ISSN 0021-9673

VOL. 636 NO. 2 APRIL 30, 1993

THIS ISSUE COMPLETES VOL. 636

JOURNAL OF CHROMATOGRAPHY

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

ELSEVIER

JOURNAL OF CHROMATOGRAPHY

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

Scope. The *Journal of Chromatography* publishes papers on all aspects of **chromatography, electrophoresis** and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental developments and their applications. The section *Biomedical Applications*, which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis or alterations during medical treatment; screening and profiling of body fluids or tissues related to the analysis of active substances and to metabolic disorders; drug level monitoring and pharmacokinetic studies; clinical toxicology; forensic medicine; veterinary medicine; occupational medicine; results from basic medical research with direct consequences in clinical practice. In *Symposium volumes*, which are under separate editorship, proceedings of symposia on chromatography, electrophoresis and related methods are published.

Submission of Papers. The preferred medium of submission is on disk with accompanying manuscript (see *Electronic manuscripts* in the Instructions to Authors, which can be obtained from the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, Netherlands). Manuscripts (in English; *four* copies are required) should be submitted to: Editorial Office of *Journal of Chromatography*, P.O. Box 681, 1000 AR Amsterdam, Netherlands, Telefax (+31-20) 5862 304, or to: The Editor of *Journal of Chromatography, Biomedical Applications*, P.O. Box 681, 1000 AR Amsterdam, Netherlands. Review articles are invited or proposed in writing to the Editors who welcome suggestions for subjects. An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation. Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere. For copyright regulations, see below.

Publication. The *Journal of Chromatography* (incl. *Biomedical Applications*) has 40 volumes in 1993. The subscription prices for 1993 are:

J. Chromatogr. (incl. *Cum. Indexes, Vols. 601–650*) + *Biomed. Appl.* (Vols. 612–651):
Dfl. 8520.00 plus Dfl. 1320.00 (p.p.h.) (total *ca.* US$ 5622.75)

J. Chromatogr. (incl. *Cum Indexes, Vols. 601–650*) only (Vols. 623–651):
Dfl. 7047.00 plus Dfl. 957.00 (p.p.h.) (total *ca.* US$ 4573.75)

Biomed. Appl. only (Vols. 612–622):
Dfl. 2783.00 plus Dfl. 363.00 (p.p.h.) (total *ca.* US$ 1797.75).

Subscription Orders. The Dutch guilder price is definitive. The US$ price is subject to exchange-rate fluctuations and is given as a guide. Subscriptions are accepted on a prepaid basis only, unless different terms have been previously agreed upon. Subscriptions orders can be entered only by calender year (Jan.–Dec.) and should be sent to Elsevier Science Publishers, Journal Department, P.O. Box 211, 1000 AE Amsterdam, Netherlands, Tel. (+31-20) 5803 642, Telefax (+31-20) 5803 598, or to your usual subscription agent. Postage and handling charges include surface delivery except to the following countries where air delivery via SAL (Surface Air Lift) mail is ensured: Argentina, Australia, Brazil, Canada, China, Hong Kong, India, Israel, Japan*, Malaysia, Mexico, New Zealand, Pakistan, Singapore, South Africa, South Korea, Taiwan, Thailand, USA. *For Japan air delivery (SAL) requires 25% additional charge of the normal postage and handling charge. For all other countries airmail rates are available upon request. Claims for missing issues must be made within six months of our publication (mailing) date, otherwise such claims cannot be honoured free of charge. Back volumes of the *Journal of Chromatography* (Vols. 1–611) are available at Dfl. 230.00 (plus postage). Customers in the USA and Canada wishing information on this and other Elsevier journals, please contact Journal Information Center, Elsevier Science Publishing Co. Inc., 655 Avenue of the Americas, New York, NY 10010, USA, Tel. (+1-212) 633 3750, Telefax (+1-212) 633 3764.

Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Chromatography Abstracts, Current Awareness in Biological Sciences (CABS), Current Contents/Life Sciences, Current Contents/Physical, Chemical & Earth Sciences, Deep-Sea Research/Part B: Oceanographic Literature Review, Excerpta Medica, Index Medicus, Mass Spectrometry Bulletin, PASCAL-CNRS, Referativnyi Zhurnal, Research Alert and Science Citation Index.

US Mailing Notice. *Journal of Chromatography* (ISSN 0021-9673) is published weekly (total 52 issues) by Elsevier Science Publishers (Sara Burgerhartstraat 25, P.O. Box 211, 1000 AE Amsterdam, Netherlands). Annual subscription price in the USA US$ 4573.75 (subject to change), including air speed delivery. Application to mail at second class postage rate is pending at Jamaica, NY 11431. **USA POSTMASTERS**: Send address changes to *Journal of Chromatography*, Publications Expediting, Inc., 200 Meacham Avenue, Elmont, NY 11003. Airfreight and mailing in the USA by Publications Expediting.

See inside back cover for Publication Schedule, Information for Authors and information on Advertisements.

0021-9673/93/$06.00

Printed in the Netherlands

CONTENTS

(Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Chromatography Abstracts, Current Awareness in Biological Sciences (CABS), Current Contents/Life Sciences, Current Contents/Physical, Chemical & Earth Sciences, Deep-Sea Research/Part B: Oceanographic Literature Review, Excerpta Medica, Index Medicus, Mass Spectrometry Bulletin, PASCAL-CNRS, Referativnyi Zhurnal, Research Alert and Science Citation Index)

REGULAR PAPERS

Column liquid chromatography

Gas chromatography

Supercritical fluid chromatography

Journal of Chromatography, 636 (1993) 189–202
Elsevier Science Publishers B.V., Amsterdam

CHROM 24 869

The shock layer thickness, a new approach to the study of column performance in non-linear chromatography

I. Optimum linear velocity in frontal analysis

Jie Zhu and Georges Guiochon*

Department of Chemistry, University of Tennessee, Knoxville, TN 37996-1600 (USA) and Division of Analytical Chemistry, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6120 (USA)

(First received July 22nd, 1992; revised manuscript received December 16th, 1992)

ABSTRACT

In non-linear chromatography, it is common to observe very steep profiles. This happens for overloaded elution bands, for frontal analysis breakthrough curves and for the band profiles of the isotachic train in displacement chromatography. These regions where the concentration vary very rapidly are called shock layers. The relationship between the thickness of the shock layer in frontal analysis and the coefficients of the conventional terms of the plate height equation were studied experimentally. The shock layer theory of Rhee and co-workers permits the simple determination of the optimum linear velocity for minimum shock layer thickness in the case when the adsorption behavior of the feed components is described by the Langmuir model. The optimum linear velocity in frontal analysis is not only a function of the coefficients of the axial dispersion and the mass transfer resistance terms, and the retention factor (k'_0), as in linear chromatography, but also a function of the plateau concentrations and the second Langmuir parameter of the isotherm, b. Depending on the retention factor, the optimum velocity in frontal analysis may be larger, but is most often much smaller than in linear chromatography. Experimental results are in excellent agreement with the prediction of the theory. If they could be extended to displacement chromatography, these findings would explain some apparent contradictions found in the literature regarding the influence of the mobile phase flow velocity on the degree of separation between bands achieved in displacement chromatography, and clarify certain controversies.

INTRODUCTION

In spite of a number of investigations [1–12], there is still a profound misunderstanding of the exact influence of the column efficiency on the band profiles in non-linear chromatography. Some workers are still mistaking the effects of thermodynamics caused by the non-linear behavior of the isotherm for a source of band broadening similar to axial dispersion [1,2]. Others have attempted to contrive empirical approaches which lack a fundamental background and have failed [2–4]. Knox and Pyper [5] suggested calculating the band width in non-linear chromatography by using the rule of variance additivity and applying it to two independent contributions, of kinetic and thermodynamic origin, respectively. The former contribution is derived from the column efficiency, the latter from an approximate solution of the ideal model [5]. This procedure gives a reasonable estimate of the band width [6–8] and a very good approximation of the profile of the dispersive boundary of the profile. However, it is not correct because the convolution of the thermo-

* Corresponding author. Address for correspondence: Department of Chemistry, University of Tennessee, Knoxville, TN 37996-1600, USA.

0021-9673/93/$06.00

dynamic band profile by an axial dispersion is a shift-variant convolution [9], and the rule of variance additivity does not apply. It would fail entirely if it were used to predict the steep parts of band profiles.

We know that when the adsorption isotherm is convex upwards the band profile has a steep front and a rear diffuse boundary. (The converse is true for a convex downwards isotherm; we shall not discuss here this infrequent case.) The mechanism of the formation of such a profile was elucidated long ago [13,14], and the work done in this area was reviewed recently [10,11]. The formation of a shock in the case of an infinitely efficient column is easily explained [10–14]. When the column has a finite efficiency, the concentration discontinuity is relaxed and replaced by a steep front which is also a constant pattern, *i.e.*, propagates at the same velocity as the shock, without changing shape, and most notably without broadening [15,16]. Similarly, in frontal analysis the breakthrough curve has a very steep front, and in displacement chromatography the boundary between two successive zones is very steep. These steep boundaries are called shock layers. The theory of shock layers has been reviewed by Vermeulen *et al.* [16], who pioneered its development [17]. The simplest and most useful model has been derived and studied by Rhee and co-workers [18–21]. This theory results in a simple expression for the shock layer thickness (SLT) in the case of Langmuir adsorption behavior [18,22,23].

We present here the results of a theoretical and experimental investigation of the dependence of the SLT in frontal analysis on the two main parameters which control it, the mobile phase velocity and the height of the concentration step injected into the column. At this stage, the shock layer theory applies only in cases where a constant state, *i.e.*, a constant concentration, is achieved behind the shock layer, and thus the ideal shock would move at a constant velocity. This is the case in frontal analysis, after the time needed for the constant state to be established [22]. This is also the case in displacement chromatography, when the isotachic train is formed, but the theory of shock layer in two-component cases is more complex [21], and this topic will be discussed in a future paper [24]. However, our present results and conclusions cannot be extended directly to the study of band profiles in overloaded elution.

