05 M phosphate buffer (pH 6.5). Each point represents the mean of three experiments.

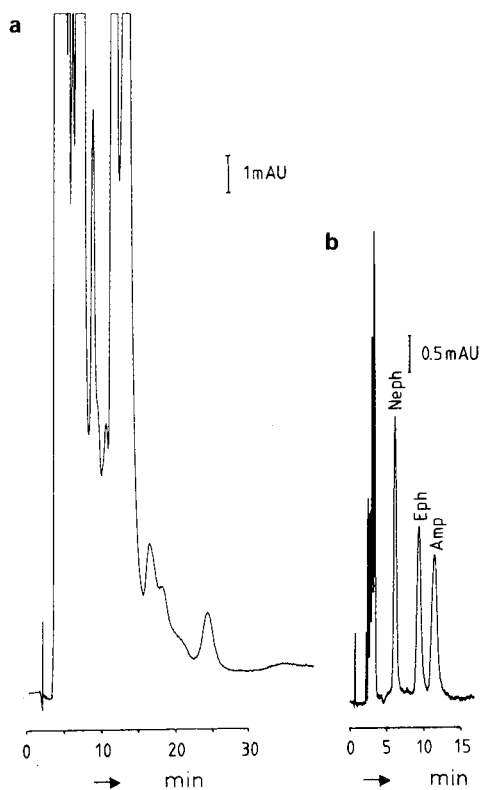


Fig. 7. Chromatogram of a urine sample spiked with amphetamine (Amp), norephedrine (Neph) and ephedrine (Eph) ($5 \cdot 10^{-5}$ M). (A) Untreated sample; (B) after 29 min of electrophoresis (1350 V). For experimental conditions, see text.

negative electrode. Dilute urine samples were spiked with amphetamine, ephedrine and norephedrine, all at $5 \cdot 10^{-5} M$.

Fig. 6 presents the recovery curve of amphetamines from buffer solutions. The optimum electrophoresis time is 29 min under these conditions for the three compounds. In comparison with untreated urine samples the selectivity of the determination is considerably improved (Fig. 7). No interfering substances were found in urine although a non-selective wavelength of 210 nm is used.

CONCLUSIONS

ZEST can be a powerful pretreatment method for isolating anionic and cationic analytes from biological samples prior to liquid chromatography. The ZEST procedure can be automated and is therefore suitable for routine analysis.

For the analysis of serum or plasma samples an important feature of the ZEST method is that proteins can be removed quantitatively on-line. Only for the isolation of very weak acids ($pK_a \gg 7$) or very weak bases ($pK_a \ll 7$) for which an electrophoresis buffer with a pH strongly different from physiological pH should be used can carry-over of proteins not be avoided. In comparison with other techniques that allow direct injection of serum or plasma, such as precolumn techniques^{7,8} or chromatography with internal-surface stationary phases⁹, ZEST offers an increase in the selectivity for ionic compounds. This selectivity is also maintained in the analysis of urine samples.

It has been shown² that the sample capacity and the time necessary for electrophoresis in ZEST are interrelated. Preconcentration of compounds from large volumes of samples will generally not be possible. However, owing to the selectivity of the pretreatment, detection can often be performed at low wavelength, with a high sensitivity. However, instrumental improvements are necessary to decrease the time of electrophoresis and/or increase the sample capacity. Investigations in this direction are now being carried out.

Measurements of the recovery of a compound from a biological sample could give an indication of the extent of protein binding. However, for the establishment of quantitative relationships between recovery and protein binding further investigations are necessary.

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HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY OF OLIGORIBONUCLEOTIDES USING LINEAR AND HYPERBOLIC SALT GRADIENTS

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SUMMARY

A method for separation and chain length determination of oligo- and polynucleotides by high-performance anion-exchange chromatography was developed, which allows resolution of individual fragments according to their chain length n , up to $n \approx 10$ by linear gradient of sodium chloride and up to $n \approx 30$ by an hyperbolic gradient of this salt. The hyperbolic relationship between n and the salt concentration at which elution of the fragment occurs allows determination of the degree of polymerization of oligo- and polynucleotides with unknown n . The method proposed can be used also for estimation of the effective charge of nucleic acids with complex structure.

INTRODUCTION

Fractionation of poly- and oligonucleotides is often used in studies of the synthesis, degradation and structure of nucleic acids, gene engineering experiments, etc.¹⁻⁹. Modern fractionation methods based on high-performance liquid chromatography (HPLC) allow the reduction of separation times and achieve good resolution⁷⁻¹⁰. These newly developed methods are mainly used for the fractionation of restriction fragments^{2,4,7-10}. However, the separation of homopolynucleotides with different chain lengths may be useful for the determination of the lengths of primers and products of DNA-polymerases⁵, and for chemically synthesized homopolynucleotides¹¹. The present work describes the use of the high-performance ion-exchange chromatography on a Mono Q column (Pharmacia) for the rapid fractionation and chain length determination of oligo- and polyribonucleotides.

EXPERIMENTAL

Separation was made with a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden) and a Mono Q HR 5/5 anion-exchange column (5 cm

$\times 0.5$ cm I.D.). Elution was performed with a gradient of NaCl in 25 mM Tris-HCl (pH 7.1 at 20°C), 1 mM MgCl₂. All chemicals were of analytical grade (Reakhim, U.S.S.R.), flow-rates were from 0.1 to 1.5 ml/min. Two kinds of gradient (total volume 30 ml) were used: linear (0–1 M) and hyperbolic $[\text{NaCl}] = 0.61 \cdot V_{\text{el}} / (6.1 + V_{\text{el}})$ where V_{el} is the eluent volume. The absorbance of the eluate was monitored at 254 nm. The dependence between n and $[\text{NaCl}]_{\text{el}}$ was linearized in double reciprocal coordinates.

Preparations of polyA and polyU were from Reanal (Budapest, Hungary): 4.6–5.6 S and 3.4–6.0 S respectively. Polynucleotides were hydrolyzed in 0.3 M KOH at 37°C. The polymerization degrees of polyA and polyU were determined by the end-phosphorus method as described by Seamen¹².

RESULTS

Typical elution patterns of polyA- and polyU-hydrolyzates are presented in Fig. 1. Fragments up to $n = 10$ are completely resolved, but peaks of oligonucleotides with higher chain lengths are also clearly visible. Analysis of the relationship between the eluting salt concentration and n shows that it can be linearized in double reciprocal coordinates ($1/[\text{NaCl}]_{\text{el}}$ vs. $1/n$) and therefore is hyperbolic (Fig. 2).

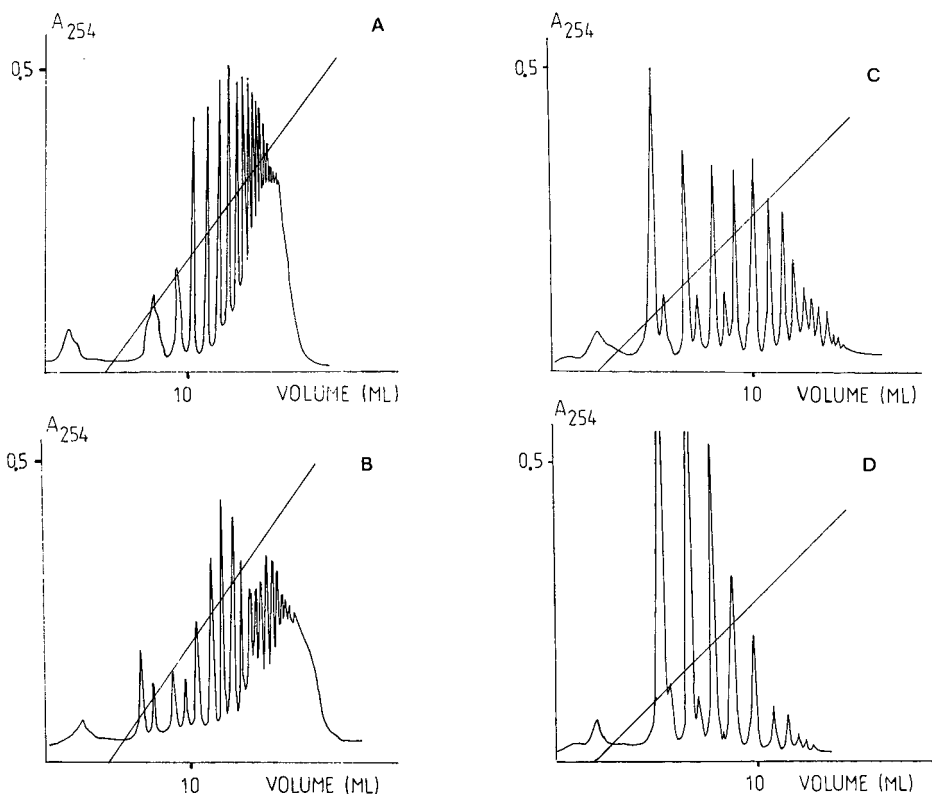


Fig. 1. Separation of hydrolytic fragments of: (A) polyA (hydrolysis time 45 min); (B) polyU (hydrolysis time 10 min); (C) polyA (hydrolysis time 3 h); (D) polyU (hydrolysis time 40 min). Flow-rate 0.1 ml/min.

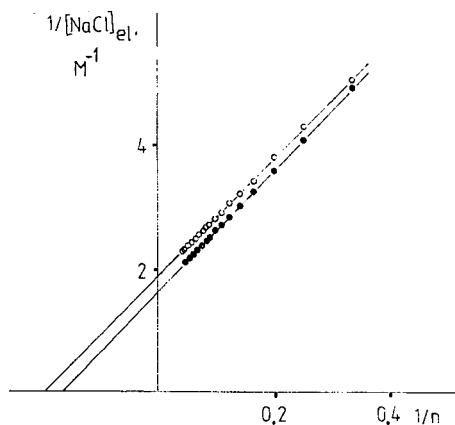


Fig. 2. Double reciprocal plots of the dependence between n and $[\text{NaCl}]_{\text{el}}$: $1/[\text{NaCl}]_{\text{el}} = a + b \cdot 1/n$. 45-min hydrolyzed polyA: $a = 1.89 \pm 0.01$; $b = 9.81 \pm 0.06$ (○). 10-min hydrolyzed polyU: $a = 1.64 \pm 0.01$; $b = 10.00 \pm 0.08$ (●).

We suggest that the relationship between $[\text{NaCl}]_{\text{el}}$ and n may be presented in the analytical form $[\text{NaCl}]_{\text{el}} = [\text{NaCl}]_{\text{lim}}n/(n_{1/2} + n)$ where $[\text{NaCl}]_{\text{lim}}$ and $n_{1/2}$ are parameters of the hyperbola; $[\text{NaCl}]_{\text{lim}}$ is the salt concentration which elutes the polymer of infinite length, and n is the polymerization degree of the oligonucleotide, eluted by $[\text{NaCl}] = [\text{NaCl}]_{\text{lim}}/2$. The dependences shown in Fig. 2 have $[\text{NaCl}]_{\text{lim}}$ and $n_{1/2}$ values of 0.61 M and 6.1 for polyU and 0.53 M and 5.2 for polyA.

Satellite peaks, which appear in the chromatograms of 10-min hydrolyzates of polyU are not taken into account, since the 40-min hydrolyzates showed no satellite peaks and they most probably correspond to the end-phosphate isomers¹³.

The proposed equation allows one to determine (analytically or graphically) molecular masses of poly- and oligonucleotides by the use of $[\text{NaCl}]_{\text{el}}$ values. The polymerization degrees of polyA and polyU (Fig. 3) determined by this method are $4.7 \cdot 10^2$ and $3.5 \cdot 10^2$ respectively. Corresponding values obtained by the end-phosphorus method are $4.9 \cdot 10^2$ and $3.8 \cdot 10^2$. The sedimentation coefficients correspond to degrees of polymerization of about $(4-5) \cdot 10^2$.

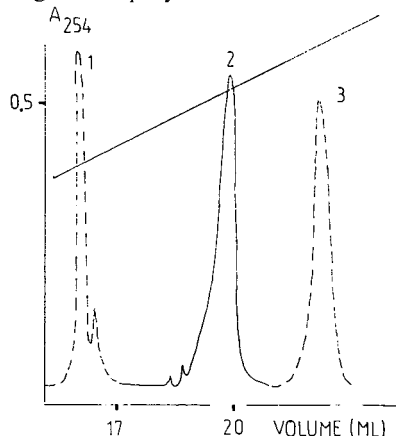


Fig. 3. Elution patterns of tRNA (1), polyA (2) and polyU (3). Flow-rate 0.1 ml/min.

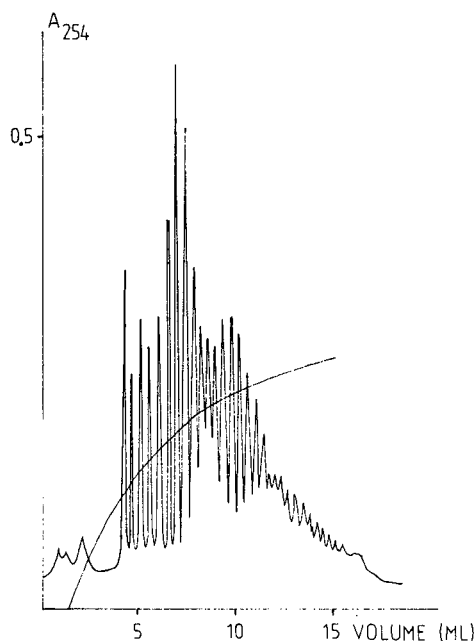


Fig. 4. Elution pattern of hydrolyzed polyU in the hyperbolic salt gradient. Flow-rate 0.25 ml/min.

The hyperbolic dependence between $[\text{NaCl}]_{\text{el}}$ and n also allows one to improve the resolution of "long" oligonucleotides. For this purpose a gradient with an hyperbolic dependence between $[\text{NaCl}]$ and volume was designed. The chromatographic pattern of a polyU hydrolyzate with this gradient is presented in Fig. 4. Practically complete resolution of the oligonucleotides up to $n \approx 30$ is achieved. However, in contrast to the expected dependence, the elution volumes are not proportional to n : short fragments are eluted later, whereas long ones appear earlier than was expected.

DISCUSSION

The analytical expression of the dependence between $[\text{NaCl}]_{\text{el}}$ and n allows one to determine chain lengths of oligo- and polynucleotides even beyond the mass-range of "hydrolytic standards". This method may be useful for experiments on the enzyme synthesis and degradation of nucleic acids, because it allows estimation of the concentration of each individual fragment in the mixture. High sensitivity and short time of analysis (for the mixture of $n \approx 10$, 2.5 h at flow-rate 0.1 ml/min and 15 min at 1 ml/min) make this procedure useful for kinetic experiments with the enzymes of nucleic acid metabolism.

This method may be applied not only to homopolynucleotides but also to nucleic acids of mixed composition and complex structure when their charges are in part inaccessible for the matrix of opposite charge (protein, ion exchanger). To verify this assumption the chromatography of total tRNA was developed in the system described (see Fig. 3). This estimation of the effective charge (*ca.* 20) is reasonable

taking into account the ternary structure of tRNA and number of bound magnesium ions¹⁴.

The results shown in Figs. 2 and 3 demonstrate the main limitations of the proposed method. First, the hyperbolic relationship doesn't allow the determination of the chain length of polynucleotides with $n \geq 500$ with acceptable accuracy. Secondly, the difference in parameters for the n vs. $[\text{NaCl}]_{\text{el}}$ dependence for polyA and polyU shows that these polynucleotides have different affinities for the ion exchanger (see also refs. 1, 13 and 15). In our experiments this difference leads to earlier elution of longer polyA than polyU molecules. This chromatographic behaviour may result from differences in the secondary structures and affinities for magnesium ions of these molecules. Addition to the eluent of compounds which destroy the secondary structure of nucleic acids may result in the "unification" of n vs. $[\text{NaCl}]_{\text{el}}$ dependences.

The hyperbolic gradient designed for the given homooligoribonucleotides on the basis of $[\text{NaCl}]_{\text{el}}$ vs. n dependence allows optimum resolution of both short and long fragments.

Long oligonucleotides ($n \geq n_{1/2}$) as shown above have lower $[\text{NaCl}]_{\text{el}}$ in the hyperbolic gradient than in a linear one. The probable cause of this effect is prolonged washing of the column by the buffer of "sub-eluting" ionic strength, insufficient for complete desorption of long fragments but quite close to it. Therefore, a practically isocratic elution which takes place under such conditions will distort the expected pattern.

Chromatography on Mono Q is also convenient for the semipreparative separation of oligonucleotide mixtures, because the capacity of this ion exchanger allows loads to 10 mg of mixture on the 1-ml column and the separation time in a linear gradient at a flow-rate of 1 ml/min is within 40 min. The flow-rate used in most of our experiments (0.1 ml/min) was chosen to ensure complete equilibration between phases, but the effect of an increase in flow-rate up to 1.0 ml/min on the resolution of fragments with $n \leq 20$ was negligible (data not shown). "Long" fragments can be separated in the hyperbolic gradient at flow-rates less than 0.5 ml/min. The use of Mono Q HR 16/10 column (gel volume 20 ml) allows scale-up of this separation (up to 200 mg).

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SEPARATION OF WATER-SOLUBLE VITAMINS BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY

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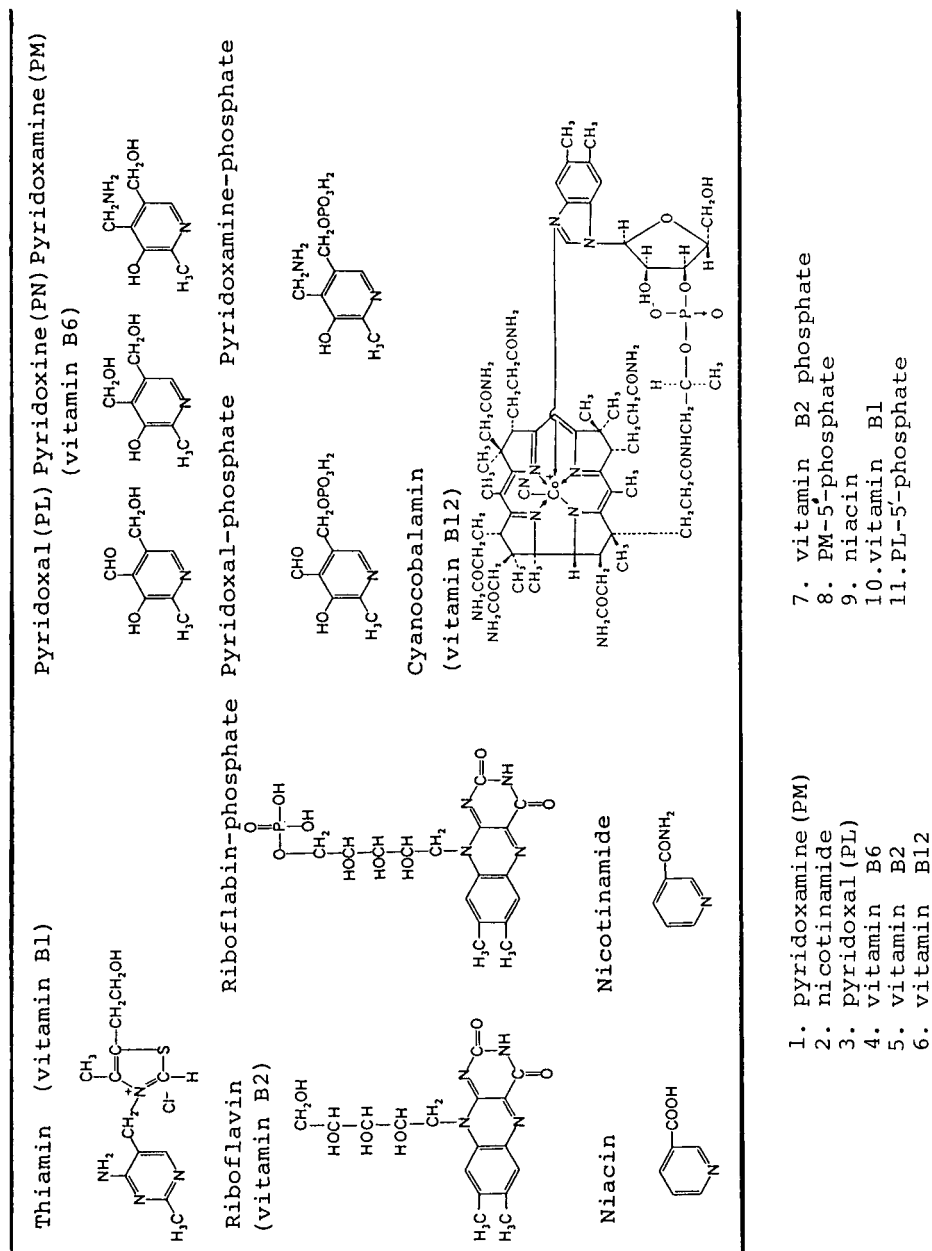
SUMMARY

The retention behaviour of eleven water-soluble vitamins in micellar electrokinetic chromatography (micellar EKC) was investigated in comparison with capillary zone electrophoresis. Sodium dodecyl sulphate (SDS) and sodium lauroylmethyl taurate were used as the anionic surfactants at concentrations of 0.05–0.2 *M* in micellar EKC. The retention times of cationic substances increased more rapidly with increasing concentration of the anionic surfactant than those of other substances. This result suggests that ion-pair formation between cationic substances and anionic surfactants contributes to the retention of the former. The difference in the structures of the two surfactants affects the retention behaviour of solutes, especially cationic substances. To clarify the effect of the micelle, an ion-pairing agent that does not form the micelle structure was employed. All solutes were successfully separated within 15 min by using a 650 mm × 0.05 mm I.D. fused-silica tube with a 0.05 *M* SDS solution (pH 9.0) to give theoretical plates ranging from 100 000 to 350 000.

INTRODUCTION

Capillary zone electrophoresis (CZE) has many advantages for the separation of ionic substances^{1–5}. In CZE, ionic substances are separated according to their electrophoretic mobilities under the influence of an high potential field which creates a strong electroosmotic flow within a tube. This strong electroosmotic flow which has a flat velocity profile causes every kind of solute including cationic, neutral and anionic substances to elute at one end of the tube with high resolution. On-column detection also allows microanalysis of a sample without any loss of column efficiency.

Although CZE has been shown to be a very powerful separation technique for ionic substances, it is lacking in ability to separate non-ionic substances. Because the migration velocity of such non-ionic substances is not affected by the electric field and



1. pyridoxamine (PM)
2. nicotinamide
3. pyridoxal (PL)
4. vitamin B6
5. vitamin B2
6. vitamin B12
7. vitamin B2 phosphate
8. PM-5-phosphate
9. niacin
10. vitamin B1
11. PL-5-phosphate

Fig. 1. Structures of eleven water-soluble vitamins. Solute numbers are also indicated.

pH, they migrate with the same velocity as that of the electroosmotic flow and no separation is achieved.

Recently, a new technique was introduced to improve the selectivity in the separation of non-ionic substances in CZE. This technique is based on micellar solubilization and was first reported in 1984⁶. A wide variety of applications to the separation of non-ionic substances are well documented⁷⁻⁹. In this system, the separation of non-ionic substances is based on the difference in the solute distribution between the aqueous phase and the micellar phase. So this technique belongs to a branch of chromatography which is probably similar to liquid-liquid partition chromatography. We term this kind of technique "electrokinetic chromatography (EKC)", in which electrokinetic migration of the ionic carrier that interacts with the solute is employed. For example, distribution mechanisms such as solubilization by micelles, host-guest interaction by cyclodextrins¹⁰ and ionic interaction^{11,12} have been explored. In the use of surfactants mentioned in this paper, we term this mode "micellar EKC".

A wide variety of applications of micellar EKC will be possible other than the separation of non-ionic substances, and it is expected to improve the selectivity in the separation even of ionic substances. We are interested in small scale analysis of pharmaceutical drugs. So we will first demonstrate the capabilities of micellar EKC for the separation of water-soluble vitamins in comparison with CZE. The effect of the structural differences of surfactants and the effect of adding an ion-pairing agent will be also discussed. There are two reports on micellar EKC separation of water-soluble vitamins^{13,14}. We have found that ion-pair formation occurs in some cases and partition also participates in distribution even for ionic substances.

EXPERIMENTAL

Apparatus

A schematic diagram of the EKC system employed has been shown previously³. Separations were performed in a 650 mm × 0.05 mm I.D. fused-silica capillary tube (Scientific Glass Engineering), with a Model HJLL-25PO high-voltage d.c. power supply (Matsusada Precision Devices, Otsu, Japan) delivering up to +25 kV. Each end of the capillary tube was dipped into a solution in a small glass beaker (10 ml) with a silicone-rubber stopper having two small-bore holes, one for a platinum electrode and the other for the capillary tube. The electric current was monitored between the negative terminal of the power supply and the negative electrode with an ammeter throughout the operation. At 150 mm from the negative end of the tube, the on-column measurement of UV absorption at 210 or 220 nm was carried out with an UVIDEC-100-VI spectrophotometric detector (Jasco, Tokyo, Japan). Peak areas and peak heights were measured by a Shimadzu Chromatopak C-R2AX data processor.

Reagents

Water-soluble vitamins were obtained from Katayama (Osaka, Japan), Nakarai (Kyoto, Japan), Wako (Tokyo, Japan) or Tokyo Kasei Kogyo (Tokyo, Japan). These test samples are listed in Fig. 1. As anionic surfactants, sodium dodecyl sulphate (SDS) from Nakarai (Kyoto, Japan) and sodium lauroylmethyl taurate (LMT) from Nikko Chemicals (Tokyo, Japan) were used as received. Sodium pentanesul-

phonate used as an ion-pairing agent was from Katayama. All other reagents were of analytical reagent grade from Katayama. Test samples were dissolved in water at concentrations 0.5–2 mg/ml to give suitable peak heights. Buffer solutions used for EKC were prepared by mixing a 0.02 *M* sodium dihydrogenphosphate solution or a 0.02 *M* ammonium acetate solution with a 0.02 *M* sodium tetraborate solution in appropriate proportions to give suitable pH values (6–9). The buffer solution was filtered through a 0.45- μ m membrane filter prior to use.

Procedure

A capillary tube was filled with a buffer solution by use of a microsyringe and about 10 ml each of the same buffer solution were introduced into the two beakers placed at the same level. To introduce a sample solution into the tube, the positive end of the tube was moved rapidly to a vessel containing a sample solution and the level of the sample solution was raised about 5 cm higher than the level of the buffer solution to allow the sample solution to flow downward into the capillary tube. After 5–10 s, the end of the tube was returned to the previous beaker of the buffer solution, then an high voltage was applied. The injection volume in this system was of the order of a nanolitre. All experiments were performed in the laboratory air-conditioned at *ca.* 27°C.

RESULTS AND DISCUSSION

Electrokinetic separation

A schematic illustration of micellar EKC is shown in ref. 11. An high d.c. voltage up to +25 kV in the present system is applied between both ends of the capillary tube containing an aqueous buffer solution of an anionic surfactant. The whole solution is transported toward the negative end by the electroosmotic flow, while the anionic micelle migrates toward the positive end with a different velocity because of an additional electrophoretic migration. However, since the electrophoretic velocity of the micelle is lower than that of the electroosmotic flow under the usual experimental conditions, the micelle also migrates toward the negative end.

When non-ionic solutes are introduced to the positive end of the tube they are separated by the difference in distribution between the micellar phase and the aqueous phase, and detected at the negative end of the tube. In the case of an ionic solute, the retention time is determined by its own electrophoresis in addition to micellar solubilization and ionic interaction.

So we can summarize the parameters that affect the separation of the solute as follows: (1) applied voltage (current)^{2,15,16}; (2) composition, concentration and pH of the buffer¹⁷; (3) structure and concentration of the surfactant¹⁶; (4) additives (for example, organic solvent)¹⁸; (5) length, diameter and characteristics of the inner surface of the capillary^{19,20}; (6) composition and concentration of the sample solution^{15,21}.

Concerning parameter (1), a linear relationship was observed between the current and migration velocity of the solute^{2,15}. We examined the migration velocity of some solutes under various applied voltages with a 0.02 *M* phosphate–borate buffer (pH 9.0) without a surfactant. The result is shown in Fig. 2. The same result as previously reported¹⁶ was obtained, and the observed plate height, *H*, values of those solutes under various applied voltages are shown in Fig. 3. It was also reported that

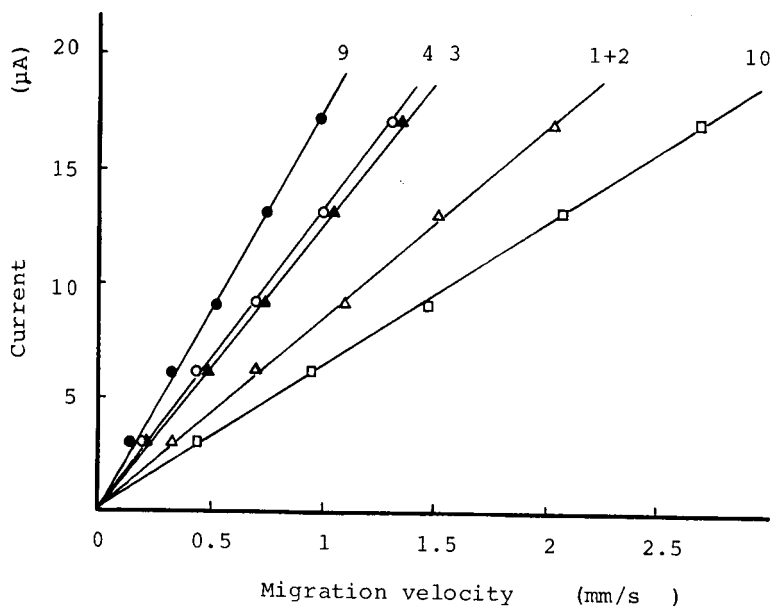


Fig. 2. Velocities of solutes as a function of the current. Solute numbers are indicated by the numbers given in Fig. 1. Conditions: buffer, 0.02 M phosphate-borate (pH 9.0) without a surfactant; separation tube, 650 mm $\times$ 0.05 mm I.D.; length of the tube used for separation, 500 mm; temperature, ambient; detection wavelength, 210 nm; attenuation, 0.08 a.u.f.s.

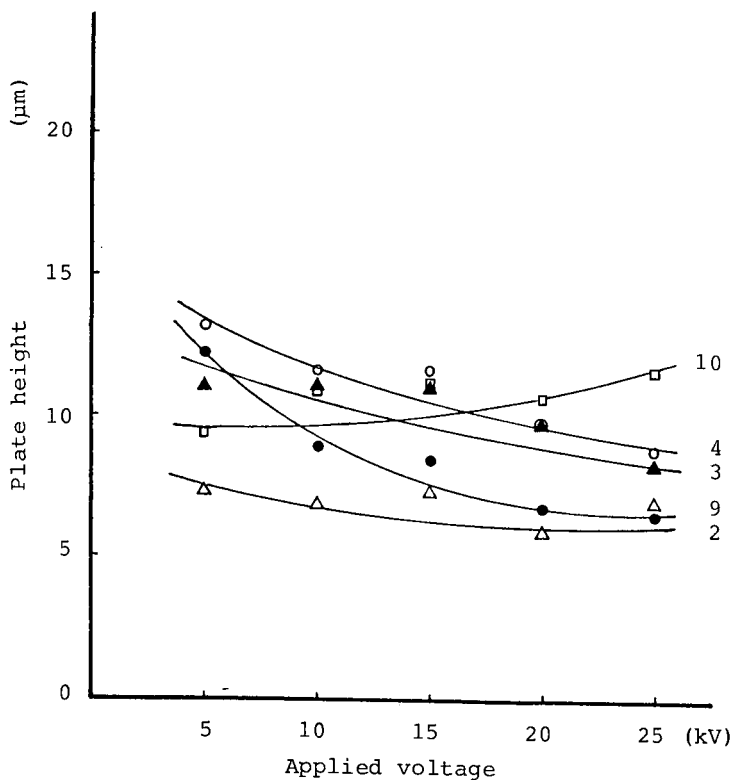


Fig. 3. Dependence of H values of solutes on applied voltage. Solute numbers and conditions as in Fig. 2.

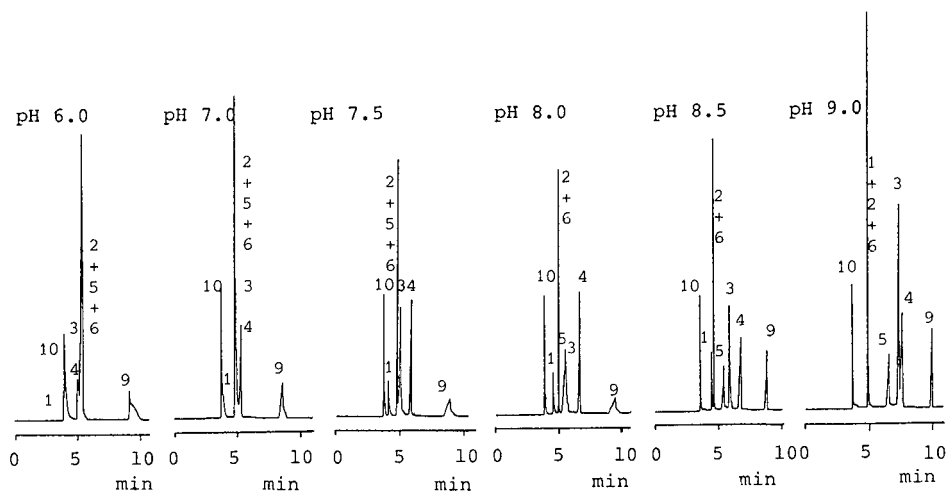


Fig. 4. Effect of pH on retention times and peak shapes of eight water-soluble vitamins. Conditions: applied voltage, 20 kV. Other conditions as in Fig. 2 except for the pH.

the higher the applied voltage is the lower is the H value¹⁹. We decided to operate the power supply at 20 or 25 kV in the following experiments. Concerning parameter (5), we selected 0.05 mm I.D. fused-silica capillary tube of length about 65 cm.

CZE separation

We investigated the separation of eight water-soluble vitamins with a buffer solution containing no SDS. In CZE, one of the effective parameters in separation is the pH value of the buffer. The pH dependence of the retention time and the peak shape in the range pH 6–9 are shown in Fig. 4. At low pH, the retention times of some solutes were very close to each other and some peaks were very broad. However, the peak shape and separation were gradually improved and retention times increased with increasing pH value, although the electroosmotic flow whose velocity was evaluated from the retention time of methanol or that of neutral compounds such as nicotinamide or vitamin B₁₂ in this case was not greatly changed in this pH range. The increase in the retention times of some solutes at high pH is due to the increase in ionization ratios of the solutes at the specified pH. That is, negatively charged solutes were more strongly retained by the electrophoretic effect. In general, the elution order of a solute in CZE can be explained by its ionization characters. In the case of cationic solutes, they are eluted first because the electroosmotic flow and its electrophoretic migration are in the same direction to the negative end of the tube in this system. Electrically neutral solutes migrate with the same velocity as the electroosmotic flow. The pH dependence of the retention times shown in Fig. 4 is in good agreement with the above mentioned behaviours: cationic vitamin B₁ which has a positively ionized nitrogen atom was eluted first and niacin which has a carboxyl group was eluted last.

In CZE, the selection of the buffer composition is also important in order to obtain symmetrical peaks. Instead of the phosphate–borate buffer, we also used an acetate–borate buffer in the above experiments, and chromatograms with the two

buffer solutions are compared in Fig. 5. The higher column efficiency was achieved by use of the phosphate-borate buffer. From these results, we employed this buffer solution at pH 9.0 in subsequent experiments, except when different pH conditions were investigated.

Micellar EKC separation

The effect of SDS concentrations on the retention time is shown in Fig. 6. Vitamin B₁₂, nicotinamide and pyridoxamine were eluted at the same position as methanol which was a tracer of the electroosmotic flow when the SDS concentration is zero, moreover the peak shape of pyridoxal 5'-phosphate was seriously distorted. However, the addition of SDS to the buffer, by which micellar EKC was effected, improved the separation of solutes. A typical chromatogram of the separation of eleven water-soluble vitamins by micellar EKC with a 0.05 M SDS solution at pH 9.0 is shown in Fig. 7. The number of theoretical plates are in the range of 100 000 (pyridoxal 5'-phosphate)–350 000 (pyridoxamine 5'-phosphate) calculated from the equation $N = 2\pi(t_R h/A)^2$, where t_R , h and A are the retention time, peak height and peak area obtained by using a Shimadzu C-R2AX.

The retention times of nicotinamide and pyridoxamine became slightly longer than that of methanol (electroosmotic flow) as the SDS concentration increased. This was due to the gradual increase in solubilization by the micelle with increasing SDS concentrations. On the other hand, large variations of the retention time were observed for vitamins B₁, B₁₂ and B₂. The increase of the retention time of vitamin B₂ is explained in terms of its high lipophilicity. For vitamins B₁ and B₁₂, which are cationic solutes having N⁺ or Co²⁺, an ion pair might be formed between the cation-

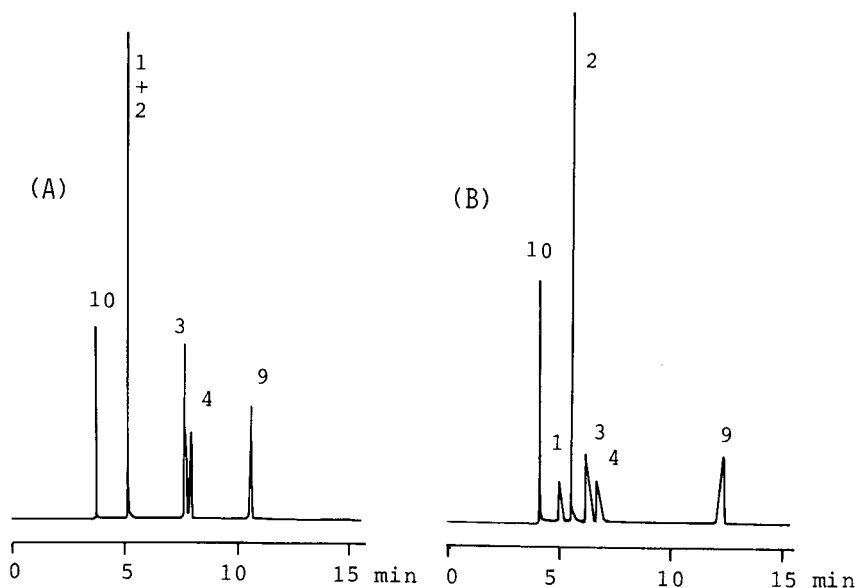


Fig. 5. Effect of the buffer composition on peak shapes: (A) 0.02 M phosphate-borate buffer (pH 9.0); (B) 0.02 M acetate-borate buffer (pH 9.0). Solutes are indicated by the numbers given in Fig. 1.

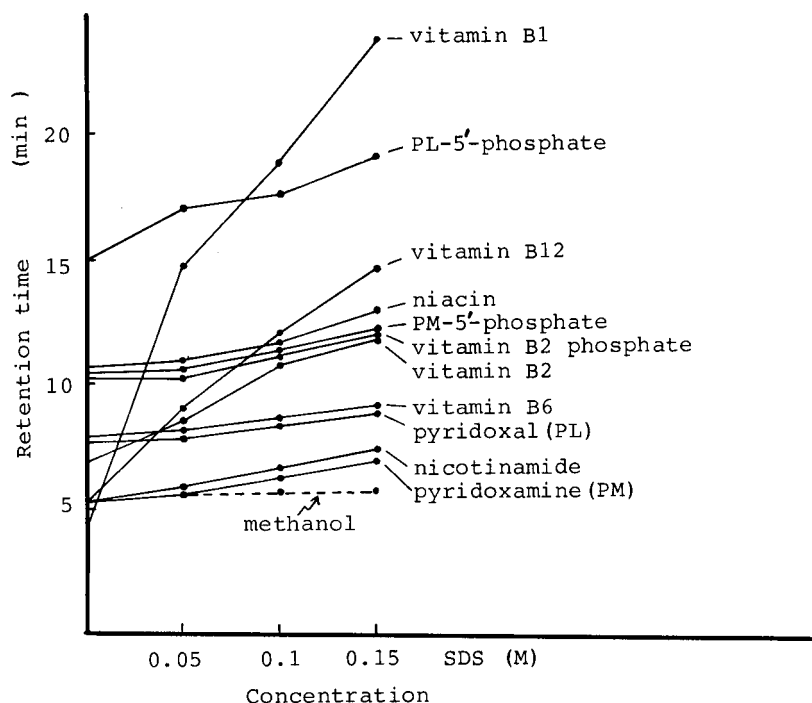


Fig. 6. Effect of SDS concentrations on the retention time of eleven water-soluble vitamins. Applied voltage: 20 kV. Other conditions as in Fig. 2 except for the addition of SDS.

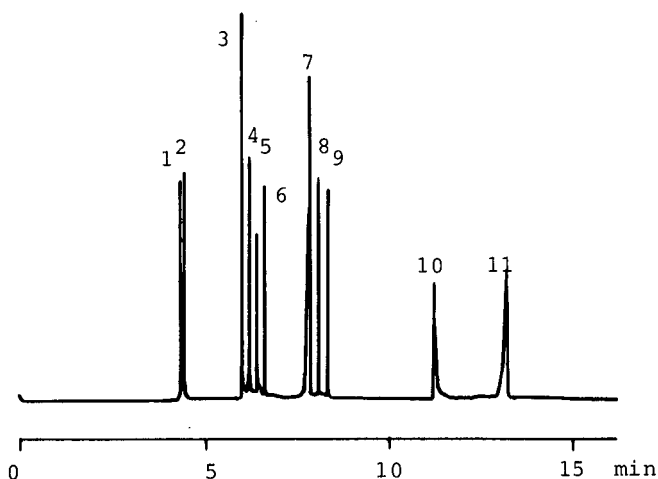


Fig. 7. Micellar EKC of 11 water-soluble vitamins. Conditions: applied voltage, 25 kV; SDS concentration, 0.05 M. Solutes are indicated by the numbers given in Fig. 1. Other conditions as in Fig. 2.

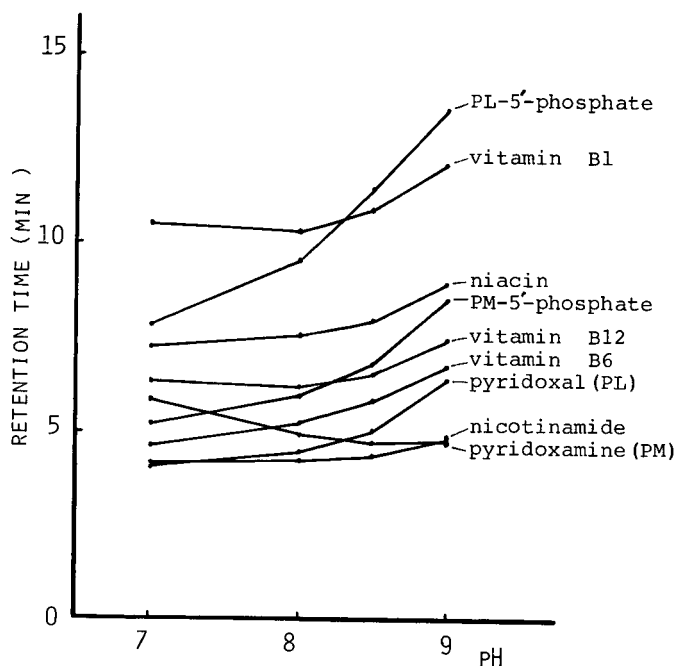


Fig. 8. Effect of pH on the retention time of nine water-soluble vitamins in micellar EKC. Conditions: applied voltage, 25 kV; SDS concentration, 0.05 *M*. Other conditions as in Fig. 2.

ic group of the solutes and the polar group of anionic surfactants (SO_4^- in the case of SDS) and its formation contributes to the large variation of the retention time. This mechanism will be discussed in the following sections. The effect of the pH on the retention time is shown in Fig. 8, which was obtained by keeping the SDS concentration constant at 0.05 *M*. The retention times of pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate increased gradually with increasing pH; on the contrary, that of pyridoxamine decreased and the retention behaviour observed might be explained in terms of the repulsive ionic interaction between solutes and anionic surfactants.

Instead of SDS, LMT was also employed as an anionic surfactant and the effect of its concentration on the retention times of seven solutes was examined. The results are shown in Figs. 9 and 10. When the effects of the two surfactants were compared a difference was observed in the retention behaviours of vitamins B_1 and B_{12} . Vitamin B_1 was eluted between vitamin B_6 and niacin in a 0.1 *M* LMT solution although its retention time was the longest in a 0.1 *M* SDS solution. This marked difference can be attributed to the difference in physico-chemical properties of the two surfactants. Some physical parameters and the structures of the two surfactants are summarized in Table I, where it is seen that the physical parameters are almost the same. The separation of seven parabens as a neutral sample with the two surfactants at 0.05 *M* is shown in Fig. 11. For these neutral solutes, the difference was not so distinct and almost the same chromatograms were obtained. However, in the case of ionic samples, especially cationic samples, in anionic surfactant solutions just like the present experimental conditions, the difference was obvious as mentioned above. The reason for this difference is probably the difference in the polar group of the surfactant, *i.e.*,

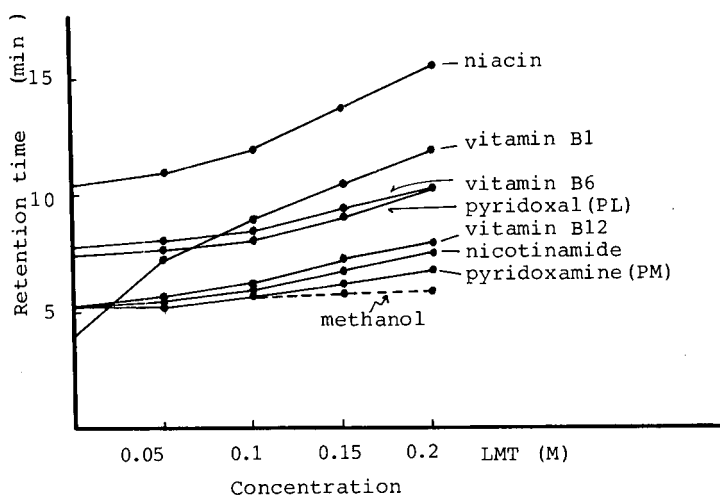


Fig. 9. Effect of LMT concentration on the retention time of seven water-soluble vitamins. Applied voltage, 20 kV. Other conditions as in Fig. 2 except for the addition of LMT.

the sulphate group for SDS and the sulphonate group for LMT. In addition to the difference in charge-bearing groups, the neighbouring groups are different: LMT has $-\text{N}(\text{CH}_3)-\text{C}(=\text{O})-$ near the ionic group, in comparison with the linear hydrocarbon chain structure of SDS. Therefore, ion-pair formation between solutes and the ionic group of LMT might be partially blocked by steric hindrance.

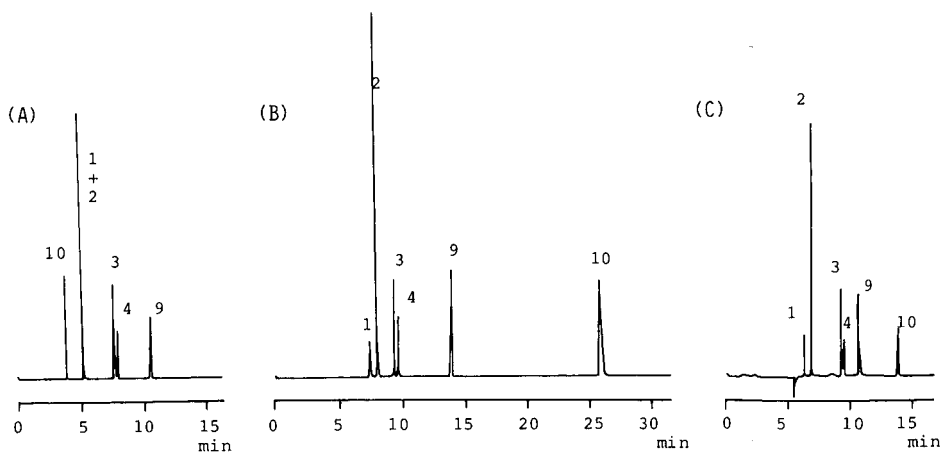


Fig. 10. Comparison between CZE separation and micellar EKC separations. Applied voltage; 20 kV. (A) CZE separation, 0.02 M phosphate-borate buffer (pH 9.0), 210 nm; (B) micellar EKC separation, 0.15 M SDS in the above buffer, 210 nm; (C) micellar EKC separation, 0.15 M LMT in the above buffer, 220 nm.

TABLE I
PHYSICOCHEMICAL PROPERTIES OF SDS AND LMT

SDS <chem>CCCCCCCCCCCCCCCCO[S](=O)(=O)[Na]</chem> LMT <chem>CCCCCCCCCCCCCCCC(=O)N(C)CC[S](=O)(=O)[Na]</chem>			
Surfactant	Mol.wt.	Kraft point (°C)	CMC (mmol/l)
SDS	288.38	ca. 8–9	8.1
LMT	343.46	<0	8.7

To clarify the effect of the micelle, we investigated the effect of an ion-pairing reagent that is often used in reversed-phase high-performance liquid chromatography. As an ion-pairing reagent, sodium pentanesulphonate was employed at 0.1 *M*. The result is shown in Fig. 12. The retention times of all the solutes were increased in the presence of the ion-pairing reagent because of the decrease in electroosmotic flow. However, the elution orders observed in 0.1 *M* SDS were not altered in this system. In conclusion, the drastic change in the elution orders of vitamins B₁ and B₁₂ in micellar EKC mentioned above was due to the effect of the micelle because sodium pentanesulphonate does not form the micelle structure. The marked increase in retention times was probably the result of ion-pair formation between the Stern layer of the micelle and the solute.

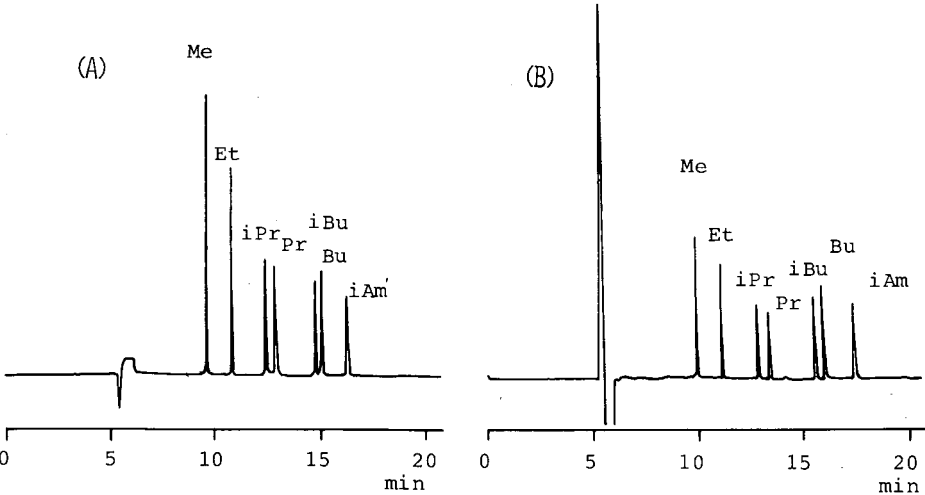


Fig. 11. Micellar EKC separation of seven parabens. Applied voltage: 20 kV. Buffer solution: 0.02 *M* phosphate–borate buffer (pH 9.0) containing 0.05 *M* SDS, 210 nm (A) and 0.05 *M* LMT, 220 nm (B). Me = Methyl paraben; Et = ethyl paraben; iPr = isopropyl paraben; Pr = propyl paraben; iBu = isobutyl paraben; Bu = butyl paraben; iAm = isoamyl paraben. Other conditions as in Fig. 2.

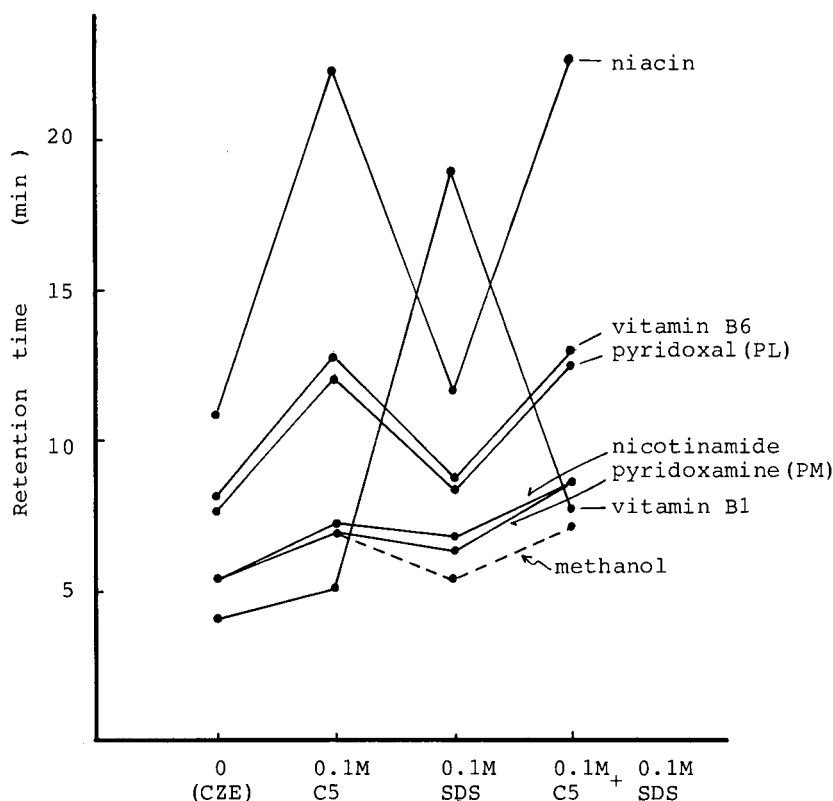


Fig. 12. Effect of the ion-pairing reagent on the separation of six water-soluble vitamins. (0) 0.02 *M* phosphate-borate buffer (pH 9.0) CZE separation; (C₅) sodium pentanesulphonate. Applied voltage; 20 kV. Other conditions as in Fig. 2.

We also expect an improvement in selectivity in separation by mixing an additive with a surfactant to separate a complex mixture if a successful separation is not obtained by changing the surfactant concentrations, although the addition of 0.1 *M* sodium pentanesulphonate to a 0.1 *M* SDS solution was not so effective in case of Fig. 12.

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SEQUENTIAL HYDROCARBON AND NITROGEN DETECTION IN THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF OIL SHALE BITUMEN

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SUMMARY

Sequential flame ionization detection (FID) and chemiluminescent nitrogen detection (CLD) were used to determine semiquantitatively the hydrocarbon and nitrogen compound types in fossil fuel sources fractionated by thin-layer chromatography (TLC). The design of the TLC–FID/CLD system achieved high specificity for organic nitrogen over a wide dynamic range. The FID mass responses for different hydrocarbon types were near unity except for heteroatom-containing concentrates which were less than unity. A method of TLC adsorbent treatment was used in differential nitrogen-type analysis by CLD. The results from TLC–FID/CLD analysis of high-boiling bitumens from western oil shale indicated that the bitumen composition changes with reaction time during the thermal decomposition of the kerogen.

INTRODUCTION

Chromatographic techniques together with element specific detectors allow complex mixtures to be characterized for compound classes and molecular mass size ranges. Multiple detectors provide characterization data and profiling of chemical species which may differ in physical and chemical properties. This study describes the development and application of an on-line flame ionization detection (FID) and chemiluminescent nitrogen detection (CLD) system for the thin-layer chromatographic (TLC) analysis of fossil fuel bitumens.

Hydrocarbon-type analysis by TLC–FID has been applied to many fossil fuel materials including: coal-derived materials^{1,2}, tar sand bitumens^{3,4}, heavy oils^{5,6}, and products from the combustion of these materials⁷. The TLC–FID analytical technique has been shown to be complementary to the classical liquid chromatographic methods used for compound-class determination and solvent-extraction methods used for the determination of asphaltene content. In addition, the application of TLC–FID in the fields of biology, food science, surfactants, and polymers has been reviewed⁸.

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The TLC-FID technique provides for the automated analysis of sample replicates in less than four hours, the reuse of the chromatographic supplies, the semi-quantitation of compound classes in high-boiling ($> 250^{\circ}\text{C}$) materials, and the versatility in choosing chromatographic conditions. However, the different FID response factors for different compound classes and the susceptibility of the sample to alteration caused by component volatility, thermal lability, oxidation, or photodegradation has limited the accuracy of the data obtained from using this technique.

An extension of the TLC-FID technique was the development of element specific detection modes for TLC analysis. One detection mode of interest is the capability to analyze selectively for nitrogen-containing species. Because nitrogen is concentrated in heavy fractions of bitumens and is detrimental to the processing of these heavy oils⁹, information regarding the qualitative and quantitative nature of the nitrogen species is beneficial to the heavy oil processor. The amount and type of organic nitrogen in materials from pyrolysis have been related to the fossil fuel source¹⁰.

An improvement in the design of the TLC-FID together with the development of an on-line flame thermionic ionization detection (FTID) system was reported by Patterson¹¹. These modifications allowed both hydrocarbon detection and the detection of nitrogen and halogen compounds such as nitroaromatics and chloro- and nitrophenols. However, FTID does not have the high specificity for nitrogen present at concentrations of less than 0.1–4 wt.% in fossil fuel materials. The interferences from hydrocarbon responses can be significant.

As an alternative to using FTID for nitrogen detection, CLD has been shown to be a highly specific nitrogen detector. McGregor *et al.*¹² developed a totally specific pyro-chemiluminescent nitrogen detector for gas chromatography that was used successfully in the analysis of nitrogen compounds in jet fuels¹³. The minimum detection limit was in the order of 0.5 ppm nitrogen with a signal-to-noise ratio of 6. Relative response factors were near one for aromatic nitrogen and slightly greater than one for non-aromatic nitrogen. Thus, it appeared that the development of CLD on-line to the TLC-FID system may be applicable in the analysis of fossil fuel material.

The development of a TLC analyzer with sequential FID and CLD is described. High sensitivity over a wide dynamic range for both hydrocarbon and nitrogen species is achieved. One application of the TLC-FID/CLD analyzer is described for determining the composition of oil shale bitumens. The data from this application provide information regarding the reaction mechanisms during a pyrolysis process.

EXPERIMENTAL

Description of the TLC-FID analyzer

An Iatroscan TH-10, MK-IV TLC-FID analyzer from Iatron Laboratories was used in this work. The FID flame was generated with 160 ml/min hydrogen gas flow and 2.5 l/min air flow sustained from compressed gas cylinders. The FID collector was designed for greater collection efficiency for the ionization produced by the flame. Samples were developed under TLC conditions on 5 μm silica gel, Chromarods SII and scanned at 40 s/scan.

Description of CLD interfaced to the TLC-FID

The Antek Model 771 pyroreactor and Model 720 chemiluminescent digital detector were modified for interfacing to the TLC-FID analyzer. The stand-alone Antek instrument achieved $\pm 3\%$ reproducibility in determining low to high concentrations ($1\text{--}10^4$ ppm) of nitrogen in fossil fuel materials. The pyroreactor combustion tube comprised a concentric-designed, quartz tube which allowed make-up oxygen to mix with the combustion gases from the TLC-FID analyzer. The detector response time was decreased by lowering the capacity of the photomultiplier current of the digital detector so that the peak width was 4 s at 10% peak height.

The chemiluminescent nitrogen detector was placed in series with the FID system via a PTFE line which was heated above the dew point to eliminate moisture condensation. The FID tower incorporated a 0.64-cm stainless-steel tube which was connected to the inlet of the PTFE line. The outlet of the PTFE line was connected by a stainless-steel union to the heated inlet of the quartz tube of the pyroreactor. The outlet of the digital nitrogen detector was connected to a mechanical vacuum pump having an in-line air bleed needle valve for vacuum control at the detector of $3.1 \cdot 10^4$ Pa (225 Torr). Make-up oxygen to the combustion tube was 10 ml/min, and oxygen flow to the ozone generator was 100 ml/min. Pumping capability provided a volume displacement of $985\text{ cm}^3/\text{min}$.

The FID and CLD response data were reduced using a Hewlett-Packard 21MX minicomputer equipped with a 3354 B/C Laboratory Automation system. The integration procedures incorporated gas chromatographic software with peak integration every 0.25 s. Data were corrected for the background responses obtained by the scanning of blank chromarods. Component retention times were used to determine R_F values and area percentages were converted to detector mass response factors using known concentrations of compound-type fractions. Each chromarod was scanned twice to ensure complete combustion of the sample material.

Chromatographic conditions

Each analysis run was conducted using a set of ten chromarods (Chromarods SII from Iatron Laboratories). Two different sets of chromarods were prepared, one was unmodified and one was treated with chromic acid. The unmodified set of chromarods was hydrated overnight prior to each day's use. After hydration, the ten chromarods were activated by scanning twice in the TLC-FID analyzer. Modification of a second set of chromarods was achieved by immersing the rods in Pierce RB5-35 cleaning solution for 1 h at 50°C , rinsing them in water, immersing them in chromic acid solution, and rinsing them with distilled water followed by blank scanning. A temperature of about 430°C was experienced by these chromarods during blank scanning which was sufficient for Cr(VI) oxide to decompose to Cr(III) oxide at 197°C .

A microsyringe with a syringe pump was used to deliver accurately $1\text{ }\mu\text{l}$ of a 1–2 vol.% solution of sample in benzene to each chromarod which was then solvent developed. The solvent system used in the TLC work consisted of the development with *n*-heptane to 10 cm, the development with *n*-heptane–toluene (30:70, v/v) to 7 cm, and the development with toluene–ethanol (90:10, v/v) to 3.5 cm. The sample application and TLC development were performed in a laboratory equipped with gold fluorescent bulbs which excluded light below 500 nm to minimize photodegrada-

tion of the sample. Drying of the solvents was accomplished using a nitrogen flow-through oven with mechanical pumping at 90°C for 6 min. Prior to analysis, the developed chromarods were rotated 180° in the holder.

In each set of ten chromarods, samples and calibrants were analyzed in duplicate, and two chromarods were used for background corrections. Model compounds and preparative-sized fractions from an oil shale bitumen were used in the calibration of TLC-FID/CLD. These fractions were generated by using adsorption chromatography on neutral alumina and silica gel^{14,15}.

Preparation of samples of bitumens from oil shale

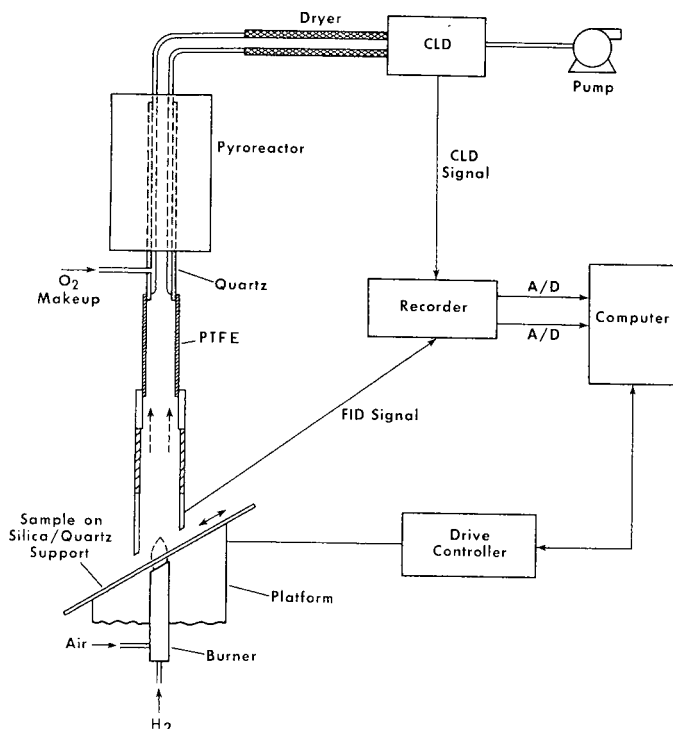
Samples of bitumen from western reference oil shale (Colorado-Green River) and from eastern reference oil shale (Kentucky-New Albany) were obtained by the isothermal heating of these shales at different reaction times. The shale particles were sized from -20 to +45 mesh and heated at 400°C at different reactor (reaction) times ranging from 5 to 480 min. The reactor design and tests are described in detail by Miknis *et al.*¹⁶. Following rapid quenching of the reactor, the heat-treated shales were withdrawn and extracted with benzene for a 24-h period using a Soxhlet extractor. The benzene solutions were filtered through 0.4 μm Nylon-66 filters, and the volume was reduced to approximately 2 vol.% bitumen in benzene. The TLC-FID/CLD analyses of these sample solutions were performed within a week of production to minimize sample alteration. After analyses, the solutions were taken to dryness at 60°C and a pressure of 5 mmHg by using a rotary evaporator. Accurate sample weights were obtained, and the concentrations of the benzene solutions were determined. Product gases and oils were collected separately and are described in other studies.

RESULTS AND DISCUSSION

Performance of the TLC-FID/CLD analyzer

The TLC-FID analyzer was used initially to obtain the hydrocarbon compositional data for bitumens produced under different process conditions. The total nitrogen contents of these bitumens were different for different bitumens. Therefore, nitrogen distributions as well as hydrocarbon distributions in these bitumens were being affected by different process conditions. A CLD unit, offering high sensitivity and specificity for nitrogen, was selected for interfacing to the TLC-FID analyzer (Fig. 1). In general application, the sample material on the chromatographic support is combusted in a hydrogen-air flame, and a mechanical vacuum pump draws the combustion gases through the dual detection system which is placed in series. The vaporized hydrocarbons are ionized in a gas-phase ionization process and detected by the flame ionization detector. Neutral products of this combustion include nitric oxide and chemically bound nitrogen which is converted by oxidative pyrolysis in the 950°C pyroreactor to additional nitric oxide and reacted with ozone in the CLD unit to form NO_2^* in the excited state. The relaxation of NO_2^* to NO_2 causes the emission of photons which are measured.

Both the hydrocarbon and organic nitrogen species in the TLC chromatographed fractions were determined semiquantitatively. By making different sized injections of nitric oxide diluted with air, a linear ($r^2 = 0.995$) dynamic range of 500



ppm to 20 wt.% nitrogen was determined for the CLD. A lower signal-to-noise ratio was observed at concentrations < 500 ppm nitrogen; this lower ratio was caused by gas flow fluctuations. The peak width at 10% peak height for nitric oxide was about 3.6 s compared to 2.4 s for that of pyrene detected by FID. A delay time of approximately 1 s was observed between the FID and CLD responses for the nitrogen-containing organic species. Good reproducibility (relative standard deviation, R.S.D. 2%) for the FID responses was observed for both model compounds and fossil fuel samples. The precision in the quantitation of detector responses for the TLC developed fractions (about 15 μg) was R.S.D. 4% and 5% for the FID and CLD, respectively; however, this compared well with that reported for FID (R.S.D. 10%)³.

Observed R_F values and detector response factors

Several model compounds and fractions from bitumen were analyzed by TLC-FID/CLD to determine R_F values, retention times, and mass response factors. Model

compounds representing species in fossil fuel materials were selected for this study. Liquid adsorption chromatography was used to generate four preparative-size fractions from a representative oil shale bitumen. These saturate, polyaromatic hydrocarbon (PAH), nitrogen polyaromatic hydrocarbon (NPAH), and polar fractions were analyzed by ultraviolet and infrared spectroscopy and determined to be comprised of numerous compound-type structures. The saturate fraction was comprised of alicyclic and cyclic saturated hydrocarbons. The PAH fraction was comprised of alkyl-substituted compounds of one to five aromatic rings. The NPAH fraction was primarily a concentrate of pyridine derivatives, and the polar fraction consisted of multifunctional, heteroatom-containing aromatic and aliphatic structures.

The R_F values were determined from the retention times of model compounds and compound-type fractions. As expected, the elution order was saturate (near the *n*-heptane solvent front), PAH, NPAH, and polar fractions ($R_F = 0.00$ at the origin). In Table I, the well-separated, narrow ranges for both the retention times and calculated R_F values indicate adequate separation of the compound-type fractions.

The TLC-FID/CLD analysis of different nitrogen compound classes in samples of complex mixtures of compound types was demonstrated. Several different nitrogen-containing model compounds had similar R_F values (Table I) which indicated their inadequate separation. However, the separations of the nitrogen species in the compound-type fractions gave distinct R_F values for specific nitrogen-type classes. This suggested that the solubility and association characteristics of nitrogen classes adsorbed on silica gel strongly influence their isolation by TLC. From the R_F values, carbazole derivatives are present in the PAH fraction, pyridine derivatives are in the NPAH fraction, and bifunctional nitrogen/oxygen species are in the polar fraction.

The effect of a sample's concentration on its associative properties was explored

TABLE I

RETENTION TIMES AND OBSERVED R_F VALUES FOR HYDROCARBON AND NITROGEN-CONTAINING SPECIES

Compound-type fraction or model compound	FID		CLD	
	Retention time (s)	R_F	Retention time (s)	R_F
Saturate	11-14	0.65-0.74	—	—
PAH	18-20	0.47-0.53	21-22	0.44-0.47
NPAH	24-28	0.24-0.35	27-29	0.24-0.29
Polar	34-36	0.00-0.06	37-38	0.00
Pyrene	19	0.50	—	—
Ethylcarbazole	20	0.47	21	0.47
Benzocarbazole	26	0.29	27	0.29
Tetrahydrocarbazole	29	0.21	30	0.21
Benzoacridine	30	0.18	31	0.18
Benzoquinoline	30	0.18	31	0.18
Formanilide	30	0.18	31	0.18
Toluamide	31	0.15	33	0.12
Hydroxyquinoline	35	0.00	37	0.00

TABLE II

RELATIVE TLC-FID MASS RESPONSES FOR 1- μ g QUANTITIES OF FRACTIONS FROM DIFFERENT BITUMENS

Compound-type fraction	TLC-FID ^a		TLC-FID/CLD ^b			
	Oil shale		Tar sand		Oil shale	
	Area	Relative response	Area	Relative response	Area	Relative response
Saturate	6790	0.81	5160	0.41	8560	0.84
PAH	7170	0.85	6040	0.48	8610	0.84
NPAH	8430	1.00	12590	1.00	10190	1.00
Polar	3830	0.45	4700	0.37	4650	0.46

^a No vacuum pump was used in this configuration.^b A mechanical vacuum pump was used in this configuration.

by using pyridine as a solvent. Solutions of different bitumen concentrations were applied to the Chromarod SII. Pyridine without sample was readily removed in the drying oven. But, the pyridine could only be removed from samples with less than 4 mg/ml of pyridine using similar drying oven conditions. Greater concentrations resulted in strong intermolecular associations of bitumen, pyridine, and silica gel.

The relative mass responses by the FID were determined for 1- μ g quantities of the saturate, PAH, NPAH, and polar fractions. The FID signals were corrected for background and integrated. Each area was normalized to the highest chromatographic peak area that was determined to be that for the NPAH fraction (Table II). These data for the compound-type fractions were dependent upon the composition of the sample (oil shale or tar sand) and on the configurations of the TLC-FID and TLC-FID/CLD systems. The FID detector mass responses for the fractions from the oil shale bitumen ranged from 0.8–1.00 compared with 0.4–1.00 for the fractions from the tar sand bitumen. The ionization of hydrocarbons was greater than that for compounds containing large amounts of oxygen, sulfur, or nitrogen which accounted for the low mass responses of the polar fractions. By varying the hydrogen–air ratio and therefore changing the flame temperature, it was observed that the volatility of the polar fractions was less than for the other fraction types. The volatility of the saturate fraction was the greatest. Because these relative FID mass responses are dependent upon the composition of the fraction and the configuration of the TLC-FID/CLD, the correlation of these data with FID data from gas chromatographic analysis should be limited. It is obvious that the FID calibration for each fossil fuel source is required.

The integrated areas of the FID responses for each compound-type fraction by TLC-FID and by TLC-FID/CLD were compared. A more effective analysis of the fractions was obtained by using the latter system's configuration, which incorporated a mechanical vacuum pump. By using reduced pressure, an area that was about 20–26 percent larger was obtained for each fraction. Reduced pressure decreased the probability of ion–ion recombination prior to the ion contact with the anode (collector electrode). In addition, the off gases from the analyzer are removed by the pump from

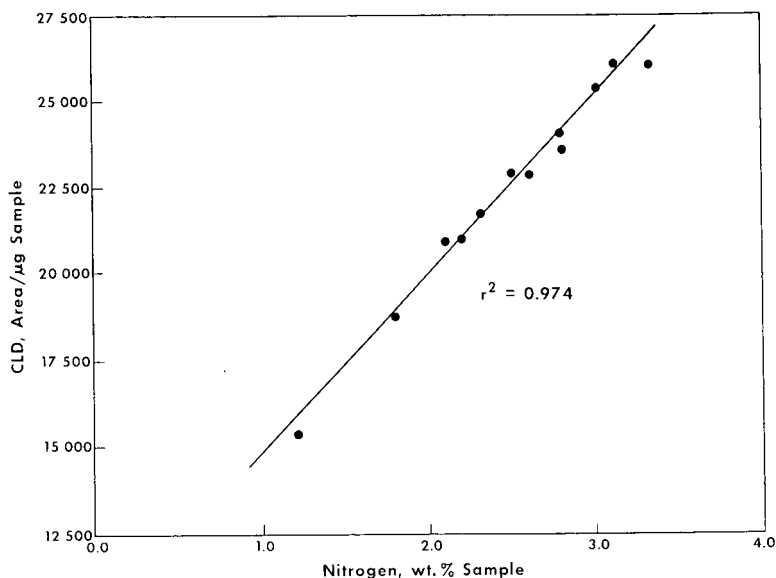


Fig. 2. Correlation for the CLD responses for nitrogen in western shale bitumens (400°C).

the laboratory environment in an acceptable manner. As a consequence of using TLC-FID/CLD, the chromatographic peaks had better resolution. Numerous reuses of the chromatographic support are obtained by using the TLC-FID/CLD configuration.

The CLD mass responses could not be determined for model nitrogen compounds because of the irreproducibility in the integrated areas. This was attributed, in part, to the thermal lability of model nitrogen compounds on the chromatographic support. However, the nitrogen species participating in the intermolecular interactions of complex mixtures such as those found in fossil fuel chromatographic fractions were amenable to TLC-FID/CLD analysis. The CLD measurements for these fractions were reproducible to within 2%.

The amount of organic nitrogen detected by chemiluminescence was independent of the nitrogen compound class. Fig. 2 demonstrates this linearity ($r^2 = 0.974$) for the CLD responses from samples of oil shale bitumens with the wt.% of nitrogen as determined by pyrolytic techniques. The total CLD response for a sample is additive of each CLD response from the PAH, NPAH, and polar fractions, and the CLD mass response for each fraction from these bitumens was assumed to be unity. The TLC-FID/CLD analysis of a fossil fuel sample provides both the distribution of nitrogen compound types and the semiquantitation of nitrogen by using a calibration curve similar to that in Fig. 2.

Differential nitrogen analysis by TLC-FID/CLD

Differential nitrogen analysis of shale bitumens by TLC-FID/CLD was accomplished by using both unmodified and chromic acid-treated Chromarod SII. The latter allowed the determination of compound types which selectively complexed to

TABLE III

EFFECT OF CHROMIC-ACID TREATMENT ON CHROMAROD SII TO DISTINGUISH COMPOUND-TYPES IN SHALE BITUMENS

Reaction time (min)	Modification	FID (area %)				CLD (area %)			Total nitrogen (Wt.% sample)
		Saturate	PAH	NPAH	Polar	PAH	NPAH	Polar	
5	None	3	6	84	7	—	92	8	1.2
	Acid	6	11	57	26	3	63	34	
30	None	4	6	78	12	<1	90	9	2.1
	Acid	5	5	36	54	(2) ^a	(39)	(59)	
60	None	3	5	71	21	<1	77	22	2.3
	Acid	6	2	27	65	(2)	(29)	(69)	
120	None	4	4	64	28	—	67	33	2.8
	Acid	5	2	25	68	1	29	70	
240	None	7	9	77	7	—	94	6	2.6
	Acid	18	8	54	20	1	68	31	
480	None	17	7	68	8	—	90	10	2.2
	Acid	22	3	57	18	3	68	29	

^a Values in parentheses were not determined directly on the chromic acid-treated chromarods but were inferred from the FID area %.

positive chromium metal centers. Separate analysis was carried out by using the different sets of chromarods, and the data were compared. The impregnation of Cr in the silica gel allowed ligand exchange/complexation reactions¹⁷ with organics during TLC development. The basic primary amine and pyridinic compound types selectively complexed to cationic metal centers. Also, π -electrons in polyaromatics allowed weak coordination to metal centers. This complexation effectively retarded the movement of such organic species when weakly polar, organic solvents were used as elements.

Several oil shale bitumens which were produced at different reactor residence (reaction) times at 400°C were analyzed using the unmodified and the chromic acid-treated Chromarod SII. The CLD area percentages for the NPAH fractions decrease as the area percentage for the polar fractions increase upon using the chromic acid-treated chromarods compared to using the unmodified chromarods (Table III). Similar trends are indicated for the FID area percentages. Because the R_F values of some carbazole derivatives are similar to pyridinic derivatives (Table I) using unmodified chromarods, the selective retardation of pyridinic derivatives on the chromic acid-treated chromarods will enable these species to be concentrated in the polar fraction. The carbazole derivatives will remain in the NPAH fraction. The summation of the CLD area percentages for the PAH and NPAH fractions using chromic acid-treated chromarods represents the relative amount of nitrogen in carbazole derivatives. The relative amounts of nitrogen in pyridinic derivatives were determined as the differences in the CLD area percentages of polar fractions using chromic acid-treated chromarods and that in the polar fractions using the unmodified chromarods. The CLD area percentage of the polar fraction determined by using only the unmodified chromarods represents the relative amount of bifunctional nitrogen compounds, which include amidic derivatives.

For maximum oil shale bitumen production between the 30 and 60 min reaction times, the relative distributions of nitrogen are in the range 31–41% carbazole, 47–50% pyridinic, and 9–22% bifunctional or amidic derivatives (Table III). These data compare favorably with the results from liquid chromatographic analysis of a retorted western shale oil having 2.2 wt.% total nitrogen: 36% carbazole, 50% pyridinic, and 14% amidic nitrogen¹⁸.

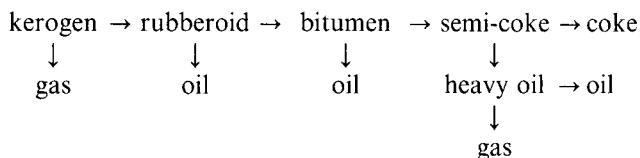
The results from differential nitrogen analysis of eastern oil shale bitumens indicated only negligible differences in the compositional distributions by using either type of chromarod. There was less complexation of the compound types in the eastern oil shale bitumens compared with that of in the western oil shale bitumens. In a retorted eastern shale oil, there was only 28% pyridinic nitrogen but 54% carbazole nitrogen¹⁸. The character of the nitrogen in the bitumen and the soil from eastern oil shale was considerably more aromatic and less basic than that from the western oil shale. Less complexation of the nitrogen compound types in eastern shale bitumen was expected.

Relative FID area percentages for the PAH and saturate fractions were erroneously measured using chromic acid-treated chromarods. Impregnation of metals in the chromarods may block the pore structure of the silica gel as well as inactivate the Si–O–Si and Si–OH active sites needed to retard the elution of aromatic structures. This would cause the PAHs to elute with saturates on chromic acid-treated chromarods, and the measured saturate content would be exaggerated. Therefore, the amounts of saturates and PAHs should be determined from the calibrated FID mass responses using unmodified Chromarod SII.

Cleaning Chromarod SII with chromic acid cleaning solution is not recommended. The previous work suggested that metal impregnation occurred when chromic acid was used. It was found that the chromic acid-treated chromarods could not be reconverted to the unmodified character of the TLC adsorbent. Because multiple sample analyses on Chromarod SII reduced cost and allowed consistency in measurements, the reuse of unmodified Chromarod SII was achieved by cleaning with 4 *M* nitric acid followed by copious washing with distilled water⁸. Nitric acid was used for cleaning without modifying the TLC adsorbent. This suggested that the chromatographic process is one of complexation and not necessarily acid–base interaction with the chromic acid-treated materials.

TLC–FID/CLD analysis of reaction intermediates

By understanding the kinetics of the thermal decomposition of shale, the efficiency of conversion processes can be improved and novel approaches to shale pyrolysis can be developed. Various models of decomposition processes have been proposed in attempts to predict the product yields for different types of oil shale. Several models have indicated that the bitumen is an intermediate in the pathway of kerogen decomposition to oil and gas as shown below¹⁹



Kerogen is defined as that portion of organic matter in oil shale that is insoluble in common organic solvents. Bitumen is defined as unvaporized benzene-soluble organic matter that is formed during the heating period and that remains in the shale sample. However, bitumen formation may depend on the kerogen structure, and there may be a variety of intermediate products²⁰. Because certain physicochemical properties of the bitumen changed with temperature and reaction time, Turner *et al.*²¹ have suggested that the varying bitumen composition was responsible for the lack of fit of the bitumen data to the kinetic model of kerogen decomposition shown above.