The determination of SLT is a new approach to the study of column performance in non-linear chromatography, which is valid when the adsorption behavior of the components considered is closely enough approximated by the Langmuir model. There is in this instance a simple relationship between the SLT and the column height equivalent to a theoretical plate (HETP). Thus, we can derive simply the optimum linear velocity for minimum SLT, u_{opt}^{S}, which is related to the optimum velocity for minimum HETP, u_{opt}^{L}. The SLT, SLT_{min} and u_{opt}^{S} will play in frontal analysis (and probably also in displacement chromatography [24]) a role similar to that of H, H_{min}, and u_{opt}^{L} in elution chromatography.

Although constant pattern behavior, the characteristics of the breakthrough curves and the thickness of shock layers have been actively studied in chemical engineering [13–21], there has been little application of the shock layer theory so far in chromatography regarding the optimization of the experimental conditions in order to improve the steepness of the breakthrough curves in single-component frontal analysis. This is not surprising, as these fronts are already very steep anyway; the only practical use of frontal analysis is in the determination of the retention time of the inflection point of the breakthrough curve, to calculate the integral mass balance of adsorption in the column; in practice, the accuracy of this determination does not depend much on the front steepness. As shown previously, this is not entirely true in two-component frontal analysis [22].

It is more surprising that there has been as little investigation regarding the optimization of the experimental conditions to minimize the SLT in displacement chromatography and to improve zone separation in the isotachic trains. We know that the side profiles of these zones cannot be vertical, as predicted by the ideal model. Because of the axial dispersion and the finite rate of mass transfer, mixed regions appear between successive bands of pure components. These regions are shock layers. Obviously, the smaller

the shock layer thickness, the less is the degree of overlap between the bands of the isotachic train and hence the better is the separation. Actually, there are contradictory reports regarding the influence of the linear velocity on the quality of the separations achieved in displacement chromatography [25–31]. Although this paper does not address this important problem, it is the first and necessary step in this direction. The solution of single-component problems is simpler and easier to study than that of multicomponent problems.

THEORY

This work is based on the shock layer theory of Rhee and co-workers [18–21]. The model they used considers the constant pattern behavior [15–21], which is an asymptotic solution, *i.e.*, is achieved only after an infinitely long migration along the column. One of the problems encountered in the application of this theory will be to determine whether a constant pattern is achieved at elution. The basic assumptions of the model are (i) the additivity of the dispersive contributions from finite mass transfer rates and from axial dispersion (axial and eddy diffusions); and (ii) the approximation of the mass transfer kinetics by the solid film linear driving force model. Because of the high column efficiency, *i.e.*, of the fast rate of the mass transfer kinetics, the error introduced by the first assumption is certainly small. It seems that the second assumption is also valid in most cases of importance in chromatography [32].

We have recently shown how this theory can be applied to the study of the very steep fronts observed in high-concentration chromatography [22,23]. We first recall the definition of the SLT, then summarize the previous results and finally derive the relationships between the SLT, the mobile phase flow velocity and the displacer concentration in the case of Langmuir isotherm behavior.

Definition of shock layer thickness

In frontal analysis, a concentration step of constant height propagates along the column. The concentration profile at the column exit, or breakthrough curve, is the column response to a step input. It becomes ideally flat only at infinite distances from the center of the shock layer while we are interested in the part of the profile within a finite region. Thus, it is useful to define two concentrations bounds C_r^* and C_l^*, and an auxiliary variable θ, *as*:

$$\theta = \frac{C^l - C_l^*}{C^l - C^r} = \frac{C_r^* - C^r}{C^l - C^r} \tag{1}$$

where C^l and C^r are the concentrations in the column at $x = -\infty$ and $x = +\infty$, respectively. The definition of θ is illustrated in Fig. 1. The thickness of the shock layer is defined as the distance between the concentration bounds C_r^* and C_l^*:

$$\Delta\eta_x = x(C_l^*) - x(C_r^*) \tag{2}$$

$\Delta\eta_x$ depends on the choice of the arbitrary number θ, which in frontal analysis plays the same role as the relative peak height in elution. In this work we have taken $\theta = 0.02$. In displacement chromatography (which we shall discuss in a future paper [24]) and in many frontal analysis experiments (including all those performed in this work), $C^r = 0$ (the column does not contain any solute before the experiment starts), and $C^l = C_0$. Then, $C_r^* = \theta C_0$ and $C_l^* = (1 - \theta)C_0$.

Rhee and co-workers [18–20] considered reduced (*i.e.*, dimensionless) variables, including a moving coordinate, $\xi = x - \lambda\tau$, with $x = z/L$, reduced distance, $\tau = ut/L$, reduced time, and λ, reduced shock velocity $\{\lambda = 1/[1 + F(\Delta Q/\Delta C)]\}$. They calculated the reduced SLT, $\Delta\xi$, along the direction ξ of migration of the shock in the x,t plane. The SLT at the column exit ($z = L$) is given by [23]:

$$\Delta\eta_x = L\,\Delta\xi \tag{3a}$$

where L is the column length. The SLT in time units at the column exit (elution) is given by:

$$\Delta\eta_t = \frac{L}{U_s} \cdot \Delta\xi \tag{3b}$$

where U_s is the shock velocity:

$$U_s = \frac{u}{1 + F \cdot \dfrac{\Delta Q}{\Delta C}} \tag{4}$$

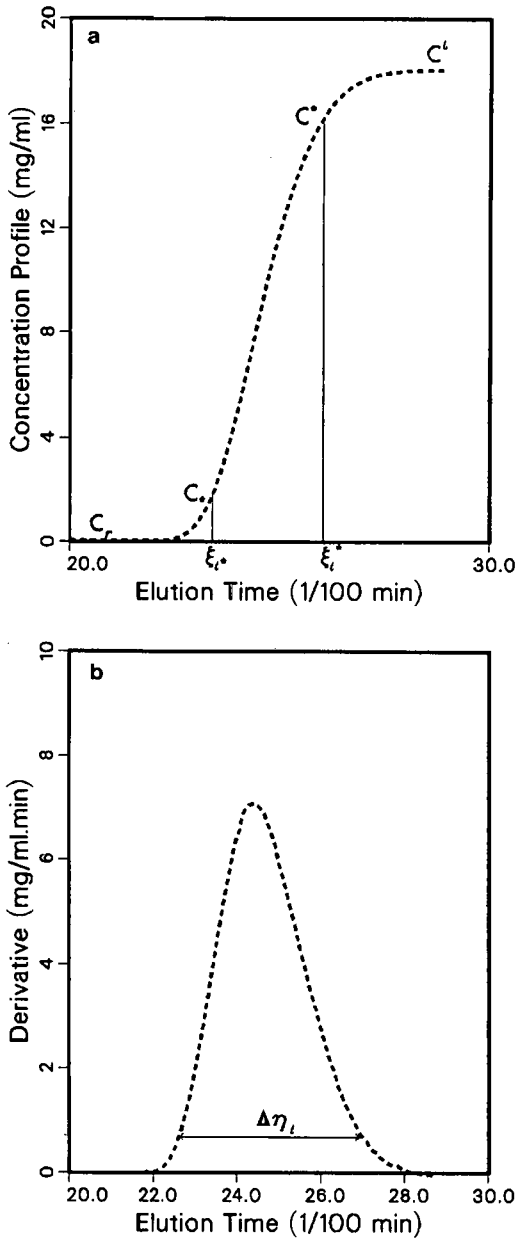


Fig. 1. Definition of θ and other parameters used to study the shock layer. (a) Breakthrough curve corresponding to a single component step. (b) Differential of the breakthrough curve corresponding to a single-component step.

where u is the mobile phase velocity and ΔQ and ΔC are the amplitude of the concentration jumps in the stationary and the mobile phases, respectively. Q and C are related by the isotherm equation, $Q = f(C)$, so the amplitudes of the concentration jumps are $\Delta Q = Q_l - Q_r = f(C_l) - f(C_r)$ and $C_l - C_r$, respectively, if C_l and C_r are the mobile phase concentrations after and before the jump, respectively.

Obviously, the shock layer thickness is a function of θ.

Thickness of the shock layer

Rhee and Amundson [19] assumed that the zone dispersion in the column results from two phenomena, axial dispersion due to molecular and eddy diffusion and characterized in chromatography by the first two terms of the Van Deemter [33] or the Knox [34] equations, and mass transfer resistance characterized by the third term of these equations. They showed that a constant pattern (shock layer) forms and propagates at the same velocity as the shock of the ideal model. They then derived an equation giving the profile of concentration in the shock layer of a pure component [19]. In the case of a Langmuir isotherm [18], this equation gives for the shock layer obtained when the concentration varies from 0 to C_0:

$$\Delta\xi = \left[\frac{D_L(1+K)}{KuL} + \frac{u}{(1+K)k_f L}\right]\frac{\Gamma_0+2}{\Gamma_0} \cdot \ln\left|\frac{1-\theta}{\theta}\right| \quad (5a)$$

$$\Delta\eta_x = \left[\frac{D_L(1+K)}{Ku} + \frac{u}{(1+K)k_f}\right]\frac{\Gamma_0+2}{\Gamma_0} \ln\left|\frac{1-\theta}{\theta}\right| \quad (5b)$$

$$\Delta\eta_t = \left[\frac{D_L(1+K)^2}{Ku^2} + \frac{1}{k_f}\right]\frac{\Gamma_0+2}{\Gamma_0} \cdot \ln\left|\frac{1-\theta}{\theta}\right| \quad (5c)$$

where D_L is the axial dispersion coefficient, including the effects of molecular axial diffusion, tortuosity and eddy diffusion, and k_f is the rate constant of mass transfer (solid film driving force model [19]). Note that in the equilibrium–dispersive model, the apparent dispersion coefficient denoted D_a and used in previous papers [32] includes also the effect of mass transfer kinetics; here it does not, which is why we use a different symbol. Eqn. 5a is dimensionless and eqns. 5b

and c give the SLT in length and time units, respectively. The equation 5b is the most practical for comparison with experimental results.