The compound-type characterization of these high-boiling, high-molecular-weight bitumens by TLC-FID/CLD allowed the investigation of bitumen composition changes caused by the reaction conditions in kerogen decomposition. The 400°C isothermal pyrolysis tests of western oil shale samples at 5–480 min reactor residence times were performed. The amounts of bitumen recovered on the basis of dry shale sample from each test ranged from 1.35 wt.% (5 min) to 6.26 wt.% (60 min). Average molecular weights determined by vapor phase osmometry of these bitumens ranged from 1030 (5 min) to 500 (360 min). By using the TLC-FID/CLD mass response factors (Table II) for the different compound-type fractions and weighting each fraction on the basis of weight percentage of recovered bitumen, the relative amounts of saturate, PAH, NPAH, and polar compound-type fractions were determined. The weight percentage of each fraction of the oil shale was plotted *versus* the $\log_e$ of the reaction time (Fig. 3). Significantly different bitumen compositions are indicated at the different reaction times. In particular, different amounts of the NPAH and polar fractions were recovered in the bitumen at different reaction times, whereas the amounts of saturated compounds and PAHs were generally constant. The amounts

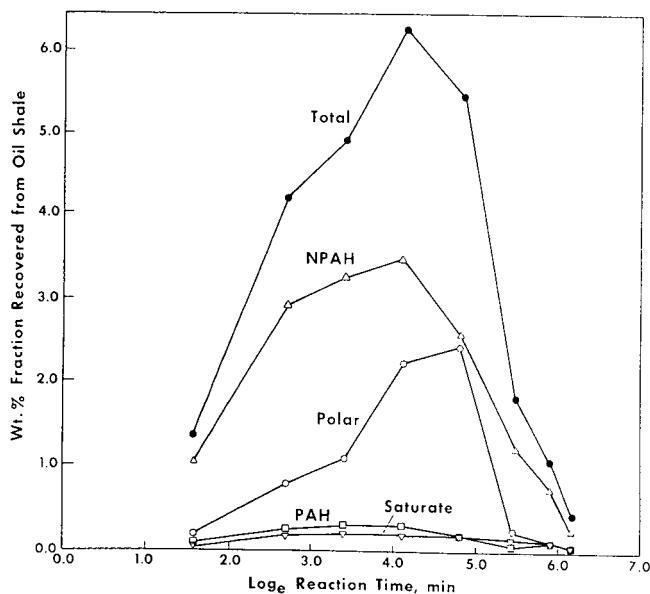


Fig. 3. Hydrocarbon distributions in bitumen recovered from oil shale at different reaction times at 400°C.

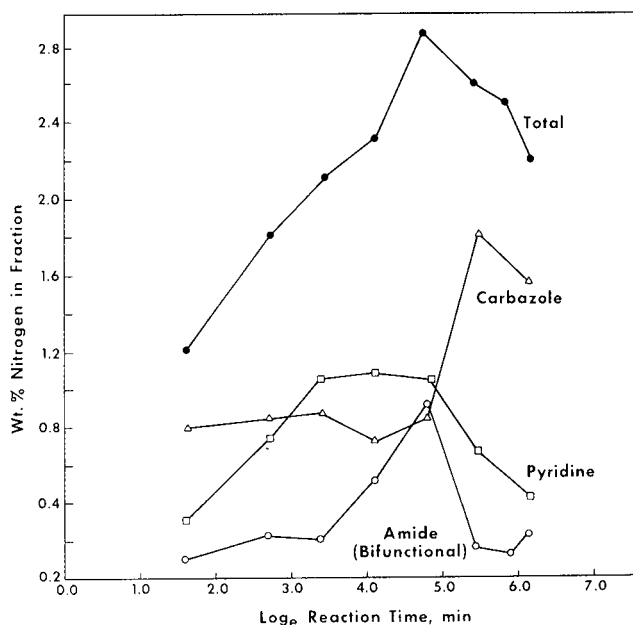


Fig. 4. Nitrogen distributions in shale bitumens produced at different reaction times at 400°C.

of the recovered NPAH fractions increased steadily from 5 min to 60 min (maximum bitumen recovery) at 400°C; the NPAH amounts decreased sharply at longer reaction times. The amounts of polars increased slowly from 5 to 30 min, rapidly increased from 30 to 120 min, and decreased sharply after 120 min.

The differential TLC-FID/CLD analysis of the bitumens was used to determine the relative amounts of nitrogen in carbazole, pyridinic, and amidic derivatives. The total nitrogen content increased from 1.2 wt.% (5 min) to 2.8 wt.% (120 min) and decreased to 2.2 wt.% (480 min). These data (Fig. 4) indicate that different bitumen compositions were obtained at different reaction times, which suggested multiple bitumen intermediates during kerogen decomposition. For each nitrogen type, the maximum concentration was observed at different reaction times: pyridinic nitrogen (60 min), amidic nitrogen (120 min), and carbazole nitrogen (240 min). Carbazole derivatives have been generally considered the most refractory (unreactive) nitrogen species, and as expected at long reaction times, these refractory species were noted to have survived isothermal heating. Lesser amounts of bifunctional species including amides and hydroxypyridine derivatives resulted from their susceptibility to thermal decomposition. Also, interconversion of nitrogen functionalities may occur during the decomposition of kerogen.

Western oil shale bitumens contained more aromatic carbons and correspondingly less aliphatic carbons as the pyrolytic reaction times increased. This was attributed to the aromatization of aliphatic carbons as thermal decomposition proceeded¹⁶. These TLC-FID/CLD data suggested that at times longer than 60 to 120 min, the increase in aromatization resulted, in part, from the presence of relatively large amounts of carbazole compounds and other dealkylated aromatic ring systems.

The TLC-FID/CLD analysis of bitumens produced by the 400°C pyrolysis of eastern oil shale indicated a more aromatic but less polar bitumen composition compared to the western oil shale bitumens. The maximum production of eastern oil shale bitumen occurred at 15 min rather than at 60 min as for the western oil shale bitumen. Again, the TLC-FID/CLD data provided compound-type information consistent with the observation that the bitumen composition was dependent on the reaction time. From the thermal decomposition of kerogen, a bitumen intermediate as a single entity was not indicated. These data suggest that bitumen and thermal decomposition formation were also dependent on the different kerogen structure for the two different shales.

Presumably, at long reaction times, secondary reactions such as the cracking and coking of the organic material occurred; these reactions effectively decreased the amounts of NPAHs and polar compounds recovered from the bitumen. The amounts of saturated compounds in the bitumen were either unaffected by the pyrolysis process or were formed as products from these secondary reactions. Also, the amounts of the recovered PAH and NPAH fractions at these reaction conditions generally increased and decreased at similar rates.

Low amounts of polar components at long reaction times result from coke formation on the spent shale as well as increased gas production. The different bitumen compositions are affected by the original components in the kerogen being converted at different times to bitumen. Because thermal decomposition of the components comprising the bitumen result in gas, oil, and coke products, the different components of the bitumen have different susceptibilities to product formation. In kerogen decomposition both a reaction time and temperature dependence is observed and a range of activation energies must be considered.

CONCLUSIONS

The TLC-FID/CLD analyzer was shown to be a versatile analytical tool. The sequential TLC-FID/CLD analysis of high-boiling, high-molecular-weight fossil fuel materials provided hydrocarbon and nitrogen compound-type distributions. The reaction mechanisms ongoing in bench-scale process studies were investigated by relating the compound-type distributions of the reaction intermediates with process conditions. In the present study of the thermal decomposition of kerogen, the TLC-FID/CLD demonstrated that the composition of the bitumen as a reaction intermediate is dependent on the source of the shale and reaction time.

The FID mass response factors were shown to depend on the configurations of the hydrogen-air flame, the detector, and the TLC support. Mass response factors were dependent on heteroatom content and component volatility. The different mass response factors for tar sand bitumen and for oil shale bitumen indicated that the FID must be calibrated for different source materials. The lower mass responses for saturates and PAHs compared to NPAHs suggested that the associations and interactions of these components on the silica gel were different. The TLC support affected elution behavior as demonstrated by the impregnation of chromium ions on the Chromarod SII. This particular Chromarod SII treatment was shown to provide a differential nitrogen-type analysis.

An advantage of TLC-FID/CLD analysis was the ability to characterize very

small quantities of material containing large amounts of high boiling material. The vacuum-assisted analysis allowed improvement in the FID response detection by increasing the samples' ionization with lower pressures. This requirement of less sample improved the resolution of the components. The rapid analysis of hydrocarbon and nitrogen species (four samples in duplicate done in less than 4 h) was performed with fair reproducibility (R.S.D. 4 and 5%, respectively). The nitrogen-specific CLD was found to be the nitrogen detector of choice with high sensitivity over a wide dynamic range.

ACKNOWLEDGEMENTS

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SEPARATION AND DETECTION OF BIS(ALKYLTHIO)SELENIDES OF PENICILLAMINE AND GLUTATHIONE BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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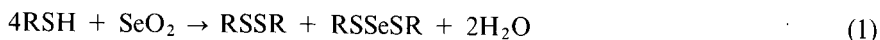
SUMMARY

Sensitive and selective methodology is described for the simultaneous determination of thiols, disulfides and bis(alkylthio)selenides in mixtures formed by reaction of selenious acid with thiols. The methods are based on separation of the various compounds by reversed-phase ion pairing chromatography followed by detection with an electrochemical detector equipped with dual mercury/gold amalgam electrodes in series in the eluent stream. Thiols are detected directly at the downstream electrode. Bis(alkylthio)selenides and disulfides are detected indirectly by first reducing them at the upstream electrode followed by detection of the reduction products at the downstream electrode. Bis(alkylthio)selenides are reduced at less negative potentials than disulfides, making it possible to selectively detect them in mixtures with disulfides. Detector response to bis(penicillamine)selenides is linear from $1.1 \cdot 10^{-7} M$ up to at least $1.2 \cdot 10^{-4} M$; the detection limit is estimated to be < 2 pmol bis-(penicillamine)selenide.

INTRODUCTION

Selenium supplements to animal diets consist largely of sodium selenite¹. Ganthier has shown that selenite is reduced metabolically by animals by a process which requires glutathione (GSH) and is stimulated by nicotinamide-adenine dinucleotide phosphate (reduced, NADPH) and anaerobic conditions². The reductive metabolism involves a series of reactions, beginning with the reaction of GSH with selenite to form the bis(alkylthio)selenide GSSeSG, which is then enzymically reduced by glutathione reductase and NADPH to yield H_2Se ^{3,4}.

The formation of bis(alkylthio)selenides by reaction of aqueous selenium dioxide with organic thiols at a combining ratio of 4:1 was first proposed in 1941 by Painter⁵.



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The 4:1 stoichiometry was confirmed by Ganther in a detailed study of the reaction of selenious acid with several thiols, including GSH⁶, and the mechanism of the reaction of 1-butanethiol and 2-methyl-2-propanethiol with selenite in acidic dioxane solution has been investigated by Kice *et al.*⁷.

In view of the importance of bis(alkylthio)selenides in the metabolism of selenite, we have initiated studies on their formation and chemical properties. As the first part of this study, we have developed methodology for the simultaneous determination of bis(alkylthio)selenides and disulfides formed by reaction 1, including the symmetrical and mixed bis(alkylthio)selenides and disulfides which are formed when selenite is reacted with a mixture of two thiols. The various bis(alkylthio)selenides and disulfides are separated by reversed-phase ion pairing liquid chromatography with detection by an electrochemical detector equipped with dual mercury/gold amalgam electrodes in series. Both the bis(alkylthio)selenide and the disulfide are reduced at the upstream electrode and then detected as thiols at the downstream electrode. The electrochemical behavior of bis(alkylthio)selenides at the mercury/gold amalgam electrode was characterized, and mobile phase conditions were established for separation of several mixtures of bis(alkylthio)selenides and disulfides by reversed-phase ion pairing liquid chromatography.

EXPERIMENTAL

Chemicals

Reduced and oxidized glutathione, GSH and GSSG, respectively, and D-penicillamine (PSH) were obtained from Sigma. Penicillamine disulfide (PSSP) was obtained from ICN Pharmaceuticals and sodium selenite and selenious acid were from Aldrich. Bis(penicillamine)selenide (PSSeSP) was prepared by reaction of penicillamine and H_2SeO_3 following the procedure reported by Nakagawa *et al.*⁸. A solution containing bis(glutathione)selenide and GSSG was prepared by reaction of $4 \cdot 10^{-4} M$ GSH with $1 \cdot 10^{-4} M$ H_2SeO_3 at pH 2.6; the combining ratio for the reaction of thiols with selenite is 4:1 over the pH range 1.2–6.4 (ref. 6). A solution containing a mixture of PSSeSP, GSSeSG, the mixed bis(alkylthio)selenide PSSeSG, and PSSP, GSSG and PSSG was prepared by reaction of $1 \cdot 10^{-4} M$ GSH, $1 \cdot 10^{-4} M$ PSH and $5 \cdot 10^{-5} M$ H_2SeO_3 at pH 2.5. Formation of PSSeSG by reaction of H_2SeO_3 with a mixture of PSH and GSH has been established by ^{77}Se -NMR⁹.

Apparatus

All chromatographic analyses were performed on a Bioanalytical Systems BAS 200 liquid chromatograph equipped with a dual-electrode detector. The detector cell consisted of two 2.3-mm diameter gold electrodes, which were converted to a mercury/gold amalgam surface by placing triply-distilled mercury onto the highly polished gold surface and then, after 2–3 min, removing the excess mercury. The dual electrodes were positioned in series in the flow channel. A BAS RE-3 Ag/AgCl reference electrode was used. Hydrodynamic voltammograms were measured for the various compounds to determine the appropriate potential settings for the two electrodes. The electrodes typically could be used for about two weeks before they needed to be reconditioned.

All tubing was stainless steel to exclude oxygen. A Rheodyne Model 7125 sam-

ple injector was used with a 20- μ l sample loop; injections were made by overloading the loop with 70 μ l of sample. Mobile phase was sparged with helium gas and then maintained under a helium positive pressure during use. Separations were done on a 3- μ m diameter particle size Biophase ODS column (100 $\times$ 3.2 mm I.D.).

d.c. and differential pulse polarograms were measured with a Princeton Applied Research Model 174A polarographic analyzer.

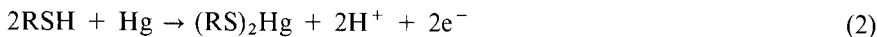
Mobile phase

The mobile phases were prepared from NaH_2PO_4 (Fisher Scientific), H_3PO_4 (Mallinckrodt), sodium octylsulfate (SOS; Aldrich), spectrophotometric grade acetonitrile (Mallinckrodt) and distilled water. For each disulfide-bis(alkylthio)selenide mixture studied, the effect of the percent acetonitrile, the concentrations of SOS and NaH_2PO_4 , and the pH on retention time was studied to identify a mobile phase which resulted in a satisfactory separation. The mobile phases used are given in the figure legends. The mobile phases were filtered through a 0.45- μ m cellulose nitrate filter membrane (Whatman F2848-24) and were pumped at a flow-rate of 0.5 ml/min.

RESULTS AND DISCUSSION

Electrochemical detection

The dual-electrode detector was operated with the downstream electrode set to a potential at which thiols give rise to an anodic current¹⁰. The detector reaction involves oxidation of the mercury electrode itself:



Because of the high stability of $(\text{RS})_2\text{Hg}$, oxidation takes place at less positive potentials than required for oxidation of mercury alone¹¹. Bis(alkylthio)selenides and disulfides were detected by reduction at the upstream electrode, followed by detection of the reduction products at the downstream electrode.

Selection of optimum potentials for the upstream and downstream electrodes requires a knowledge of the electrochemical properties of thiols, disulfides and bis(alkylthio)selenides at mercury/gold amalgam electrodes. The electrochemical properties of PSH, GSH, PSSP, GSSG and PSSG at such electrodes have been characterized previously¹². The hydrodynamic voltammograms for both PSH and GSH, determined by measuring the chromatographic peak current as the potential of the downstream electrode was varied, indicated that a setting of +0.15 V vs. the Ag/AgCl reference electrode is optimum for the detection of thiols¹². To determine the optimum potential setting for the upstream electrode, hydrodynamic voltammograms were determined for PSSeSP, PSSeSG and GSSeSG by measuring the chromatographic peak current detected by the downstream electrode (E_2) as the potential of the upstream electrode (E_1) was varied over the range +0.20 to -0.90 V. For comparison, hydrodynamic voltammograms were also measured for PSSP and GSSG by the same procedure.

The hydrodynamic voltammograms for PSSeSP and PSSP are presented in Fig. 1. For both, reduction reactions take place at the upstream electrode, the products of which give rise to an anodic current at the downstream electrode. The hydrodynamic

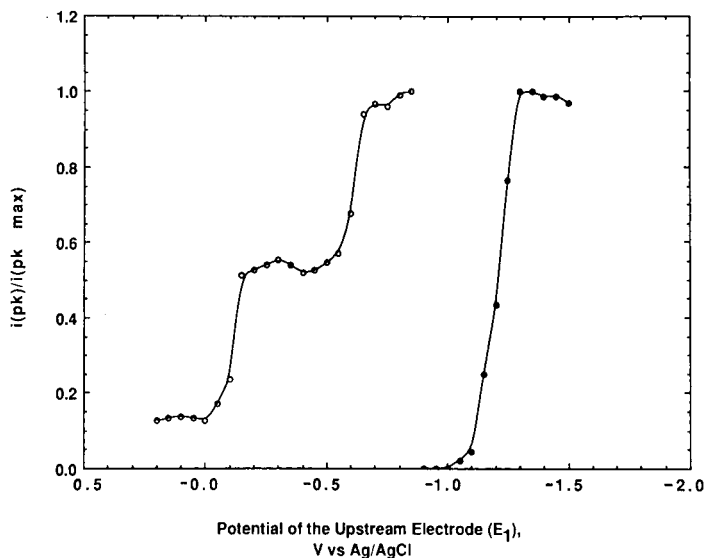


Fig. 1. Hydrodynamic voltammograms for PSSeSP (○) and PSSP (●) at the mercury/gold amalgam electrode. The voltammograms were obtained by measuring the anodic peak current from the downstream electrode (E_2) as a function of the potential applied to the upstream electrode (E_1); the potential of the downstream electrode was held constant at +0.15V. The response is plotted as the ratio of the chromatographic peak current at a particular potential to the maximum chromatographic peak current. The aqueous mobile phase contained 6% CH_3CN , 30 mg SOS/l, 0.075 M NaH_2PO_4 and sufficient H_3PO_4 to give a pH of 2.9. The flow-rate was 0.5 ml/min.

voltammograms in Fig. 1 are plotted as the ratio of the anodic chromatographic peak current at a given potential to the maximum peak current ($i_{\text{pk}}/i_{\text{pk, max}}$) vs. potential. For PSSP, the hydrodynamic voltammogram consists of a single wave corresponding to a single two-electron reduction at the upstream electrode¹³.



For PSSeSP, the hydrodynamic voltammogram consists of two waves, both at potentials somewhat more positive than for PSSP. Also, the peak current is finite at potentials more positive than the potential of the first wave.

To elucidate the electrochemical behavior of PSSeSP at the mercury/gold electrode, the hydrodynamic voltammograms in Fig. 2 were measured. In Fig. 2, the actual chromatographic peak current from the downstream electrode is plotted vs. potential. The hydrodynamic voltammogram obtained by measuring peak current as a function of the potential of the downstream electrode, with the upstream electrode set to 0.000 V, is plotted in Fig. 2A. The voltammogram shows clearly that the two waves for PSSeSP in Fig. 1 are due to reductive processes at the upstream electrode, and furthermore that PSSeSP gives rise to an anodic current at potentials more positive than approximately +0.10 V. Thus, the current over the potential range +0.20 to -0.05 V in Fig. 1, where PSSeSP is not being reduced at the upstream electrode, is due to the electrochemical activity of PSSeSP at the downstream electrode, which was set to +0.15 V.

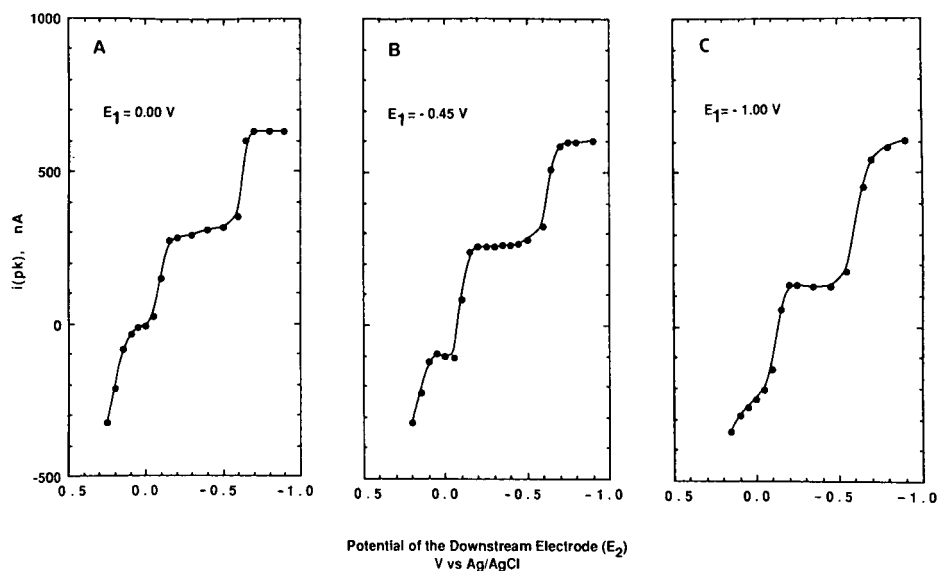


Fig. 2. Hydrodynamic voltammograms determined for PSSeSP by measuring the current from the downstream electrode as a function of the potential applied to the downstream electrode (E_2). The potential applied to the upstream electrode (E_1) was held constant at 0.00 V (A), -0.45 V (B) and -1.00 V (C). The actual peak current is plotted to show that the electrode process switches from anodic to cathodic as the potential of the downstream electrode is varied from 0.25 V to -0.9 V. The aqueous mobile phase contained 7% CH_3CN , 30 mg SOS/l, 0.075 M NaH_2PO_4 and H_3PO_4 to give pH 3.0.

The hydrodynamic voltammograms in Fig. 2B and C were obtained by measuring the chromatographic peak current as a function of the potential of the downstream electrode, with the potential of the upstream electrode set to -0.45 V and -1.00 V, respectively. There are several features of interest in Fig. 2A–C. First, when the downstream electrode is set in the range $+0.1$ – 0.05 V, the anodic peak current increases as the potential of the upstream electrode is changed from 0.00 to -0.45 to -1.00 V corresponding to greater reduction of PSSeSP to electroactive forms which are then detected at the downstream electrode. The potential of the oxidation wave suggests that it is due to PSH (eqn. 2)¹². Also, the peak currents on the first two reduction plateaus are less as the potential of the upstream electrode is made more negative due to conversion of some of the PSSeSP at the upstream electrode to forms which are not electroactive at the downstream electrode when it is more negative than -0.15 V. The results also indicate, however, that only a small fraction of the PSSeSP is reduced at the upstream electrode when it is set to potentials more negative than -0.15 V. Thus, the peaks obtained with the downstream electrode set to $+0.15$ V and the upstream electrode more negative than -0.15 V are the sum of the currents due to the electrochemical activity of the PSSeSP not reduced at the upstream electrode and the reduction products (eqn. 2).

Hydrodynamic voltammograms obtained for GSSeSG and GSSG and for PSSeSG by measuring the chromatographic peak current at the downstream electrode, set to $+0.15$ V, as a function of the potential of the upstream electrode are

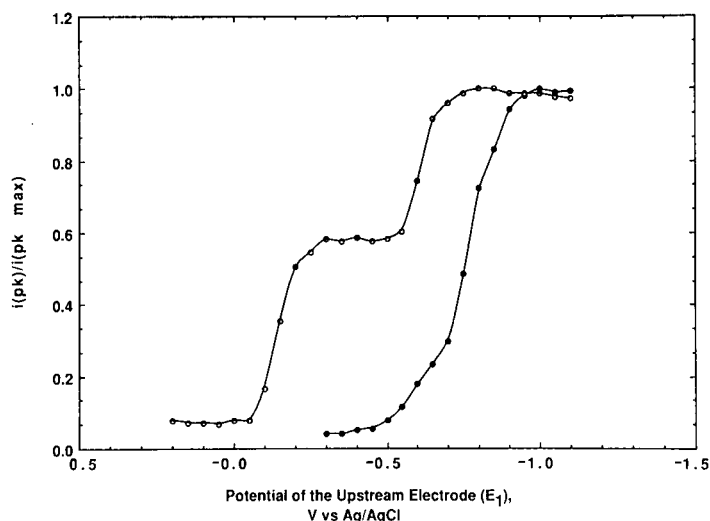


Fig. 3. Hydrodynamic voltammograms obtained for GSSeSG ($\circ$) and GSSG ($\bullet$) by measuring the anodic peak current from the downstream electrode ($E_2 = +0.15$ V) as a function of the potential applied to the upstream electrode (E_1). The response is plotted as the ratio of the peak current to the maximum peak current. The aqueous mobile phase contained 6% CH_3CN , 40 mg SOS/l, 0.075 M H_3PO_4 and sufficient H_3PO_4 to give pH 2.6.

shown in Figs. 3 and 4, respectively. The voltammograms for GSSeSG and PSSeSG are similar to those for PSSeSP (Fig. 1), with two reduction waves at essentially the same potentials and with a finite current over the potential range $+0.2 - 0$ V due to the electrochemical activity of GSSeSG and PSSeSG at the downstream electrode set to $+0.15$ V.

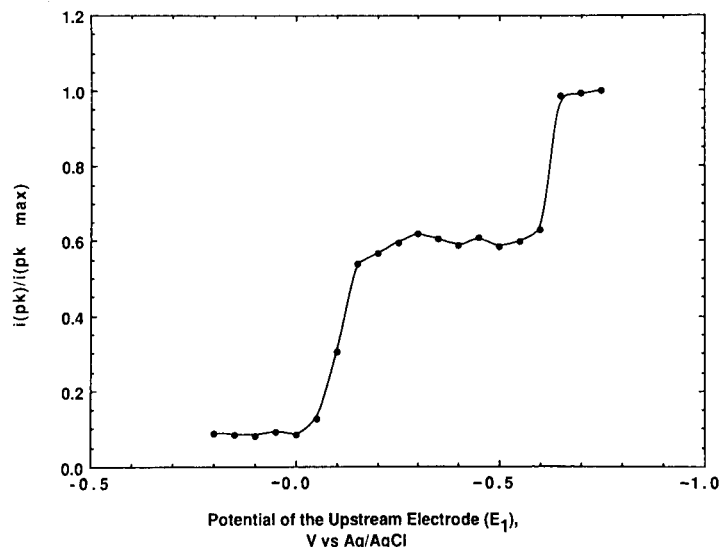
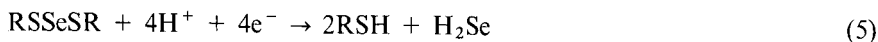
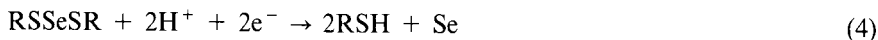


Fig. 4. The hydrodynamic voltammogram for GSSeSP measured using the same procedure and mobile phase as in Fig. 3.

Although the nature of the electrode reactions giving rise to the two reduction waves has not been elucidated, the results are consistent with the reactions in eqns. 4 and 5 giving rise to the first and second waves, respectively.



Because the number of electrons in the two reactions is different, they give different limiting peak currents (Fig. 2A). This was confirmed by measuring d.c. and differential pulse polarograms for PSSeSP. The d.c. polarogram consists of two waves, at essentially the same potentials as in Figs. 1 and 2, and the ratio of the limiting currents for the first and second waves varies from 1.7 to 1.3 over the pH range 1.8 – 5.6. Two peaks were observed in the differential pulse polarogram, and the ratio of the peak currents varied from 2.2 to 1.4 over the pH range 1.8 – 5.6.

Of the two products formed in eqn. 4, presumably only the RSH will give rise to an anodic current whereas both products from eqn. 5 will be electroactive. Thus, the peak current is expected to be larger when the second reaction occurs at the upstream electrode. The formation of RSH at potentials on the first reduction plateau was confirmed by measuring ^1H -NMR spectra of a solution containing $5 \cdot 10^{-3} M$ PSSeSP as it was electrolyzed at a mercury pool electrode set to $-0.45 V$. At the start of electrolysis, resonances were observed only for PSSeSP. After two hours, resonances were observed for both PSSeSP and PSH. No PSSP was detected. A black layer formed on the surface of the mercury, perhaps due to reaction of mercury with the Se formed in eqn. 4.

Selection of detector electrode potentials using the results in Fig. 1–4 is discussed in the following section.

Chromatographic separation

Several mixtures of thiols, disulfides and bis(alkylthio)selenides were chromatographed to demonstrate the feasibility of analyzing such mixtures by high-performance liquid chromatography (HPLC) with electrochemical detection (ED). The mixture studied in most detail contained PSH, PSSP and PSSeSP. Based on previous work on the chromatographic separation of PSH and PSSP by reversed-phase ion pairing chromatography^{12,14,15}, we chose a mobile phase of aqueous CH_3CN containing SOS for ion pairing and NaH_2PO_4 and H_3PO_4 for ionic strength and pH control. Capacity factors were measured for PSH, PSSP and PSSeSP as the CH_3CN content, the concentrations of SOS and NaH_2PO_4 , and the pH were systematically varied. To summarize the results, the capacity factor for PSSeSP is consistently larger than those for PSH and PSSP, and it is affected much more by changing the amount of CH_3CN , the pH and the concentrations of SOS and NaH_2PO_4 than are those for PSH and PSSP. The capacity factor for PSSeSP decreased as the CH_3CN content was increased from 1 to 4% and as the NaH_2PO_4 concentration was increased from 0.02 to 0.08 M , but it increased as the concentration of SOS was increased from 0 to 90 mg/l. From these results, a mobile phase containing 20 mg SOS/l, 3% CH_3CN , 0.075 M NaH_2PO_4 and sufficient H_3PO_4 to give a pH of 2.7 was selected. A flow-rate of 0.5 ml/min was used.

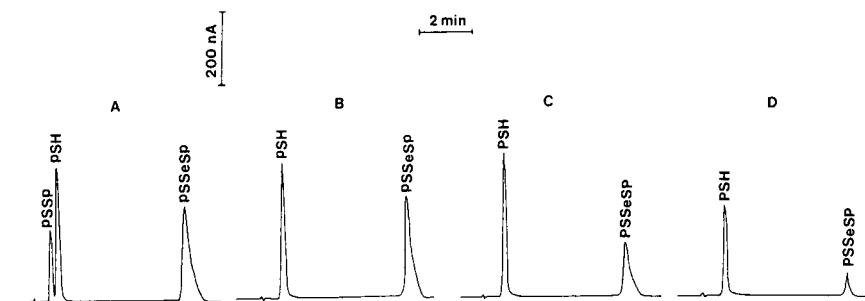


Fig. 5. Chromatograms of a solution containing $4.4 \cdot 10^{-5} M$ PSSH, $4.4 \cdot 10^{-5} M$ PSSP and $6.7 \cdot 10^{-5} M$ PSSeSP. The mobile phase composition is given in Fig. 1. The downstream electrode (E_2) was set to $+0.150 V$, vs. Ag/AgCl, and the upstream electrode (E_1) was set to (A) $-1.300 V$, (B) $-0.900 V$, (C) $-0.450 V$ and (D) $0.000 V$.

A series of chromatograms obtained for a mixture containing $4.4 \cdot 10^{-5} M$ PSSH, $4.4 \cdot 10^{-5} M$ PSSP and $6.7 \cdot 10^{-5} M$ PSSeSP is presented in Fig. 5. All were measured with the downstream electrode set to $+0.15 V$. Chromatogram A was measured with the upstream electrode set to $-1.3 V$. The hydrodynamic voltammograms in Fig. 1 indicate that, at this potential, the upstream electrode will reduce both PSSP and PSSeSP to products which can be detected at the downstream electrode, and thus all three components of the mixture are detected.

The voltammograms in Fig. 1 also indicate that additional selectivity can be achieved through the potential applied to the upstream electrode. To illustrate, chromatogram B in Fig. 5 was obtained with the upstream electrode set to $-0.90 V$. At this potential, only the PSSeSP is reduced at the upstream electrode and thus PSSH and PSSeSP can be selectively detected in the presence of PSSP. Chromatograms C and D in Fig. 5 were obtained with the upstream electrode set to $-0.45 V$ and $0.00 V$ respectively. At $-0.45 V$, PSSeSP is reduced, but the anodic current resulting at the downstream electrode is less (Fig. 1). At $0.00 V$, PSSeSP is not reduced, however it still gives an anodic current at the downstream electrode because the electrode was set to $+0.15 V$.

To characterize the response of the electrochemical detector to bis(alkylthio) selenides, a standard curve was prepared by plotting peak current (nA) vs. concentration for PSSeSP with the upstream electrode set to $-0.90 V$. The response is linear from the concentration of $1.13 \cdot 10^{-7} M$ to a concentration of at least $1.2 \cdot 10^{-4} M$. The slope and intercept were determined by linear least square calculations to be $3.97 (\pm 0.09) \cdot 10^6$ nA/mol PSSeSP/l and $8.9 (\pm 4.3)$ nA, respectively, with a correlation coefficient of 0.9974. Detection limits are estimated to be $< 1 \cdot 10^{-7} M$ for PSSeSP or < 2 pmol.

Chromatograms for a mixture containing $1 \cdot 10^{-4} M$ GSSeSG and $1 \cdot 10^{-4} M$ GSSG are shown in Fig. 6. The mobile phase selected for this separation contained 6% CH_3CN , 40 mg SOS/l, $0.075 M$ NaH_2PO_4 and sufficient H_3PO_4 to give a pH of 2.6. Chromatogram A was obtained with the downstream and upstream electrodes set to $+0.15 V$ and $-1.0 V$, respectively. At a potential of $-1.0 V$, both GSSeSG and GSSG are reduced (Fig. 3). Chromatogram B in Fig. 6 demonstrates that GSSeSG can be selectively determined by setting the upstream electrode to $-0.55 V$.

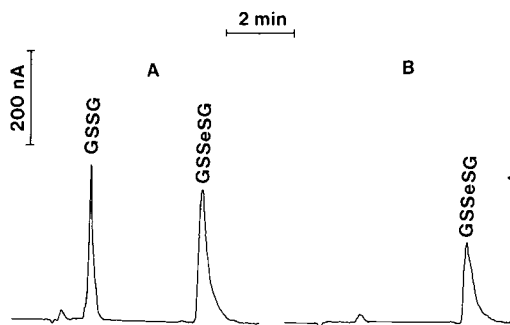


Fig. 6. Chromatograms of a solution containing $1 \cdot 10^{-4} M$ GSSG and $1 \cdot 10^{-4} M$ GSSeSG. The mobile phase was the same as in Fig. 3. The downstream electrode (E_2) was set to $+0.150V$, vs. Ag/AgCl, and the upstream electrode (E_1) was set to (A) $-1.000 V$ and (B) $-0.55 V$.

Finally, the chromatogram for a mixture containing PSSeSP, PSSeSG, GSSeSG, PSSP, PSSG and GSSG is shown in Fig. 7. To achieve a satisfactory separation in a reasonable time, it was necessary to use gradient elution. Two mobile phase mixtures were used: (A) 4% CH_3CN , 80 mg SOS/l, 0.1 M NaH_2PO_4 and H_3PO_4 to give pH 2.5; (B) 50% CH_3CN , 80 mg SOS/l, 0.1 M NaH_2PO_4 and H_3PO_4 to give pH 2.5. The gradient program used to achieve the separation in Fig. 7 consisted of changing the mobile phase linearly from 100% A at the time of injection to 80% A and 20% B 4 min after injection and then linearly back to 100% A at 40 min after injection.

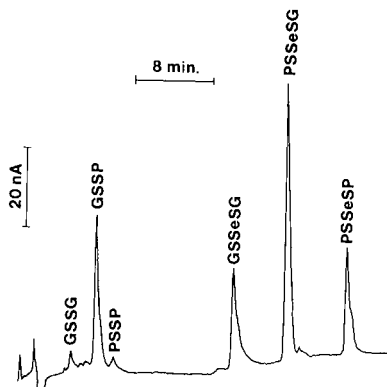


Fig. 7. Chromatogram of a solution containing $4.7 \cdot 10^{-6} M$ GSSG, $4.5 \cdot 10^{-5} M$ GSSP, $4.1 \cdot 10^{-6} M$ PSSP, $1.1 \cdot 10^{-4} M$ GSSeSG, $2.3 \cdot 10^{-5} M$ PSSeSG and $1.5 \cdot 10^{-5} M$ PSSeSP. The upstream (E_1) and downstream (E_2) electrodes were set to $-1.300 V$ and $+0.150 V$, respectively. Gradient elution was used; the mobile phases and the gradient program are described in the text.

CONCLUSION

Even though the importance of selenium as both an essential and a toxic element has long been recognized¹⁶, relatively few methods have been reported for the

speciation analysis of organic and biological selenium-containing compounds¹⁷. The majority of the reported methods for the determination of selenium in organic and biological matrices give the total selenium content rather than the concentration of specific selenium-containing compounds¹⁸⁻²⁰. In a previous study, we demonstrated that HPLC-ED is a sensitive and selective method for the determination of selenols, diselenides and selenenylsulfides²¹. The results of the present study indicate this technique to also be sensitive and selective for the determination of bis(alkylthio)selenides. By appropriate choice of detector electrode potential, both bis(alkylthio)selenides and disulfides can be determined or the bis(alkylthio)selenides can be detected selectively. These methods are predicted to be of use in studies of bis(alkylthio)selenides, *e.g.* in studies of the kinetics of their formation by reaction of thiols with selenious acid.

ACKNOWLEDGEMENTS

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RAPID ANALYSIS OF TRIACYLGLYCEROLS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH LIGHT SCATTERING DETECTION

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SUMMARY

This paper details a high-performance liquid chromatography (HPLC) method for the separation of triacylglycerols, using a 3- μ m, 15 cm $\times$ 4.6 mm I.D. Spherisorb ODS column and gradient elution with dichloromethane and acetonitrile. The triacylglycerols are detected using a light scattering detector (mass detector). Separations of a number of different edible oils and fats are reported. The procedure offers a possible method for determining cocoa butter equivalents and the adulteration of edible oils and fats by other non-generic fats and oils.

INTRODUCTION

Since Pei *et al.*¹ applied reversed-phase high-performance liquid chromatography (HPLC) to the separation of triacylglycerols a number of reversed-phase systems have been developed as rapid and efficient resolution of complex triacylglycerol mixtures can be achieved. El-Hamdy and Perkins² and Plattner³ evaluated several packing materials and concluded that reversed-phase C₁₈ columns of small particle size gave the most efficient separations. Most authors^{4,5} have used 5 μ m, 25 cm columns, often two in series, to obtain detailed triacylglycerol profiles.

A number of papers^{4,6–8} have detailed isocratic systems combined with refractive index (RI) detectors, often with acetonitrile–acetone mobile phases. Such solvent systems continued to be used even though the lack of solubility of triacylglycerols with carbon numbers greater than 46 in this mobile phase has been noted⁹. Solvent gradients which would be required for optimum separations of complex triacylglycerol mixtures are not compatible with RI detection. Ultraviolet (UV) detectors have also been used^{10–12} but the range of mobile phases is limited since triacylglycerols absorb only in the far UV range.

Detectors compatible with gradient elution systems have been used. Mass spectrometric (MS) detection has been used with solvent gradients of acetone–acetonitrile and propionitrile–acetonitrile^{13,14}. Phillips *et al.*^{5,15} used their own design flame ion-

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isation detection (FID) system with solvent gradients of methylene chloride and acetonitrile. Different gradients were used for different oils. More recently Herslöf and Kindmark¹⁶ used a light scattering detector with gradients of acetonitrile-ethanol-hexane. Both of these systems produced efficient separations of complex triacylglycerol mixtures but retention times were long. There has been no report of one gradient system that can be used to separate all known natural triacylglycerol mixtures regardless of their carbon number distribution.

This work was carried out to separate triacylglycerols by HPLC using a 3- μ m, 15 cm $\times$ 4.6 mm I.D. reversed-phase column and light scattering detection. A rapid separation using a single gradient system was sought to enable the method to be adopted for routine analytical work, for identification of oils and possible detection of adulteration.

MATERIALS AND METHODS

Analysis of triacylglycerols using HPLC

The HPLC system consisted of a Spectra Physics SP8700 extended range pump with a Spherisorb ODS-2 column, 150 $\times$ 4.6 mm I.D., 3 μ m particle size and 12% carbon loading, obtained from Phase Separations (Queensferry, U.K.). Chromatography was carried out using a stepped gradient elution system of 30–70% dichloromethane in acetonitrile over 60 min, with 45% dichloromethane after 20 min and 70% after 60 min. The flow-rate was 0.6 ml/min. The detector used was an Applied Chromatography Systems mass detector and peak areas were measured using a Spectra Physics SP4270 integrator.

Standard triacylglycerol and sample preparation

Triacylglycerol standards were stated as 99% pure (carbon numbers and units of unsaturation are indicated in brackets): tricaprylin (24:0), trionanoin (27:0), tricaprillin (30:0), trilaurin (36:0), trimyristin (42:0), tripalmitin (48:0), tristearin (54:0), triolein (54:3), trilinolenin (54:9) and trionadecanoin (57:0) were obtained from Sigma Chemical (Poole, U.K.), triundecanoin (33:0) from Fluka (Buchs, Switzerland), *rac*-glycerol-1,3-palmitate-2-oleate (50:1) from PL Biochemicals (WI, U.S.A.) and *rac*-glycerol-1-palmitate-2-oleate-3-stearate (52:1) and *rac*-glycerol-1,3-stearate-2-oleate (54:1) from Supelco (Bellefonte, PA, U.S.A.).

Vegetable oils were obtained from consignments imported into the U.K. Samples of cocoa butter were obtained from British Cocoa Mills (Hull, U.K.) and a cocoa butter equivalent was obtained from Loders and Nucoline plc. Solutions of 0.05% (w/v) of each standard triacylglycerol and 1% (w/v) oil and fat samples in dichloromethane-acetonitrile (1:1) were filtered through a 0.45- μ m filter and injected onto the HPLC system using a 20- μ l injection loop.

Argentation TLC

Argentation TLC was carried out according to the IOCCC Method No. 111/B-1985¹⁷, with the following amendment: 20 $\times$ 20 cm glass plates coated with 0.25 mm silica gel were dipped in a 10% aqueous solution of silver nitrate and dried at 100°C in the dark.

Capillary gas chromatography (GC)

Fatty acid methyl esters were prepared by the IUPAC Method No. 2.301¹⁸. Capillary GC was carried out using a Carlo Erba Vega gas chromatograph fitted with a 50 m $\times$ 0.32 mm I.D. WCOT Silar-5 column and a flame ionisation detector.

RESULTS AND DISCUSSION

A rapid method for separating a standard mixture of triacylglycerols by HPLC, based on the procedure of Phillips *et al.*¹⁵, was devised. Resolution of the standard triacylglycerols was achieved with a linear gradient of 30–70% dichloromethane in acetonitrile with a flow-rate of 0.6 ml/min but optimum resolution of the complex triacylglycerol mixtures present in natural oils and fats requires a step in this gradient. Using the stepped gradient the resolution and retention times of these standards were found to be reproducible. The method was then applied to a range of edible oils and fats. Trinonadecanoin was used, at a level of 0.01%, as an internal standard. This compound was chosen as it is not found naturally in the oils and it elutes after all the other triacylglycerols. Since trinonadecanoin had been subjected to all the conditions of the gradient, reproducible relative retention times for each triacylglycerol were obtained. Figs. 1–3 show examples of triacylglycerol profiles obtained by this method. Triacylglycerols elute from this system in order of increasing carbon number. The presence of double bonds reduces the retention time of the triacylglycerol, *e.g.*, triolein elutes before tristearin and trilinolein before triolein.

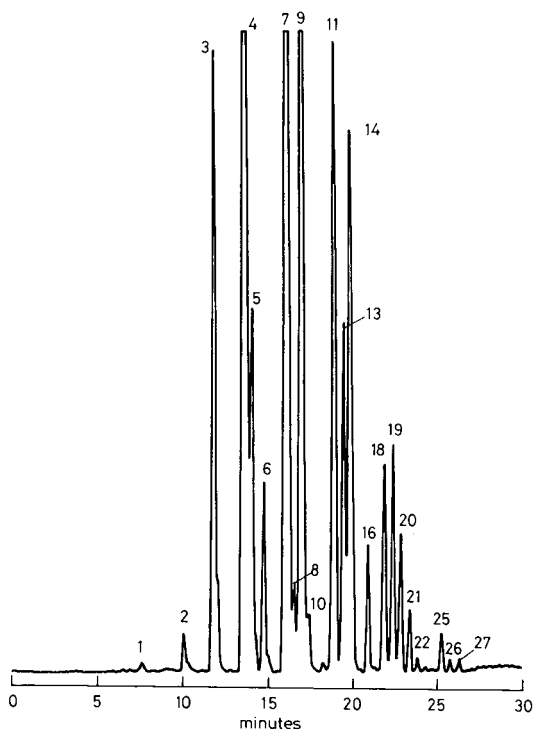


Fig. 1. Triacylglycerol profile of soyabean oil. For peak identification see Table I.

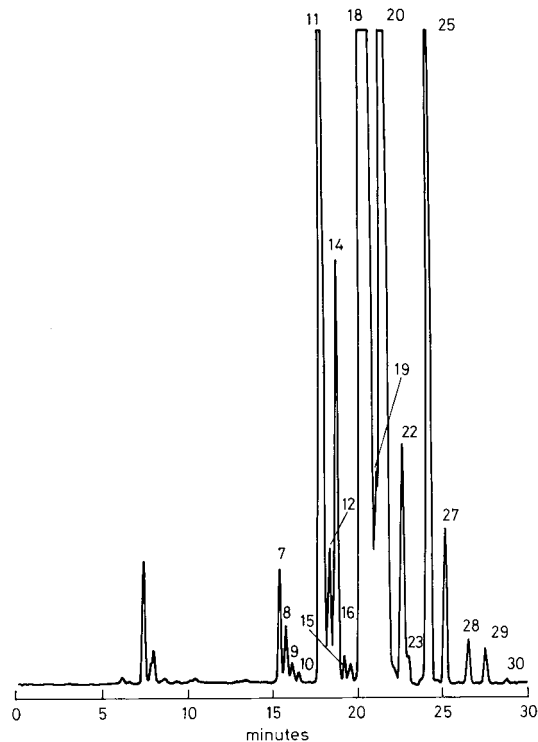


Fig. 2. Triacylglycerol profile of olive oil. For peak identification see Table I.

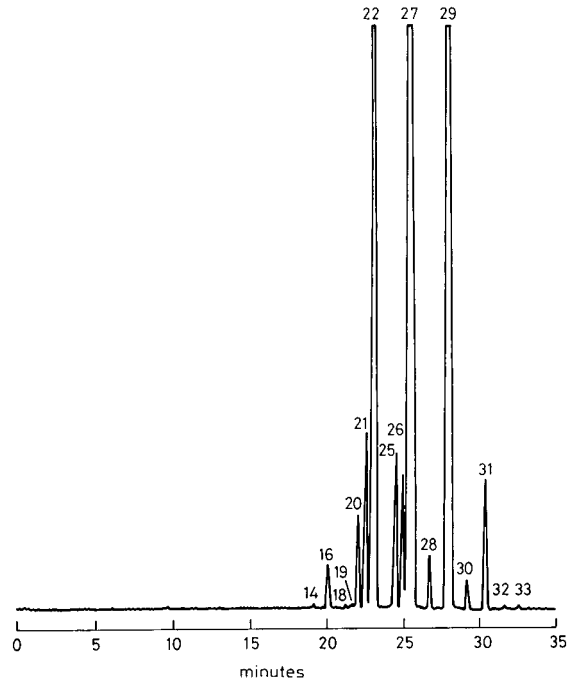


Fig. 3. Triacylglycerol profile of cocoa butter. For peak identification see Table I.

The triacylglycerol profile of cocoa butter (Fig. 3) shows sixteen triacylglycerols being separated. Cocoa butter is known to consist of three major triacylglycerols with carbon numbers 50, 52 and 54. Standards of glycerol-1,3-palmitate-2-oleate (POP) (50), glycerol-1-palmitate-2-oleate-3-stearate (POS) (52) and glycerol-1,3-stearate-2-oleate (SOS) (54) were chromatographed separately under the same conditions in order to identify the major peaks by comparison of relative retention times. As this method does not differentiate positional isomers such as glycerol-1,3-palmitate-2-oleate and glycerol-1,2-palmitate-3-oleate, the position of unsaturation in the cocoa butter triacylglycerols was confirmed to be in the "2" position using argentation TLC¹⁷.

This information was initially used for identifying the triacylglycerol peaks along with the triacylglycerol labels used by Phillips *et al.*⁵ For confirmation, the eluent containing each component triacylglycerol was collected from the HPLC system using a stream splitter. Capillary GC of the fatty acid methyl esters prepared from each of the collected fractions was used to confirm their identity (*e.g.*, methyl palmitate and methyl oleate in a ratio of 2:1 are obtained from the POP fraction of cocoa butter). The peaks of the triacylglycerol profiles (Figs. 1–6) are numbered, the peak identification is shown in Table I. The early eluting components in Figs. 2 and 4–6 are diacylglycerols.

In earlier work¹⁹ the author has looked at the natural variation within six cocoa butters of different origins. Normalising the percentages of the three major cocoa butter peaks (POP, POS, SOS) gives a very similar composition for all the cocoa butters analysed, the range from the mean percentage being 1.4 for POP, 1.7 for POS

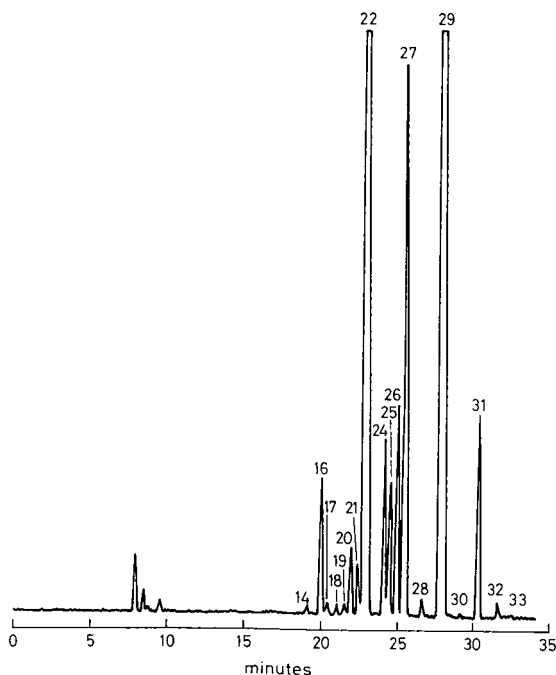


Fig. 4. Triacylglycerol profile of cocoa butter equivalent. For peak identification see Table I.

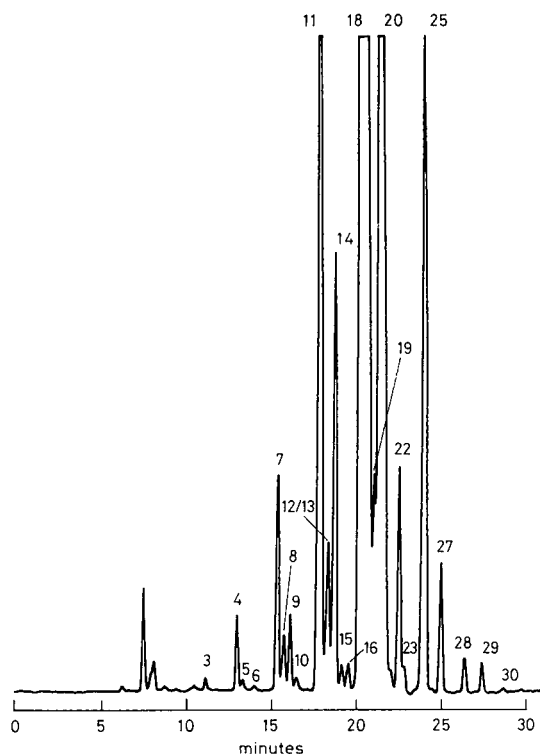


Fig. 5. Triacylglycerol profile of 5% soyabean oil in olive oil.

and 1.1 for SOS. The triacylglycerol composition of a cocoa butter equivalent (Fig. 4) shows a marked difference from that of cocoa butter. This would probably enable the addition of 10% of this cocoa butter equivalent to be easily detected. Using the triacylglycerol composition for detecting this cocoa butter equivalent offers an alternative to using fatty acid profiles which are often used for the identification of oils and fats. There are no significant differences between the fatty acid profiles of this cocoa butter equivalent and cocoa butter¹⁹.

The triacylglycerol profiles for soyabean oil (Fig. 1) and olive oil (Fig. 2) are significantly different. Olive oil contains no triacylglycerols eluting before LLO whereas soyabean oil contains a large proportion of triacylglycerols eluting before LLO. This difference could be used to detect the addition of low levels of soyabean oil in olive oil. Figs. 5 and 6 illustrate the effect of blending 5 and 10% of soyabean oil with olive oil. The changes in the composition of olive oil with the addition of soyabean oil are detailed in Table II. Soyabean oil has been used to illustrate the possibilities for the method to detect adulteration of olive oil. Any oil containing a significant percentage of early eluting triacylglycerols, *e.g.*, zero erucic rapeseed oil could be detected in the same manner.

Detailed work on the natural variation in triacylglycerol composition of a range of oils is required before very low levels of adulteration could be accurately assessed.

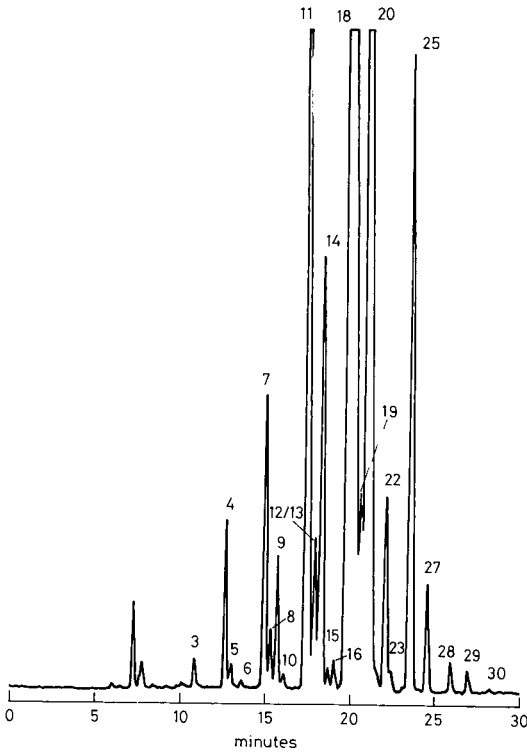


Fig. 6. Triacylglycerol profile of 10% soyabean oil in olive oil.

TABLE I

IDENTIFICATION OF TRIACYLGLYCEROL PEAKS FOR FIGS. 1-6

P = palmitic acid, Pa = palmitoleic acid, S = stearic acid, O = oleic acid, L = linoleic acid, Ln = linolenic acid, A = arachidic acid.

Peak No.	Triacylglycerol	Peak No.	Triacylglycerol
1	LnLnLn	18	OOO
2	LLnLn	19	OLS
3	LLnL	20	OOP
4	LLL	21	PLS
5	LLnO	22	POP
6	LLnP	23	PPaS
7	LLO	24	PPP
8	OLnO	25	OOS
9	LLP	26	SLS
10	OLnP	27	POS
11	OLO	28	PPS
12	OPaO	29	SOS
13	LLS	30	SPS
14	OLP	31	SOA
15	OPaP	32	SSS
16	PLP	33	SSA
17	SLnP		

TABLE II

TRIACYLGLYCEROL COMPOSITIONS SHOWING THE CHANGE IN OLIVE OIL COMPOSITION WITH THE ADDITION OF SOYABEAN OIL

Each composition is the mean of 3 determinations.

Peak No.	Triacylglycerol	Triacylglycerol composition			
		100% Olive oil	5% Soya in olive oil	10% Soya in olive oil	100% Soya-bean oil
1	LnLnLn	—	—	—	0.1
2	LLnLn	—	—	—	0.4
3	LLnL	—	0.1	0.2	7.3
4	LLL	—	0.5	1.3	18.9
5	LLnO	—	0.1	0.2	5.4
6	LLnP	—	0.1	0.1	2.1
7	LLO	0.8	1.8	2.6	18.8
8	OLnO	0.5	0.5	0.6	1.0
9	LLP	0.2	0.6	1.2	14.2
10	OLnP	0.1	0.2	0.1	0.7
11	OLO	11.0	10.8	10.9	9.0
12	OPaO	1.2	—	—	—
13	LLS	—	1.4 ^a	1.5 ^a	3.8
14	OLP	4.4	4.8	4.8	9.2
15	OPaP	0.2	0.2	0.2	—
16	PLP	0.2	0.3	0.3	1.3
18	OOO	41.1	39.8	39.0	2.2
19	OLS	1.6	1.8	1.4	2.6
20	OOP	25.2	24.6	24.1	1.6
21	PLS	—	—	—	0.7
22	POP	2.6	2.5	2.2	0.1
23	PPaS	0.2	0.2	0.2	—
25	OOS	8.6	8.0	7.5	0.4
26	SLS	—	—	—	0.1
27	POS	1.3	1.1	1.0	0.1
28	PPS	0.4	0.3	0.3	—
29	SOS	0.3	0.2	0.2	—
30	SPS	0.1	0.1	0.1	—

^a Combination of OPaO and LLS.

It should be noted that the detector does not respond equally for all triacylglycerols and is not quantitative²⁰. Therefore the compositions quoted in this paper are not absolute compositions. However reproducible responses were obtained for this work so the compositions can be used for comparative purposes.

CONCLUSIONS

The method described is a rapid method utilising a simple solvent regime. The component triacylglycerols of a range of oils and fats are separated within 35 min. The method is reproducible and capable of providing detailed information on the triacylglycerol composition of oils and fats containing triacylglycerols of carbon numbers 12 to 57.

The short 3- μ m, 15 cm column used gives rapid separations with no loss in resolution of individual triacylglycerols when compared to the separations of Phillips *et al.*⁵, where two 5- μ m, 25 cm columns in series were used giving 80-min elution times.

The method offers the possibility of detecting and determining adulteration of oils and fats, particularly in cases where the triacylglycerol profiles of two oils show greater differences than their fatty acid profiles.

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Note

Capillary gas chromatography of some polycyclic aromatic compounds on several stationary phases

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The analytical study of polycyclic aromatic compounds (PACs) is of great interest as (1) they are widespread in the environment, in natural water, in foods and in oil- and coal-derived materials¹, (2) many of these compounds are suspected mutagens and/or carcinogens² and (3) their presence and concentration in some coal-derived materials, such as pitches, plays a very important rôle with regard to the future use of these materials³.

The determination of PACs has been carried out by different chromatographic techniques^{4,5}, but the most important tools are high-performance liquid chromatography and gas chromatography (GC)¹; with the latter the best separations of PACs are achieved with temperature programming. However, to the best of our knowledge, only linear-temperature-programmed retention indices of PACs on the slightly polar stationary phase SE-52 are available^{6–9}.

In this paper linear-temperature-programmed retention indices of a group of 25 PACs, obtained on OV-101, SE-54 and OV-1701 as stationary phases, are reported. The influence of the polarity of the stationary phase and the temperature programme on the chromatographic retention of these PACs is discussed. Also relationships between retention indices on the above mentioned stationary phases and some properties of these compounds are discussed.

EXPERIMENTAL

Retention times were determined with a Model 8320 gas chromatograph (Perkin-Elmer, Beaconsfield, U.K.) equipped with flame ionization detection (FID). The separation was carried out on three fused-silica capillary columns coated with OV-101, SE-54 and OV-1701 stationary phases respectively. The characteristics of these columns, the polarity of the stationary phases on the McReynolds scale¹⁰ and working conditions are given in Table I.

The compounds were co-chromatographed with naphthalene, phenanthrene and crysene, which were used as internal standards for calculation of the retention indices. The latter were calculated by using a linear retention index scale for linear-temperature-programmed GC, suggested by Van Den Dool and Kratz¹¹ and used by others^{6–9}. The equation was

TABLE I
CAPILLARY COLUMNS AND WORKING CONDITIONS

Parameter	Column		
	1	2	3
Stationary phase (McReynolds polarity)	OV-1701 ($P_M=789$)	OV-101 ($P_M=229$)	SE-54 $P_M=337$)
Length (m)	25	25	20
Internal diameter (mm)	0.22	0.22	0.22
Carrier gas (hydrogen) flow-rate (ml/min)	1.8	0.7	1.4
Splitting ratio	1:120	1:64	1:64
Detector and injector temperatures ($^{\circ}\text{C}$)	300	300	300
Temperature programme from 50 to 300 $^{\circ}\text{C}$ ($^{\circ}\text{C}/\text{min}$)	4	4	4

$$I = 100 \left[n + \frac{t_{R(\text{compound})} - t_{R(n)}}{t_{R(n+1)} - t_{R(n)}} \right] \quad (1)$$

where I is the retention index, n is the number of rings in the hydrocarbon standard eluted before the substance of interest, $t_{R(\text{compound})}$ is the measured retention time of the substance for which the retention index is to be determined and $t_{R(n)}$ and $t_{R(n+1)}$ are the measured retention times of the hydrocarbon standards eluted just before and after the compound studied. The solutes were obtained from Fluka, Aldrich, Merck and Janssen. The calculated retention indices are given in Table II.

Table III shows the boiling points, T_b , molecular polarizabilities, α , molecular connectivities, $^1\chi$, Van der Waals volumes, V_w , and molecular weights, W_M , of these compounds. The boiling points were taken from the literature^{12,13}; α , $^1\chi$ and V_w were calculated as indicated in refs. 14, 15 and 16, respectively.

RESULTS AND DISCUSSION

Table II shows the retention indices obtained on OV-101 (McReynolds polarity = 229), SE-54 (McReynolds polarity = 337) and on OV-1701 (McReynolds polarity = 789). Also in Table II the difference, δ , between the retention indices on OV-1701 and on OV-101 are given, indicating that the retention of the PACs is clearly affected by the stationary phase polarity.

The nitrogenated compounds are in general more strongly retained on the more polar stationary phase, especially the indole derivatives (compounds 21 and 23), 2-aminoanthracene and diphenylamine (compounds 13 and 24); among the pyridine derivatives, compounds 1, 2, 6, 8 and 18, are the more influenced in their retention by the stationary phase polarity.

The behaviour of the oxygenated compounds shows two trends: the ethers (compounds 7, 10 and 11) are more strongly retained on the less polar stationary phases, whereas this effect is opposite for aromatic ketones (compounds 19, 22 and 25).

The sulphur compounds (14 and 20) show a very similar chromatographic relative retention on the three stationary phases. However, they show the highest relative retention on SE-54.

TABLE II

RETENTION INDICES OF SOME PACs ON SEVERAL STATIONARY PHASES

Compound number	Name	$I^{OV-1701}$ $P_M = 789$	I^{SE-54} $P_M = 337$	I^{SE-52} $P_M = 334$	I^{OV-101} $P_M = 229$	δ^a
1	Quinoline	215.90	210.95	210.26 ^b	209.51	+ 6.39
2	Isoquinoline	220.35	214.49	214.14 ^b	213.19	+ 7.16
3	8-Methylquinoline	226.51	225.86	225.18 ^b	225.49	+ 1.02
4	2-Methylquinoline	227.38	224.12	224.13 ^b	223.47	+ 3.91
5	7-Methylquinoline	235.36	231.80	231.37 ^b	230.99	+ 4.37
6	6-Methylquinoline	235.85	232.19	229.82 ^c	230.75	+ 5.10
7	Diphenyl ether	236.94	240.83	—	241.16	— 3.82
8	4-Methylquinoline	241.41	236.82	235.77 ^b	235.54	+ 5.87
9	2,2'-Bipyridyl	248.27	246.71	247.15 ^b	245.68	+ 2.59
10	Dibenzofuran	257.68	259.14	258.77 ^b	259.57	— 1.89
11	Dibenzyl ether	280.99	281.12	—	281.19	— 0.20
12	5H-Indeno[1,2- <i>b</i>]pyridine	282.77	279.70	279.85 ^b	278.75	+ 4.02
13	Diphenylamine	283.77	276.68	—	275.74	+ 8.03
14	Dibenzothiophene	296.00	296.15	295.39 ^b	295.95	+ 0.05
15	Acridine	306.41	303.79	304.04 ^b	303.18	+ 3.23
16	7,8-Benzoquinoline	306.90	303.45	301.94 ^b	302.57	+ 4.33
17	Phenanthridine	311.81	308.01	307.94 ^b	307.45	+ 4.36
18	5,6-Benzoquinoline	313.25	308.01	307.94 ^b	306.96	+ 6.29
19	9-Xanthenone	318.12	314.04	—	313.05	+ 5.07
20	Thianthrene	328.61	329.88	330.13 ^c	328.58	+ 0.03
21	Carbazole	330.07	311.18	311.71 ^b	309.63	+ 20.44
22	Anthraquinone	336.30	332.19	—	331.38	+ 4.92
23	2-Phenylindole	361.97	343.80	346.18 ^b	343.02	+ 18.95
24	2-Aminoanthracene	379.92	366.78	367.45 ^b	365.89	+ 14.03
25	Phenanthrenequinone	380.49	363.39	—	362.04	+ 18.45

^a $\delta = I^{OV-1701} - I^{OV-101}$.^b Retention indices taken from ref. 6.^c Retention indices taken from ref. 9.

Therefore, the elution order of this group of compounds on the moderately polar stationary phase OV-1701 is not the same as on the less polar stationary phases SE-54 and OV-101. Inversions in the elution order can be observed: 8- and 2-methylquinoline (compounds 3 and 4), 7- and 6-methylquinoline (compounds 5 and 6), diphenyl ether and 4-methylquinoline (compounds 7 and 8), dibenzyl ether and diphenylamine (compounds 11 and 13), acridine and 7,8-benzoquinoline (compounds 15 and 16), phenanthridine and 5,6-benzoquinoline (compounds 17 and 18), 2-aminoanthracene and phenanthrenequinone (compounds 24 and 25), and carbazole (compound 21) which on OV-101 is eluted after 5,6-benzoquinoline. The elution order on the moderately polar stationary phase OV-1701 follows the increasing order of the boiling point more closely than the elution order on the less polar stationary phases. That is, the chromatographic process on OV-1701 is more similar to distillation, than on the less polar stationary phase OV-101 or SE-54 (see data in Tables II and III).

Table II also shows retention indices of 19 PACs obtained on SE-52 (McReynolds polarity = 334) at 2°C/min^{6,9}. These data are in very good agreement

TABLE III
PROPERTIES OF PACs UTILIZED IN THIS STUDY

Compound number	Name	$T_b(^{\circ}\text{C})$	$\alpha(\text{\AA}^3)$	W_M	$^1\chi$	$V_w(\text{ml/mol})$
1	Quinoline	238.0	17.11	129	3.0145	71.39
2	Isoquinoline	243.0	17.11	129	3.0044	71.39
3	8-Methylquinoline	—	18.96	143	3.6811	82.54
4	2-Methylquinoline	247.6	18.96	143	3.6852	82.54
5	7-Methylquinoline	257.6	18.96	143	3.6752	82.54
6	6-Methylquinoline	258.6	18.96	143	3.6752	82.54
7	Diphenyl ether	259.0	20.31	170	4.2296	94.88
8	4-Methylquinoline	264.2	18.96	143	3.6811	82.54
9	2,2'-Bipyridyl	272.5	18.20	156	3.7909	86.54
10	Dibenzofuran	285.0	22.47	168	4.3129	86.64
11	Dibenzyl ether	298.0	24.36	198	5.1058	115.84
12	5H-Indeno[1,2- <i>b</i>]pyridine	306.0	20.22	167	4.4716	94.30
13	Diphenylamine	302.0	21.14	169	4.3213	99.76
14	Dibenzothiophene	332.3	24.24	184	4.9339	94.24
15	Acridine	346.0	24.79	179	4.6792	96.99
16	7,8-Benzoquinoline	—	24.79	179	4.6752	96.99
17	Phenanthridine	349.0	24.79	179	4.6752	96.99
18	5,6-Benzoquinoline	—	24.79	179	4.6752	96.99
19	9-Xanthene	—	24.27	196	4.7671	98.34
20	Thianthrene	364.6	26.23	216	5.2132	105.04
21	Carbazole	355.0	23.36	167	4.4047	91.52
22	Anthraquinone	379.8	25.86	208	5.0629	106.84
23	2-Phenylindole	—	25.32	193	5.0653	109.24
24	2-Aminoanthracene	—	26.82	193	5.0087	107.58
25	Phenanthrenequinone	—	25.86	208	5.0629	106.84

with those obtained on SE-54 (McReynolds polarity = 337) at 4°C/min. The relationship between the two sets of data shows a correlation coefficient of 0.9998, indicating that the variation from 2 to 4°C/min in the temperature programme does not affect, in any appreciable way, the relative retention of these compounds.

Relationships between retention indices of PACs and some of their properties have been reported. Retention indices of a group of PACs have been related to molecular connectivity, but the equations obtained have no value because of their high standard deviations¹⁷. More rigorous contributions have been made by other authors¹⁸⁻²⁰.

The properties selected in this paper in order to study their relationships with the chromatographic retention have a clear meaning and are as follows. The boiling point, T_b , is the temperature at which the vapour pressure of a compound reaches 1 atm. This property gives information about the magnitude of the intermolecular attractions in each compound, and can be measured experimentally. The molecular polarizability, α , provides information about the intermolecular dispersive and inductive forces in the interactions with other compounds²¹. It is usually obtained from experimental data on the density and refractive index, but unfortunately these data are not available for

most of the compounds studied here and were calculated additively¹⁴. The molecular weight, W_M , is the sum of the atomic weights of all atoms in a molecule. The first order molecular connectivity, $^1\chi$, is a quantification of the branching, atom connections, shape and size of the molecule, and is determined by additive calculation¹⁵. Finally the Van der Waals volume, V_w , is the volume occupied by a molecule impenetrable to other molecules with thermal energies at ordinary temperatures. Its determination is by additive calculation¹⁶. The Van der Waals volume and the first order connectivity have been related previously with dispersive solute-stationary phase interactions²²⁻²⁵.

Table IV gives the correlation coefficients between these properties for 25 and 18 compounds, respectively. The more and the less correlated parameters with T_b are α and V_w respectively, but each one has a different physico-chemical meaning, and therefore provides different kinds of information; among the other parameters the least close relationship is between α and V_w , and the closest is between W_M and $^1\chi$.

First, the relationships between retention indices and each of the above-mentioned properties were studied. Table V shows the statistics of the $I = f(\theta)$ equations, where $\theta = T_b, \alpha, W_M, ^1\chi$ or V_w . The parameter most closely connected with the chromatographic retention is the boiling point, T_b . The relationships between retention indices and the other parameters $\alpha, W_M, ^1\chi$ or V_w are very poor, and the equations obtained have no predictive value, in accordance with similar previous results¹⁷.

However the different rôles of these parameters on the several stationary phases is noteworthy. The additively calculated parameters $\alpha, W_M, ^1\chi$ or V_w show a closer relationship with retention indices obtained on the slightly polar stationary phases, than with the retention indices obtained on the moderately polar stationary phase. On the other hand, the T_b values are more closely related with retention indices obtained on the moderately polar stationary phase, than with retention indices obtained on the less polar stationary phase. This behaviour is not an exception, and has been observed previously for other groups of compounds^{26,27}. The equations $I = f(T_b)$ were obtained with only 18 compounds, owing to the fact that the boiling point data of the other compounds were not available in the literature^{12,13}.

Although retention indices on OV-1701 are very closely connected with boiling point values, T_b , the former are not a linear function of the latter (see compounds 12

TABLE IV

CORRELATION COEFFICIENTS BETWEEN SEVERAL PROPERTIES FOR 18 AND 25 COMPOUNDS RESPECTIVELY

$N = 18$						$N = 25$				
	T_b	α	W_M	$^1\chi$	V_w		α	W_M	$^1\chi$	V_w
T_b	1.0000	0.9178	0.8449	0.8538	0.7243	α	1.0000	0.9277	0.9489	0.8669
α		1.0000	0.9289	0.9426	0.8333	W_M		1.0000	0.9674	0.9295
W_M			1.0000	0.9674	0.9258	$^1\chi$			1.0000	0.9452
$^1\chi$				1.0000	0.9330	V_w				1.0000
V_w					1.0000					

TABLE V

STATISTICAL DATA FOR THE $I = f(\theta)$ EQUATIONS, WHERE $\theta = T_b, \alpha, W_M, {}^1\chi$ OR V_w R = Correlation coefficient, s = standard deviation, N = number of experimental points and F = F-test.

Stationary phase	P_M	T_b	α	W_M	${}^1\chi$	V_w
OV-101	229	$R = 0.9897$ $s = 5.51$ $N = 18$ $F_{1,16}^a = 766.6$	$R = 0.9438$ $s = 15.27$ $N = 25$ $F_{1,23}^b = 187.4$	$R = 0.8997$ $s = 20.16$ $N = 25$ $F_{1,23} = 97.7$	$R = 0.9067$ $s = 19.47$ $N = 25$ $F_{1,23} = 106.4$	$R = 0.8494$ $s = 24.37$ $N = 25$ $F_{1,23} = 59.6$
SE-54	337	$R = 0.9909$ $s = 5.19$ $N = 18$ $F_{1,16} = 866.3$	$R = 0.9414$ $s = 15.59$ $N = 25$ $F_{1,23} = 179.3$	$R = 0.8970$ $s = 20.43$ $N = 25$ $F_{1,23} = 94.8$	$R = 0.9033$ $s = 19.83$ $N = 25$ $F_{1,23} = 101.9$	$R = 0.8461$ $s = 24.64$ $N = 25$ $F_{1,23} = 57.9$
OV-1701	789	$R = 0.9921$ $s = 4.92$ $N = 18$ $F_{1,16} = 1001.0$	$R = 0.9142$ $s = 20.04$ $N = 25$ $F_{1,23} = 117.0$	$R = 0.8581$ $s = 25.39$ $N = 25$ $F_{1,23} = 64.2$	$R = 0.8664$ $s = 24.70$ $N = 25$ $F_{1,23} = 69.2$	$R = 0.8185$ $s = 28.41$ $N = 25$ $F_{1,23} = 46.7$

^a $F_{1,16}(\alpha=0.01) = 8.53$.^b $F_{1,23}(\alpha=0.01) = 7.88$.

TABLE VI

EQUATIONS $I = f(T_b)$ AND $I = f(T_b, \theta)$, WHERE $\theta = \alpha, W_M, {}^1\chi$ OR V_w

Stationary	P_M	No.	Equation	R	s	N	F
OV-101	229	1	$I = 13.38 + 0.85T_b$	0.9897	5.51	18	$F_{1,16}^a = 767.6$
		2	$I = 4.45 + 0.68T_b + 2.78\alpha$	0.9934	4.42	18	$F_{2,15}^b = 564.8$
		3	$I = 3.39 + 0.68T_b + 0.35W_M$	0.9971	2.91	18	$F_{2,15} = 1311.4$
		4	$I = 6.19 + 0.67T_b + 14.73{}^1\chi$	0.9983	2.22	18	$F_{2,15} = 2250.2$
		5	$I = -9.51 + 0.73T_b + 0.63V_w$	0.9980	2.46	18	$F_{2,15} = 1839.9$
SE-54	337	6	$I = 14.23 + 0.85T_b$	0.9909	5.19	18	$F_{1,16} = 866.3$
		7	$I = 6.51 + 0.70T_b + 2.40\alpha$	0.9937	4.33	18	$F_{2,15} = 586.4$
		8	$I = 5.03 + 0.70T_b + 0.33W_M$	0.9972	2.87	18	$F_{2,15} = 1340.4$
		9	$I = 7.67 + 0.68T_b + 13.44{}^1\chi$	0.9980	2.38	18	$F_{2,15} = 1949.9$
		10	$I = 7.16 + 0.74T_b + 0.59V_w$	0.9981	2.37	18	$F_{2,15} = 1975.4$
OV-1701	789	11	$I = 12.52 + 0.87T_b$	0.9921	4.92	18	$F_{1,16} = 1001.0$
		12	$I = 12.52 + 0.87T_b - 0.001\alpha$	0.9921	4.92	18	$F_{2,15} = 469.2$
		13	$I = 10.92 + 0.84T_b + 0.06W_M$	0.9923	4.86	18	$F_{2,15} = 480.6$
		14	$I = 10.53 + 0.82T_b + 4.08{}^1\chi$	0.9927	4.72	18	$F_{2,15} = 511.0$
		15	$I = 1.58 + 0.81T_b + 0.30V_w$	0.9939	4.32	18	$F_{2,15} = 611.6$
OV-1701	789	16	$I = 17.25 + 0.85T_b$	0.9953	3.74	16	$F_{1,14}^c = 1473.1$
		17	$I = 15.38 + 0.74T_b + 8.09{}^1\chi$	0.9977	2.58	16	$F_{2,13}^d = 1440.7$
		18	$I = 4.64 + 0.77T_b + 0.40V_w$	0.9984	2.14	16	$F_{2,13} = 2087.9$

^a $F_{1,16}(\alpha=0.01) = 8.53$.^b $F_{2,15}(\alpha=0.01) = 6.36$.^c $F_{1,14}(\alpha=0.01) = 8.86$.^d $F_{2,13}(\alpha=0.01) = 6.70$.

and 13 and 20 and 21 in Tables II and III). In order to find better predictive equations, biparametric regressions were tested (Table VI).

For slightly polar stationary phases satisfactory predictive biparametric equations $I = f(T_b, \theta)$, where $\theta = {}^1\chi$ or V_M (eqns. 4, 5 and 9, 10), have been found for all kinds of compounds. The statistics of these equations are better than those of the monoparametric ones and their standard deviations are very close to the experimental values.

For the moderately polar stationary phase none of the biparametric equations improves the statistics of the monoparametric ones; examination of the retention indices predicted with eqns. 12–15 indicates that the compounds that show the highest deviations from experimental data are those whose heteroatoms support hydrogen atoms, *i.e.*, diphenylamine and carbazole. Biparametric equations obtained without the introduction of these compounds show satisfactory statistical data (eqns. 17 and 18). The PACs, whose heteroatoms support hydrogen atoms, give rise to hydrogen-bonding interactions. The dipole–dipole and hydrogen-bonding interactions of these compounds with the stationary phase must be taken into account in order to find valuable predictive equations for calculating approximate retention indices of all kinds of PACs on a polar or moderately polar stationary phase.

These results agree with previous quantitative structure–retention relationship studies on solutes which have the capacity to give rise to hydrogen-bonding interactions²⁷.

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Note

Determination of the molecular weights of alginates by agarose gel filtration

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The molecular weight of alginic acid has been determined by several techniques such as light scattering, sedimentation, diffusion and viscosity measurements^{1–3}. On the other hand, there are few reports on determination of molecular weights by gel filtration chromatography, although chromatography is a much more simple and rapid technique than those mentioned above. In chromatography, it is well known that molecular weights are determined on the basis of the elution volumes of the test samples. However, in the case of acidic substances, the elution volumes are found to be affected by the ionic strength of the eluents employed. Skalka⁴ showed that in chromatography on a Sephadex G-200 column, heparin (mol. wt. 6000–20 000) was eluted at the void volume with 0.02 mM phosphate buffer solution, and also that as the ionic strength of the buffer increased so did the elution volume. Gelotte⁵ demonstrated that acidic amino acids were excluded with pure water from the gel phase of Sephadex G-25. Similar results have been reported for humic and fulvic acids^{6,7}, D-galacturonic acid oligomers⁸, lignins⁹ and lignosulphonates¹⁰. Thibault⁸ demonstrated that as the ionic strength, I , of the eluent increased from 0 to 0.075 M so did the partition coefficient (K_{av}) of D-galacturonic acid from 0.05 to 0.46, whereas that of D-galactose remained constant (0.63) under the same conditions. These results indicate that the elution behaviour of acidic substances is different from that of neutral substances in the gel phase, and also that, for the determination of the molecular weights of acidic substances by gel filtration chromatography, eluents with ionic strengths high enough to make K_{av} constant are required.

Recently, we found¹¹ that the purification of alginic acid (mol. wt. 33 000, by viscosity measurement) can be achieved not by gel filtration on a Sephadex G-100 column (mol. wt. 1000–100 000) but by gel filtration on a Sepharose 4B column (mol. wt. 30 000 – $5 \cdot 10^6$), because on the former column the polysaccharide was eluted at the void volume. Thus, purification of alginic acid has been achieved, but its molecular weight remains to be determined reliably by gel filtration. It is considered that if the molecular weight of alginic acid can be obtained reliably by chromatography, the relationships between its physical and chemical properties and biological activities and molecular weight will be more easily and rapidly investigated.

So, in an attempt to determine the molecular weight of alginic acid reliably, we first examined the effect of the ionic strength of the eluents employed for the chromatography. As a result, although reproducible molecular weights of alginic acid were

obtained, the values were higher than those obtained by physicochemical methods, which gave almost the same values. So, we examined the relationship between molecular weights obtained by gel filtration chromatography and by viscosity measurements of several purified alginic acids. As a result, we found a linear relationship between the molecular weights obtained by both procedures.

In the present paper, we describe the effect of the ionic strength of eluents employed for gel filtration chromatography, and a relationship between molecular weights obtained by chromatography and by viscosity measurements of purified alginic acids. By use of this relationship, the molecular weight of alginic acid will be more easily obtained from its K_{av} or elution volume in gel filtration chromatography.

EXPERIMENTAL

Alginate sample a, prepared from *Lessonia nigrescens*, was kindly donated by Kimitsu Chemical Industrial Co. (Tokyo, Japan). Samples b and c were prepared as follows: an aqueous solution (0.5%, w/v) of sample a was heated at 100°C for 4 h (sample b) or 8 h (sample c) in an oil-bath, and the by-products produced were then removed by extraction from lyophilized preparation with 70% aqueous ethanol. Alginate sample d was prepared from the fronds of *Sargassum fulvellum* as described previously¹¹. Samples e and f were prepared as follows: 300 mg of sample d were partially hydrolyzed with 100 ml of 0.3 M hydrochloric acid at 35°C (sample e) or 40°C (sample f) for 1 h. A half-volume of 95% aqueous ethanol was added to each aqueous solution of the hydrolysates obtained (1.4%, w/v) and the precipitates formed were used in the present investigation. These alginate samples were shown to be homogeneous by electrophoresis, performed on a cellulose acetate paper, Separax (11 cm × 6 cm, Fuji Film) by the following systems: (1) 0.1 M pyridine-acetic acid (pH 3.5), 200 V, 15 min; (2) 0.1 M hydrochloric acid, 16.5 V, 2.5 h and (3) 0.1 M zinc acetate (pH 6.6), 200 V, 1 h.