In these equations, the Langmuir isotherm is written as

$$q = \frac{ac}{1+bC} = \frac{bq_sC}{1+bC} = \frac{q_s\Gamma}{1+\Gamma} = q_s\Lambda \quad (6a)$$

$$\Lambda = \frac{\Gamma}{1+\Gamma} \quad (6b)$$

where Q and C are the actual concentrations of the compound in the stationary and the mobile phases, respectively, q_s is the specific saturation capacity of the adsorbent, a and b are numerical coefficients and $\Lambda = q/q_s$ and $\Gamma = bc$ are dimensionless concentrations; when $\Gamma = 1$, the amount adsorbed at equilibrium is $\Lambda = 0.5$. Accordingly, $\Gamma_0 = bC_0$. The parameter K in the eqns. 5 is

$$K = \frac{k'_0}{1+\Gamma_0} \quad (7)$$

where k'_0 is the retention factor, proportional to the initial slope of the isotherm ($k'_0 = Fa = Fbq_s$; see eqn. 6a). Finally, we note that the shock velocity in the conventional case when $C_r = 0$ and $C_1 = C_0$ can be written as

$$U_s = \frac{u}{1+\dfrac{Fbq_s}{1+\Gamma_0}} = \frac{u}{1+K} \quad (8)$$

As shown by Rhee and Amundson [19], and discussed recently [32], the band profiles calculated with the equilibrium–dispersive model and the solid film driving force model are the same provided that we use the following equation for the HETP:

$$H = \frac{2D_a}{u} = \frac{2D_L}{u} + 2\cdot\frac{K}{(1+K)^2}\cdot\frac{u}{k_f} \quad (9)$$

where D_a is the apparent dispersion coefficient [32,35] and K is given by eqn. 7. Eqn. 9 provides a relationship between the apparent dispersion coefficient and the concentration. The assumptions on which it is based have been discussed above. The study of the dependence of the shock layer thickness on the velocity, u, and the concentration, C_0, provides the only direct method of determination of H. In the case of linear chromatography, this equation becomes a form of the classical plate height equation:

$$H = \frac{2D_L}{u} + \frac{2k'_0u}{(1+k'_0)^2k_f} \quad (10)$$

Depending on the relationship between D_L and u which is used to account for eddy diffusion, we obtain the Van Deemter [33] or the Knox [34] plate height equations:

$$H = A + \frac{B}{u} + Cu \quad (11a)$$

$$H = \frac{B}{u} + Au^{1/3} + Cu \quad (11b)$$

with

$$2D_L = Au + B \quad (12a)$$

or

$$2D_L = B + Au^{4/3} \quad (12b)$$

and

$$C = \frac{2k'_0}{(1+k'_0)^2k_f} \quad (12c)$$

In eqns. 11 and 12, B is usually assumed to be equal to $2\gamma D_m$ [33,34], where γ is the packing tortuosity and D_m the mobile phase diffusivity of the solute. We note that eqn. 12c was derived by Giddings [36] using a completely different approach.

EXPERIMENTAL

Equipment

The modular liquid chromatograph used for the measurements of the SLT was assembled from two Gilson (Middleton, WI, USA) Model 302 pumps, a Valco (Houston, TX, USA) ten-port pneumatically actuated valve connected with a 1-ml loop and a Spectroflow 757 variable-wavelength UV detector (Kratos, Ramsey, NJ, USA). The detector output signal was connected to a DATA Master Model 621 (Gilson) for discretization of the response. This response was then acquired on a microcomputer, handled using the Gilson 715 software, and uploaded, when needed, on one of the computers of the University of Tennessee Computer Center.

Columns and chemicals

A 5 × 0.46 cm I.D. Vydac (Separation Group, Hesperia, CA, USA) 5-μm Protein & Peptide C_{18} column, and two other columns, 5 × 0.21 cm I.D. and 25 × 0.21 cm I.D., laboratory packed with 10-μm Protein & Peptide C_{18}, were used.

2-Phenylethanol was purchased from Fluka (Buchs, Switzerland), 4-*tert.*-butylphenol from Aldrich (Milwaukee, WI, USA) and HPLC-grade water and methanol from Burdick and Jackson (Muskegon, MI, USA). All these chemicals were used as received.

Procedures

Chromatographic experiments. The experiment was designed to reduce the amount of sample needed, so the injection of a wide rectangular pulse was substituted for a step injection. The columns were first equilibrated for 10 min with a mobile phase stream [methanol–water (50:50)] originating from one of the pumps. Then, the ten-port valve was actuated for the time needed to let the other pump push the sample plug contained in the 1-ml loop through the column and the pure mobile phase stream was resumed. Although the retention volume of the breakthrough curve exceeds 1 ml, a plateau at the injected concentration was always reached before the negative breakthrough curve started. The operation was repeated at different flow-rates and with the three columns.

Measurement of column efficiency. The efficiencies of the two 5-cm long columns were measured under linear conditions as a function of the mobile phase flow velocity. The results are reported in the Figs. 2 (Vydac column, with 2-phenylethanol) and 3 (laboratory packed column, with 4-*tert.*-butylphenol). The experimental data were fitted to the Van Deemter equation [33] (eqn. 11), which gave a smaller residual than the Knox equation [34] (eqn. 11b) in this instance. The values of the coefficients $D_L = 0.5(A + B/u)$ and $k_f = 2k'_0/(1 + k'_0)^2 C$ derived from the fit, together with the retention factors, k'_0, are given in Table I.

Measurement of adsorption isotherm. The adsorption isotherms of 2-phenylethanol and 4-*tert.*-butylphenol on the three columns used were measured by frontal analysis, as described

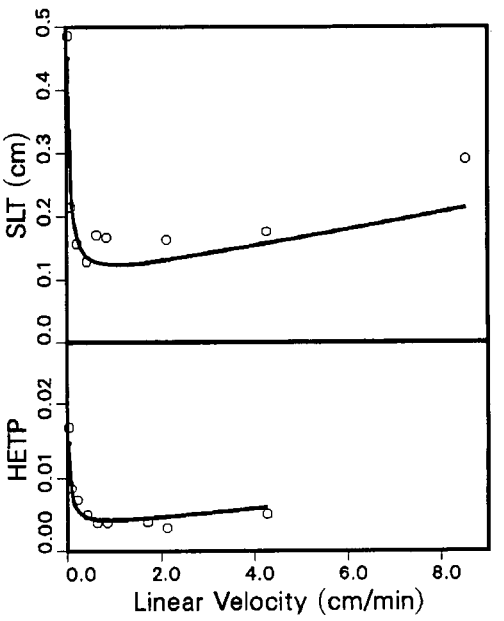


Fig. 2. Comparison between the dependences of the HETP (cm) measured under linear elution conditions and of the SLT (cm) for 2-phenylethanol on the mobile phase flow velocity. Top: plot of the SLT *versus* the mobile phase flow velocity. Experimental data (symbols) and prediction of eqn. 5b (solid line). Bottom: plot of the HETP *versus* the mobile phase velocity. Experimental data (symbols) and best fit to the Van Deemter equation (solid line). Experimental conditions (both plots): 5 cm long Vydac column; mobile phase; methanol–water (50:50), detection at 270 nm; sample, 2-phenylethanol; height of the concentration step in frontal analysis, 20 mg/ml; sample size for linear elution peaks, 40 μg (0.2 μl of a 20 mg/ml solution).

previously [37]. The experimental data were fitted to a Langmuir isotherm. There was excellent agreement between the experimental data and the Langmuir model in both instances. The retention factors of 2-phenylethanol and 4-*tert.*-butylphenol are 0.88 and 10.0, respectively.

Measurement of shock layer thickness. In principle, two procedures are available for these measurements. In the direct procedure, following the definition of the shock layer, we measure the distance between the moments when the signal reaches two selected fractions (*e.g.*, 5 and 95%) of the baseline shift corresponding to the elution of the concentration step. Alternately, the breakthrough curves could be differentiated and their widths at a certain fractional height (*e.g.*, half-height) taken as a measure of the

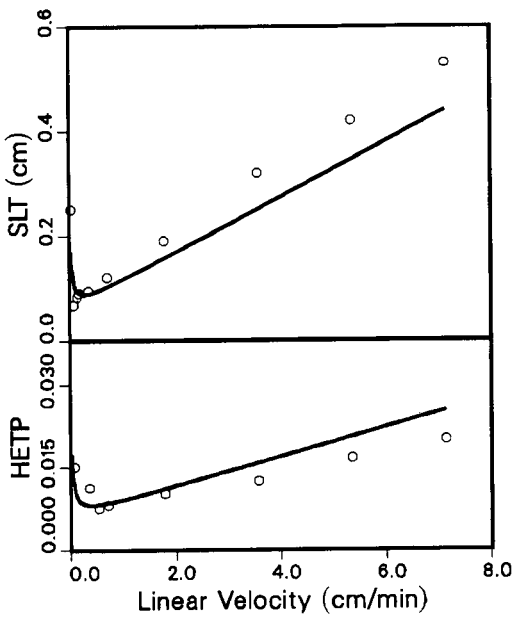


Fig. 3. Comparison between the dependences of the HETP (cm) measured under linear elution conditions, and of the SLT (cm) for 4-*tert.*-butylphenol on the mobile phase flow velocity. Plots as in Fig. 2. Experimental conditions (both plots): 5 cm long laboratory made column; mobile phase, methanol–water (50:50); detection at 276 nm; sample, 4-*tert.*-butylphenol; height of the concentration step in frontal analysis, 20 mg/ml; sample size for linear elution peaks, 0.2 μg.

shock layer width. Both procedures require smoothing of the response signal before the measurement is carried out, in order to eliminate the signal noise.