Preliminary experiments were carried out for alginate sample d on a Sepharose CL-4B column (96.4 cm × 1.2 cm) with pure water, 0.01, 0.05, 0.1, 0.2 and 0.5 M sodium chloride as the eluents. On the basis of these experiments, determination of the molecular weights of the above samples was performed by gel filtration chromatography on a Sepharose 2B column (95.5 cm × 1.2 cm) for sample a on a Sepharose CL-4B column (99.7 cm × 1.2 cm) for samples b, c and d and on a Sepharose CL-6B column (97.6 cm × 1.2 cm) for samples e and f with 0.2 M sodium chloride as an eluent. Alginate was dissolved in 0.2 M sodium chloride (1–2 mg/ml) and the solution applied to each column. The standard pullulan samples used for the determination were as follows: P-20, P-50, P-100, P-200, P-400 and P-800, having molecular weights of 20 800, 46 700, 95 400, 194 000, 338 000 and 758 000. The molecular weights of the alginate samples were estimated from the K_{av} vs. log(mol. wt.) curve obtained by using the pullulans; K_{av} was calculated from the elution volume, V_e , by using the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$, where K_{av} , V_0 and V_t are the partition coefficient, the void volume and total volume of the column, respectively¹². However, as suitably high-molecular-weight standard samples for the estimation of V_0 were not available, the molecular weight of sample a was estimated by extrapolating its elution volume to that corresponding to a molecular weight of more than 758 000 (mol. wt. of P-800) using the V_e vs. log(mol. wt.) curve for the pullulans. Viscosity measurements on the

alginate samples (0.08–4.8 mg/ml) were performed in 0.1 *M* sodium chloride with an Ostwald type viscosimeter (flow time: 159 s for water) at $25.00 \pm 0.02^\circ\text{C}$. The degree of polymerization (DP) of each sample was calculated from the intrinsic viscosity, which was obtained from the measurements, using the formula of Donnan and Rose³. The molecular weight was estimated from the DP.

RESULTS AND DISCUSSION

Preliminary experiments were carried out in order to find the ionic strength of the eluent suitable for chromatography of an alginate sample (sample d) on a Sepharose CL-4B column. When pure water was used as the eluent, the K_{av} value of sample d was shown to be 0.09. However, as the ionic strength of the eluent increased, so did K_{av} (0.09 to 0.45) to a constant (0.45) at > 0.05 *M* sodium chloride. Therefore, in order to avoid the effect of the ionic strength of the eluent on K_{av} , we employed 0.2 *M* sodium chloride for the present study.

The respective molecular weights, determined by gel filtration, of alginate samples a, b, c, d, e and f were $18.7 \cdot 10^5$, $7.8 \cdot 10^5$, $4.6 \cdot 10^5$, $2.9 \cdot 10^5$, $1.4 \cdot 10^5$ and $0.6 \cdot 10^5$. On

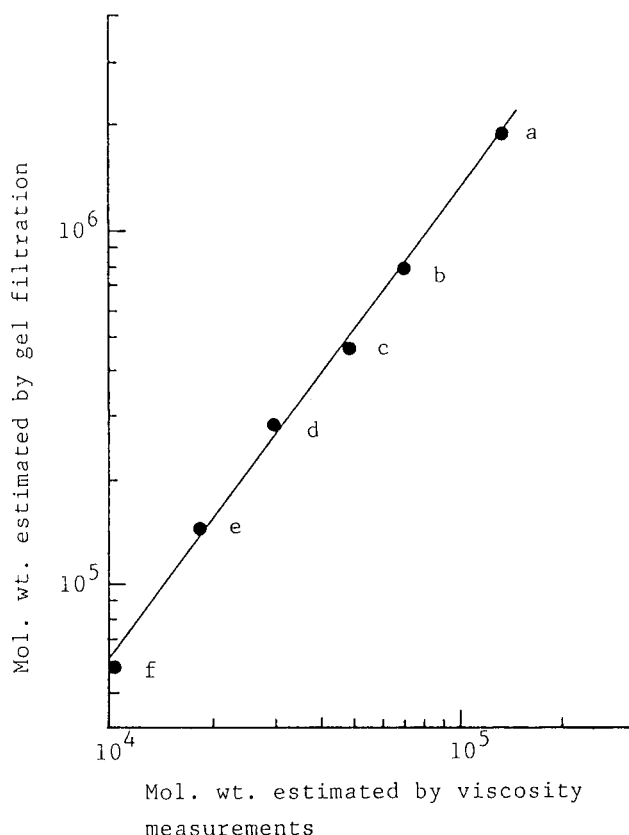


Fig. 1. Relationship between molecular weights of alginate determined by both gel filtration and viscosity measurements. For samples a–f, see text.

the other hand, the molecular weights determined by viscosity measurements were $13.4 \cdot 10^4$, $7.0 \cdot 10^4$, $4.9 \cdot 10^4$, $2.9 \cdot 10^4$, $1.9 \cdot 10^4$ and $1.0 \cdot 10^4$, respectively. These differences may be caused by the use of neutral polysaccharides as molecular weight standards in the chromatography. So we examined the relationship between the molecular weights obtained by the two methods. The results are shown in Fig. 1. The linear relationship found had a correlation coefficient of 0.998.

$$\log B = 1.31 \log A - 0.440$$

where A and B are the molecular weights estimated by viscosity measurements and by gel filtration, respectively. Donnan and Rose³ determined the intrinsic viscosity, $[\eta]$, and osmotic pressure of alginates in order to estimate their molecular weights and found that $[\eta]$ was proportional to the molecular weight determined by osmotic pressure. Other workers also reported the presence of a linear relationship between $[\eta]$ and the molecular weight of alginate obtained by sedimentation-viscosity measurement² or by light scattering¹. These results indicate that molecular weights determined by the viscosity measurements are reliable. However, viscosity measurements are somewhat troublesome to perform.

In the present study, it was shown that the relationship between molecular weights determined by gel filtration and those obtained by viscosity measurements is linear. Therefore, by using the above equation, reliable molecular weights of alginates can be obtained not only rapidly but also precisely. Polyuronic acids such as pectic acid and polygalacturonic acid may interact with agarose gel in a manner similar to that of alginate. Therefore, the molecular weights obtained by gel filtration may not be precise. If a calibration graph is obtained in the same way as described above, reliable molecular weights of acidic polysaccharides may be also determined by gel filtration chromatography.

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Note

Capillary gas chromatography–mass spectrometry of very-long-chain α,ω -dicarboxylic acid dimethyl esters from *Equisetum* (horsetail)

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The occurrence of α,ω -dicarboxylic acids in nature is much smaller than that of fatty acids. Moreover, among these dicarboxylic acids are those having hydrocarbon chain lengths greater than C_{22} as a minor component¹. There are several types of plant material in which α,ω -dicarboxylic acids represent an important component, e.g., in the bark of needle-leaved trees^{2,3} or leafy trees⁴ and fossil sediments like peat⁵ or shales⁶. Only the last two kinds of materials contain α,ω -dicarboxylic acids with chain lengths up to C_{28} and C_{32} , respectively.

It was concluded that the only available material containing very small amounts of long-chain α,ω -dicarboxylic acids is horsetail, a precursor of fossil sediments^{7,8}.

In our previous work⁹ it was mentioned that only Ryhage and Stenhagen^{10,11} had dealt systematically with the mass spectrometry of dimethyl esters of α,ω -dicarboxylic acids (DEDAs), however, only up to the C_{22} chain. In another paper⁸, solely the DEDA- C_{30} mass spectrum was shown. For this reason we have characterized other very-long-chain DEDAs up to C_{34} .

EXPERIMENTAL

Preparation of DEDAs

The cones from *Equisetum arvense* L. were collected about 30 km south of Prague in the first half of April 1988. Further isolation from spores was carried out according to the method of Adams and Bonnett⁸. The total gain was 47 mg of waxy substance, from which the methyl esters were prepared by treatment with diazomethane. DEDAs were separated by thin-layer chromatography (TLC) on silica gel G using the solvent system hexane–diethyl ether (7:3, v/v); the R_F was 0.63 and the total gain was 31.3 mg.

Gas chromatography–mass spectrometry (GC–MS) of DEDAs

The mixture of DEDAs was chromatographed on a non-polar capillary column

60 mm $\times$ 0.32 mm I.D.) SPB-1 (Supelco, Bellefonte, PA, U.S.A.) using splitless injection and helium as a carrier gas. The oven temperature was programmed from 100 to 300°C at 4° min⁻¹. Mass detection was carried out by a coupled mass spectrometer, QP 1000 (Shimadzu, Kyoto, Japan) which uses a glass separator as a connection between the chromatograph and mass spectrometer. The ionization energy was 70 eV and the electron multiplier voltage was 2.5 kV.

RESULTS AND DISCUSSION

Capillary GC

Fig. 1. shows the baseline separation of the homologous saturated DEDAs on a non-polar capillary column. Previously we have demonstrated⁹ a certain discrimination of higher homologues, *i.e.* of DEDAs with chain lengths higher than C₂₀. To avoid such losses, splitless injection was used, which helps decrease the discrimination of higher boiling components. By this method, even DEDA-C₃₄ could be detected.

The effective chain length (ECL) of DEDAs between C₂₀ and C₃₄ is about 3.8 units higher than that of the methyl esters of the respective fatty acids (FAMES), see Fig. 2. The data for the lower-chain-length DEDAs have been extracted from our previous measurements⁹. Fig. 2 shows an increasing Δ (ECL) for higher mass molecules which causes a gradual deterioration in separation quality between DEDAs and FAMES.

The distribution of DEDA homologous is shown in Fig. 1; in horsetail, α,ω -dicarboxylic acids were found ranging between C₁₀ and C₃₄. Adams *et al.*^{7,8}, however, reported that more than 90% of the total dicarboxylic acids was DEDA-C₃₀. Dicarboxylic acids with hydrocarbon chains longer than C₃₀ were not found; among the

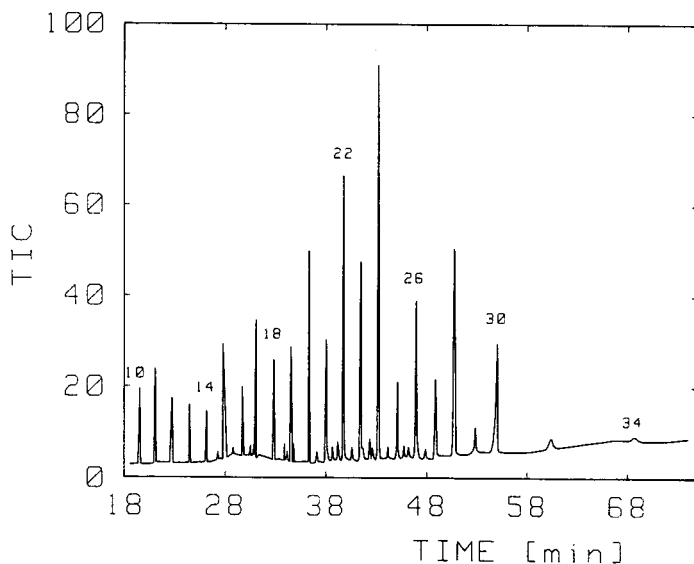


Fig. 1. Capillary GC-MS of DEDAs (the numbers at the peaks indicate the carbon chain lengths) from *Equisetum arvense* L. on a SPB-1 column (Supelco), 60 mm $\times$ 0.32 mm I.D. splitless injection, oven programmed from 100 to 300°C at 4° min⁻¹. TIC = total ion current.

TABLE I
ELECTRON IMPACT MASS SPECTRA OF DEDAs OBTAINED BY MEANS OF A QUADRUPOLE MASS SPECTROMETER

Acid	Mol.wt.	M-31	M-32	M-63	M-64	M-73	M-91	M-92	M-105	M-106	M-123	111	98	97	87	84	83	74	73	69	59	57	56	55	
C ₂₁	384	14.3	3.7	2.8	5.2	6.8	0.9	1.0	9.5	7.1	3.8	31.5	10.9	100	20.8	14.7	45.7	10.7	29.4	5.8	10.5	14.7	20.1	7.7	54.6
C ₂₂	398	10.9	4.3	3.7	4.1	7.0	1.8	2.5	7.6	4.9	3.6	32.3	14.3	100	27.6	12.1	42.6	27.2	37.0	5.4	35.7	18.3	23.9	8.7	63.9
C ₂₃	412	10.1	3.9	6.7	11.7	5.2	1.6	3.8	6.5	5.3	4.1	29.1	18.6	100	36.6	13.8	50.7	39.8	29.3	6.5	44.9	18.5	17.2	13.8	64.9
C ₂₄	426	7.3	3.7	2.5	2.9	4.8	0.7	1.2	5.5	4.7	2.4	28.3	14.3	100	27.3	5.5	41.8	26.3	26.3	5.5	36.0	16.7	21.3	8.4	63.3
C ₂₅	440	1.7	0.9	6.2	12.9	0.5	0.8	1.5	9.5	7.9	1.5	29.1	5.6	100	12.1	8.4	32.3	25.8	25.1	4.8	29.9	12.7	17.4	7.3	51.6
C ₂₆	454	9.7	4.0	3.6	5.2	5.2	3.5	3.1	6.9	4.2	3.4	25.7	11.1	100	21.6	4.5	38.6	21.4	27.3	4.2	27.3	11.6	7.5	5.5	45.8
C ₂₇	468	7.9	4.4	6.4	13.4	3.9	1.6	1.7	5.5	4.7	2.5	21.3	24.2	100	44.6	4.9	29.7	49.1	22.7	4.8	60.9	16.7	7.6	11.4	19.2
C ₂₈	482	10.4	8.9	4.8	8.1	4.1	1.1	2.9	8.2	6.0	2.4	26.6	7.3	100	10.6	7.0	37.3	23.8	36.4	6.5	17.6	11.4	8.1	3.5	12.2
C ₂₉	496	8.1	9.1	2.2	4.3	1.1	0.8	3.7	9.8	6.1	1.1	29.3	8.4	100	24.2	3.8	42.1	14.3	29.8	3.2	35.2	10.3	7.8	9.8	10.9
C ₃₀	510	13.4	9.5	2.4	3.8	0.8	2.1	3.0	9.7	11.5	0.5	28.0	8.9	100	14.0	6.7	35.3	15.8	31.3	5.0	9.0	7.2	6.9	7.2	14.1

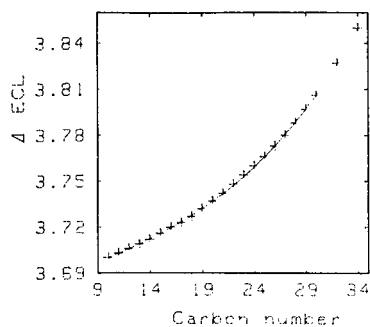


Fig. 2. The relationship between the carbon number of DEDAs and the difference in ECL between DEDA and FAME.

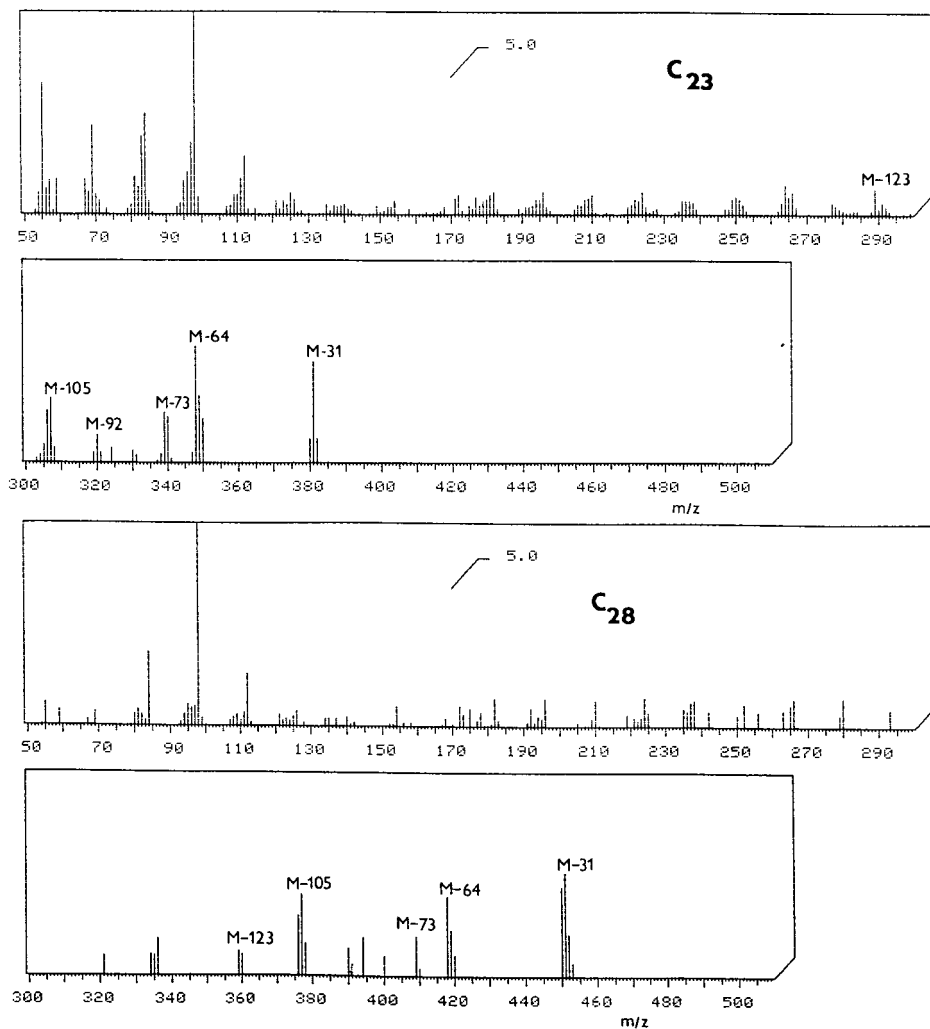


Fig. 3. Electron impact mass spectra of DEDA-C₂₃ (top) and -C₂₈ (bottom).

others the content of DEDA-C₂₈ lay between 1.7 and 5%, and the contents of C₂₂ and C₂₄ were at most 2%. Our results substantially differ from this distribution, which can be attributed to different varieties of horsetail.

Mass spectra of long-chain DEDAs

The most characteristic feature of the mass spectra of long chain DEDAs (distinguishing them unambiguously from short chain DEDAs up to C₁₀ as well as from the FAME analogues) are the homologues ions at m/z 84, 98 and 112 found in almost constant abundance ratio, approximately 4:10:3, the ion at m/z 98 being the base peak of the mass spectra for all DEDAs starting with C₁₁; the structure of these ions was discussed elsewhere^{9,11,12}.

In the absence of a molecular ion (ions of abundance lower than 0.5% of the base peak were not recorded) there are two important ions in the high mass region: $[M - 31]^+$ corresponding to the loss of one methoxy group similarly to FAMES and a rearrangement ion $[M - 64]^+$ formed by the loss of two molecules of methanol.

Mass spectra of rarely occurring (C₂₃ and C₂₈) long chain DEDAs are displayed as an example in Fig.3 and the most significant ions of all C₂₁-C₃₀ DEDAs are summarized in Table I. Some differences in ion abundance especially in the high mass region of C₂₁-C₂₄ DEDAs, if compared to our previous report⁹, are largely due to the different instrumentation (HP5995 and Shimadzu QP 1000) and experimental conditions employed.

CONCLUSIONS

Very-long-chain α,ω -dicarboxylic acids in the form of dimethyl esters can be successfully analyzed by capillary GC on a non-polar column (because high temperatures are necessary) in combination with mass spectrometric detection, which enables besides qualitative analysis also quantitative estimates of the long-chain DEDAs, based upon the characteristic ions (m/z 84, 98, 112, $M - 123$, $M - 146$) for this group of compounds. If the analyzed material contains FAMES along with DEDAs, there is hardly another analytical method available because of the similar chemical character of these two groups of compounds, especially for the C₂₀-C₃₄ DEDA region, where the effective chain length difference from the interfering FAME decreases substantially.

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Note

Ion chromatographic determination of cyanide compounds by chloramine-T and conductivity measurement

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Ion chromatography (IC) is a powerful technique for the separation and determination of anions and cations¹. However, cyanide ion cannot be directly detected by the conductivity detector due to its low dissociation constant ($pK=9.2$). Therefore, other means such as electrochemical detection (ED)^{2–6}, or derivatization to a conducting form^{7–10}, should be employed. Though ED methods are sensitive and selective for cyanide ion, the detectors are too expensive. Derivatization methods are time consuming and troublesome.

One of us previously reported an IC method for the determination of free cyanide and metal cyanide complexes using a conductivity detector¹¹, and applied it to plating solutions and waste-waters¹². It was based on the oxidation of cyanide ion by sodium hypochlorite to give cyanate ion (CNO^-)^{10,11}. Since the dissociation constant of hydrogen cyanate is fairly high ($pK=3.66$), cyanate ion can be measured by a conventional conductivity detector. However, a rather large chloride ion peak was observed, and the resin in the column was damaged during analysis.

Then, we attempted to replace sodium hypochlorite by a weaker oxidizing agent, chloramine-T, which is generally used in colorimetry^{13–15}. The resin in a separator column is less damaged and the chloride ion peak is smaller than with sodium hypochlorite. Since the present method does not need buffer solution, colour developing agent and long standing time, it is much simpler than the usual one^{13–15}.

Various parameters such as the amount of chloramine-T, temperature, standing time, and the stability of CNO^- formed were optimized to provide a quantitative conversion of CN^- into CNO^- . The calibration graph for CN^- , interferences from diverse anions and reducing agents and the applicability to the determination of metal cyanide complexes were also investigated. The method was applied successfully to the determination of cyanide compounds in waters and waste-waters.

EXPERIMENTAL

Apparatus and reagents

Chromatography was performed on a Dionex system 2010i ion chromatograph equipped with a conductivity detector. A separator column (HPIC-AS4A) with a guard column (HPIC-AG4) and a fibre suppressor (AFS-1) were used. The volume of the sample loop was 50 μ l. The reagents used, except for chloramine-T solution, were as in ref. 11. Chloramine-T solution (5 or 10% w/v) was prepared daily.

Procedure

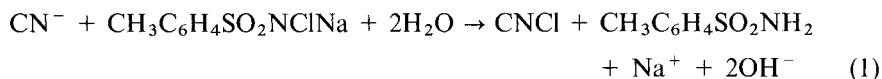
In a 50-ml volumetric flask were placed 5.0 ml of cyanide solution (10 mg/l, 0.01 *M* sodium hydroxide) and 4.5 ml of 0.1 *M* sodium hydroxide solution and the volume was made up to 50 ml with deionized water. A 50- μ l volume of chloramine-T solution was added and mixed thoroughly. The mixture was kept at 80°C for 10 min, then cooled in a water-bath (5 min). An aliquot of the solution was injected into the ion chromatograph.

For real sample analysis, an aliquot of sample solution and 0.1 *M* sodium hydroxide solution were mixed and diluted to 50 ml in deionized water (pH 12). Then, the procedure mentioned above was followed.

RESULTS AND DISCUSSION

Chromatogram of cyanate ion

The overall reactions leading to cyanate ion may be written as follows^{13,16,17}:



Cyanide ion reacts with chloramine-T to produce cyanogen chloride (CNCl) (reaction 1). When heated at alkaline pH, CNCl is hydrolyzed to CNO^- (reaction 2). Typical chromatograms of cyanate ion are shown in Fig. 1.

A small peak, corresponding to only 5.4% of CN^- , appeared when the reaction was performed at room temperature (25°C) for 10 min (A). However, when heating at 80°C is carried out for 10 min (B) the reaction proceeds quantitatively. The chromatogram of a blank is shown in Fig. 1 (C).

As shown in Fig. 1, a positive peak, called the "pseudopeak", appears first. Peaks of chloride and cyanate ions (A,B) follow. Their retention times are *ca.* 2.3 and 3.0 min, respectively. In this case, the peak of chloride ion was smaller than that produced when sodium hypochlorite was used¹¹. The size is dependent on the concentration of cyanide ion (reaction 2).

Optimum oxidizing condition

The volume of chloramine-T solution for the conversion of CN^- into CNO^- was optimized by using 5% chloramine-T solution for 1 mg/l cyanide solution and 10% solution for 10 mg/l cyanide solution. The results are shown in Fig. 2.

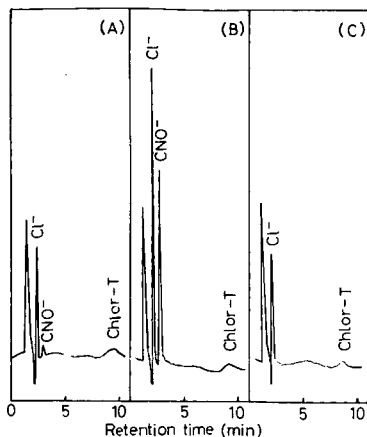


Fig. 1. Chromatogram of cyanate ion: (A) CN^- (1 mg/l, 50 ml) + chloramine-T (Chlor-T) (5%, 50 μl), 25°C, 10 min; (B) CN^- (1 mg/l, 50 ml) + chloramine-T (5%, 50 μl), 80°C, 10 min; (C) water (pH 12, 50 ml) + chloramine-T (5%, 50 μl), 80°C, 10 min. Column: HPIC-AS4A. Eluent: 2.2 mM Na_2CO_3 . Detector: conductivity; full scale, 3 μS .

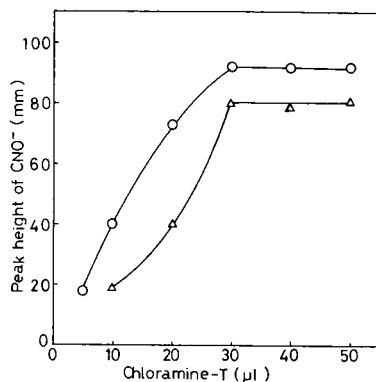


Fig. 2. Effect of the volume of chloramine-T solution added: ○ = CN^- (1 mg/l, 50 ml) + chloramine-T (5%, 10 – 50 μl); Δ = CN^- (10 mg/l, 50 ml) + chloramine-T (10%, 10 – 50 μl). Conditions: 80°C, 10 min stand; Column: HPIC-AS4A; eluent, 2.2 mM Na_2CO_3 ; detector, conductivity, full scale, 3 μS (○), 30 μS (Δ).

In both cases, when 30 – 50 μl of the solution were added the peak heights were almost constant. Therefore, the volume of chloramine-T solution was fixed at 50 μl .

The effect of temperature on the conversion of CN^- into CNO^- was examined. Results are shown in Fig. 3. The peak height of cyanate ion increases with temperature to a maximum at 70°C and was almost constant between 70 and 90°C.

The effect of reaction time was examined at 80°C (Fig. 4). No differences were observed for reaction times of 10 – 30 min. Cyanate ion formed at pH 12 was stable for 45 min. Therefore, 80°C and 10 min were chosen.

Calibration graphs

Two calibration graphs for cyanide ion concentration ranges of 0.05 – 1.0 and 1.0 – 10.0 mg/l, are shown in Fig. 5. Both plots are essentially linear, which indicates that the oxidation reaction is almost stoichiometric. The correlation coefficients were 0.9996 (for 0.05 – 1.0 mg/l) and 0.9992 (for 1.0 – 10.0 mg/l).

Interferences

The effects of diverse anions and reducing agents on the cyanide analysis were examined. The results are presented in Table I.

Common anions do not interfere. When the concentration of chloride ion is 50 times larger than that of cyanide ion the separation of cyanate ion from chloride ion is incomplete, which gives a negative error. The retention time of nitrite ion (2.7 min) almost coincides with that of cyanate ion (3.0 min), which gives a positive error. The latter can be avoided by decomposing nitrite ion with amidosulphuric acid¹¹.

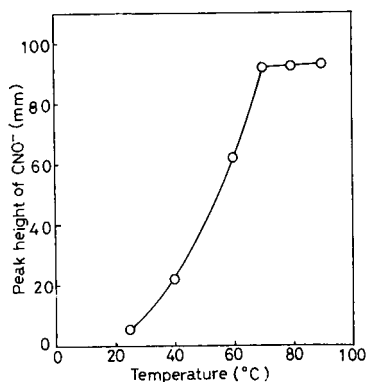


Fig. 3. Effect of temperature (25–90°C). Conditions: CN^- (1 mg/l, 50 ml) + chloramine-T (5%, 50 μl), 10 min; Column: HPIC-AS4A. Eluent: 2.2 mM Na_2CO_3 . Detector: conductivity: full scale, 3 μS .

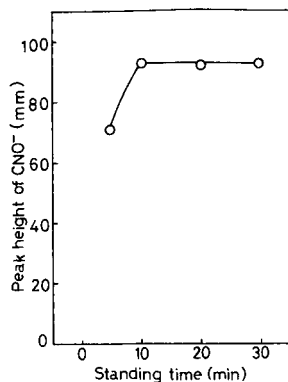


Fig. 4. Effects of reaction time (5–30 min). Conditions: CN^- (1 mg/l, 50 ml) + chloramine-T (5%, 50 μl), 80°C; Column: HPIC-AS4A. Eluent: 2.2 mM Na_2CO_3 . Detector: conductivity: full scale, 3 μS .

Large amounts (10 mg/l) of reducing agents such as sulphide, thiosulphate and ascorbic acid cause serious interferences. However, at less than 1 mg/l, interference only from sulphide ion is considerable. Sulphite, hypophosphite, phosphite and arsenite ions cause no interference, even when the amount is 10 times larger than that of cyanide ion.

Thiocyanate ion gives cyanate ion upon reaction with chloramine-T¹⁷ and heating. Therefore, even a small amount of this ion causes serious interference.

Analysis of metal cyanide complexes

In order to study the applicability to metal cyanides, solutions of zinc, cad-

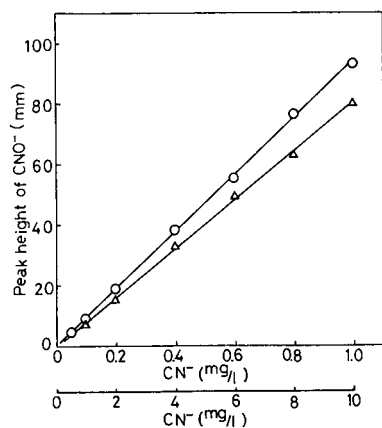


Fig. 5. Calibration graphs: ○ = CN^- (0.05–1 mg/l, 50 ml) + chloramine-T (5%, 50 μl); △ = CN^- (1–10 mg/l, 50 ml) + chloramine-T (10%, 50 μl). Conditions: 80°C, 10 min stand; Column: HPIC-AS4A. Eluent: 2.2 mM Na_2CO_3 ; detector, conductivity, full scale, 3 μS (○), 30 μS (△).

TABLE I

INTERFERENCES FROM ANIONS AND REDUCING AGENTS

Volumes of cyanide solutions: 1 mg/l, pH 12, 50 ml, full scale, 3 μ S; 10 mg/l, pH 12, 50 ml, full scale, 30 μ S. Volume of chloramine-T (5 or 10%) solution: 50 μ l. Conditions: 80°C, 10 min stand; column, HPIC-AS4A; eluent, 2.2 mM Na₂CO₃; detector, conductivity.

Anion or reducing agent	Retention time (min)	ppm ratio (CN ⁻ /A ⁿ⁻)	Recovery (%)
F ⁻	1.6	1/10	101.0
Cl ⁻	2.3	1/10	100.0
Cl ⁻	2.3	1/50	92.6
Cl ⁻	2.3	10/100	102.0
Cl ⁻	2.3	10/500	82.3
NO ₂ ⁻	2.7	1/1	101.0 ^a
NO ₂ ⁻	2.7	1/10	98.0 ^a
NO ₂ ⁻	2.7	10/10	101.0 ^a
HPO ₄ ²⁻	7.6	1/10	100.0
Br ⁻	3.7	1/10	96.9
NO ₃ ⁻	4.3	1/10	101.0
SO ₄ ²⁻	7.8	1/10	100.0
S ²⁻		1/1	64.5
S ²⁻		1/10	0.0
SO ₃ ²⁻	7.7	1/1	101.0
SO ₃ ²⁻	7.7	1/10	97.8
S ₂ O ₃ ²⁻		1/1	100.0
S ₂ O ₃ ²⁻		1/10	10.8
PO ₂ ³⁻		1/1	102.0
PO ₂ ³⁻		1/10	102.0
PO ₃ ³⁻	5.3	1/1	101.0
PO ₃ ³⁻	5.3	1/10	101.0
C ₆ H ₈ O ₆		1/1	101.0
C ₆ H ₈ O ₆		1/10	89.2
AsO ₂ ⁻		1/1	101.0
AsO ₂ ⁻		1/10	100.0
SCN ⁻		0/1	34.3
SCN ⁻		1/1	130.2

^a Measured after the decomposition of NO₂⁻ (ref. 11).

mium, copper, silver, nickel, iron, gold and cobalt cyanides were examined. The results are presented in Table II together with those obtained by using 1% sodium hypochlorite solution.

Weak cyanide complexes (Category 1) such as Zn(CN)₄²⁻, Cd(CN)₄²⁻ and Cu(CN)₄²⁻ can be quantitatively determined as CNO⁻ after oxidation with chloramine-T.

Cyanate ion peaks were detected from moderately strong cyanide complexes (Category 2) such as Ag(CN)₂⁻ and Ni(CN)₄²⁻. The peak height of CNO⁻ from Ag(CN)₂⁻ is about 38% of that with sodium hypochlorite.

Strong cyanide complexes (Category 3) such as Fe(CN)₆⁴⁻, Fe(CN)₆³⁻, Au(CN)₂⁻, Au(CN)₄⁻ and Co(CN)₆³⁻ are stable. Therefore, almost no CNO⁻ peaks were detected even after heating at 80°C, except for Au(CN)₄⁻. The reason why the

TABLE II

OXIDATION YIELDS OF METAL CYANIDE COMPLEXES WITH CHLORAMINE-T AND SODIUM HYPOCHLORITE

Volumes: cyanide ion (ca. 1 mg/l, pH 12) 50 ml; chloramine-T (5%) solution 50 μ l; NaClO (1%) solution 50 μ l. Standing time: 80°C, 10 min. Column: HPIC-AS4A, AFS. Eluent: 2.2 mM Na₂CO₃. Detector: conductivity: full scale; 3 μ S.

Species	Formation Constant	Chloramine-T(5%)	NaClO(1%)
Zn(CN) ₄ ²⁻	16.7	88.0 ^a	86.5 ^a
Cd(CN) ₄ ²⁻	18.8	98.0	96.0
Cu(CN) ₄ ³⁻	30.3	91.0	94.0
Ag(CN) ₂ ⁻	21.2	36.0	95.0
Ni(CN) ₄ ²⁻	31.3	106.0	106.0
Fe(CN) ₆ ⁴⁻	35	1.0	13.0
Fe(CN) ₆ ³⁻	42	0.0	0.0
Au(CN) ₂ ⁻	38.3	0.0	0.0
Au(CN) ₄ ⁻	—	21.0	20.0
Co(CN) ₆ ³⁻	64	0.0	0.0

^a Peak height of CNO⁻ formed (mm).

CNO⁻ peak was detected from Au(CN)₄⁻ is considered to be that a part of Au(CN)₄⁻ was decomposed to Au(CN)₂⁻ and CN⁻. Therefore, it was oxidized to CNO⁻ by chloramine-T at 80°C.

Real sample analysis

The proposed method was applied to the determination of cyanide ion in drinking water, river-water and plating waste-waters. As no detectable amount of cyanide

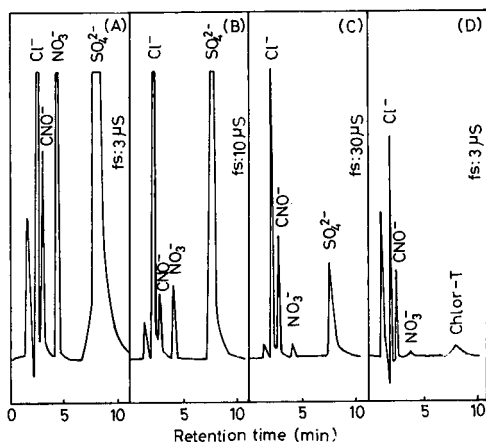


Fig. 6. Determination of cyanide in real samples: (A)=Drinking water spiked with 1.0 mg/l cyanide ion; (B)=River-water spiked with 1.0 mg/l cyanide ion; (C)=Plating waste-water before treatment; (D)=Plating waste-water after treatment, distillation was performed. Conditions: 80°C, 10 min stand; column: HPIC-AS4A; eluent: 2.2 mM Na₂CO₃; detector: conductivity. fs = Full scale.

TABLE III
DETERMINATION OF CYANIDE IN REAL SAMPLES

Sample	CN ⁻ (mg/l) mean ^a	S.D.	C.V. (%)
Drinking water ^b	0.98	0.008	0.8
River-water ^b	0.95	0.022	2.3
Plating waste-water ^c	173.0	1.24	0.7
Plating waste-water ^d	0.46	0.004	0.9

^a Five samples at each concentration. Column: HPIC-AS4A. Eluent: 2.2 mM Na₂CO₃. Detector: conductivity. Chloramine-T, 50 µl; 80°C, 10 min stand.

^b Spiked with 1.0 mg/l cyanide ion.

^c Before treatment of cyanide by alkaline chlorination.

^d After treatment of cyanide by alkaline chlorination; distillation was performed (total cyanide).

ion was found, a small amount of cyanide ion (50 µg) was added to 50 ml of drinking water (A) and river-water (B), and the recoveries were checked. Plating waste-water (C) was diluted 25 times in deionized water. Waste-water (D) was distilled and this distillate was directly measured for its cyanide concentration. The results are presented in Table III and Fig. 6.

The recoveries of cyanide ion from the spiked drinking water and river-water were 98 and 95%, respectively. The coefficients of variation (%) of samples A (1.0), B (1.0), C (173) and D (0.96 mg/l, CN⁻) were 0.8, 2.3, 0.7 and 0.9%, respectively.

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Note

Reversed-phase ion-pair liquid chromatographic separation of weak analgesics and related drugs

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Many freely available popular drugs contain one or several pain relievers such as paracetamol, acetylsalicylic acid, codeine, aminopyrine, phenacetin and salicylamide in combination with various other drugs, including hypnotics (carbromal, bromural), stimulants (caffeine), sedatives (barbiturates) and spasmolytics (tiemonium iodide, hyoscyamine). Several commercially available pharmaceuticals combine no less than six active ingredients. The analysis of such mixtures by high-performance liquid chromatography (HPLC) involves serious separation problems as basic, acidic and quasi-neutral compounds with widely different lipophilic properties are involved (Table I). In addition, different UV absorption characteristics and widely varying

TABLE I
COMPOUNDS STUDIED AND pK_a VALUES

pK_a values taken from refs. 10 and 11.

Compound	Abbreviation	pK_a
Acetylsalicylic acid	ASA	3.5
Paracetamol	PAR	9.5
Codeine phosphate	COD	8.2
Caffeine	CAF	1
Carbromal	CAR	—
Bromural	BRO	10.8
Aminopyrine	AMI	5.0
Phenacetin	PHE	2.2
Salicylamide	SAA	8.2
Phenobarbital	PHO	7.4
Hyoscyamine	HYO	9.7
Dipyrene	DIP	—
Salicylic acid	SAL	3.0
Tiemonium iodide	TIE	—

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concentrations of the ingredients in multi-component pharmaceuticals further complicate the analysis.

To our knowledge, no method capable of separating all of the commonly used drugs in commercially available weak analgesic multi-component pharmaceuticals has been reported, although the HPLC analysis of weak analgesics is well documented¹⁻⁵. However, these papers cited deal with the separation of only a few drugs and none of the separation methods used was applicable to more complex analgesic formulations.

Das Gupta⁶ reported an excellent separation of a standard mixture of ASA, PAR, CAF, COD, PHE and SAA by reversed-phase (RP) HPLC. Unfortunately, similar commercial drug mixtures containing also CAR and BRO or spasmolytics of the quaternary ammonium type could not be separated unless excessive retention times and/or severe peak tailing were accepted. Cockaerts *et al.*⁷ achieved both gradient and isocratic separations of ASA, PAR, BRO, CAR, CAF and COD. The isocratic method gave a good separation of all active components of the pharmaceutical dosage form and even allowed the separation of potential impurities such as SAL, acetylcodeine and diacetyl-*p*-aminophenol. However, the use of a column-switching technique involving two analytical columns and the long analysis time of about 1 h make the method unsuitable for routine analysis.

This paper reports a method for the isocratic liquid chromatographic separation and determination of weak analgesics and related drugs in complex pharmaceutical mixtures, based on the simultaneous use of an anionic and a cationic modifier in the mobile phase^{8,9}.

EXPERIMENTAL

Chemicals and solvents

All reference drugs were of pharmacopoeial grade (Table I). Sodium chloride (UCB, Belgium) and 85% orthophosphoric acid (Merck, F.R.G.) were of analytical-reagent grade.

N,N-Dimethyloctylamine (DMOA) was obtained from Aldrich (Milwaukee, WI, U.S.A.), anhydrous sodium 1-octanesulphonate (SOS) from Janssen (Belgium) and analytical-reagent grade methanol from UCB. Water was purified by ion-exchange chromatography and subsequent distillation. All other chemicals used were of analytical-reagent grade.

HPLC equipment

Chromatography was performed on an SP 8000 liquid chromatograph (Spectra-Physics, Darmstadt, F.R.G.) equipped with a Model 770 variable-wavelength detector (Spectra-Physics) and a BD 8 single-channel recorder (Kipp & Zonen, Delft, The Netherlands).

Chromatographic conditions

The mobile phase was prepared by dissolving the required amounts of SOS and DMOA in *ca.* 990 ml of methanol-water in various proportions. The mixture was adjusted to pH 3.0 with orthophosphoric acid and diluted to exactly 1000 ml. Before chromatography, the mobile phase was filtered through a 5- μ m filter and degassed with helium.

The flow-rate, column temperature, detection wavelength and injection volume were 1 ml/min, 25°C, 220 nm and 10 μ l, respectively.

The following columns were used: RSil C₁₈, 5 μ m (150 $\times$ 4.1 mm I.D.) and RSil C₁₈, 10 μ m (250 $\times$ 4.6 mm I.D.) from Alltech-RSL (Eke, Belgium) and LiChrosorb RP-18, 5 μ m (150 $\times$ 4.6 mm I.D.), RP-18, 10 μ m (250 $\times$ 4.6 mm I.D.) and RP-8, 10 μ m (250 $\times$ 4.6 mm I.D.) from Chrompack (Middelburg, The Netherlands).

RESULTS AND DISCUSSION

Chromatography on RSil C₁₈

Considerable efforts were made to separate isocratically all active ingredients of Perdolan® dosage forms, as this pharmaceutical is among the most complex in a long series of similar commercial products. The tablets combine ASA, PAR, COD, CAF, BRO and CAR; PHO was also included in the test mixture as this compound is extensively administered in combination with the other drugs. SAL was considered to be the only potential degradation product as a previous study confirmed that acetyl-codeine and diacetyl-*p*-aminophenol were not formed in such mixtures under normal conditions of storage over a period of 5 years⁷.

The effect of mobile phase composition (*i.e.*, the volume fraction of water, ϕ , the DMOA and SOS concentrations and the pH) on the capacity factors and selectivities was investigated. The results are shown in Fig. 1. SAL, the main degradation

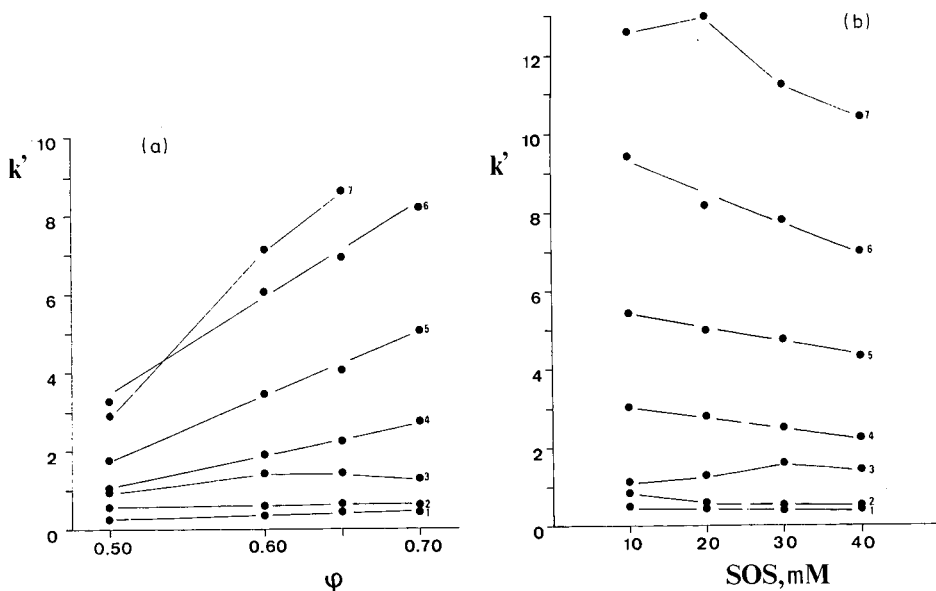


Fig. 1. Retention as a function of (a) the volume fraction of water (ϕ) and (b) the SOS concentration in the eluent. Mobile phase: (a) methanol-water containing 20 mmol/l of SOS and 5 mmol/l of DMOA (pH 3.0); (b) methanol-water (30:70) containing 5 mmol/l DMOA (pH 3.0). Column RSil C₁₈ (5 μ m) (150 $\times$ 4.1 mm I.D.); column temperature, 25°C; flow-rate, 1 ml/min; detection, 220 nm. Peaks: 1 = paracetamol; 2 = caffeine; 3 = codeine phosphate; 4 = acetylsalicylic acid; 5 = bromural; 6 = carbromal; 7 = salicylic acid; 8 = phenobarbital.

product of ASA, does not interfere with any of the active ingredients at sufficiently high water concentrations and under the conditions indicated in Fig. 1a, with $\phi = 0.60$, all compounds could be baseline resolved except CAF-PAR. As the selectivity for this peak pair decreases with increasing ϕ whereas ASA and COD coelute and SAL interferes with the elution of CAR at lower ϕ values, other eluent parameters were varied.

As expected, increasing the apparent pH of the eluent decreased the retention of the acids SAL and ASA. The retention of all other compounds was independent of pH changes, except COD, which showed an increased retention at pH 6. However, the selectivity changes that resulted from increases in pH were such that the separation of the seven compounds of interest became even more critical compared with that obtained at pH 3.0. Hence, further experiments were performed at pH 3.0.

The variation of retention with SOS concentration revealed two important selectivity changes (Fig. 1b): the resolution between ASA-COD and CAF-PAR in-

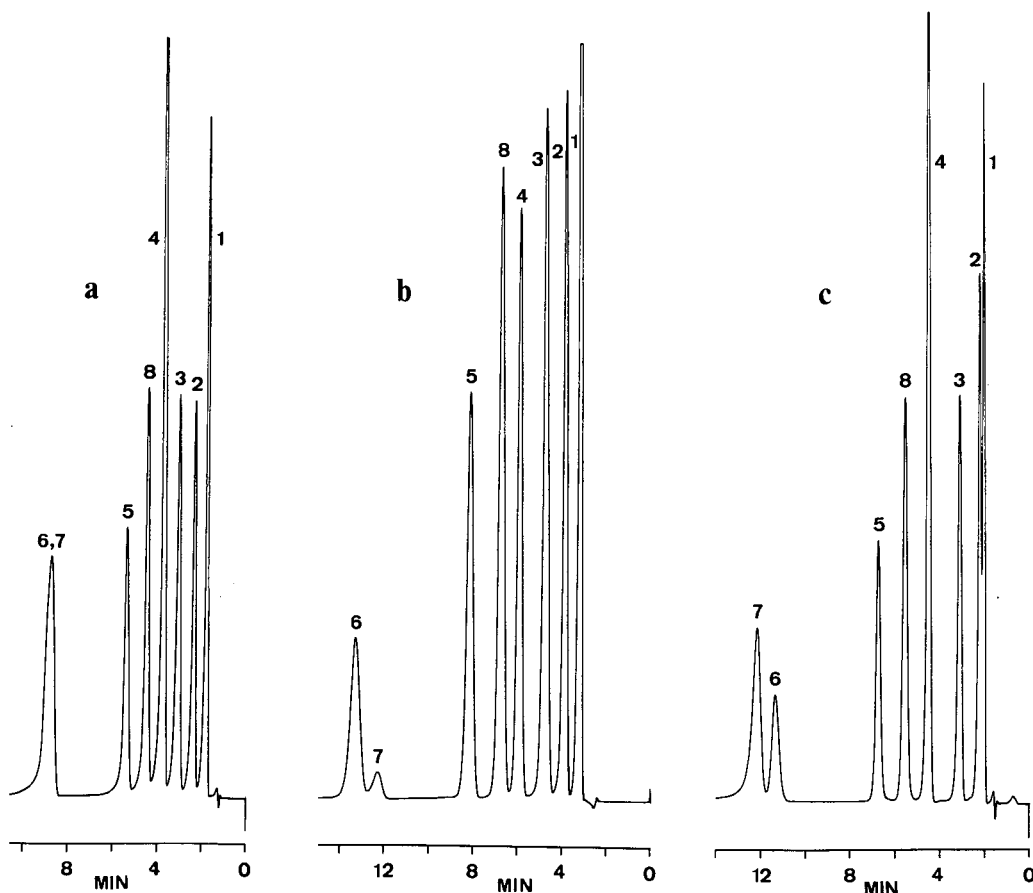


Fig. 2. Separation of a standard mixture of weak analgesic, hypnotic, sedative and stimulant drugs. Mobile phase: (a and c) methanol-water (40:60) containing 12.5 mmol/l of SOS and 5 mmol/l of DMOA (pH 3.0); (b) as (a) and (c), except $\phi = 0.50$. Column: (a) as in Fig. 1; (b) RSil C₁₈ (10 μ m) (250 $\times$ 4.6 mm I.D.), (c) LiChrosorb RP-18 (5 μ m) (150 $\times$ 4.6 mm I.D.). Chromatographic conditions and peaks as in Fig. 1.

creased with decreasing SOS concentration. In general, the retention decreased with increasing SOS content, except for COD, which was more strongly retained whereas the elution of PAR was not affected by the SOS content of the eluent.

The influence of DMOA concentration on retention was only briefly investigated as a concentration less than 2.5 mmol/l could not be used in order to avoid unacceptable peak tailing for COD and concentrations higher than 5 mmol/l reduced the resolution between CAF, COD and PAR. It should be stressed, however, that increasing the DMOA concentration can introduce important selectivity changes by increasing the retention of the acidic compounds and decreasing that of the basic compounds.

Attempts to use the described chromatographic conditions were successful in resolving all active components of the mixture but under such conditions CAR coelutes with SAL (Fig. 2a). No better results could be obtained using various combinations and concentrations of mobile phase modifiers.

From previous results it could be deduced that the maximum overall selectivity could be expected at a relative low volume fraction of water, ϕ , and a low SOS concentration in the eluent. Such chromatographic conditions, however, could not be used on a short column (150 $\times$ 4.1 mm I.D.) as the retention became so low that PAR eluted near the dead volume of the column and insufficient separation was obtained for COD-CAF and ASA-COD. It was therefore decided to use a column with appropriate dimensions, containing the same stationary phase material, but with 10- μ m instead of 5- μ m particle diameter. Fig. 2b shows the resulting chromatogram of a separation of the analgesic mixture using an appropriate eluent composition. It is apparent that the baseline separation obtained and the sufficient separation of PAR from the dead volume of the reversed-phase column will permit the simultaneous determination of these drugs in pharmaceuticals.

Chromatography on other RP stationary phases

Commercially available reversed phases can differ considerably in their carbon load and degree of derivatization and the contribution to retention of residual polar groups may significantly affect the resolving power of a chromatographic system^{12,13}. To evaluate such effects, LiChrosorb RP material was selected, as it is a non-end-capped RP silica and increased polar interactions can be expected in comparison with RSil RP silica, which is end-capped. Elution was performed on LiChrosorb RP-18 (5 and 10 μ m) and on LiChrosorb RP-8 (10 μ m) stationary phases, using the chromatographic conditions in Fig. 2a. The elution sequence of the solutes investigated was the same on all of the supports tested. Chromatograms obtained on LiChrosorb RP-18 and RP-8 (both 10 μ m) columns (250 $\times$ 4.6 mm I.D.) were very similar with respect to both the retention capacity and selectivity: SAL was not resolved from CAR in either instance and CAF-PAR were incompletely separated on RP-8, unlike on RP-18. Decreasing ϕ to 0.5 as in Fig. 2b results in insufficient resolution of the early eluting compounds in both instances.

The separation obtained on LiChrosorb RP-18 (5 μ m) is shown in Fig. 2c to facilitate comparison with that obtained on RSil (Fig. 2a). The overall increase in retention observed on LiChrosorb might be due to the different column packing material, showing a higher retention for the compounds studied. CAR and SAL were sufficiently resolved but CAF and PAR almost coeluted. These results suggest that

polar activities do not significantly affect the separation selectivity or at least the combined effects of DMOA and SOS can adequately eliminate such interactions, as (weakly) basic compounds are extremely sensitive to such interactions, mostly resulting in long elution times and tailing peaks. We conclude that the separation can be repeated on LiChrosorb RP-18 (5 μ m) using appropriate eluent compositions. No attempt was made, however, to optimize further the separation presented in Fig. 2c.

Applications

The liquid chromatographic behaviour of several other drugs frequently administered simultaneously with one (or several) of the drugs mentioned in Fig. 2. was studied. It is not possible to give exact chromatographic data about the separation of all these drugs, as the chromatographic conditions for a particular compound are partly determined by the accompanying drugs in the pharmaceutical preparation under investigation. However, it can be stated that any mixture containing a realistic

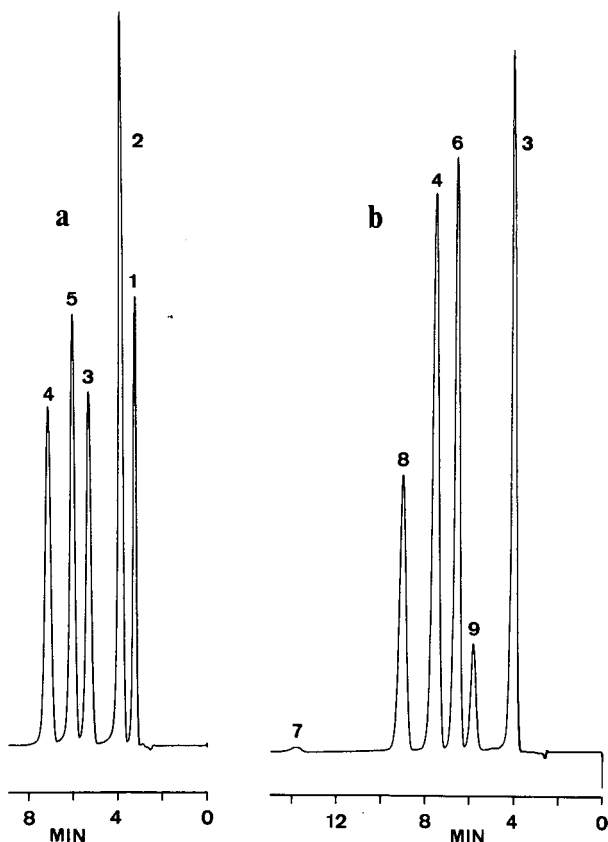


Fig. 3. Separation of all active drugs in (a) Gentarol® and (b) Salydent®. Mobile phase: (a) methanol-water (50:50) containing 15 mmol/l of SOS and 5 mmol/l of DMOA (pH 3.0); (b) as in (a) but containing 5 mmol/l of SOS and 5 mmol/l of DMOA. Column and chromatographic conditions as in Fig. 2b. Peaks: 1 = PAR; 2 = CAF; 3 = COD; 4 = PHO; 5 = SAA; 6 = ASA; 7 = SAL; 8 = PHE; 9 = HYO.

number of the drugs discussed above and allobarbitol, butalbital, phenazone, aminophenazone, phenacetin, hyoscyamine, salicylamide and quinine can be separated such that simultaneous determination becomes possible. This statement includes about twenty commercially available pharmaceuticals, *e.g.*, Perdolan®, Veganine®, Codis®, Dolviran® and Neuridon®. An illustration of the separation possibilities with the proposed approach for two commercially available pharmaceuticals is given in Fig. 3.

Special attention was also paid to the analysis of pharmaceuticals containing quaternary ammonium drugs (QADs) and weakly basic analgesics. The separation of such multi-component pharmaceuticals requires a sufficiently high water content ($\phi > 0.5$) to avoid coelution and/or elution in the dead volume of the early eluting drugs, and a low SOS content (2.5 mmol/l) and a relatively high DMOA concentration (5 mmol/l) to ensure fast elution of the QADs. As under these conditions COD and CAF are not completely resolved at pH 3.0, the pH was adjusted to 5.7 to provide

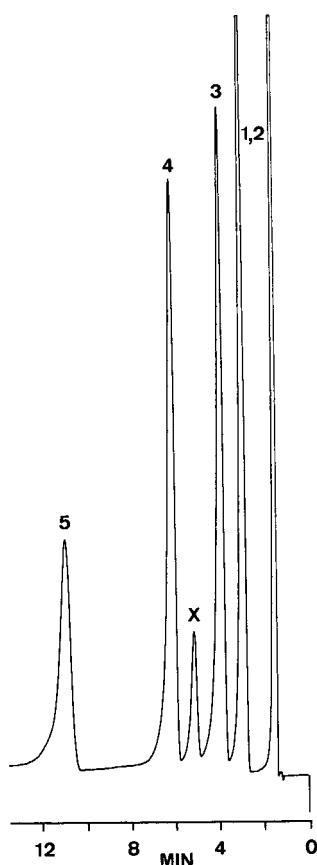


Fig. 4. Separation of a standard mixture of quaternary ammonium and weak analgesic drugs. Mobile phase: methanol-water (35:65), containing 2.5 mmol/l of SOS and 5 mmol/l of DMOA (pH 5.7). Chromatographic conditions and column as in Fig. 1. Peaks: 1 = dipyrone; 2 = caffeine; 3 = codeine phosphate; 4 = aminopyrine; 5 = tiemonium iodide; X = unidentified impurity of DIP. CAF, COD, AMI, TIE and DIP, COD, TIE are the active ingredients of Regisan® and Visceralgine® Compositum, respectively.

adequate selectivity. The resulting separation will allow the simultaneous determination of all components in the pharmaceuticals studied (Fig. 4). The coelution of DIP and CAF was not of practical importance because to our knowledge no pharmaceuticals containing DIP, CAF and QADs are marketed. All drugs shown in Fig. 4 can be eluted as pure compounds using 2.5 mmol/l DMOA in the eluent but the analysis time then increases to about 20 min.

CONCLUSIONS

The simultaneous addition of SOS and DMOA to RP liquid chromatographic systems can provide adequate resolution of mixtures containing basic, acidic and neutral drugs. About twenty commercially available pharmaceuticals, each containing several weak analgesics and related drugs, including hypnotics, sedatives, spasmolytics and barbiturates, were successfully analysed by this method.

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Note

Automated on-line trace enrichment and determination of organic compounds in ground and tap water by high-performance liquid chromatography

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There is great need for analytical methods suitable for determining concentrations in water of less than $0.1 \mu\text{g/l}$ of trace constituents, which is the EEC maximum allowed concentration of pesticides¹.

Sample loading for high-performance liquid chromatographic (HPLC) trace enrichment is effected on-line by changing the bottle containing the sample by the operator^{2–5} or by filling an adequately dimensioned loop with a syringe⁶, or off-line after preconcentration of the sample to a few millilitres and subsequent injection with a commercial liquid sampler.

An automated system to feed the enrichment column with the necessary large sample volume is described. The instrument setup allows the selection of the sample volume to be concentrated and the unattended processing of up to 32 water samples.

EXPERIMENTAL

Materials

All six port valves are pneumatically activated Valco type AC6W (Valco, Houston, TX, U.S.A.). The 16-port sampling valves are also pneumatically activated models (Valco type ASD16P), one of which is equipped with a binary encoder to indicate the position of the valve, corresponding to the sample number. The second valve is connected in parallel. The data system decodes the sample number from the injection sequence and activates the selection valve to direct the sample on to the concentration column. A magnetic micro gear pump (P1 in Fig. 1) is used to flush and fill the connecting tubes (Micropump Series Paragon P-11-361-500, 0–24 V d.c., flow range 0–6 ml/min; Micropump, Concord, CA, U.S.A.). The enrichment pump P2 is a constant-flow pump (Model LC 410; Kontron, Zürich, Switzerland) with remote flow-rate control. The eluent pump (P3) of the separation column is a constant-flow pump (Model A 300 CS; Gynkotec, Munich, F.R.G.). The timed switching events are delivered by the laboratory data system (Model 3357 LAS; Hewlett-Packard, Avondale, PA, U.S.A.)⁷ It is equipped with electronic control modules (ECM Model 18653 B) and analogue-to-digital converters (A/D Model 18652 A) for data acquisition. The ECM provides a binary input of the sampling valve position and seven 110-V a.c. power outputs used to control the solenoid valves. These outputs are switched on and off independently by the system software (the chromatographic integration software)

or by post-analytical user programs initiated by the chromatographic integration system software.

The binary encoder of the sampling valve (V-1) allows random access of the samples and is used to access the bottle of the calibration standards several times within an analytical sequence by the laboratory data system.

A programmable sequence timer with eight times exits of 110 V a.c. (Alpha Electronics, Stutensee, F.R.G.) can also be used to control the switching times without restriction of the overall chromatographic behaviour of the system if no adequate data system is available. Each solenoid valve may be electrically connected in parallel with 110 V a.c. relays to control external devices or functions. The programmable electronic timer restricts the use of the system to only 16 samples without random sample access.

A UV detector Model LC 786 (Kratos, Westwood, NJ, U.S.A.) was used.

Chromatographic conditions

The chromatographic columns (stainless steel, 4–12 cm $\times$ 4.6 mm I.D.) are packed with Nucleosil 100 C₁₈, particle size 5 or 10 μ m (Macherey, Nagel & Co., Düren, F.R.G.). The eluents are mixtures of acetonitrile (E. Merck, Darmstadt, F.R.G.) and water containing 15–50% of the modifier at a flow-rate of 1 ml/min. The detector wavelength is set at 220 μ m.

Procedure

The exits of the 16-port sampling valves (V-1A and V-1B) are connected through valve V-4 to the enrichment valve (V-2) (Fig. 1a and b).

The micro gear pump (P1-E) at the exit of the enrichment valve (V-2) sucks the sample through the 16-port valve directly from the bottle to waste and flushes the connecting tubes (Fig. 1b). After rotating the enrichment valve (V-2) into the ON position (Fig. 1c), the sample stream is directed through a constant-flow low-dead-volume pump (P2) and the switching valve (V-3) on to the enrichment column (column 1). During the enrichment step the flow of the pump P2 is set to a higher rate which reduces the sampling and overall analysis time. After the enrichment step, the valve V-2 is rotated back into the OFF position and the enrichment column is rinsed for a preset time period with a rinsing solvent(s) (Fig. 1d). The polarity of the rinsing solvent is selected so as not to elute the analytes from column 1. After rotating the transfer valve V-3 (ON position) the flow (P3) of the separation column 2 is directed on to the enrichment column 1 for a short transfer period (Fig. 1e). The analytes are transferred on to column 2. Finally, the analytes are eluted and detected after rotating the transfer valve (V-3) back into the OFF position (Fig. 1f). Fig. 1 illustrates the arrangement in the indirect transfer technique⁸.

Standard preparation. Water completely free from organic substances in detectable amounts (Nanopure HPLC water purifier; Barnstead, subsidiary of Sybron, Newton, MA, U.S.A.) is used to prepare the standard solutions. Stock solutions of the compounds are first prepared at concentrations of 1 mg/ml in ethanol and thereafter diluted with water to the concentration range 0.05–1 ng/ml (0.05–1 ppb).

Sample analyses. Samples of ground and tap waters are analysed without any pretreatment. The sample containers are directly connected to the sampling valve by immersing 1/8-in. PTFE tubing. An immersible filter is connected to the PTFE tube if the samples are contaminated with particulates.

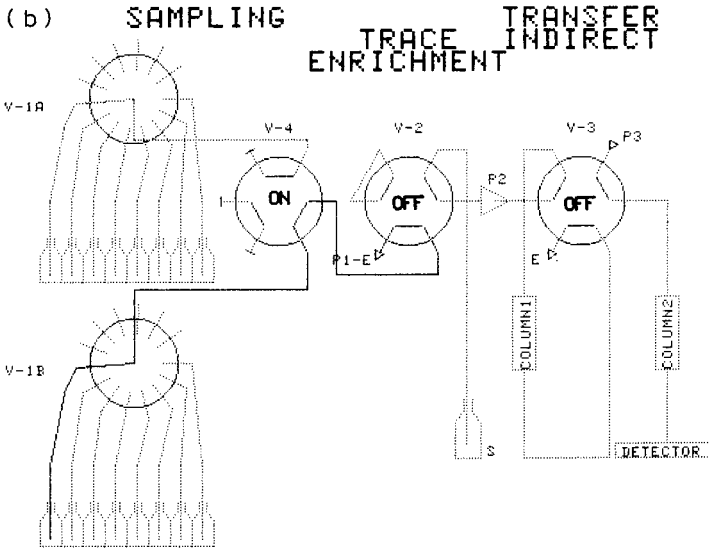
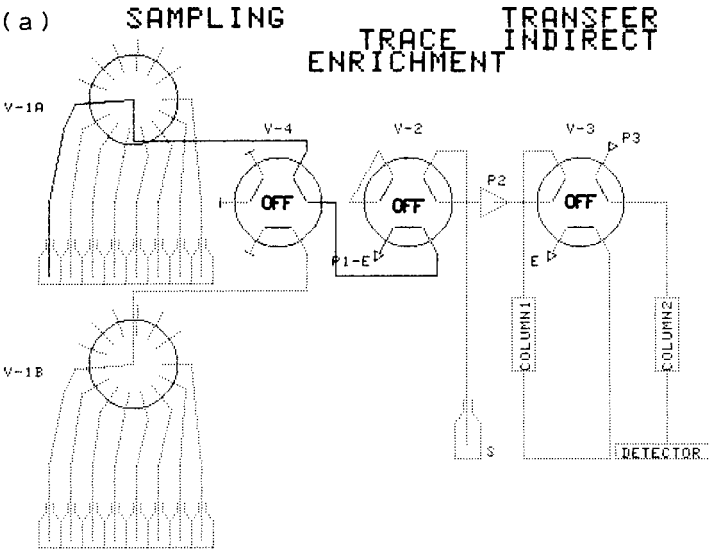


Fig. 1.

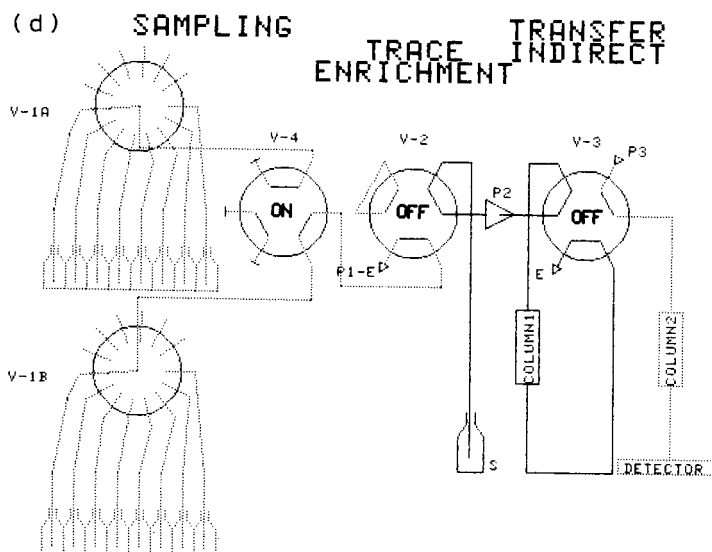
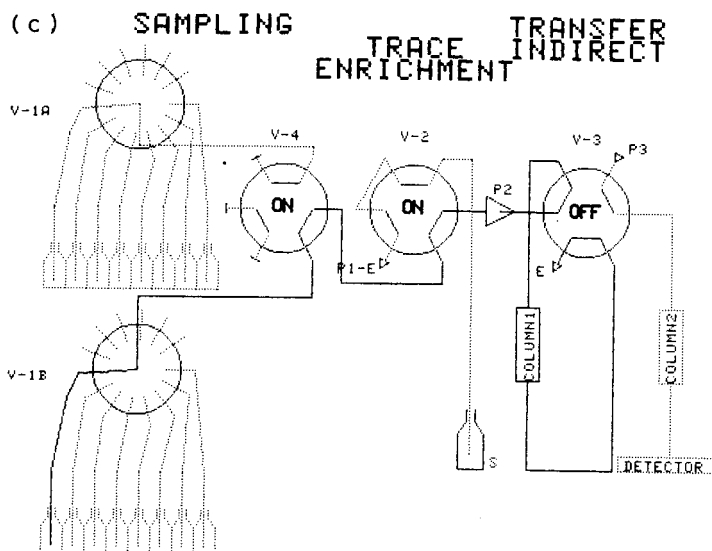


Fig. 1.

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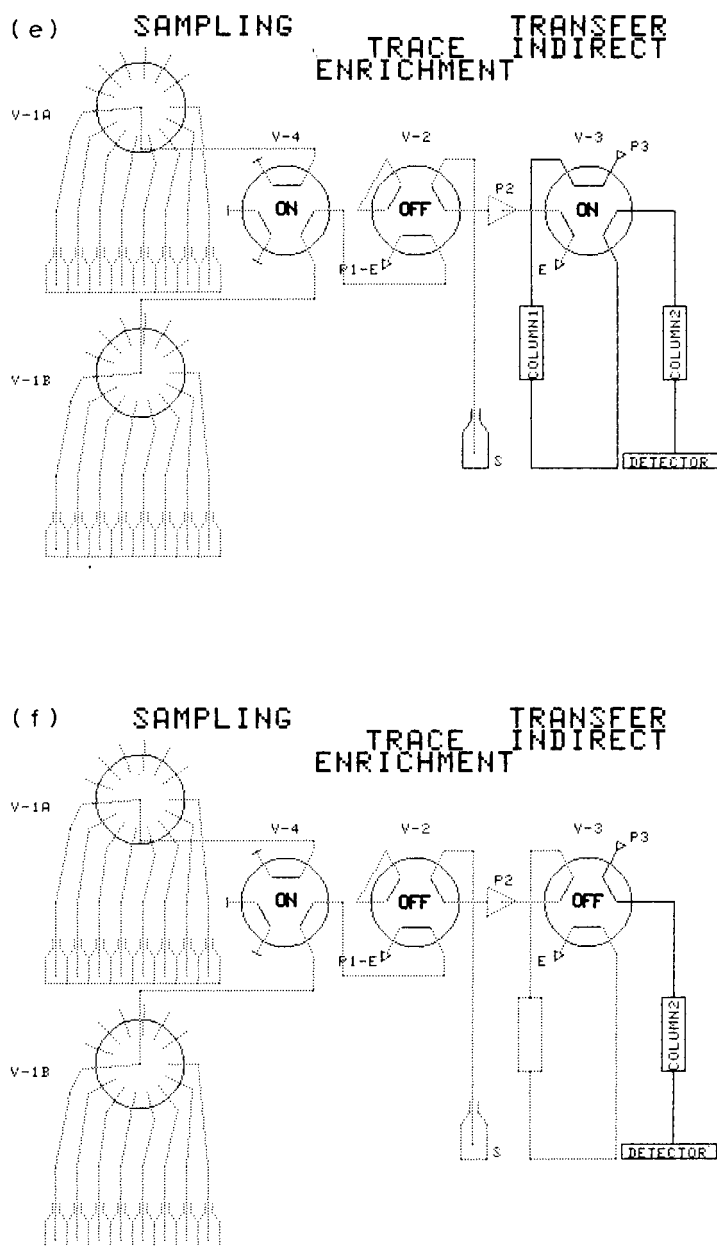


Fig. 1. Flow diagram of the automated enrichment and separation network designed to handle up to 32 samples. Dotted lines are standby positions; solid lines show the actual sample flow. V-1 to V-4, pneumatically actuated high-pressure valves; OFF = standby positions, ON = transfer positions. Valve V-4 is used to switch the flow from the sampling valves V-1A or V-1B. (a), (b) Standby position, sample container connected to the rinsing pump (P1-E); (c) injection pump (P2) in-line with the sample container and the enrichment column; (d) injection valve (V-2) in standby position, enrichment column and pump (P2) are flushed with rinsing solvent; (e) transfer indirect with the mobile phase of the analytical column directed to the enrichment column and the analytical column; (f) elution of the analytical column.

Calibration. The chromatographic system is calibrated by enriching a preselected volume of the aqueous standard solutions. The resulting peak heights are used to calculate a linear regression curve.

RESULTS

Table I shows the structure of the compounds used to evaluate the performance of the enrichment and the chromatographic separation system. All these compounds are used as herbicides or are degradation products thereof.

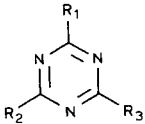
The relative standard deviations (R.S.D.) of the measured peak heights from the calculated linear calibration graphs are given in Table II, and range from 1.5 to 19.8%. Four values from 39 calibration graphs show R.S.D. values higher than 10%. About 30 min per sample are required for the enrichment and subsequent on-line chromatographic determination.

The linear calibration graphs were calculated from four concentrations in the range 1–20 ng in 20 ml of water. Each standard solution was measured at least twice. Metolachlor calibration graphs were calculated from standard concentrations of 2.5–50 ng in 50 ml of water.

DISCUSSION

Cross-contamination of the system is minimized as the sample feeding lines are flushed with the sample itself by the pump (P1-E) at the valve V-3 before directing the flow to the enrichment column. The enrichment pump and all connecting lines which are flushed with the sample are washed with the rinsing solvent (see also Fig. 1d).

TABLE I
COMPOUNDS EXAMINED



Compound	R ₁	R ₂	R ₃
Atrazine	Cl	NHCH ₂ CH ₃	NHCH(CH ₃) ₂
Simazine	Cl	NHCH ₂ CH ₃	NHCH ₂ CH ₃
Terbutylazine	Cl	NHCH ₂ CH ₃	NHC(CH ₃) ₃
Terbutryn	SCH ₃	NHCH ₂ CH ₃	NHC(CH ₃) ₃
GS 26379	Cl	NH ₂	NHC(CH ₃) ₃
G 28279	Cl	NH ₂	NHCH ₂ CH ₃
G 30033	Cl	NH ₂	NHCH(CH ₃) ₂

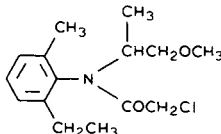


TABLE II
CALIBRATION EXPERIMENTS

Compound	Range of R.S.D. (%)	n
Atrazine	1.9- 6.7	9
Simazine	1.5-10.8	5
Terbutylazine	4.5- 7.6	4
Terburyn	5.2- 5.6	2
GS 26379	1.7- 6.4	5
G 28279	2.8-14.2	5
G 30033	4.8-19.8	5
Metolachlor	3.6-12.5	4

Adsorption of the components to be analysed in the PTFE tubes or other components of the system was not detected. Standard and sample analyses were run under identical conditions (concentration and enrichment).

Depending on the separation problems, additional switching functions may be included in the chromatographic network to improve the separation⁸.

CONCLUSION

This automated on-line enrichment and desorption technique maintains reproducible chromatographic adsorption conditions, as the same enrichment column is used throughout the analyses and reconditioning of this column is standardized.

To achieve the same sensitivity with on-line as with off-line enrichment techniques, a smaller volume of water sample had to be treated and the time required for concentration can therefore be proportionately reduced.

Calibration samples and analytical samples are run under identical conditions. The linearity of the calibration graph shows the integrity of the overall system, including the adsorption, desorption and final chromatographic separation. As demonstrated, the relative standard deviations of the calibration curves are in an acceptable range for trace analysis.

The method of on-line enrichment with an automated sampling device is obviously advantageous from the viewpoint of sensitivity, rapid sample handling and costs when large monitoring programmes are to be performed. About 600 samples of potable water of different origins were analysed, and demonstrated the superiority of this automated on-line procedure over the previously used labour-intensive off-line techniques with prepacked cartridges for sample enrichment.

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Note

High-performance liquid chromatography–electrochemical detection of singlet oxygen by reaction with 2,2,6,6-tetramethyl-4-piperidone

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Singlet molecular oxygen ($^1\text{O}_2$) is a potentially toxic oxidant. During the past 10 years, the characteristic emission band at 1270 nm has been used to measure directly this reactant^{1–3}, but it must be monitored with an ultrasensitive spectrometer equipped with a liquid-nitrogen-cooled germanium detector because the near-infrared band is very weak. An indirect detection of $^1\text{O}_2$ using electron spin resonance (ESR) spectrometry has been proposed^{4,5}. The stable nitroxide radicals generated by reaction of sterically hindered amines with $^1\text{O}_2$ were detectable down to 10 nM. ESR spectrometry is a well established method for detecting free radicals, however the spectrometer is an expensive instrument. An high-performance liquid chromatography (HPLC)–electrochemical detection (ED) system has been used to measure superoxide- and hydroxyl-generated DMPO adducts though they have short lifetimes^{6,7}. The oxidation of sterically hindered heterocyclic amines by $^1\text{O}_2$ produces more stable nitroxide radicals which are known as spin labels.

We have tried to detect and quantify the $^1\text{O}_2$ adduct of 2,2,6,6-tetramethyl-4-piperidone (TEMP) using the corresponding spin label, namely 2,2,6,6-tetramethyl-4-piperidone-N-oxyl (TEMPO), as a standard by an HPLC–ED method. The HPLC system attached to a silicon polymer-coated silica gel column enabled us to measure the spin adduct as sensitively as by ESR spectrometry. The method was applied to the measurement of $^1\text{O}_2$ induced by a porphyrin-photosensitized oxidation.

EXPERIMENTAL

TEMPO and TEMP were obtained from Aldrich, catalase from Sigma. The concentration of TEMPO was calculated from the absorbance at 235 nm due to the $>\text{N}^{\cdot}\text{O}$ group, using $\epsilon_{235} = 2500 \text{ M}^{-1} \text{ cm}^{-1}$ (ref. 8). α , β , γ , δ -Tetrakis(4-N-methylpyridyl)porphine (TMPyP) from Dojindo (Kumamoto, Japan) was used for the photosensitized production of $^1\text{O}_2$. The photoirradiation was carried out at 25°C in an oxygen electrode cell, YSI Model 5301 (Yellow Springs Instrument, Yellow Springs, OH, U.S.A.), placed at the centre of a 28 W toroidal lamp of the daylight fluorescent type. The oxygen consumption during photoirradiation was measured with a Clark oxygen electrode YSI 5331. The sample solution was prepared in calcium- and magnesium-free Dulbecco phosphate-buffered saline (PBS), pH 7.5 or pD 7.5, made using water or $[\text{D}_2]\text{water}$. The HPLC analysis was performed on a LC-6A HPLC in-

strument (Shimadzu, Kyoto, Japan) equipped with a Capcell Pak C_{18} ($5\ \mu\text{m}$) $150 \times 4.6\ \text{mm}$ I.D. column (Shiseido, Tokyo, Japan) packed with silicon polymer-coated silica gel, a Shimadzu L-ECD-6A electrochemical detector and a $10\text{-}\mu\text{l}$ sample loop. The detector potential was set at $+0.8\ \text{V}$ versus an Ag/AgCl reference electrode so as to get the maximum response for TEMPO (Fig. 1). The retention time and peak integration were recorded by a Shimadzu C-R4A chromatograph integrator. The eluent consisted of $0.05\ \text{M}$ citric acid, 10% acetonitrile and 4% methanol adjusted to pH 3.5 with $10\ \text{M}$ sodium hydroxide, containing $10\ \text{mg}$ $\text{Na}_2\ \text{EDTA}$ per litre. Elution was accomplished with a flow-rate of $1\ \text{ml/min}$ through an ERC-3312 degasser (Erma, Tokyo, Japan).

RESULTS AND DISCUSSION

Fig. 2 shows a typical chromatogram of the spin label, TEMPO. The electrochemical response was linear over three orders of magnitude ranging from $50\ \text{nM}$ to $50\ \mu\text{M}$. The linearity between the peak area and TEMPO concentration (μM) was represented by the following equation: $y = 108\,747x - 1373$, $r = 0.9999$. When a LiChrocart, Supersphere RP-18 ($4\ \mu\text{m}$), $125 \times 4\ \text{mm}$ I.D. column (Merck) was used in place of the Capcell Pak column, the detection limit of TEMPO was increased $0.2\ \mu\text{M}$ because of the broadening of the peak.

The oxygen consumption during the reaction between TEMP and $^1\text{O}_2$ was measured in water and $[\text{}^2\text{H}_2]\text{water}$ (Fig. 3). The rate of oxygen consumption by the control system was 17.5 times faster in $[\text{}^2\text{H}_2]\text{water}$ than in water. Sodium azide added in $[\text{}^2\text{H}_2]\text{water}$ effectively inhibited the oxygen consumption as shown in Fig. 3 (right). These results show that TEMP reacts directly with $^1\text{O}_2$ since the lifetime of $^1\text{O}_2$ is about 17 times longer in $[\text{}^2\text{H}_2]\text{water}$ than in water⁹ and azide ion is a physical quencher of $^1\text{O}_2$ (ref. 10).