In the first procedure, the recorded chromatogram is first smoothed using a thirteen-point floating average algorithm. Then, the times at which the filtered breakthrough curve reaches the fractions 2% and 98% of the value corresponding to the plateau are determined by interpolation. These values correspond to $\theta = 0.02$ in eqns. 5. The distance between these two times is taken as the thickness of the shock layer. This choice of θ is arbitrary and made for the sake of convenience: any value between 0 and 0.5 would be possible. A compromise has to be found between the choices of a small value of θ, giving a large shock layer thickness, potentially more accurate, and of a large value of θ, corresponding to values of the detector signal which are sufficiently different from 0 and C_0 and can be measured more precisely, because the slope of the signal is more important.

In the second procedure, the differential of the chromatogram is calculated by taking the difference between successive data points on the recorded chromatogram and dividing by the data acquisition period. The differential chromatogram is smoothed using a thirteen-point floating average algorithm. The points on the differential profile corresponding to the fractional heights θ and $1 - \theta$ on the breakthrough curve are obtained at time τ_1 and τ_2, respectively, so that

$$\int_0^{\tau_1} y(t)\,\mathrm{d}t = \theta \tag{13a}$$

$$\int_0^{\tau_2} y(t)\,\mathrm{d}t = 1 - \theta \tag{13b}$$

Calibration has shown that the values of τ_i that correspond to a given value of θ correspond approximately to a fractional height of 10% of the derivative signal. This value changes little with the characteristics of the shock layer, *e.g.*, the height of the concentration step. Hence, this procedure, which is attractive because of its

TABLE I

PARAMETERS OF THE VAN DEEMTER EQUATION

Column	C_0 (mg/ml)	k'_0	D_a	k_f
Vydac (with 2-phenylethanol)	0	0.88	$0.0016u_0 + 0.00025$	800
	20		$0.0016u_0 + 0.00024$	542
Laboratory-made (with 4-*tert.*-butylphenol)	0	10	$0.003u_0 + 0.00032$	83
	20		$0.003u_0 + 0.00035$	48

simplicity and good reproducibility, was used in this work.

As the band width in linear chromatography, the thickness of the shock layer can be expressed in either time or volume units. As we study the influence of the flow velocity on this thickness, it is more appropriate to use a distance or volume unit, which accounts automatically for the trivial effect of the change in the time scale when the mobile phase velocity is adjusted. As the procedure described gives $\Delta\eta_t$, we obtain $\Delta\eta_x$ by multiplying the former by the shock velocity, $U_s = u/(1+K)$.

RESULTS AND DISCUSSION

We determined the SLT in single-component frontal analysis using two compounds and three columns of different lengths, packed with the same stationary phase. Shock layers are much narrower than either the small sample size peaks, or the breakthrough curves of small concentration steps which are recorded under the same experimental conditions, but correspond to linear chromatography. We see in eqn. 5 that, for values of Γ_0 between 0.1 and 1, corresponding to moderate to heavy column loading, the SLT is between 50 and 7 times the efficiency contribution, itself of the same order as the column HETP. Accordingly, SLTs are more difficult than band widths to measure accurately. For this reason, some discrepancies are noted between the experimental results and theoretical predictions. Such discrepancies are more prone to take place at high velocities, because of the difficulty in eliminating extra-column band broadening.

It should be noted that the thickness of the shock layer cannot become larger than the width of the breakthrough curve under linear conditions. This width is of the order of $q\sigma$ or, in distance terms, $q\sqrt{HL}$, q (a numerical parameter) depending on the value selected for θ, with $\mathrm{erf}(q) = \theta$. In this work, q was equal to 2.15, corresponding to the width of a Gaussian curve at 10% of its height. However, eqns. 5 predict that SLT tends towards infinity when Γ_0, and hence C_0, tends towards 0 (since K tends towards k'_0). This incorrect result comes from the fact that eqns. 5 are valid only when the shock layer is fully formed. In linear chromatography, there is no shock or shock layer, and eqns. 5 are not valid. In the transition region, when the radius of curvature of the isotherm is very large but no longer infinite, a shock layer could exist, but it takes a very long column for this shock layer to form. If the actual column used is too short, the breakthrough curve recorded is not the shock layer profile, it is narrower, and the experimental result cannot be expected to fit with the theory.

Depéndence of the thickness of the shock layer on the mobile phase velocity

Experimental determination of the shock layer is difficult. The signal must be recorded precisely and sources of signal noise carefully controlled. The study also requires an accurate measurement of the adsorption isotherm (to derive the isotherm parameter b), and of the dependence of the HETP on the mobile phase velocity (to derive the parameters of the Van Deemter equation, eqn. 11a). As a consequence, a significant amount of random fluctuations is expected.

In Figs. 2 and 3, we show for each of the two columns studied the plots of the column HETP *versus* the mobile phase flow velocity and of the shock layer thickness for a constant concentration step height *versus* the same velocity. The experimental results (symbols) are compared with the prediction of eqn. 5b (solid line), which is overlaid. In all instances, the agreement between the experimental results and the prediction of eqn. 5b is satisfactory over the whole range of velocities of interest in practical applications. The slightly faster rate of increase of the shock layer thickness at high flow velocities may be attributed to an instrumental contribution (response time of the detector). This result demonstrates the validity, in the experimental cases studied, of eqn. 5b.

The plots of SLT and HETP *versus* the flow velocity are similar. Both exhibit a minimum. We observe, however, that the minima of the two curves are not obtained for the same velocity. This can be explained by comparing eqns. 9 and 10. HETP in linear chromatography is given by eqns. 11a and b. It includes two terms. The first term of eqn. 10 is the sum of the axial diffusion, $2\gamma D_m/u$ (where γ is the tortuosity coefficient and

D_m the molecular diffusivity of the solute in the mobile phase) and the eddy diffusion (A in the Van Deemter equation, $Au^{1/3}$ in the Knox equation; see eqns. 11 and 12). The second term in eqn. 10 accounts for the rate of mass transfer [32,35,36]. As k_f is a lumped coefficient, this term includes the effects of diffusion through the stationary phase particles and the rate of adsorption/desorption.

Assuming a Van Deemter plate height equation (eqns. 11a and 12a), the first term is constant, the second term is proportional to $1/u$ and the third term is proportional to u. Hence there is an optimum value of the mobile phase velocity for which the H is minimum and the column efficiency maximum:

$$u_{opt}^{L} = \sqrt{\frac{\gamma D_m (1 + k_0')^2 k_f}{k_0'}} \quad (14)$$

This is a classical result. If we consider now eqn. 5b, we see that, assuming that D_L is again given by eqn. 12a, only the first factor on the right-hand side depends on the mobile phase velocity. Differentiating it with respect to u yields an optimum value of the velocity:

$$u_{opt}^{S} = \sqrt{\frac{\gamma D_m (1 + K)^2 k_f}{K}} \quad (15)$$

The two equations are obviously very similar, but because of the non-linear thermodynamics of phase equilibrium, the second one depends on the height of the concentration step and also on the second isotherm parameter. Therefore, for a given system, there exists an optimum linear velocity which gives the smallest shock layer thickness for a given step concentration. This optimum velocity is a function of the concentration and differs from the optimum velocity under linear conditions.

We note also that when the mobile phase velocity increases indefinitely, the shock layer in time units, $\Delta\eta_t$ (eqn. 5c), tends towards a finite limit. This limit is inversely proportional to k_f.

Dependence of the optimum mobile phase velocity u_{opt}^{S} on the height of the concentration step

Figs. 4 (2-phenylethanol) and 5 (4-*tert*-butylphenol) show the plots of the optimum flow

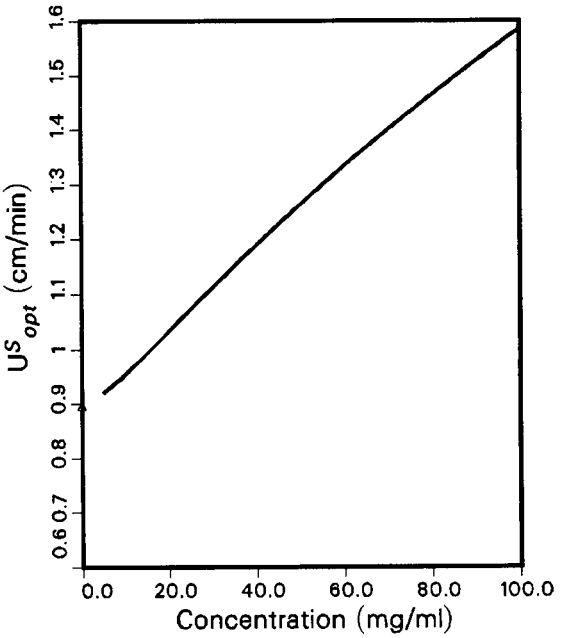


Fig. 4. Plot of the optimum velocity for minimum shock layer thickness *versus* the height of the concentration step (eqn. 15). Sample, 2-phenylethanol ($k_0' = 0.88$). Experimental conditions as in Fig. 2. The symbol △ gives u_{opt}^{L}, the optimum velocity for minimum HETP.

velocity *versus* the height of the concentration step for the two compounds studied. Both curves have a limit equal to u_{opt}^{L} for $\Gamma_0 = C_0 = 0$, which is obvious from eqns. 14 and 15. However, these two curves are strikingly different. One exhibits a well defined minimum, whereas the other is steadily increasing with increasing concentration.

Eqn. 15 shows that the optimum velocity for minimum shock layer thickness is a function of the height of the concentration step. Differentiation of eqn. 15 with respect to Γ_0 shows that the optimum velocity, u_{opt}^{S}, passes through a minimum, achieved at the concentration

$$\Gamma_0^* = k_0' - 1 \quad (16a)$$

$$C_0^* = \frac{k_0' - 1}{b} = F q_s \cdot \frac{k_0' - 1}{k_0'} \quad (16b)$$

The difference between the curves in Figs. 4 and 5 is now easily explained. There is obviously no minimum of u_{opt}^{S} when k_0' is smaller than unity; then the optimum velocity for minimum SLT increases regularly with increasing concentration step height. For the value of Γ_0 given by eqn.

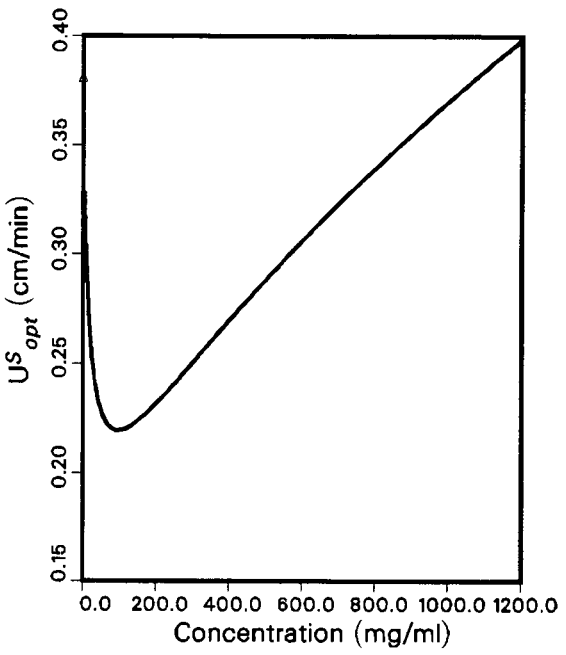


Fig. 5. Plot of the optimum velocity for minimum shock layer thickness *versus* the height of the concentration step. Sample, 4-*tert.*-butylphenol ($k'_0 = 10$). Experimental conditions as in Fig. 3. The symbol △ gives u^L_{opt}, the optimum velocity for minimum HETP. The concentration for which $u^S_{opt} = u^L_{opt}$ is 97 mg/ml.

16a, K is equal to 1 and u^S_{opt} is smaller, or much smaller, than u^L_{opt}.

By equating eqns. 14 and 15, we obtain the concentration step height at which the optimum linear velocity for minimum SLT is equal to that for minimum HETP. We have two solutions, $\Gamma_0 = 0$ (a trivial solution) and

$$\Gamma_0^{**} = k_0'^2 - 1 \tag{17a}$$

$$C_0^{**} = Fq_s\left(k'_0 - \frac{1}{k'_0}\right) \tag{17b}$$

We have shown above that the velocity for which the shock layer thickness is minimum depends on the height of the concentration step and is given by eqn. 15. We have also shown that the two optimum velocities, u^S_{opt} for minimum shock layer thickness and u^L_{opt} for minimum HETP, are equal for $\Gamma_0 = k_0'^2 - 1$. At lower concentrations, we have $u^S_{opt} < u^L_{opt}$, and the column should be operated at lower velocities in frontal analysis than under linear conditions. When the concentration step exceeds $k_0'^2 - 1$, however, the converse is true, and the column should be operated at a higher flow velocity in frontal analysis than under linear conditions.

These results are illustrated in Figs. 4 and 5. It can be seen that part of or the whole of the concentration range discussed above is often inaccessible. The only exception is when the retention factor of the component selected is smaller than about 2 and the column saturation capacity is large. For example, for 2-phenylethanol (Fig. 4) the retention factor is lower than unity, the optimum mobile phase velocity for minimum SLT always increases with increasing concentration step height and u^S_{opt} always markedly exceeds u^L_{opt}. In contrast, for 4-*tert.*-butylphenol (Fig. 5), which has a large retention factor ($k'_0 = 10$), the optimum velocity for minimum shock layer thickness decreases with increasing concentration until $C_0 = 97$ mg/ml, where it is minimum, and then increases. With this compound, u^S_{opt} is lower than u^L_{opt} until C_0 reaches the impractical concentration of 1080

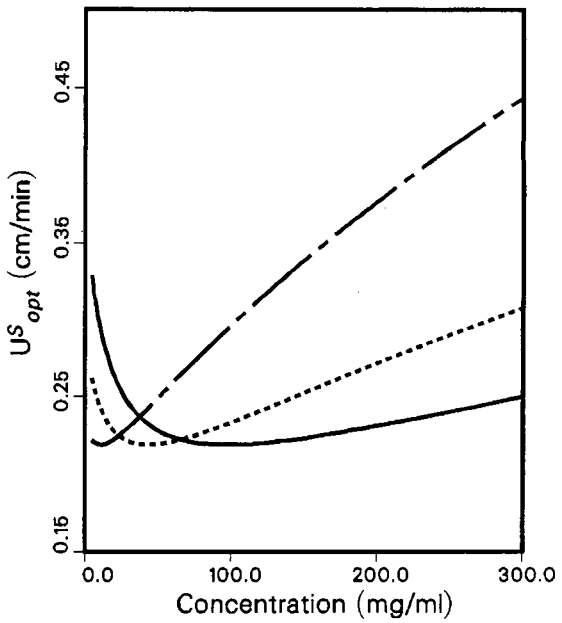


Fig. 6. Plot of the optimum velocity for minimum shock layer thickness *versus* the height of the concentration step (eqn. 15). Dash-dotted line, $k'_0 = 2$; dotted line, $k'_0 = 5$; solid line, $k'_0 = 10$.

mg/ml. In the range of concentrations accessible to experiments, u_{opt}^{S} will be much lower than u_{opt}^{L}.

Fig. 6 illustrates the important differences between the behavior of the SLT of different compounds with the height of the concentration step. When the retention factor, k_0', is low, the optimum velocity for minimum SLT increases constantly with increasing concentration. When the retention factor exceeds about 2, the optimum velocity exhibits a minimum for a certain concentration, and this concentration increases with increasing value of the retention factor.

Dependence of the thickness of the shock layer on the height of the concentration step

The SLT depends on the height of the concentration step. However, differentiation of eqn. 5b with respect to Γ_0 and setting the differential equal to zero gives a fourth-degree equation without simple roots. We can have an idea of the variation of $\Delta\eta_t$, however, by studying separately the two terms.

Differentiation of the first term $[(1+K)/K][(\Gamma_0+2)/\Gamma_0]$, with respect to Γ_0 shows that it is minimum for

$$\Gamma_{0,1} = \sqrt{2(1+k_0')} \tag{18a}$$

This concentration is high. For $k_0' = 1$, which is a very small value of the retention factor by displacement standards, the value of $\Gamma_{0,1}$ is 2, for which value we achieve a coverage of two-thirds of a monolayer at equilibrium (see eqn. 6b). For $k_0' = 7$, a surface coverage of 80% would be obtained at the optimum concentration of 4. As a consequence, we can expect that this term will decrease with increasing height of the concentration step in the entire range of practical interest.

Similarly, differentiation of the second term of eqn. 5b, $[1/(1+K)][(\Gamma_0+2)/\Gamma_0]$, shows that it is minimum for

$$\Gamma_{0,2} = \frac{2+\sqrt{4+2(k_0'^2-2)(k_0'+1)}}{k_0'^2-2} \tag{18b}$$

For $k_0' > \sqrt{2}$, the value of $\Gamma_{0,2}$ is large. For $k_0' = 2$, $\Gamma_{0,2} = 3$, for which the surface coverage at equilibrium is 75%. For $k_0' < \sqrt{2}$, the second term of eqn. 5b decreases monotonically with increasing concentration.

As both terms of eqn. 5b decrease with increasing value of C_0 until they are minimum for impractically large concentrations, we can conclude that the same is true for the SLT, which in practice will decrease steadily with increasing height of the concentration step.

We measured the shock layer thickness of a series of breakthrough curves recorded for the injection of solutions of increasing concentrations in a column previously swept with pure mobile phase, so the height of the concentration step is always C_0. Between two successive experiments, the column is properly swept with pure mobile phase during the time needed to purge it from the solute introduced previously. The experimental data obtained with the Vydac column are shown in Fig. 7a (symbols). They are overlaid in this figure with the curve derived from eqn. 5b (solid line). There is very good agreement between the experimental results and the prediction of eqn. 5b at high concentrations. At low concentrations, this equation predicts a shock layer thickness that is thicker than the width of a breakthrough front under linear conditions, and significant deviations take place.

The dotted and dashed lines in Fig. 7a correspond to the two contributions to the shock layer thickness in eqn. 5b. These contributions result from the axial dispersion and the mass transfer resistance, respectively. They were calculated from the sum of the A and B terms and the C term of the Van Deemter equation [33] (eqns. 12a and c and Table I). Whereas the contribution of the mass transfer resistance decreases monotonically with increasing concentration, the contribution of the axial dispersion passes through a minimum, which explains the minimum in the shock layer thickness. We note that, under the experimental conditions of Fig. 7a, eqns. 5 and 16 predict a minimum for the thickness of the shock layer for a concentration step of *ca.* 20 mg/ml, in agreement with the experimental data.