TEMP was treated with photochemically generated $^1\text{O}_2$ in the presence of sodi-

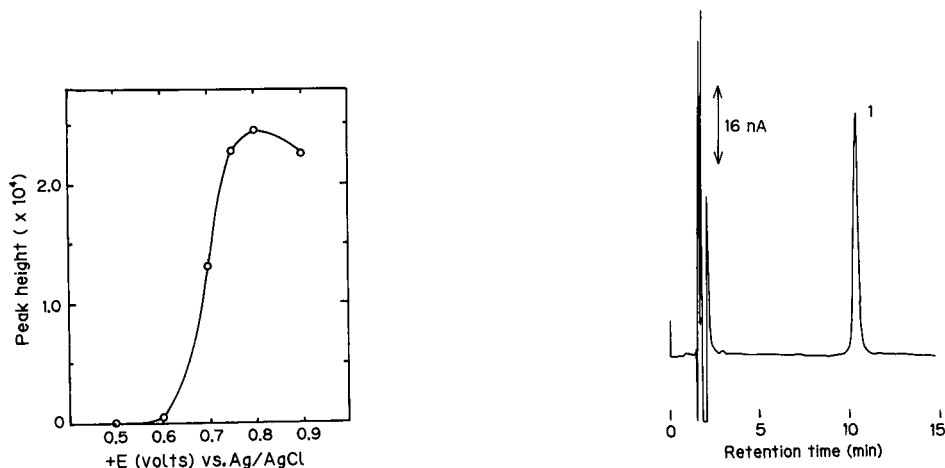


Fig. 1. Voltammogram of TEMPO ($10\ \mu\text{M}$) measured by an electrochemical detector.

Fig. 2. Chromatogram of authentic TEMPO measured by an electrochemical detector. Peak 1 = $1\ \mu\text{M}$ TEMPO.

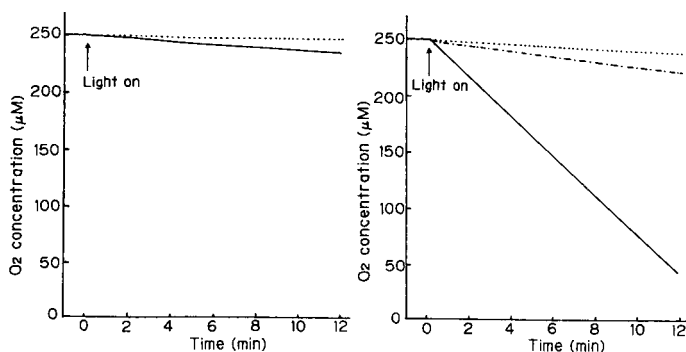


Fig. 3. Oxygen uptakes of TEMP during the TMPyP-photosensitized oxidation. The control solution contained 50 mM TEMP and 10 μM TMPyP in 3 ml PBS buffer, pH 7.5 or pD 7.5, made from water (left) or [²H₂]water (right). The oxygen concentration of the air-saturated solution at 25°C was estimated as 250 μM. After thermal equilibration for 5 min, the sample solution was irradiated with a 28-W fluorescent lamp. (—), Control system; (···), minus TMPyP; (---) plus 1 mM sodium azide.

um azide and mannitol in aerated water and [²H₂]water. The chromatograms of the reaction mixture in [²H₂]water are shown in Fig. 4. The peak near 10.5 min decreased dramatically in the presence of azide ion, a ¹O₂ quencher, but was unchanged in the presence of mannitol, a specific hydroxyl radical scavenger. The peak was, therefore, due to TEMPO formed from TEMP and ¹O₂.

Table I shows the amount of TEMPO calculated from the peak area using a calibration graph for authentic TEMPO. The TEMPO yields from both control systems were much lower than the 610 nmol in [²H₂]water and 35 nmol in water expected on the basis of the molecular oxygen consumed, as shown in Fig. 3. The ratio of the TEMPO found in [²H₂]water to that in water was 4.5. After the irradiation, TEMPO, which itself is very stable even upon photoirradiation for 90 min, decreased rapidly

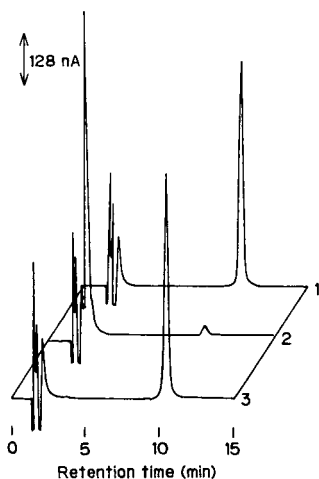


Fig. 4. Effect of sodium azide and mannitol on the TEMPO production during the TMPyP-photosensitized oxidation in PBS made from [²H₂]water. 1 = Control prepared as described in Fig. 3; 2 = plus 1 mM sodium azide; 3 = plus 10 mM mannitol. Aerated sample solutions were irradiated at 25°C for 12 min with a 28-W fluorescent lamp.

with time. Externally added TEMPO also reduced through the TMPyP-photosensitized oxidation together with TEMP. However, it did not change in the absence of TEMP and was also uninfluenced by TEMP added immediately after cessation of the irradiation. The results suggest that photoinduced TMPyP radicals¹¹ and their TEMP adducts have little effect on the decrease of TEMPO and that TEMP causes the degradation of TEMPO formed during the photosensitization of TMPyP. Sodium azide surprisingly inhibited the formation of TEMPO in [²H₂]water, but resulted in the lower inhibition in water. This is due to hydrogen peroxide produced by the dismutation reaction



of a small amount of superoxide anion ($\text{O}_2^{\cdot -}$) generated by electron transfer from excited states of porphyrin to molecular oxygen, since hydrogen peroxide is used as the oxidizing agent for the synthesis of TEMPO from TEMP¹² and $\text{O}_2^{\cdot -}$ is unreactive with TEMP¹³. Catalase reduced the formation of TEMPO to 87% in water, suggesting the participation of hydrogen peroxide, but did not do so in [²H₂]water. Mannitol scarcely inhibited the formation of TEMPO in both solvents. Although TEMP also reacts with hydroxyl radical generated via hydrogen peroxide to give TEMPO¹³ and since mannitol is a specific quencher of hydroxyl radical, the results show that little hydroxyl radical is generated under the present experimental conditions. Therefore, the amounts of TEMPO resulting from the reaction of TEMP with ¹O₂ were 6.07 nmol in water and 31.33 nmol in [²H₂]water.

When using the TEMP spin-trapping to monitor ¹O₂ and hydroxyl radical yields, one should study the solvent and quencher effects for nitroxide production to verify any active oxygen species involved. Our HPLC-ED system was easily capable of determining TEMPO with a 10-μl sample solution and within 15 min. Moreover,

TABLE I

EFFECTS OF SOLVENTS AND QUENCHERS ON THE TEMPO PRODUCTION DURING THE TMPyP-PHOTOSENSITIZED OXIDATION OF TEMP

Experiments were carried out as described in the legend to Fig. 4. Before irradiation, the amounts of nitroxide radicals present in the water and [²H₂]water systems were 0.08 and 0.11 nmol, respectively. These amounts were subtracted from amounts of nitroxide radical obtained from the systems used. Catalase added was 39.6 units per 3 ml of reaction mixture.

System	Amount of TEMPO			
	Water		[² H ₂]water	
	nmol	%	nmol	%
Control	6.99 ± 0.14	100	31.54 ± 0.84	100
Control-10 μM TMPyP	0.06 ± 0.03	0.1	0.23 ± 0.12	0.7
Control + 1 mM sodium azide	2.27 ± 0.01	32.5	1.36 ± 0.03	4.3
Control + 1.25 nM catalase	6.07 ± 0.43	86.8	31.33 ± 0.10	99.3
Control + 1.25 nM heated catalase ^a	6.69 ± 0.01	100	31.47 ± 0.07	99.8
Control + 10 mM mannitol	6.69 ± 0.24	95.7	31.23 ± 0.13	99.0

^a Catalase was heated to 95°C for 5 min.

the system can detect TEMPO at 10 nM range as sensitively as ESR spectrometry. The present method, which is reproducible and simple, may be very useful in the elucidation of $^1\text{O}_2$ and/or hydroxyl radical involvement in chemical and biological systems.

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Note

Liquid chromatographic separation of positional isomers of suprofen on a cyclodextrin-bonded phase

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Suprofen, [(±)- α -methyl-4-(2-thienylcarbonyl)benzene acetic acid], is a potent peripherally active analgesic with both anti-inflammatory and antipyretic properties. The acid is a white to off-white powder with a pK_a value of 3.91 and a melting point of 123.5°C.

Two positional isomers of suprofen may be formed during synthesis. Since these isomers are not completely removed by crystallization, a method was needed to identify and quantitate these compounds. These materials are potential process impurities. They need to be observed and quantified if present to ensure the quality of the drug substance. Although suprofen can be assayed by conventional reversed-phase high-performance liquid chromatography (HPLC) and/or gas chromatography, neither of these methods can adequately resolve the two isomers from each other or from suprofen. Additionally, the gas chromatographic assay requires a derivatization step involving the use of potentially hazardous diazomethane.

A number of methodological approaches were tried, *e.g.*, different stationary phases (phenyl, C_8 , cyano), combinations of methanol, acetonitrile and aqueous buffers at various pH values on reversed-phase packings, and the addition of metal cations to the buffer in an attempt to form chelated species as suggested by Karger *et al.*¹. All of these approaches proved to be ineffective. A β -cyclodextrin column was subsequently investigated.

β -Cyclodextrin is a helical arrangement of seven gluco-pyranose units. Because of its inherent chirality (each glucose unit is chiral), β -cyclodextrins have been employed as enantioselective stationary phases². However, these columns can also be used to separate structural isomers³. Cyclodextrins are known for their ability to form selective inclusion complexes for a variety of organic molecules. Therefore, as a stationary phase in HPLC, these materials should offer separation possibilities different from conventional packings. The mechanism of separation may involve many factors including molecular size or bulk, dipole-dipole interaction, hydrogen bonding and the hydrophobicity of the solute. These inclusion complexes are formed in aqueous media and therefore, chromatography is usually performed using high concentrations of water. The degree and strength of complex formation, owing to the physical orientation of the solute in the toroid structure and the symmetrical arrangement of hydrogen bonding sites of the cyclodextrin, enhances retention of symmetrical structures over those found for traditional alkyl-bonded phases.

A facile separation was developed using a β -cyclodextrin column and a mobile phase of acidified water and acetonitrile. No special sample preparation is required and all three isomers are baseline resolved.

EXPERIMENTAL

Materials, equipment and liquid chromatographic conditions

A Waters Associates HPLC system with a fixed-wavelength (254 nm) detector and Hewlett-Packard Model 3354 laboratory data system was used. The HPLC column was a Cyclobond I, β -cyclodextrin, 5- μ m particle size, 25 cm $\times$ 4.6 mm I.D. (Advanced Separations Technologies) and the mobile phase was water-acetonitrile (70:30). The water was prepared by adding 1 ml triethylamine to 1 l of water and adjusting to pH 4.5 ± 0.1 with glacial acetic acid. The acetonitrile was HPLC grade as were the water and its additives. The column was maintained at 35°C and the flow-rate was maintained at 2.0 ml/min.

Solution, standard and sample preparation

Acidified water. HPLC-grade water was adjusted using 1.0 ml triethylamine and dropwise addition of glacial acetic acid until pH 4.5 ± 0.1 was reached as determined by a pH meter.

Sample solvent. A volume of 300 ml of acetonitrile was added to 700 ml of HPLC-grade water, thoroughly mixed and degassed.

Mobile phase. A volume of 300 ml of acetonitrile was added to 700 ml of acidified water, thoroughly mixed and degassed.

Sample or standard. About 40 mg of sample and standard were accurately weighed into separate 100-ml volumetric flasks, dissolved and diluted to volume with sample solvent.

Resolution mixture. About 50.0 mg of standard was accurately weighed into a 100-ml volumetric flask. About 25 mg of the isomer eluting closest to suprofen was accurately weighed into a separate 100-ml volumetric flask, and diluted to volume with sample solvent. A 5.0-ml aliquot of this solution was transferred to the volumetric flask containing the suprofen standard, and the mixture dissolved and diluted to volume with sample solvent.

Diluted resolution mixture. Accurately measured 10.0-ml aliquots of the resolution solution and sample solvent were transferred to a suitable container.

Assay procedure

The instrument was set as previously indicated and the column equilibrated for at least 20 min with the mobile phase flowing. The system suitability was determined prior to sample analysis. The resolution solution and the diluted resolution solution were injected and the chromatograms and integrated areas for the components obtained. The resolution between suprofen and its isomer should be at least 1.0 using the standard resolution equation. The integrated area of suprofen in the diluted resolution mixture should be between 48 and 52% of that of suprofen in the resolution mixture. The precision of the system was evaluated using the relative standard deviation of the response factors (area/ μ g) for the injections of the standard solution. Typically, the relative standard deviation was less than 2.0%.

Calculations

Integrated sample area counts were normalized for the amount of compound injected (area/ μg sample) divided by normalized standard area counts and multiplied by the percent purity of the standard to determine the percent assay. The isomers were calculated in a similar fashion, with a sensitivity factor (F) added; this factor accounts for the difference in sensitivity between the major compound and a known isomer under analytical assay conditions. Unidentified peaks were assigned a sensitivity factor of 1.0.

RESULTS AND DISCUSSION

Suprofen and each of the positional isomers were used as racemic mixtures. The HPLC procedure described was used to assess the positional isomeric purity of suprofen. The suprofen was quantified using a standard of known purity, while sensitivity factors were employed in quantifying the amount of isomers. These sensitivity factors were obtained experimentally by chromatographing known concentrations of authentic samples of the isomers and dividing their response factor (area/ μg injected) by the suprofen response factor.

The structures, retention times, sensitivity factors and detection limits of suprofen and its positional isomers are presented in Table I, demonstrating the procedure to be specific and sensitive. A typical chromatogram is shown in Fig. 1.

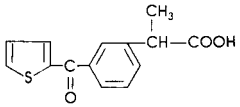
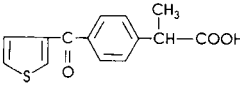
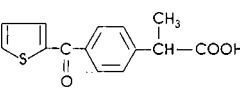
The linearity of response for each compound is shown in Fig. 2. The suprofen plot represents up to 110% of the normal amount chromatographed, while the isomer plots range from 0.05% to 2.2% of the nominal suprofen injection. The lower limit of detection was determined from these plots.

Precision was evaluated using twelve injections of a suprofen sample spiked with each of the two isomers. The concentration of each impurity isomer component was adjusted to represent approximately 0.5% of the nominally injected suprofen.

TABLE I

STRUCTURES, RETENTION TIMES, SENSITIVITY FACTORS AND DETECTION LIMITS OF SUPROFEN AND ITS ISOMERS

NA = Not applicable.

Compound	Structure	Retention time (min)	F	Detection limit (%)
McN-JR-26283		8.8	0.87	0.04
McN-JR-44379		10.9	1.70	0.04
Suprofen		12.2	1.0	NA

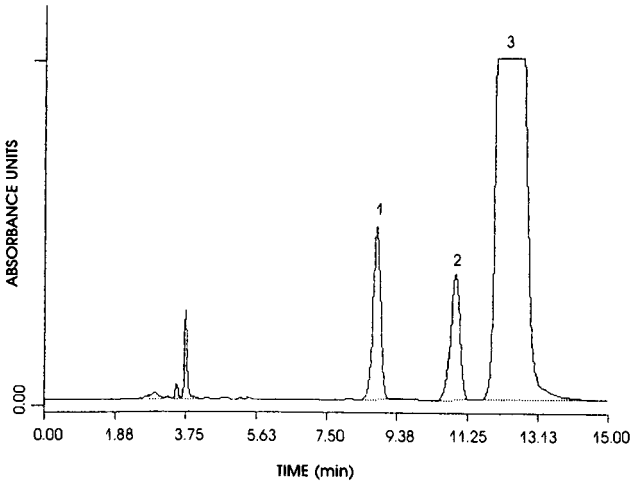


Fig. 1. Typical chromatogram of a spiked suprofen sample. Peaks: 1 = McN-JR-26283; 2 = McN-JR-44379; 3 = suprofen.

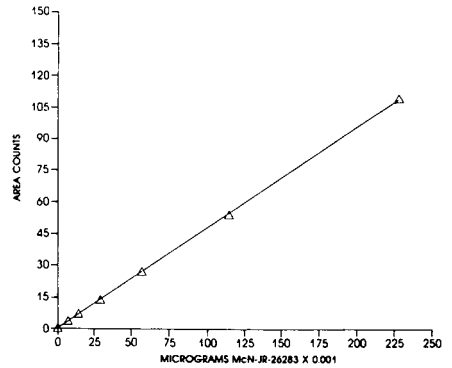
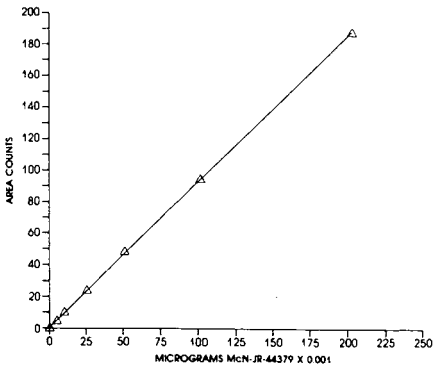
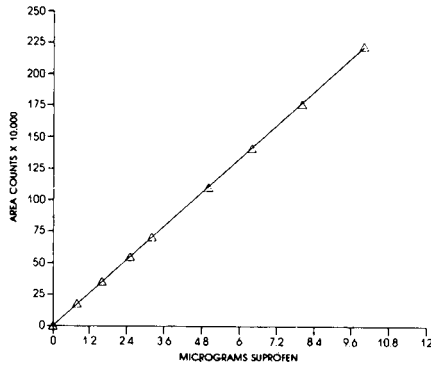


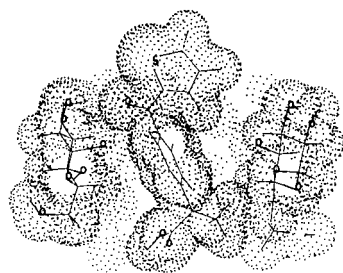
Fig. 2. Linearity plots for suprofen and each isomer.

The data indicated the integrated peak areas for each component were reproducible, with the following relative standard deviations: 0.24% for suprofen (McN-JR-25061), 1.6% for the 3-phenyl isomer (McN-JR-26283), and 1.5% for the 3-thienyl isomer (McN-JR-44379). The quantitation of and resolution between each of the two positional isomers and suprofen was found to be rugged with respect to column temperature, flow-rate, pH of the mobile phase, percent acetonitrile in the mobile phase, and the amount of triethylamine in the aqueous portion of the mobile phase. The resolution between the 3-thienyl isomer and suprofen, and the quantitation of suprofen and its two isomers with respect to changes in the above-mentioned parameters are shown in Table II. The resolution between the 3-thienyl and 3-phenyl isomers was sufficiently large that chromatographic alterations resulted in inconsequential resolution changes under the condition investigated. The mobile phase composition could be varied from 25 to 35% acetonitrile although resolution begins to decrease at 35% acetonitrile. Increasing the amount of aqueous component increased the resolution but also increased retention time. The pH of the aqueous buffer did not appear to affect the resolution within the limits investigated. However, at pH values greater than 4.5, the retention times for all components began to decrease. The amount of triethylamine did not seem to affect the resolution to any significant degree, but without the amine, none of the components eluted. At higher temperatures (*i.e.*, 50°C) the resolution decreased sharply. The flow-rate over the range investigated

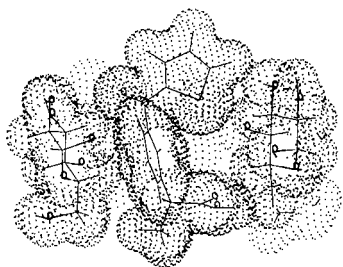
TABLE II

EFFECT OF CHANGES IN CHROMATOGRAPHIC PARAMETERS ON RESOLUTION AND QUANTITATION OF SUPROFEN AND ITS POSITIONAL ISOMERS

Parameter	Value	Resolution	% (w/w)		
			Suprofen	McN-JR-26283	McN-JR-44379
Flow-rate (ml/min)	1.0	1.21	98.93	0.83	0.71
	1.5	1.16	98.78	0.83	0.74
	2.0	1.06	98.76	0.83	0.73
Column temperature (°C)	35	1.16	98.78	0.83	0.74
	40	1.09	98.66	0.85	0.73
	45	1.03	98.58	0.83	0.73
	50	0.83	98.72	0.83	0.73
Acetonitrile content (%)	25	1.35	98.70	0.81	0.71
	30	1.16	98.78	0.83	0.74
	35	0.74	98.63	0.81	0.73
pH aqueous phase	4.0	1.06	98.73	0.83	0.73
	4.5	1.16	98.78	0.83	0.74
	5.0	1.20	98.48	0.85	0.73
Triethylamine content (ml/l)	0.0	No elution of any components			
	0.5	0.91	98.55	0.83	0.73
	1.0	1.16	98.78	0.83	0.74
	2.0	1.17	98.67	0.83	0.73



SUPROFEN in CYCLODEXTRIN



McN-JR-26283 in CYCLODEXTRIN

Fig. 3. Computer model of racemic suprofen isomers and β -cyclodextrin.

did not affect the resolution. Discussion of operational parameters, general selectivity and the ruggedness of cyclodextrins have recently been published⁴⁻⁶.

Computer modeling of the β -cyclodextrin with the racemic suprofen isomers using the program SYBAL (Tripos Assoc.) revealed interesting docking arrangements. The computer program positioned the suprofen positional isomers to minimize steric and electronic hindrances. The 3-thienyl and 4-phenyl isomers have essentially the same conformation when inserted into the β -cyclodextrin, while the 3-phenyl isomer differs. Computer-generated figures of cross-sections of the 3-phenyl and 4-phenyl racemic isomer complexes are shown in Fig. 3. Although these represent low-energy arrangements, the ease with which the isomers can attain these docking positions is unknown and solvation effects are not incorporated. Thus, strict interpretation of these data to predict elution order is currently unclear. However, the observed elution order (first to last) *m*-, *o*-, *p*- is corroborated by ref. 3.

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Note

Direct headspace gas chromatographic determination of dichloromethane in decaffeinated green and roasted coffee

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Gas chromatographic (GC) analysis of chlorinated solvents in foods and in coffee, has in recent years been the object of several investigations, and attention has been paid to the determination of dichloromethane since its content is regulated in several countries. Most methods used distillation^{1–5} to isolate the solvent followed by GC with an electron-capture or electrolytic conductivity detector for quantitation.

Headspace gas chromatography (HSGC) is at present extensively used for the determination of volatile compounds in liquid and solid matrices^{6–18}. Recently a procedure for the determination of dichloromethane in decaffeinated tea and coffee has been reported which uses a packed column and an electrolytic conductivity detector¹⁹.

The present investigation has been carried out to develop a simple procedure for the analysis of dichloromethane in decaffeinated coffee by use of a capillary column and a temperature programmed vaporizer injection system (PTV) and electron-capture detection (ECD). These devices have been applied because a variety of volatile compounds are present in green and roasted coffee and an efficient fractionation of them is required to isolate and detect dichloromethane.

EXPERIMENTAL

HSGC was carried out on a glass capillary column, 50 m × 0.30 mm I.D., precoated with Carbopack A, coated with FFAP (free fatty acid phase) and stabilized with DCUP (dicumylperoxide)²⁰. The procedure used to prepare the column is briefly as follows: 50 mg of Carbopack A were added to 25 ml of dichloromethane and 25 ml of tetrachloromethane and placed in an ultrasonic bath to obtain an uniform suspension. The suspension was made to flow four times in the capillary each time reversing the flow direction. A flow of nitrogen was then passed through the column to remove the solvents. On the carbopack layer a stationary phase consisting of 4% FFAP and 0.15% dicumylperoxide was coated by using a dynamic procedure.

The column was conditioned by a flow of hydrogen for 1 h at 250°C and is thereafter ready for use. The number of theoretical plates was 150 000 for 2,6-dimethylaniline ($k' = 4.5$) at 150°C.

A DANI (Monza, Italy) gas chromatograph Model 6500 equipped with a temperature-programmed vaporizer (PTV) injector and an electron-capture detector con-

nected with a Shimadzu integrator Model CR 3A was used. Operating conditions used for HSGC of dichloromethane: column temperature, 70°C for 5 min then programmed at 1°C/min to the end of the analysis; PTV programmed from 50 to 70°C and with the valve closed.

RESULTS AND DISCUSSION

Headspace analysis of roasted coffee yields a gas chromatogram with several peaks and in order to determine dichloromethane it has been found convenient to operate with a coffee suspension in water and to perform the headspace analysis by using an high-resolution capillary column allowing equilibrium among the three phases.

The solubility of dichloromethane and equilibrium conditions are affected by several factors (temperature, shaking time, salt concentration). In Fig. 1 are shown the results of HSGC of a decaffeinated coffee sample in water and in saline solution (0.5 *M* sodium sulphate) obtained with the capillary column described. With the saline solution an higher peak than with the aqueous solutions was measured and the interference from a compound frequently found in roasted coffe was less.

The gas-phase concentration measured by HSGC depends upon equilibria occurring among the solid; the liquid and the gas phases. In order to obtain information on the time required to reach equilibrium, a set of experiments has been carried out on roasted coffee samples to which 1.4 μg dichloromethane were added. The samples were either shaken by hand or subjected to ultrasonic vibrations: with the former

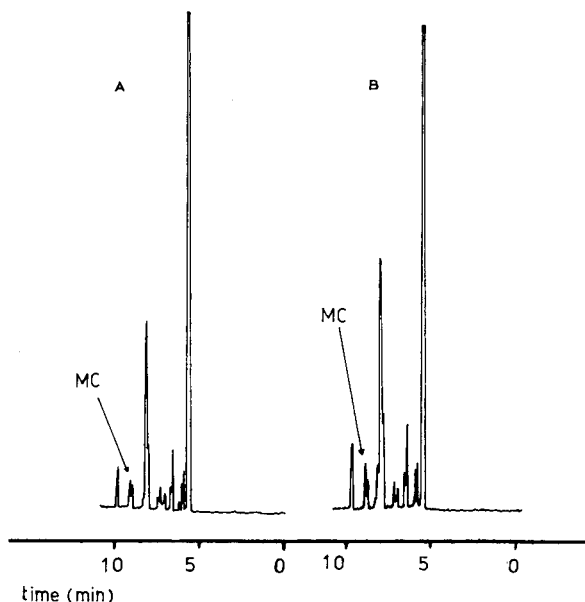


Fig. 1. Headspace gas chromatograms of a decaffeinated roasted coffee. (A) In distilled water suspension; (B) in saline solution.

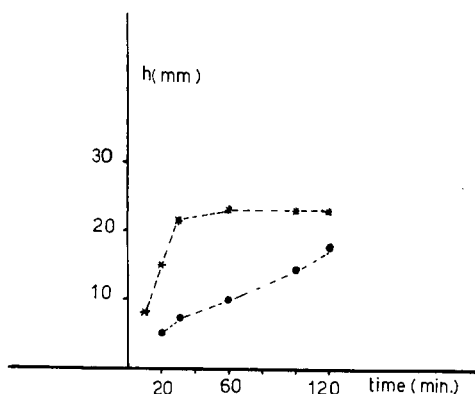


Fig. 2. Dichloromethane peak height vs. conditioning time for coffee samples containing 1.4 ppm dichloromethane (*) subjected to ultrasonic vibration; (●) shaken manually.

procedure no equilibrium is reached after mixing for 2 h, whereas with the latter a constant value is obtained after about 30 min as shown in Fig. 2.

The following procedure has been developed for the sample preparation: for each coffee sample, three 20-ml vials are taken to which 1 g of roasted or green coffee, previously dried, is transferred with 10 ml 0.5 *M* sodium sulphate solution. To each vial known amounts are added of standard dichloromethane in xylene solution (1–10 μ l dichloromethane, 1 mg ml⁻¹). The vials are closed with an aluminium capsule with a rubber septum coated with PTFE and placed in an ultrasonic bath for 10 min to obtain an uniform homogenisation. The samples are conditioned at 30°C for 60 min to obtain equilibrium among the solid, liquid and gas phases; 0.5 ml of the gas phase are injected for GC.

By operating with the PTV system set at low temperature the injection of less volatile compounds is prevented. In Fig. 3 is shown the plot of peak height *versus* concentration of dichloromethane added expressed in terms of ppm. Extrapolation of the straight line to the abscissa axis yields the dichloromethane concentration of the sample.

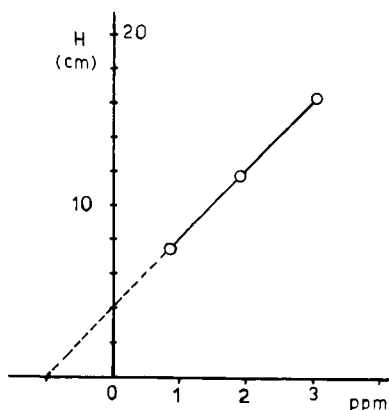


Fig. 3. Dichloromethane determination in decaffeinated samples according to the procedure described.

TABLE I

MEASUREMENT OF DICHLOROMETHANE IN GREEN AND ROASTED COFFEE SAMPLES

	Dichloromethane (ppm)	n (measurements)	S*	C.V. (%)
Roasted coffee	2.5	30	0.1	4.0
Green coffee	2.5	20	0.02	0.9
Decaffeinated roasted coffee				
A	0.9	4	0.07	7.8
B	0.6	3	0.08	13.3
C	0.5	3	0.07	14.0
D	1.1	5	0.05	4.5
E	8.7	4	0.07	0.8
Decaffeinated green coffee				
A	1.5	3	0.03	2.0
B	1.0	5	0.02	2.0
C	2.0	3	0.04	2.0
D	1.8	6	0.04	1.8

By performing the same determination on various days and by subjecting the sample under investigation to ultrasonic vibration to homogenize the suspension, the same analytical results for dichloromethane concentration is obtained though the line slope may be different.

In order to evaluate the reliability of the procedure, samples of roasted and green coffee to which 2.5 ppm of dichloromethane has been added were analyzed and the repeatability ($S^* \cdot 100/\bar{x}$) was calculated, where S^* = standard deviation. In Table I are collected also the results obtained on various samples with different dichloromethane concentrations. The coefficient of variation (C.V.) is lower for green coffee and for the sample with an higher dichloromethane concentration in the roasted coffee; it is rather high for low values of dichloromethane in roasted coffee because of the presence of a peak present in small concentration with a retention time close to that of dichloromethane.

The results indicate that the analytical procedure described permits the reliable determination of dichloromethane in decaffeinated coffee; it is rapid and simple and needs only minor manipulations, no internal standard is used and the calibration of the detector is carried out with all measurements. It requires however an high-resolution capillary column and the use of standard additions to the sample under examination.

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Note

Determination of Methyl Yellow, Sudan I and Sudan II in water by high-performance liquid chromatography

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Methyl Yellow (C.I. 11020), Sudan I (C.I. 12055) and Sudan II (C.I. 12140) are used as dyes in gasoline, shoe polish, special inks and plastics. However, Methyl Yellow has been reported to be a carcinogen^{1,2}. If these dyes are present in the environment, they would be released slowly into river and sea waters, owing to their limited solubility in water. Therefore, a microanalytical method is required to detect these dyes in environmental waters.

There have been few papers concerning analytical methods for these dyes in environmental samples. This investigation was undertaken to establish a suitable method for their simultaneous determination in environmental waters at very low concentrations. After distillation, the dyes were extracted by modified mixing extraction as they are not readily soluble in water. Mixing extraction methods^{3–5} using a very small amount of solvent permit simple and rapid operation, but the formation of emulsions often makes the extraction difficult. In this paper, the formation of the emulsion was improved by adding acetone. The separation of the dyes was possible by high-performance liquid chromatography (HPLC). The method established gives excellent sensitivity and recovery.

EXPERIMENTAL

Reagents

Methyl Yellow, Sudan I and Sudan II were obtained from Wako and recrystallized from acetone. The grades of hexane and acetone used were those suitable for the detection of pesticide residues. The grade of methanol used was that suitable for HPLC. Sodium chloride was of analytical-reagent grade. After 0.025 g of each dye had been dissolved in 50 ml of acetone, standard solutions were prepared by diluting each acetone solution with methanol.

Apparatus and measurement conditions

HPLC was carried out with a Shimadzu LC-4A instrument and an SPD-1 variable-wavelength UV-VIS spectrophotometric detector. The output of the detector was monitored with a Chromatopac C-R2AX data processor.

The HPLC conditions were as follows: column, LiChrosorb RP-18, 7 μ m (25 cm $\times$ 4.0 mm I.D.); column temperature, 40°C; mobile phase, methanol–water

(90:10, v/v); flow-rate, 1 ml/min; detection, 460 nm, 0.02 a.u.f.s.; and sample size, 20 μ l.

Standard procedure

A 500-ml sample of water was placed in a 1-l distillation flask and three boiling stones were added. The distillation flask was heated with a mantle heater. Distillation was carried out into 20 ml of distilled water in a 500-ml measuring cylinder for 80–90 min until 400 ml of distillate had been collected. The distillate was transferred to a 500-ml measuring flask equipped with a PTFE stirring bar (3 cm $\times$ 7 mm O.D.). The water-cooled condenser and the funnelled adapter were washed with 10 ml of acetone and the 500-ml measuring cylinder was washed with 5 ml of acetone and 20 ml of distilled water. The wash liquids were transferred to the measuring flask containing the distillate and 5 g of sodium chloride were added and dissolved. The solution was diluted to volume with distilled water and 3 ml of hexane were added. After mixing extraction for 10 min with a magnetic stirrer, the hexane layer was transferred to a 10-ml test-tube using a Pasteur pipette. The mixing extraction procedure was repeated twice for a total of three extractions. In the measuring flask, the distance from the base of the neck to the calibration line should be as short as possible (less than 2 cm) to make mixing easy.

The extract in the test-tube was concentrated and dried on a water-bath (below 50°C) with a flow of nitrogen. The dyes were dissolved in 1 ml of methanol using an ultrasonic bath. The solution was passed through a Millipore membrane filter (pore size 0.45 μ m), then 20 μ l of the methanol-dissolved extract was injected into the HPLC column.

RESULTS AND DISCUSSION

Examination of distillation conditions

First, an examination was conducted to establish the most suitable pH for distillation. To a 1-l distillation flask were added 500 ml of water adjusted to pH 1–13 with sodium hydroxide solution or sulphuric acid, and three boiling stones and 2 μ g of the standard mixture were added. The solution was distilled until 400 ml of distillate had been obtained and the distillate was treated according to the standard procedure. The recoveries were constant, being 87.3–91.7% in the pH range 5–13 for Methyl Yellow, 88.5–93.9% in the pH range 1–11 for Sudan I and 90.5–94.8% in the pH range 1–13 for Sudan II. Normally, the pH of river and sea water is in the range 5–11, here the pH of the water samples did not need to be adjusted.

Next, an examination was conducted to establish the most suitable distillate volume. The above procedure was employed. In this examination, the pH of the distilled water sample was not adjusted and the volume of distillate was varied between 100 and 450 ml. It was found that constant recoveries were obtained if the volume of distillate was in the range 400–450 ml. The recoveries were 87.6–87.7% for Methyl Yellow, 92.5–93.3% for Sudan I and 91.1–93.0% for Sudan II. Consequently, it was necessary to continue distillation until 400 ml of distillate had been collected.

Examination of extraction conditions

An examination of the effect of the pH of the distillate on the recovery was

carried out. To a 500-ml measuring flask equipped with a stirring bar were added 400 ml of distilled water adjusted to pH 1–13 with sodium hydroxide solution or sulphuric acid. After 2 μ g of the standard mixture and 15 ml of acetone had been added, 5 g of sodium chloride were also added and dissolved. The solution was diluted to volume with distilled water. Subsequent treatments were conducted according to the standard procedure. The recoveries were constant, being 94.3–96.2% in the pH range 5–11 for Methyl Yellow, 92.8–98.8% in the pH range 1–13 for Sudan I and 100–102% in the pH range 1–13 for Sudan II.

In the mixing extraction, the amount of hexane used for the extraction was very small. Therefore, an examination of the effect of the amount of hexane added on the recovery was carried out. The above procedure was employed and the amount of *n*-hexane was varied between 1 and 5 ml. In this examination, the pH of the distilled water for the distillate was not adjusted. Extraction was carried out only once, and therefore the residual layer of hexane in the measuring flask was recovered by the addition of 5 ml of hexane and transferring the solution to a test-tube. The addition of more than 2 ml of hexane gave constant recoveries of over 90%.

The residual dyes in the water-cooled condenser, the funnelled adapter and the measuring cylinder should be recovered by washing with acetone. Therefore, an examination of the effect of the amount of acetone used on the recovery was carried out. The same procedure as used for the examination of the effect of the pH of the distillate on the recovery was employed and the amount of acetone was varied between 0 and 100 ml. In this examination, the pH of the distilled water for the distillate was not adjusted. It was found that the recoveries were constant and over 90% when the amount of acetone was in the range 0–100 ml. Moreover, the formation of emulsion was improved when acetone was used.

Examination of HPLC conditions

The absorption maxima of the dyes dissolved in the mobile phase consisting of methanol–water (90:10, v/v) were 405 nm for Methyl Yellow, 475 nm for Sudan I and 493 nm for Sudan II. Consequently, the dyes were detected at 460 nm.

An examination was carried out with three mobile phases, with different proportions of methanol and water (95:5, 90:10 and 85:15, v/v), to determine the opti-

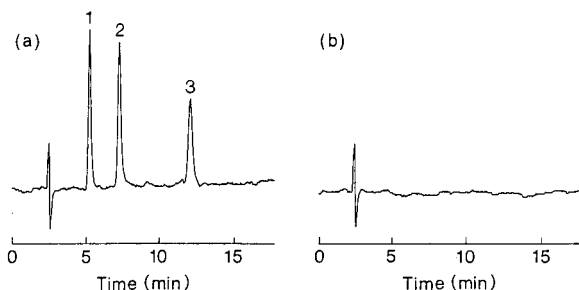


Fig. 1. Chromatogram of (a) a standard mixture containing 40 ng of each dye and (b) river water. Column, LiChrosorb RP-18, 7 μ m (25 cm $\times$ 4.0 mm I.D.); column temperature, 40°C; mobile phase, methanol–water (90:10, v/v); flow-rate, 1 ml/min; detection, 460 nm. Peaks: 1 = Methyl Yellow; 2 = Sudan I; 3 = Sudan II.

TABLE I

RECOVERY OF DYES FROM RIVER AND SEA WATERS

c.v. = Coefficient of variation ($n=5$).

Compound	River water			Sea water		
	Added (μg)	Mean recovery (%)	C.V. (%)	Added (μg)	Mean recovery (%)	C.V. (%)
Methyl Yellow	2	91.4	2.2	2	87.5	4.5
Sudan I	2	96.4	1.7	2	96.4	1.3
Sudan II	2	90.5	3.8	2	89.3	2.4

mum mobile phase composition for separation. Methanol-water (90:10) offered a good compromise between peak resolution and reasonably short analysis time. Fig. 1a illustrates the chromatogram.

Recovery experiments and detection limits

As shown in Table I, the recoveries from river and sea waters were over 87.5% with coefficients of variation below 5%.

The calibration graphs were linear in the ranges 4–80 ng for Methyl Yellow, 5–105 ng for Sudan I and 7–145 ng for Sudan II. The detection limits for a 500-ml water sample were 0.2 $\mu\text{g/l}$ for Methyl Yellow and Sudan I and 0.3 $\mu\text{g/l}$ for Sudan II.

Application

The proposed method was applied to several samples of river and sea waters. The dyes were not detected in any of the samples analysed and the chromatograms were free from interferences. A chromatogram of water from the Mikasa River in Fukuoka is shown in Fig. 1b.

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Note

Simultaneous high-performance liquid chromatographic analysis of oleic acid and simple glycerides of oleic acid

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Food analysts have tended to follow selectively those relevant applications developed by analysts in the biochemical, clinical, pharmaceutical and oleochemical fields, where interest has included blood lipids^{1,2}, steroids^{3,4}, prostaglandins^{5,6}, carotenoids^{7–10}, vitamins^{11–13} and technical emulsifiers^{14–17}.

In lipid analysis, ion-exchange high-performance liquid chromatography (HPLC) is seldom useful and high-performance gel permeation chromatography finds some applications. The most widely used mode of HPLC for lipid analysis, however, involves reversed-phase systems, where an hydrophobic stationary phase competes with an hydrophilic mobile phase for the lipid component.

Until recently, thin-layer chromatography (TLC) with flame ionization detection (FID) has been mainly used for quantification of fatty acids and their glycerides¹⁸. However, this technique has limitations such as poor resolution and artifact formation. On the other hand, the use of HPLC offers several advantages including high efficiency, selectivity and sensitivity. Although it was reported that oleic acid and its glycerides were analyzed by a refractive index detector¹⁹ or a moving-wire detector²⁰, the more sensitive UV detection has not been reported and it is necessary to analyze oleic acid and simple glycerides of oleic acid for our study of enzymatic glycerolysis.

Therefore, the purpose of this study was to develop a simple, sensitive and rapid procedure for the analysis of oleic acid, 1(3)-monoolein, 1,2-diolein, 1,3-diolein and triolein which are major components of glycerolysis carried out using triolein and glycerol as substrates and aimed at mono- or diolein production.

EXPERIMENTAL

Reagents

1(3)-Monoolein, 1,2- and 1,3-diolein (99.9%) were obtained from Sigma (St. Louis, MO, U.S.A.), oleic acid and triolein (99.9%) from Supelco (Bellefonte, PA, U.S.A.). Acetonitrile, isopropanol, hexane and chloroform of HPLC grade (Burdick & Jackson, Muskegon, MI, U.S.A.) were stored over a Type 4A molecular sieve and filtered prior to use.