The same data are shown in Fig. 7b, where the choice of a logarithmic scale for the concentration axis permits a clearer illustration of the experimental results obtained in a concentration

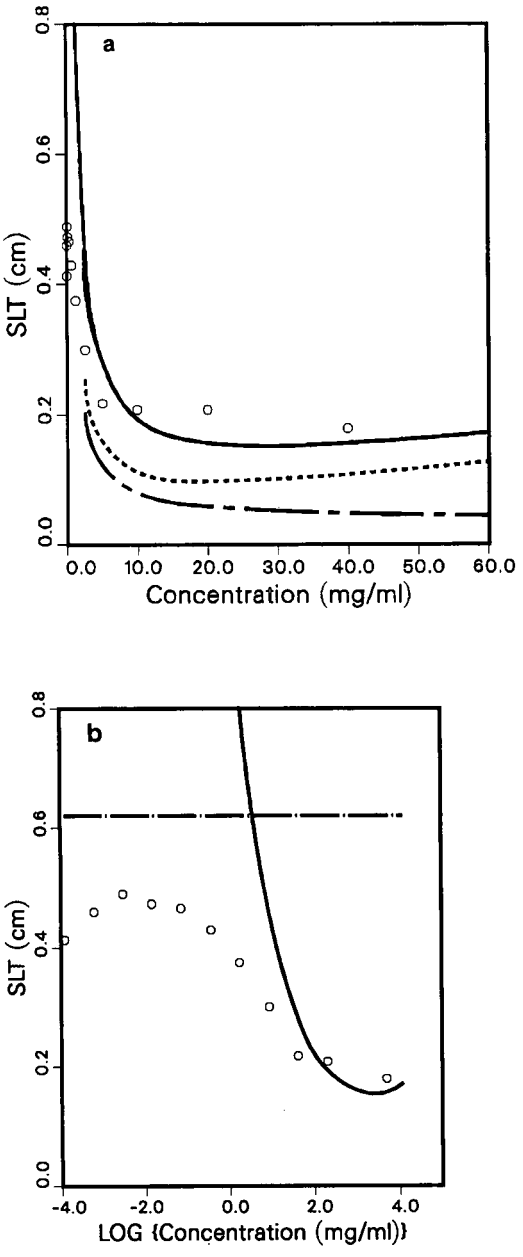


Fig. 7. Plot of the shock layer thickness *versus* the height of the concentration step of 2-phenylethanol. Experimental conditions as in Fig. 2 except the flow velocity, 0.07 cm/s. At this flow-rate, $H = 4.8 \cdot 10^{-3}$ cm, under linear conditions. (a) Symbols, experimental data; solid line, eqn. 5b; dotted line, contribution of axial dispersion to the height of the shock layer; dashed line, contribution of mass transfer resistance to the height of the shock layer. (b) Symbols, experimental data in semi-logarithmic coordinates; dot-dashed line, value predicted by the linear model ($\omega = 4\sqrt{HL} = 0.62$ cm); solid line, variation predicted by eqn. 5b.

range covering 3.5 orders of magnitude. The existence of three concentration domains is obvious. At high concentrations, eqn. 5b fits the experimental results well. At very low concentrations, on the other hand, the width of the breakthrough front is constant and equal to the value resulting from measurements of the width of the Gaussian peak obtained under linear conditions. There is an intermediate concentration range in which the width of the breakthrough curve decreases sharply with increasing height of the concentration step.

As mentioned above, eqns. 5 correspond to a constant pattern behavior, *i.e.*, to an asymptotic solution. In other words, these equations are strictly valid only for an infinitely long column. The rate at which a breakthrough profile converges towards a constant pattern decreases with decreasing step height, and hence with decreasing change in isotherm slope across the step. At the end of a finite column, we observe a breakthrough curve that is close to a constant pattern, and hence has the shock layer profile, only if the column is long enough and the concentration step high enough. If the column is too short or the step height too small, a constant pattern is not achieved, and the width of the breakthrough curve is narrower than predicted by eqn. 5b. This is in agreement with the experimental results in the figures.

CONCLUSIONS

An optimum linear velocity for minimum thickness of the shock layer exists in frontal analysis. This optimum velocity is a function of the height of the concentration step. Depending on the retention of the compound under infinite dilution, the optimum velocity increases constantly with increasing height of the concentration step, or may pass through a minimum.

Accordingly, when measuring adsorption isotherms by frontal analysis, the mobile phase velocity should be selected carefully. When the retention factor under linear conditions is much higher than unity, relatively low values of the mobile phase velocity, lower than the optimum velocity for minimum plate height, should be considered, especially for the small step heights.

However, the most important consequences of a study of the dependence of shock layer thicknesses on the mobile phase velocity and the height of the concentration step will be found in displacement chromatography [24]. As this problem is a binary problem, it must be discussed in connection with the theory of shock layers between two components.

SYMBOLS

a, b Coefficients of the Langmuir isotherm
A, B Coefficients in the plate height equation
C Concentration in the mobile phase (mg/ml)
C^{l} Concentrations in the column at $x = -\infty$
C^{r} Concentrations in the column at $x = +\infty$
C_1^* Concentration bound of the shock layer, on the upstream side
C_r^* Concentration bound of the shock layer, on the downstream side
C_0 Concentration of a step injection
D_a Apparent dispersion coefficient
D_L Axial dispersion coefficient (cm^2/s)
F Phase ratio of the column (ml/ml)
$f(C)$ Isotherm equation
HETP, H Height equivalent to a theoretical plate (cm)
K Auxiliary parameter
k_f Rate constant of mass transfer (s^{-1})
k_0' Capacity factor of the component at infinite dilution
L Column length
Q Concentration in the stationary phase (mg/ml)
q_s Column saturation capacity
SLT Shock layer thickness
t Time
U_s Shock layer velocity (cm/min)
u Mobile phase linear velocity (cm/s)
u_{opt}^{L} Optimum linear velocity in linear chromatography (cm/s)
u_{opt}^{S} Optimum linear velocity for minimum shock layer thickness (cm/s)
x Reduced distance along the column
z Distance along the column

Greek letters

Γ Dimensionless mobile phase concentration
γ Tortuosity of the packing
$\Delta\eta_x$ Shock layer thickness in length units (cm)
$\Delta\eta_t$ Shock layer thichness in time units (s)
ΔQ Concentration amplitude of the shock layer in the stationary phase
ΔC Concentration amplitude of the shock layer in the mobile phase
$\Delta\xi$ Dimensionless shock layer
λ Reduced shock velocity
Λ Dimensionless stationary phase concentation
θ Parameter defining the shock layer thickness
σ Standard deviation of a Gaussian peak
τ Reduced time
ξ Moving coordinate of the shock layer

ACKNOWLEDGEMENTS

We gratefully acknowledge the gift by the Separation Group (Hesperia, CA) of the column and the packing material used in this work. This work was supported in part by grant CHE-9201663 from the National Science Foundation and by the cooperative agreement between the University of Tennessee and the Oak Ridge National Laboratory. We acknowledge support of our computational effort by the University of Tennessee Computing Center.

REFERENCES

1 B.A. Bidlingmeyer, *Preparative Liquid Chromatography*, Elsevier, Amsterdam, 1987.
2 H. Colin, *Sep. Sci. Technol.*, 22 (1987) 1953.
3 J.E. Eble, R.L. Grob, P.E. Antle and L.R. Snyder, *J. Chromatogr.*, 384 (1987) 25.
4 J.E. Eble, R.L. Grob, P.E. Antle and L.R. Snyder, *J. Chromatogr.*, 384 (1987) 31.
5 J.H. Knox and M. Pyper, *J. Chromatogr.*, 363 (1986) 1.
6 S. Golshan-Shirazi and G. Guiochon, *Anal. Chem.*, 60 (1988) 2364.
7 S. Golshan-Shirazi and G. Guiochon, *Anal. Chem.*, 61 (1989) 462.

8 C.A. Lucy and P.W. Carr, *J. Chromatogr.*, 556 (1991) 159.
9 E.V. Dose and G. Guiochon, *Anal. Chem.*, 62 (1990) 1723.
10 A.M. Katti and G. Guiochon, *Adv. Chromatogr.*, 31 (1991) 1.
11 S. Golshan-Shirazi and G. Guiochon, in F. Dondi and G. Guiochon (Editors), *Theoretical Advancement in Chromatography and Related Separation Techniques (NATO ASI Series, Series C*, Vol. 383), Kluwer, Dordrecht, 1992, pp. 1–33.
12 B.C. Lin, S. Golshan-Shirazi, Z. Ma and G. Guiochon, *Anal. Chem.*, 60 (1988) 2647.
13 T. Vermeulen, *Adv. Chem. Eng.*, 2 (1958) 147.
14 F. Helfferich, *Ion Exchange*, McGraw-Hill, New York, 1962, Ch. 9.
15 D.O. Cooney and E.N. Lightfoot, *Ind. Eng. Chem. Fundam.*, 5 (1966) 212.
16 T. Vermeulen, M.D. LeVan, N.K. Hiester and G. Klein, in R.H. Perry, C.H. Chilton and S.D. Kirkpatrick (Editors), *Chemical Engineers' Handbook*, Academic Press, New York, 4th ed., 1963, Sect. 16.
17 N.K. Hiester and T. Vermeulen, *Chem. Eng. Prog.*, 48 (1958) 505.
18 H.-K. Rhee, B.F. Bodin and N.R. Amundson, *Chem. Eng. Sci.*, 26 (1971) 1571.
19 H.-K. Rhee and N.R. Amundson, *Chem. Eng. Sci.*, 27 (1972) 199.
20 H.-K. Rhee and N.R. Amundson, *Chem. Eng. Sci.*, 28 (1973) 55.
21 H.-K. Rhee and N.R. Amundson, *Chem. Eng. Sci.*, 29 (1974) 2049.
22 Z. Ma and G. Guiochon, *J. Chromatogr.*, 603 (1992) 13.
23 Z. Ma and G. Guiochon, *J. Chromatogr.*, 609 (1992) 19.
24 J. Zhu and G. Guiochon, in preparation.
25 Cs. Horváth, A. Nahum and J.H. Frenz, *J. Chromatogr.*, 218 (1981) 365.
26 Cs. Horváth, J. Frenz and Z. El Rassi, *J. Chromatogr.*, 255 (1983) 273.
27 J. Frenz, Ph. Van Der Schrieck and Cs. Horváth, *J. Chromatogr.*, 330 (1985) 1.
28 J. Frenz and Cs. Horváth, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography—Advances and Perspectives*, Vol. 5, Academic Press, New York, 1988, pp. 211–314.
29 F. Cardinali, A. Ziggiotti and G.C. Viscomi, *J. Chromatogr.*, 499 (1990) 37.
30 G. Subramanian, M.W. Phillips and S.M. Cramer, *J. Chromatogr.*, 454 (1988) 1.
31 G. Subramanian and S.M. Cramer, *Biotechnol. Prog.*, 454 (1989) 1.
32 S. Golshan-Shirazi and G. Guiochon, *J. Chromatogr.*, 603 (1992) 1.
33 J.J. Van Deemter, F.J. Zuiderweg and A. Klinkenberg, *Chem. Eng. Sci.*, 5 (1956) 271.
34 J.H. Knox and M. Saleem, *J. Chromatogr.* Sci., 7 (1969) 745.
35 G. Guiochon, S. Golshan-Shirazi and A. Jaulmes, *Anal. Chem.*, 60 (1988) 1856.
36 J.C. Giddings, *Dynamics of Chromatography*, Marcel Dekker, New York, 1964.
37 S. Golshan-Shirazi, S. Ghodbane and G. Guiochon, *Anal. Chem.*, 60 (1988) 2630.

Journal of Chromatography, 636 (1993) 203–212
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2653

Simultaneous optimization of pH and micelle concentration in micellar liquid chromatography☆

Andrew H. Rodgers☆☆

Chemistry Department, University of New Orleans, Lakefront, New Orleans, LA 70148 (USA)

Joost K. Strasters☆☆☆ and Morteza G. Khaledi*

Department of Chemistry, North Carolina State University, P.O. Box 8204, Raleigh, NC 27695-8204 (USA)

(First received June 30th, 1992; revised manuscript received November 20th, 1992)

ABSTRACT

A retention model for ionizable compounds in micellar liquid chromatography is derived and verified. The use of the model for the prediction of retention is illustrated and appropriate optimization strategies for the separation of ionizable compounds in Micellar Liquid Chromatography are discussed.

INTRODUCTION

Reversed-phase liquid chromatography (RPLC) is the method of choice for the analysis of ionizable compounds with adequate retention. However, the method is unable to retain hydrophylic, ionizable compounds [1].

Poorly retained ionizable solutes can be retained by the addition of submicellar quantities of ionic surfactants acting as ion-pairing agents. However, this modification suffers from the drawback of extending the time required to equilibrate the stationary phase. This is due to the direct dependency of retention on the charge density of ionic surfactant adsorbed on the stationary phase [2]. Similarly, during gradient elution, the increasing organic content of the mobile phase reduces the charge density of surfactant on the stationary phases. As a consequence, the column must be re-equilibrated with numerous column volumes of the weaker mobile phase to regenerate the same surface coverage of pairing reagent required for consistent retention. This can lead to poor retention reproducibility and makes the prediction of optimum separation conditions difficult [3].

Micellar liquid chromatography (MLC) is also capable of the retention and separation of ionizable and neutral compounds. In MLC, surfactant concentrations in excess of the critical micelle concentration (CMC) are used so that micelles are formed in the mobile phase. The presence of micelles in the mobile phase allows for the direct on-column injection of physiological fluids [4–10] and offers enhanced detection possibilities [11–16].

Ionizable compounds are retained in a manner

* Corresponding author.

☆ Presented at the *16th International Symposium on Column Liquid Chromatography, Baltimore, MD, June 14–19, 1992.* The majority of the papers presented at this symposium were published in *J. Chromatogr.*, Vols. 631 + 632 (1993).

☆☆ Present address: Witco Corporation, P.O. Box 310, Hahnville, LA 70057, USA.

☆☆☆ Present address: Sterling Winthrop, Great Valley Parkway, Malvern, PA 19335, USA.

0021-9673/93/$06.00

similar to ion-pair chromatography due to surfactant deposition on the stationary phase but here elution strength (in the isocratic and gradient modes) is related to the concentration of micelles in the mobile phase. Since in MLC the surfactant concentration is greater than the CMC, then the variation of surfactant concentration on the stationary phase will be limited. Hence, the composition of the stationary phase in MLC is less variable [17]. Consequently, the regeneration capabilities of MLC are comparatively rapid [18,19] and reproducibility of retention is attained in a shorter period of time and with a greater degree of certainty. The stable and predictable nature of MLC retention facilitates the rapid optimization of retention and prediction of optimum conditions [20].

The intent of pH manipulation in RPLC is to increase the retention (through ion suppression) and selectivity of ionizable solutes [21,22]. In addition, the quality of separation could be improved by optimizing the mobile phase organic modifier content or type. Foley and May [21] demonstrated an approach whereby selectivity enhancement can be achieved by optimizing the mobile phase pH. They verified their theory by predicting the optimum pH for the separation of a group of methylated cresols. The mobile phase pH was targeted to separate a critical peak pair based upon the effective ionization constants and self-selectivities of the two solutes in a mobile phase containing 30% acetonitrile.

One should be cautious, however, in optimizing one parameter at a time as this approach can only be effective when the parameters are not interactive. This is usually not the case for optimizing pH and micelle concentration in micellar mediated techniques such as MLC [23–28] and micellar electrokinetic capillary chromatography (MECC) [29,30]. This is mainly due to the fact that the apparent ionization constant in micellar media is a function of micelle concentration, and more importantly, the magnitude of micellar induced shift of ionization constants is a function of solute type [31]. In order to disclose the full resolving power of the method this paper reports the preliminary results of the optimization of retention in MLC using an appropriate retention model that simultaneously describes retention in terms of pH and micelle concentration.

Zwitterionic amino acids are selected as the test solutes because the thermodynamic ionization constants of these solutes are very similar and it has been shown that certain protonated amino acids in MLC are essentially unresolved at low pH [28]. Consequently, it is unlikely that the independent variation of mobile phase pH will provide the desired separation. Interaction with additional parameters such as micelle concentration will probably be required.

THEORY

Fig. 1 shows the equilibria involved in the retention of zwitterionic compounds in MLC. In the figure, anionic micelles are shown but cationic and nonionic micelles could also be considered, although there would be no immediate advantage in using nonionic surfactants for the separation of charged solutes in MLC. K_{a1} and K_{a2} represent the acid dissociation equilibrium constants between the cationic ($HABH^+$), zwitterionic ($^-ABH^+$) and anionic (^-AB) forms

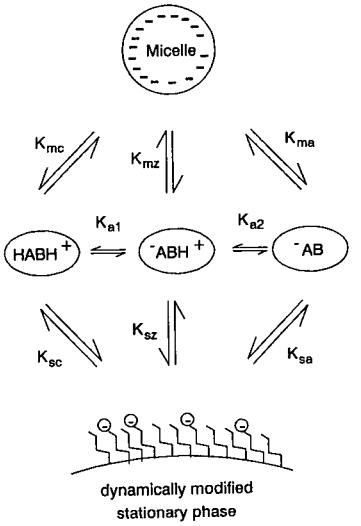


Fig. 1. Equilibria of a zwitterion in MLC with anionic surfactant.

in aqueous solution; K_{mc}, K_{mz} and K_{ma} are the corresponding solute–micelle equilibrium (or binding) constants of the cation, zwitterion and anion. K_{sc}, K_{sz} and K_{sa} are the respective binding constants of the cation, zwitterion and anion to the stationary phase ligands. Note that in MLC, the stationary phase is dynamically modified by the adsorption of surfactant monomers. Following the reasoning given in refs. 23, 25 and 28, the capacity factor is defined as:

$$k' = \{\Phi([HABH^+ - L_s] + [^-ABH^+ - L_s] + [^-AB - L_s])/\{[HABH^+] + [^-ABH^+] + [^-ABL] + [HABH_m^+] + [^-ABH_m^+] + [^-AB_m]\} \quad (1)$$

The following equilibria can be defined:

$$HABH^+ + L_s \rightleftharpoons HABH^+ - L_s \quad (K_{sc}) \quad (2)$$

$$HABH^+ + M \rightleftharpoons HABH^+ - M \quad (K_{mc}) \quad (3)$$

$$^-ABH^+ + L_s \rightleftharpoons {}^-ABH^+ - L_s \quad (K_{sz}) \quad (4)$$

$$^-ABH^+ + M \rightleftharpoons {}^-ABH^+ - M \quad (K_{mz}) \quad (5)$$

$$^-AB + L_s \rightleftharpoons {}^+AB - L_s \quad (K_{sa}) \quad (6)$$

$$^-AB + M \rightleftharpoons {}^+AB - M \quad (K_{ma}) \quad (7)$$

$$^-ABH^+ + H^+ \rightleftharpoons HABH^+ \quad (K_{a1}) \quad (8)$$

$$^-AB + H^+ \rightleftharpoons {}^-ABH^+ \quad (K_{a2}) \quad (9)$$

[M] is the micelle concentration (surfactant concentration, [S], minus the CMC). L_s is the stationary phase ligand and Φ is the phase ratio of the column. Substitution of equilibrium constants of expressions 2–9 into eqn. 1 gives:

$$k' = \{\Phi[L_s](K_{sc} + K_{sz}K_{a1}/[H^+] + K_{sa}K_{a1}K_{a2}/[H^+]^2)\}/\{1 + K_{mc}[M] + (1 + K_{mz}[M])K_{a1}/[H^+] + (1 + K_{ma}[M])K_{a1}K_{a2}/[H^+]^2\} \quad (10)$$

Note that eqn. 10 can also be derived by the phenomenological approach [27,28]. Accurate determination of the constants Φ and $[L_s]$ is difficult and so these terms are incorporated into the constants K_{sc}, K_{sz} and K_{sa} to give the factored constants K'_{sc}, K'_{sz} and K'_{sa} (i.e. $K'_{sc} = K_{sc}\Phi$ $[L_s]$ etc.). This simplification introduces column dependency in the derived values:

$$k' = \{K'_{sc} + K'_{sz}K_{a1}/[H^+] + K'_{sa}K_{a1}K_{a2}/[H^+]^2\}/\{1 + K_{mc}[M] + (1 + K_{mz}[M])K_{a1}/[H^+] + (1 + K_{ma}[M])K_{a1}K_{a2}/[H^+]\}^2 \quad (11)$$

eqn. 11 is shown in Fig. 2. Values for the constants are chosen to reflect those that may be typical for a zwitterionic amino acid with anionic surfactant such as sodium dodecyl sulfate (SDS). The figure shows the predicted retention at variable pH (0–12.5) and variable surfactant concentration ([S] = 0.03–0.074 *M*). Also shown in the figure are the derivatives of eqn. 11 used to determine the apparent ionization constant(s).