TABLE I
GRADIENT PROGRAMME

Time (min)	Solvent A (%)	Solvent B (%)	Elution mode
0.00	100	0	Isocratic elution
14.00	100	0	Isocratic elution
17.00	40	60	Convex gradient
19.00	40	60	Isocratic elution
20.00	100	0	Linear gradient

Chromatography

The analysis of triolein, 1,3-diolein, 1,2-diolein, 1(3)-monoolein and oleic acid in chloroform was performed with a Waters Assoc. high-performance liquid chromatograph equipped with a Waters Model 510 pump and connected to a Waters 740 data module. The column used was a Z-module C_{18} and the operating conditions were as follows: elution solvent A, acetonitrile-isopropanol-hexane (50:3:2, v/v/v); elution solvent B, isopropanol-hexane (5:4 v/v), sample solvent, chloroform, flow-rate, 2.20 ml/min. The gradient programme is shown in Table I.

Absorption was measured at 214 nm by a Waters Model 441 absorbance detector. The quantitative analysis was performed with the single point calibration method using an external standard, the concentration of which was confirmed by a spectrophotometric measurement.

RESULTS AND DISCUSSION

TLC-FID analysis for the separation of lipids involves a time-consuming procedure of spotting, developing, drying and scanning. Compared to this method, on the HPLC column lipid mixtures were satisfactorily separated in less than 25 min, as shown in Fig. 1. Since a reversed-phase column (Z-module C_{18}) was used, the components were eluted in the order of decreasing polarity; 1(3)-monoolein, oleic acid, 1,3-diolein, 1,2-diolein, triolein. Each peak was identified by comparison of its retention time with that of the corresponding standard compound. The purity of each standard compound was greater than 95% as determined using the HPLC peak area. This typical chromatogram of lipids was reproducible. The reproducibility of the quantification of the components is presented in Table II.

1,2-Diolein has optical isomers which have been separated by TLC²¹ but they were not detected in our system.

Although the separation of 1,3-diglyceride and 1,2-diglyceride was done using a moving-wire detector by Aitzetmüller and Koch²⁰, the specific separation of 1,3-diolein and 1,2-diolein in this study should be noted and is the key part of a study of the glycerolysis reaction mechanism.

In conclusion, reversed-phase HPLC provides an efficient method for the separation and quantification of oleic acid, 1(3)-monoolein, 1,2-diolein, 1,3-diolein and triolein. Furthermore, the method may be extended to the study of enzyme reaction mechanisms.

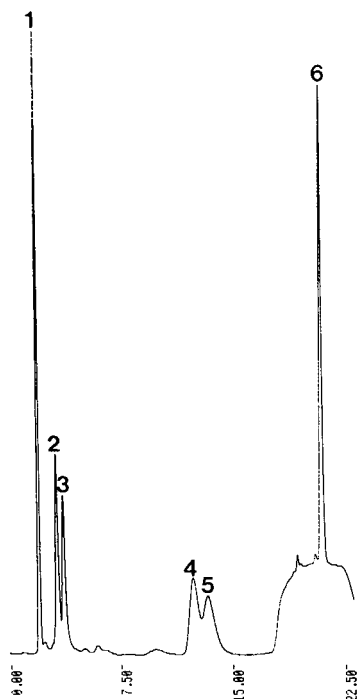


Fig. 1. HPLC analysis of lipid compounds. Concentrations (μmol) are as follows; 1(3)-monoolein, 1; oleic acid, 1; 1,2-diolein, 2; 1,3-diolein, 2; triolein, 1 in 5 μl of chloroform injected. Reaction conditions as described in Experimental. Peaks: 1 = chloroform; 2 = 1(3)-monoolein; 3 = oleic acid; 4 = 1,3-diolein; 5 = 1,2-diolein; 6 = triolein.

TABLE II

QUANTIFICATION OF THE PEAKS FOUND FOR TEN REPLICATE EXPERIMENTS
Conditions as described in Experimental.

Component	Retention time ^a (min)	Amount (μmol)	
		Actual	Found
1-Monoolein	3.24 ± 0.01	1.00	1.02 ± 0.03
Oleic acid	3.70 ± 0.02	1.00	1.04 ± 0.05
1,3-Diolein	12.29 ± 0.11	2.00	2.11 ± 0.10
1,2-Diolein	13.32 ± 0.09	2.00	2.07 ± 0.09
Triolein	21.04 ± 0.12	1.00	1.02 ± 0.04

^a Mean value $\pm$ standard deviation based on ten samples.

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Note

Quantitation of N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide and 2-amino-4-(5-nitro-2-furyl)thiazole in rodent diet

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Nitrofurans are one of the largest groups of heterocyclic nitro compounds available for human and veterinary medicinals and food additives¹. A number of these agents are known to cause cancer in a variety of animal species². Carcinogenesis is considered to be a multi-stage process and two separate events known as initiation and promotion are required for tumor production^{3,4}. N-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide (FANFT) is a potent carcinogen for rat bladder epithelium when fed in a diet at low levels³⁻⁵. It has been widely used as an initiator in studies of two-stage carcinogenesis using the rat bladder model⁶⁻⁸.

Multi-stage carcinogenesis studies in our laboratory involve the feeding of diets containing FANFT, and a method for quantitative analysis has been developed. In addition, a high-performance liquid chromatography (HPLC) method has been devised for the determination of 2-amino-4-(5-nitro-2-furyl)thiazole (ANFT), which is a contaminant produced by deformylation of FANFT. These methods for quantitation of FANFT and ANFT in the diet allow for the calculation of ingested doses of carcinogen from food consumption data, as well as determination of the extent of FANFT decomposition during preparation, storage, and feeding of the diet.

EXPERIMENTAL

ANFT was a gift from Dr. S. Swaminathan (University of Wisconsin, Madison, WI, U.S.A.). FANFT was obtained from Dr. G. T. Bryan (University of Wisconsin, Madison, WI, U.S.A.) and purified by sublimation by Dr. D. S. Williamson. It was blended at a level of 0.2% (w/w) into various diets (Prolab 3200, Agway, St. Marys, OH, U.S.A., and NIH-07, Ziegler Bros, Gardners, PA, U.S.A.) for the proposed study. Spectral-grade solvents [dichloromethane, dimethylformamide (DMF), nitromethane and light petroleum (b.p. 35–60°C)] were obtained from Midland Scientific (Omaha, NE, U.S.A.). Silica gel (60–200 mesh) for column chromatography was purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). All other materials were from commercial sources.

Pelleted diets were ground to a fine powder in a coffee grinder. Samples of powdered diet (*ca.* 1 g) were extracted with 5-ml aliquots of DMF and the insoluble material collected by centrifugation (10 min, 11 000 g). The supernatants were re-

moved and stored. After four successive extractions with DMF, the pooled supernatants were adjusted to a final volume of 25 ml by addition of DMF.

A small quantity of glass wool was placed into a 3-ml syringe barrel as support for silica gel packing material (630 mg) which was lightly tamped down. The column was conditioned by washing with two 3-ml aliquots of dichloromethane prior to application of diet extract (300 μ l) in DMF. The column was washed with 3 ml of light petroleum, and the nitrofurans eluted with nitromethane. This solution was adjusted to 5 ml with nitromethane and clarified by centrifugation (10 min, 11 000 g), prior to measuring the absorbance at 380 nm in a Perkin-Elmer Lambda 4B UV-VIS spectrophotometer. The amounts of nitrofuran present in the column eluate were determined by comparing the measured absorbance to a standard curve. The degradation of FANFT to ANFT was investigated by HPLC analysis of the DMF extract which was filtered (0.2- μ m 13-mm Nylon Acrodisk, Gelman Sciences, Ann Arbor, MI, U.S.A.) prior to injection (10- μ l aliquots). HPLC was performed on a Waters (Milford, MA, U.S.A.) system consisting of Model 510 pumps, a WISP 712 automated sample injector or U6K manual injector, temperature control unit and oven. A Waters end-capped C₁₈ column (15 cm $\times$ 0.39 cm I.D., 4 μ m particle size) was eluted isocratically at 65°C with 100 mM ammonium acetate-methanol (65:35), pH 6.0, at a flow-rate of 1.5 ml/min. The mobile phase was made using only HPLC-grade solvents and was filtered and degassed before use.

Nitrofurans were detected by using a Waters 481 UV-VIS spectrophotometer (380 nm; 0.001 a.u.f.s.) which was interfaced with a Hewlett-Packard Model 3390A integrator to provide on-line reporting of data. The absorbance wavelength was determined by use of a Perkin-Elmer Lambda 4B UV-VIS spectrophotometer. Fluorescence detection was also considered but use of the prescan facility on a Perkin-Elmer Model LS5B spectrofluorometer failed to provide data on excitation/emission maxima for the nitrofurans.

RESULTS

The standard curve for FANFT dissolved in nitromethane (10–60 μ g/ml) was linear to 40 μ g/ml (Fig. 1, top) and shows the upper limit of the assay. A second standard curve covering a lower range (0–13 μ g/ml) is also linear (Fig. 1, bottom) and shows the lower limit for detection of FANFT by absorbance to be 1.64 mg/ml. The binding capacity of the silica gel solid-phase extraction column was determined by placing varying amounts (100–800 μ l) of dietary extract (in DMF) on the columns (see Fig. 2). Binding was linear up to about 600 μ l of extract so 300 μ l of extract were applied to the solid-phase extraction column for routine analysis. The amount of nitrofuran in the NM extract was determined by interpolation from a standard curve constructed by solid-phase extraction of diet spiked with known amounts of FANFT. Extraction efficiency was determined by mixing 2 ml of FANFT (1 mg/ml in DMF) with 1 g of diet which was subject of extraction and chromatography as described above. The absorbance of the chromatographed standard was divided by that obtained from a non-chromatographed FANFT standard (4.8 μ g/ml in nitromethane); under these conditions recovery was 95–101%. The percentage of nitrofuran in the diet was calculated by dividing the determined value by the weight of diet extracted. The assay was accurate providing determined values of 0.180% $\pm$ 0.014 when 16

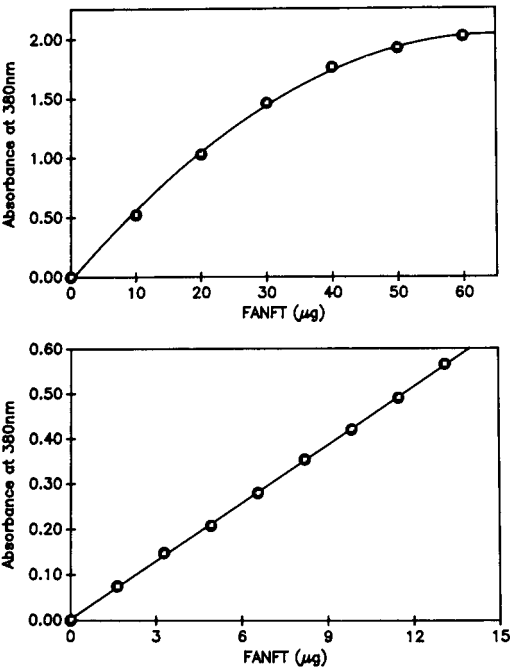


Fig. 1. Standard curves for FANFT in nitromethane. Top: 10–60 µg/ml; bottom: 0–13 µg/ml.

samples of diets containing 0.2% (w/w) FANFT were analyzed. Precision was 2.2% when one sample containing 0.2% (w/w) FANFT was subject to 10 repeat analyses ($0.205 \pm 0.005\%$). To determine the possible degradation of FANFT to ANFT, an HPLC method was employed since both nitrofurans exhibit maximum absorbance at 380 nm. The relative elution positions of FANFT and ANFT are shown in Fig. 3, and a standard curve for the quantitation of trace amounts of ANFT (5% or less of the total amount of nitrofuran injected) in FANFT is linear over the range 0–100 ng (Fig.

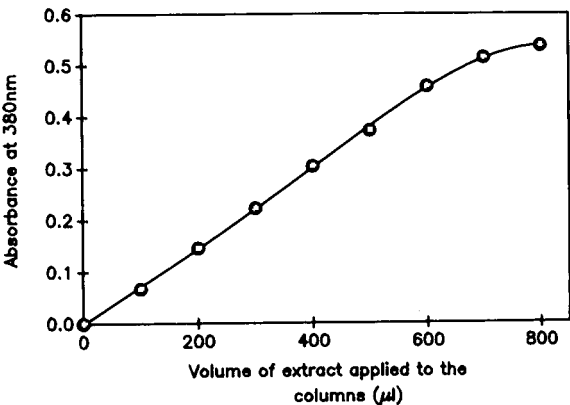


Fig. 2. Binding curve for DMF extract on the silica gel solid-phase extraction columns.

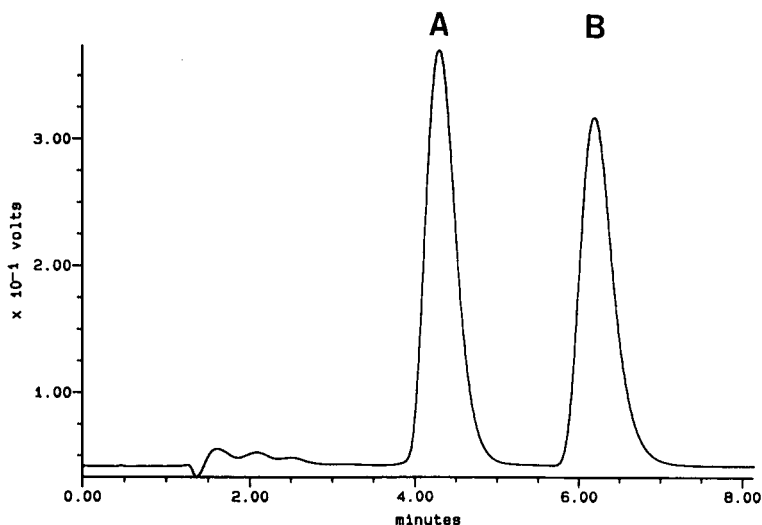


Fig. 3. HPLC analysis of a mixture of ANFT (peak A) and FANFT (peak B) to show relative elution position.

4). The lower limit for ANFT detection by HPLC was 4 ng and this amount corresponds to a contamination level of 0.5% when 10 μ l of DMF extract of diet containing 0.2% FANFT are chromatographed.

This HPLC method was used to determine the stability of FANFT in two diets, Prolab 3200 and NIH-07 (Table I). The amount of ANFT in the DMF extract was determined from the standard curve (Fig. 4) and was expressed as a percentage of the total amount of nitrofurantoin in the extract which had been determined by solid-phase extraction and spectrophotometric assay at 380 nm (Fig. 1). The amount of FANFT slowly decreases up to 10 weeks after blending and the amounts are equivalent in each of the diets.

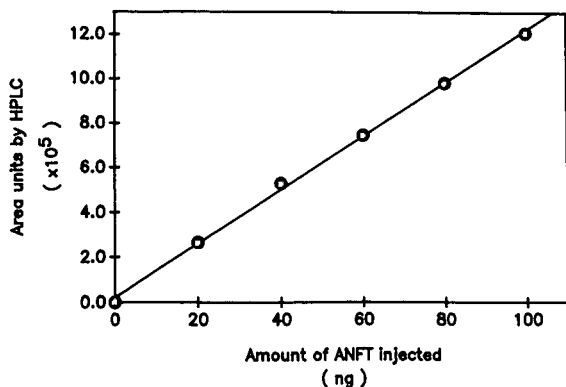


Fig. 4. Standard curve for the quantitation of ANFT (0–100 ng) in DMF extracts by HPLC. Each point is the mean of three injections.

TABLE I

HPLC ANALYSIS OF NITROFURANS IN RODENT DIETS AT VARIOUS TIMES AFTER BLENDING

Time (weeks)	Diet ^a			
	Prolab 3200		NIH-07	
	FANFT	ANFT	FANFT	ANFT
6	98.77	1.23	99.09	0.91
10	97.14	2.86	97.14	2.86

^a Amounts are expressed as a percentage of total nitrofurantoin injected.

DISCUSSION

Nitrofurans have been assayed by microbiological⁹ as well as chemical methods⁹⁻¹⁶. While the former are preferred for assessing potency they do not provide information on the purity of the agent and a chemical method is therefore preferred. Paper chromatography has been applied to the detection of many nitrofurans¹⁰ but the technique is qualitative and a number of quantitative assays for specific nitrofurans exist. Column chromatography on cationic Amberlite resin has been used in the isolation of nifurpione from urine prior to quantitation by colorimetric or spectrophotometric methods¹¹. Similar methods involving solvent extraction and formation of a chromophore have been reported for nitrofurantoin¹²⁻¹⁴. An HPLC method for nitrofurantoin requires solvent extraction and rotary evaporation prior to analysis on an octylsilane column¹⁵.

This method for the rapid extraction and sensitive quantitation of FANFT and its deformedylated derivative, ANFT, is based upon published methods¹⁶. The extremely low solubility of FANFT in aqueous media requires the use of an organic solvent, DMF, for the initial extraction prior to solid-phase extraction on silica gel columns. The wash with light petroleum during this adsorption chromatography allows for the removal of unknown contaminants which are soluble in DMF but not NM.

The quantitation of FANFT is based on the absorbance of the NM eluate at 380 nm and provides a rapid method for determining the amount of FANFT in a diet sample. Derivatization before detection was not necessary due to the strong absorbance of the nitro group at this wavelength. However, FANFT deformedylates to ANFT, which also absorbs at 380 nm and this spectrophotometric technique does not distinguish between the two nitrofurans. An HPLC method was, therefore, devised to quantitate trace amounts of ANFT (<5% of the total amount of nitrofurantoin) in the presence of FANFT. This was achieved by adapting a published procedure¹⁶ and increasing column temperature to 65°C and the flow-rate to 1.5 ml/min. This elevated temperature resulted in sharper peak profiles with reduced retention times and was found to greatly facilitate detection of ANFT in the nanogram range at high detector sensitivities (0.001 a.u.f.s.). Direct injection of a filtered DMF extract onto the HPLC allows for rapid quantitation of ANFT in an extract and is useful in determining the stability of FANFT in diets as well as the degree of contamination by ANFT. These data show that FANFT is stable in both diets (Table I) and that the diets contain equivalent amounts of ANFT at each time point.

ACKNOWLEDGEMENTS

We thank Dr. Daniel S. Williamson for purification of the FANFT; I. Ann Sysel, Jan Wehner, Martin Cano and Margaret St. John for preparation of the diets containing FANFT, and Emily Garland, Claudia Borgeson and Lori Davis for technical assistance. We are grateful for the secretarial assistance of Deborah Coleman in the preparation of this manuscript. This research was partially supported by USPHS grants CA28015, CA32513 and CA36727 and by a grant from the International Life Sciences Institute-Nutrition Foundation.

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Note

Separation of norepinephrine and metabolites as Dns derivatives by two-dimensional thin-layer chromatography

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As part of a continuing investigation of age-related changes in adrenergic chemical transmission at the cardiac neuroeffector system^{1,2} we desired to evaluate alterations in the metabolic fate of norepinephrine (NE). The isolated rat heart preparation perfused according to the method of Langendorff preloaded with exogenous tritium-labelled NE would provide the model system to assess metabolism both under basal conditions and during electrical stimulation of the isolated right cardiac sympathetic nerve. A reliable method to separate and isolate relatively low levels of [³H]NE and metabolites was necessary for these studies. Several previous studies^{3,4} employed a multiple-column chromatographic technique; others developed a thin-layer chromatographic (TLC) method employing isotopic dilution with unlabelled compounds separated on cellulose plates with colorimetric detection⁵. The latter technique fails to resolve the two acid metabolites [3,4-dihydroxymandelic acid (DOMA) and 3-methoxy-4-hydroxymandelic acid (VMA)] or the two glycols [3,4-dihydroxyphenylethylene glycol (DOPEG) and 3-methoxy-4-hydroxyphenylethylene glycol (MHPG)]. The separation of the three methoxy derivatives of NE, epinephrine and dopamine as their Dns derivatives via TLC⁶ led us to attempt a similar separation of NE and its five metabolites as Dns derivatives. The impressive work of Seiler and Wiechmann⁷ on separation of Dns derivatives of biogenic amines and selected metabolites provided a useful starting place for the assay described in this report.

EXPERIMENTAL

Materials

NE, normetaphrine (NMN), DOMA, VMA, DOPEG, MHPG, 5-N,N-dimethylamino naphthalenesulfonyl chloride (Dns-Cl), and high-performance silica gel on glass TLC plates (200 μ m layer thickness, 20 $\times$ 20 cm), were from Sigma (St. Louis, MO, U.S.A.).

TLC development chambers, solvents and other chemicals were from Fisher Scientific (King of Prussia, PA, U.S.A.); the latter were used as supplied. Fluorescent spots were visualized by long-wavelength ultraviolet (UV) light (365 nm) (Mineral light lamp UVS1-25, Ultraviolet Products, San Gabriel, CA, U.S.A.).

Methods

Formation of Dns derivatives. NE and the five metabolites were added singly and in combination to 10 ml deionized water (50–500 μg each/10 ml). Dns derivatization was accomplished basically by the method of ref. 5 using saturating Na_2CO_3 and adding 400 μl of a solution of Dns-Cl in acetone (400 $\mu\text{g}/\text{ml}$). Samples were left in the dark at room temperature overnight.

Extraction of Dns derivatives. Acetone was removed from the samples via a dry air stream, and the pH was lowered to 5–5.5 using concentrated hydrochloric acid. A 2-ml volume of ethyl acetate were added and the mixture shaken. The ethyl acetate layer was removed and dried using an air stream. The residue was redissolved in 200 μl acetonitrile.

TLC conditions. A 20- μl volume of the acetonitrile solution was spotted in one corner of a silica TLC plate. After drying, the plate was developed in the first direction with ethyl acetate–chloroform (2:1, v/v), dried and developed in the second direction with benzene–chloroform–methanol–5 *M* acetic acid (70:20:20:1, v/v). Alternatively the plate was irradiated with long-wavelength UV light (365 nm) for 3 min before development. For both, fluorescent spots were localized under UV light and circled.

RESULTS AND DISCUSSION

Table I contains the R_f values for NE and its metabolites in the TLC system described. It was found that lowering the pH of the reaction mixture before extraction improved the extraction efficiency for the two acid metabolites. With the concentration examined (50–500 μg of each compound), all six derivatives were easily visualized after spotting 20 μl of the acetonitrile solution in samples containing 250 $\mu\text{g}/10$ ml or greater. In the 100 $\mu\text{g}/\text{ml}$ and lesser samples, the mono-Dns compounds (VMA, MHPG) were not discernable. Blank samples treated in the same fashion showed no fluorescent spots other than at the origin and at the solvent front.

TABLE I

SEPARATION OF Dns DERIVATIVES BY TWO-DIMENSIONAL TLC

Solvent 1: ethyl acetate–chloroform (2:1, v/v); solvent 2: benzene–chloroform–methanol–5 *M* acetic acid (70:20:20:1, v/v).

Compound	R_f value			
	Before irradiation		After irradiation	
	Solvent 1	Solvent 2	Solvent 1	Solvent 2
Dns-Norepinephrine	0.81	0.72	0.80	0.57
Dns-Normetaphrine	0.81	0.65	0.80	0.65
Dns-3,4-Dihydroxyphenylethylene glycol	0.41	0.43	0.40	0.74
Dns-3-Methoxy-4-hydroxyphenylethylene glycol	0.33	0.48	0.33	0.48
Dns-3,4-Dihydroxymandelic acid	0.03	0.19	0.05	0.31
Dns-3-Methoxy-4-hydroxymandelic acid	0.00	0.31	0.00	0.31

Frei *et al.*⁸ have described the photochemical degradation of tri-Dns-epinephrine (adrenaline) and ascribed the reactivity to steric strain between the 3,4-bis-Dns-phenolic groups. As phenolic di-Dns derivatization could occur in NE, DOMA and DOPEG we examined the effect of UV light on R_F values by reacting each of the six compounds individually. After spotting, the TLC plate was irradiated for 3 min by long-wavelengths UV light to allow ample time for the photochemical reaction to go to completion. Development of the plates and visualization showed that the R_F values of the dihydroxy compounds were altered while those with the 3-methoxy-4-hydroxy nucleus were unchanged (Table I). Performing the reaction in the dark and working up the samples with minimal light exposure is thus important for these compounds.

The foregoing describes a straightforward method for separating norepinephrine and its metabolites. The method is currently being employed to assess age-related changes in rat heart NE metabolism. The results of these studies will be reported subsequently.

ACKNOWLEDGEMENTS

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Book Review

Practical high-performance liquid chromatography, by V. R. Meyer, Wiley, Chichester, New York, Brisbane, Toronto, Singapore, 1988, 310 pp., price US\$ 57.50, ISBN 0-471-91140-2.

In 25 chapters, high-performance liquid chromatography (HPLC) is dealt with comprehensively and from a practical point of view, including theoretical aspects, equipment design, stationary phases, separation mechanisms and analytical and preparative HPLC, and with specialized chapters on, *e.g.*, enantiomer separations and a very brief survey of prospective methods. Review papers concerned with the separation of different classes of compounds are listed, as also are the most important stationary phases that are commercially available, according to separation mechanisms and specific applications.

The book, an English translation of the fifth, updated German edition (which appeared in 1988), constitutes an introduction to practical HPLC work for the beginner. Although the compilation of so much experimental experience is a difficult task, especially when aiming at succinct presentation, this has been well achieved.

It would have been profitable to the target group for whom this book was written if more appropriate chromatograms had been chosen from the literature. Fig. 5.12 (p. 77) is an example to shock even the experienced reader, and well behind today's state-of-the-art.

The problem-solution approach that has been followed throughout most chapters is an adequate complement to the running text.

With its comprehensive review of basic concepts and current techniques in HPLC, this book will provide valuable reading for the beginner.

Innsbruck (Austria)

G. BONN

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Erratum

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Page 90, legend to Fig. 5, “(a) 1.96 ml/h; (b) = 3(a); (c) = 10(a); (d) = 15(a)” should read “(d) 1.96 ml/h; (c) = 3(d); (b) = 10(d); (a) = 15(d)”.

journal of chromatography news section

SYMPOSIUM PROGRAMME

PREP '89, 6th INTERNATIONAL SYMPOSIUM ON PREPARATIVE CHROMATOGRAPHY, WASHINGTON, DC, U.S.A., MAY 8-10, 1989

The 6th International Symposium on Preparative Chromatography will be held at the Sheraton Washington Hotel, in the heart of Washington, DC, U.S.A., from May 8 to 10, 1989. Registration information and full details of the symposium can be obtained from: Janet Cunningham, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, U.S.A., tel.: (301) 898-3772; fax: (301) 898-5596. The detailed programme of the symposium is given below.

— Chairman —
Dr. Georges Guiochon
*University of Tennessee and
Oak Ridge National Laboratory*

MONDAY, MAY 8, 1989

- 8:00 Registration**
9:00 Interference Phenomena and Mass Transfer in Large Scale Liquid Chromatography
L. WANG
Purdue University, West Lafayette, IN
9:30 The Theory of Optimization of the Experimental Conditions of Preparative Elution Using the Ideal and Semi-Ideal Models of Chromatography
S. GOLSHAN-SHIRAZI, G. Guiochon
University of Tennessee, Knoxville, TN
10:00 Product Purity as a Function of Elution Order
J.A. PERRY, J.D. Rateike
Regis Chemical Co., Morton Grove, IL
10:30 Pause
11:00 Preparative Purification of Large Biomolecules by Reversed-Phase Gradient Elution. Experimental and Computer-Modeling Studies
L.R. SNYDER, J.W. Dolan, G.B. Cox*
*LC Resources, Lafayette, CA,
E I duPont de Nemours, Glasgow, DE

- 11:30** The Utility of Sample Self-Displacement as a Routine Preparative Separation Technique
J. NEWBURGER, G. Guiochon*
*Squibb Institute for Medical Research, Princeton, NJ,
University of Tennessee, Knoxville, TN
12:00 Experimental and Theoretical Investigation of Overload Phenomena in Reversed Phase High Performance Liquid Chromatography
J.E. WADE, C. Lucy, P.W. Carr
University of Minnesota, Minneapolis, MN
12:30 Pause
2:00 POSTER SESSION
3:00 Recent Advances in the Theory and Practice of Displacement Chromatography
S.M. CRAMER, G. Subramanian, M.W. Phillips
Rensselaer Polytechnic Institute, Troy, NY
3:30 Mass Overload in Liquid Chromatography: A Theoretical and Experimental Approach
A.M. KATTI, G. Guiochon
University of Tennessee, Knoxville, TN
4:00 Efficient Preparative-Scale Chiral Separations on Cyclodextrin-Silica Columns Using Displacement Chromatography
G. Quintero, Gy. Farkas, A. Bartha, GY. VIGH
Texas A&M University, College Station, TX
4:20 Experimental Investigation of Displacement Effects in Isocratic and Gradient Mass-Overloaded Liquid Chromatography
G.B. COX, B.J. Permar, L.R. Snyder*
*E I duPont de Nemours, Wilmington, DE,
LC Resources, Orinda, CA

- 4:40 Studies on the Adsorption of Peptides and Proteins to Silica Gel: Utilization in Various Purification Strategies
J.M. DI BUSSOLO, C. Xanthopoulos*
*PQ Corporation, Lafayette Hill, PA,
West Chester State University, PA
- 5:00 **Pause**
- 5:30 **DISCUSSION SESSION I —**
Importance of Efficiency in Preparative Scale Liquid Chromatography
- 6:30 **Adjourn**
- 7:00 **Mixer**

TUESDAY, MAY 9, 1989

- 9:00 An Electron Micrographic Examination of Chromatographic Packings Used in Protein Separations
F.E. REGNIER, V.B. Yang
Purdue University, West Lafayette, IN
- 9:30 Preparative Isolation of Proteins from Plasma Membranes of Different Rat Organs
D. JOSIC, M. Raps, Y.-P. Lim, W. Hofmann, W. Reutter, J. Reusch*
*Freien Universität Berlin, WEST GERMANY,
Knauer Saulentechnik, Berlin, WEST GERMANY
- 10:00 Structural Characterization of Native Membrane Glycoproteins with Sequential High Performance Liquid Affinity Chromatography
D. RENAUER, F. Oesch, K.K. Unger, R.J. Wieser
Johannes Gutenberg-Universität, Mainz, FRG
- 10:30 **Pause**
- 11:00 Preparative Protein Purification: Prospectives, Problems and Processes
A. Velayudhan, A. Lee, F. Antia, CS. HORVATH
Yale University, New Haven, CT
- 11:30 Multi-Component Adsorption of Proteins to Ion-Exchangers
H.A. CHASE, G.L. Skidmore, B.J. Horstmann
University of Cambridge, ENGLAND
- 12:00 Protein Purification by Affinity Chromatography on Novel Immobilised Dye Adsorbents
S.J. BURTON
Affinity Chromatography Ltd., Cambridge, UK
- 12:30 **Pause**
- 2:00 **POSTER SESSION**
- 3:00 Chromatographic Engineering: Rational Purification Schemes for Antibody Isolation
S. FULTON, W. Kopaciewicz, P. O'Neil, S. Cameron
Amicon Division, W.R. Grace, Danvers, MA
- 3:30 Large Scale Synthesis and RP-HPLC Purification of a Gonadotropin Releasing Hormone (GnRH) Antagonist
C. HOEGER, J. Porter, C. Miller, D. Kirby, J. Rivier
The Clayton Foundation Laboratories for Peptide Biology, La Jolla, CA
- 4:00 The Multi-Coil Countercurrent Chromatograph for the Preparative Purification of Peptides
M. KNIGHT, S. Gluch, T. Dang, Y. Ito*
*Peptide Technologies Corporation, Washington, DC,
National Institutes of Health, Bethesda, MD
- 4:20 Preparative Chiral Liquid Chromatography of prostaglandin Precursors
L. MILLER, H. Bush, E.M. Derrico
G.D. Searle, Skokie, IL

- 4:40 Methods Development for the Preparative High Performance Liquid Chromatographic Separation and Purification of 3'-Azido-3'-Deoxythymidine-5'-Phosphate
J.G. Burcotte, N.-I. Jang, P.E. Pivarnik, H.H. Singh, D.E. MacBride*, P.R. BROWN
*University of Rhode Island, Kingston, RI,
Separations Technology, Wakefield, RI
- 5:00 **Pause**
- 5:30 **DISCUSSION SESSION II —**
Strategies for Optimization of Purifications
- 6:30 **Adjourn**

WEDNESDAY, MAY 10, 1989

- 9:00 Some Considerations on Axial Compression Columns for Preparative Liquid Chromatography
H. COLIN, P. Hilaireau
Prochrom SA, Champigneulle, FRANCE
- 9:30 Comparison of Fractionation Costs by Prep-HPLC and Prep-SFC
C. Berger, R.C. Nicoud*, M. PERRUT
*Lab. Physico-Chimie Industrielle, ENSIC, Nancy, FRANCE, *Separex, Champigneulle, FRANCE*
- 10:00 Aspects of Production Scale Chromatography
O. KAPPELI
Bideco AG, Baden, SWITZERLAND
- 10:30 **Pause**
- 11:00 Strategies for Optimization of High Performance Process Chromatography
R.D. SITRIN, P. DePhillips
Merck Sharp and Dohme Research Labs, West Point, PA
- 11:30 Modelling Methods to Aid the Design and Optimisation of Batch Stirred Tank and Packed-Bed Column Adsorption and Chromatographic Units
G.H. COWAN, I.S. Gosling, W.P. Sweetenham
BIOSEP Biotechnology Group, Oxon, UK
- 12:00 Scale-Up and Optimization in Production Liquid Chromatography
A.M. WILHELM
ENSIGC, Toulouse, FRANCE
- 12:30 **Pause**
- 2:00 Preparative Reversed-Phase Chromatography of a Natural Product Produced by Fermentation
G.T. CARTER
American Cyanamid Company, Pearl River, NY
- 2:20 Strategies for the Preparative Scale Isolation of Kadsurenone and Futoquinol from the Medicinal Plant Piper Futokadsura
M.P. STRICKLER, M.J. Stone, A.S. Kennington, D.M. Goldstein*
*Waters Chromatography Div. of Millipore, Fairfax, VA,
University of Virginia, Charlottesville, VA
- 2:40 A Novel Chromatographic Method for the Purification of Therapeutic-grade Monoclonal Antibodies
D.R. NAU
J.T. Baker, Phillipsburg, NJ
- 3:00 **Pause**
- 3:30 **DISCUSSION SESSION III —**
Costs of Preparative Liquid Chromatography Purifications
- 5:00 **Closing**

Poster Presentations

Investigations on the Use of Fibrous Beds for the Chromatographic Separation of Proteins—**A.E. Osawa, C.L. Cooney**, *Massachusetts Institute of Technology, Cambridge, MA*

New Column Design for Preparative Chromatography Utilizing Radial Compression Technology—**B. Bell-Alden**, *Waters Chromatography Division, Milford, MA*

Multi-Column Switching for Improved Throughput in Preparative HPLC—**T.M. Finn, G.D. Mack**, *Varex Corporation, Rockville, MD*

A New Look at Preparative Gas Chromatography—**T.M. Finn, G.D. Mack**, *Varex Corporation, Rockville, MD*

Large Scale Purifications on a C3 Bonded 300 Å Silica Packing—**R.C. Williams, R. Lehrer, C.D. Belles, D.M. Knapp**, *El DuPont de Nemours, Wilmington, DE*

The Utility of Very High Porosity Media in Preparative Chromatography of Proteins—**I. Mazsaroff, N.B. Afeyan, R.C. Dean, Jr.**, *Synosys Corporation, Cambridge, MA*

The Influence of Column Packing Characteristics on Preparative HPLC Throughput—**N.T. Miller, J.M. Di Bussolo, C. Xanthopoulos***, *PQ Corporation, Lafayette Hill, PA, *West Chester University, PA*

Optimization of the Hydrophobic Interaction Chromatographic Purification of Monoclonal Antibodies—**D.R. Nau, J.T. Baker**, *Phillipsburg, NJ*

Method Development and Scale-Up of Antibody Purification—**D.R. Nau, J.T. Baker**, *Phillipsburg, NJ*

Preparative Liquid Chromatography of Basic Compounds on Sodium Hydroxide Modified Silica Gel—**D. Honda, G.D. Searle**, *Research and Development, Skokie, IL*

Hydrolysis and Extraction of Bonded Phase From C4 Reverse Phase Preparative HPLC Materials—**C.D. Carr, K. Harrison**, *Separations Group, Hesperia, CA*

Porous Polymeric Packings for Production Chromatography—**P.G. Cartier, K.J. King, J.J. Maikner**, *Rohm and Haas Research Laboratories, Spring House, PA*

Preparative HPLC Purification of Proteins by Reversed Phase Chromatography—**M.P. Nowlan, M.N. Schmuck, D.L. Gooding, K.M. Gooding**, *SynChrom, Lafayette, IN*

Practical Aspects of Scale-Up Separations of Synthetic Peptides on Preparative High Performance LC Columns—**R.S. Cooley, R. Meyer, M. Knight*, S. Gluch***, *YMC, Morris Plains, NJ, *Peptide Technologies Corporation, Washington, DC*

Preparative Reverse Phase HPLC in the Synthesis of Viscosin, a Cyclic Dipeptide—**T.R. Burke, Jr., B. Chandrasekhar**, *Peptide Technologies Corporation, Washington, DC*

Microbiological Sanitization of Process Scale Chromatography Systems—**T. Pettersson, N. Adner, S. Doversten**, *Pharmacia AB, Uppsala, SWEDEN*

Isolation of Virus-Safe Transferrin by Preparative High Performance Ion-Exchange Chromatography—**D. Rudnick, N. Kothe, U. Bethke, M. Kloft, W. Moller**, *BIOTEST Pharma GmbH, Dreieich, FRG*

Use of Dyes Immobilized on Dextran Coated Silica for Purification of Proteins of Human and Plant Origin—**Y. Krowiarski, E. Algiman, S. Cochet, X. Santarelli, D. Muller, D. Dherny, O. Bertrand**, *INSERM, Cligny, FRANCE, and LRM, Villeteuse, FRANCE*

High Speed Affinity Chromatography: Measurement of Antibody-Receptor Capture Kinetics—**R. Hammen, C. Cook, D. Pang, H. Thompson**, *ChromatoChem, Missoula, MT*

Evaluation of an HPLC Affinity Support for the Preparative Purification of Antibodies—**T. McIntosh, A. Nazareth**, *Carter-Wallace, Cranbury, NJ*

Purification of Nitrophenylvaleric Acid Reaction Mixtures by Counter-current Chromatography—**W.D. Conway, J.D. Klingman, D. Greco, K. Huh**, *State University of New York at Buffalo, Amherst, NY*

Preparative and Automated Compound Class Separation of Venezuelan Vacuum Residue by High Performance Liquid Chromatography—**L. Carbognani, A. Izquierdo**, *INTEVEP SA, Caracas, VENEZUELA*

Purification of Fatty Acid Esters by Prep-HPLC—**R.M. Nicoud, M. Perrut***, *Separex, Champigneulle, FRANCE, *ENSIC, Nancy, FRANCE*

Isolation and Purification of Lecithin by High Performance Liquid Chromatography Using an Annular Expansion Preparative Column—**J.V. Amari, C.M. Grill*, D. MacBride*, P.R. Brown**, *University of Rhode Island, Kingston, RI, *Separations Technology, Wakefield, RI*

Efficient Preparative-Scale Chiral Separations on Pirkle-Type Columns Using Displacement Chromatography—**G. Quintero, Gy. Farkas, A. Bartha, Gy. Vigh**, *Texas A&M University, College Station, TX*

Preparative Scale Separations of Enantiomers by Chiral Chromatography—**M.P. Henry, J.T. Baker**, *Phillipsburg, NJ*

Purification of a Cephalosporin Analytical Reference Standard Using Preparative HPLC With Automated Column Switching—**B. Kaluzny, B. Snider, M. Dunn**, *Upjohn Company, Kalamazoo MI*

Binding Aluminate to Silica by Sonication Technique: The Use of Silica Bonded Aluminate in Isomerisation of Glucose to Fructose—**Y. Ali, M.S. Maysara**, *Agricultural College, Abu-Ghraib, IRAQ*

Purification of Lanosterol by Normal-Phase Chromatography in the Displacement Mode—**J.M. Di Bussolo, N.T. Miller**, *PQ Corporation, Lafayette Hill, PA*

Purification of Radiolabeled Pharmaceuticals—**C. Baker, C. Bowlen, D. Koharski, P. McNamara**, *Schering Plough Corporation, Bloomfield, NJ*

Scale Up from Analytical to Preparative Chromatography Using Chiral Stationary Phases—**J.N. Kinkel, K. Reichert, E. Merck**, *Darmstadt, WEST GERMANY (presented by P.A. Sienkiewicz, EM Science, Gibbstown, NJ)*

Isolation, Identification and Separation of Isomeric Truxillines in Illicit Cocaine—**I.S. Lurie, T.C. Kram, D. Cooper, J. Moore**, *Drug Enforcement Administration, McLean, VA*

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PUBLICATION SCHEDULE FOR 1989

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

MONTH	J	F	M	A	M	J	
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Bibliography Section		486/1		486/2		486/3	
Biomedical Applications	487/1	487/2	488/1 488/2	489/1 489/2 490/1	490/2	491/1	

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.)

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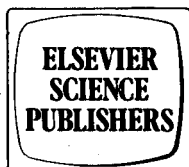
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