Individually, the parameters behave as expected, the highest retention is observed at low pH and low micelle concentration and increasing these parameters decreases retention. However, the interactive nature of these parameters is observable through the apparent ionization con-

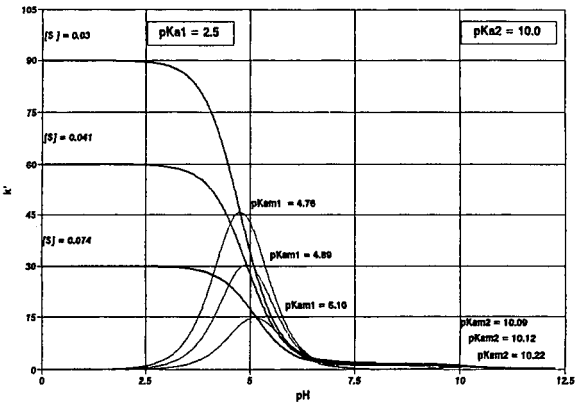


Fig. 2. Predicted retention of a zwitterion as a function of pH and micelle concentration with non-ionic micelles using eqn. 11. Surfactant concentrations: [S] = 0.03 *M*, 0.041 *M* and 0.074 *M*; [CMC] = $8.31 \cdot 10^{-3}$ *M*. Also shown are the derivatives of the double sigmoidal curves at each micelle concentration to give the apparent ionization constants at shown surfactant concentration (pK_{am1} and pK_{am2}). Constants: $K'_{sc} = 19800$, $K'_{sz} = 2$, $K'_{sa} = 0.2$, $pK_{a1} = 2.5$, $pK_{a2} = 10$, $K_{mc} = 10\,000$, $K_{mz} = 10$, $K_{ma} = 0.1$.

stant which is not constant but increases with micelle concentration.

The pH range shown in Fig. 2 (0–12.5) exceeds the pH limitation of the alkyl-bonded silica-based stationary phases (2.5–7.5). However, the pH range 2.5–7.5 is compatible with the retention of zwitterionic solutes in SDS micellar mobile phase as shown in Fig. 2 where, at pH > 7.5, the retention approaches zero and at pH < 2.5 no change in k' with pH is expected. The effect of pK_{a2} on the retention between pH 2.5–7.5 will be negligible and therefore, as this constant tends to zero, eqn. 11 reduces to:

$$k' = \frac{K'_{sc} + K'_{sz}K_{a1}/[H^+]}{1 + K_{mc}[M] + (1 + K_{mz}[M])K_{a1}/[H^+]} \quad (12)$$

Eqn. 12 should be appropriate to test the proposed model for the retention of zwitterionic amino acids with SDS micelles over the limited pH range of a silica based column.

The constants for eqn. 12 can be determined in the following manner. If K_a, K_{mc} and K_{mz} values are available from the literature [27,32,33] or can be determined [32], then K'_{sc} and K'_{sz} can be determined by combining eqn. 12 with the following equations [25,34].

$$K'_{sc} = k'_c(1 + K_{mc}[M]) \quad (13a)$$

$$K'_{sz} = k'_z(1 + K_{mz}[M]) \quad (13b)$$

to give:

$$k' = \frac{k'_c(1 + K_{mc}[M]) + k'_z(1 + K_{mz}[M])K_{a1}/[H^+]}{1 + K_{mc}[M] + (1 + K_{mz}[M])K_{a1}/[H^+]} \quad (14)$$

where k'_c and k'_z are the respective limiting capacity factors for the cationic and zwitterionic forms of the solute. This equation can be linearized:

$$k'\{1 + K_{mc}[M] + (1 + K_{mz}[M])K_{a1}/[H^+]\} = k'_c(1 + K_{mc}[M]) + k'_z(1 + K_{mz}[M])K_{a1}/[H] \quad (15)$$

A plot of the left hand side of the equation *vs.* $K_{a1}/[H^+]$ yields an intercept of $K'_{sc} = k'_c(1 + K_{mc}[M])$ and slope of $K'_{sz} = k'_z(1 + K_{mz}[M])$.

TABLE I

CAPACITY FACTORS FOR THE MLC RETENTION OF IONOGENIC SOLUTES AS A FUNCTION OF SURFACTANT CONCENTRATION AND pH

SDS micellar mobile phase, 50 m*M* sodium phosphate buffer adjusted to pH with concentrated phosphoric acid. Phe = Phenylalanine; Trp = tryptophan; Met = methionine; PPA = phenylpropionic acid.

pH	k'			
	Phe	Trp	Met	PPA
[SDS] = 0.05 M				
7.50	1.23	3.04	0.58	0.30
6.50	1.48	4.31	0.62	1.22
5.60	3.03	6.50	0.75	5.50
3.50	46.7	51.5	17.5	24.8
2.50	56.6	56.7	41.0	25.3
[SDS] = 0.10 M				
7.50	1.60	1.80	0.59	0.30
6.50	1.80	2.57	0.62	1.00
5.60	3.18	5.53	0.72	4.89
3.50	25.6	26.3	13.1	14.1
2.50	28.0	27.1	21.6	14.7
[SDS] = 0.20 M				
7.50	1.00	1.19	0.56	0.31
6.50	1.10	1.71	0.56	0.87
5.60	1.60	2.62	0.65	3.46
3.50	12.9	13.4	7.50	8.33
2.50	13.6	13.6	12.6	8.46

EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, MA, USA) liquid chromatographic system was used to collect the chromatographic measurements. The system consisted of a 6000A and an M45 pump, an M680 solvent programmer, a U6K universal liquid chromatograph injector and a Varian UV 50 variable-wavelength detector set at 200 nm. The column was an Ultrasphere, ODS analytical column (Altex, USA, 15 cm × 0.46 cm, d_p 5 μm) protected with a silica precolumn before the injector to saturate the mobile phase with silicates. The silica precolumn and the analytical column were water jacketed and thermostated at 25°C with a Model 1268-02 constant-temperature

recirculating bath (Cole-Palmer). The pH of the mobile phases was measured with Model 231 (Orion) pH meter and 13-639-104 combination electrode (Fisher Scientific).

Reagents

SDS (puriss grade) was obtained from Fluka and used as received. Phosphoric acid (HPLC grade) and the mono- and divalent sodium salts were obtained from Fisher Scientific (NJ, USA). The solutes were purchased from Sigma (St. Louis, MO, USA).

The void volume, V_0, of the system, before exposure to SDS micellar eluents, was measured from the time of injection of $NaNO_3$ to the first deviation of the baseline. A mean value of 0.92 ml ($n = 7$) was used for all subsequent k' calculations. The requisite weight of SDS and 50 m*M* of the sodium salt, were dissolved in doubly distilled, deionized water and filtered through 0.45-μm nylon-66 membrane filters (Rainin). The mobile phase was titrated with concentrated phosphoric acid to pH. The mobile phase was then passed through the system until the column effluent pH equalled the input pH. After the retention volumes were measured the pH of the mobile phase was decreased to the next value and the measurements repeated (Table I).

RESULTS AND DISCUSSION

Regression of eqn. 14 for K'_{sc} and K'_{sz}

The results are shown in Table II where the mean values of K'_{sc} and K'_{sz} are calculated. For each solute the limiting capacity factors are reported with the 95% confidence intervals. For

TABLE II

NON-LINEAR REGRESSION RESULTS OF EQN. 14

Data from Table I; K_m and pK_a values from ref. 29. CMC taken as 0.0081 *M*. ±95% Confidence intervals (CI) reported for limiting capacity factors.

[SDS]	k'_c + 95% CI	k'_c	k'_c − 95% CI	K'_{sc}	k'_z + 95% CI	k'_z	k'_z − 95% CI	K'_{sc}
Solute: Phe; $K_{mc} = 2100$; $K_{mz} = 1.8$; p$K_a = 2.18$								
0.05	58.2	58.0	57.9	5161	1.36	1.25	1.15	1.34
0.10	28.7	28.6	28.6	5548	1.62	1.59	1.57	1.85
0.20	14.4	13.7	12.9	5535	1.36	0.74	0.12	1.00
Mean				5415				1.40
Solute: Trp; $K_{mc} = 7210$; $K_{mz} = 6.5$; p$K_a = 2.35$								
0.05	59.8	55.9	52.0	16 943	5.45	2.22	−1.00	2.82
0.10	28.3	27.1	25.8	17 984	2.57	1.48	0.40	2.36
0.20	15.5	13.4	11.4	18 554	2.36	0.62	−1.11	1.39
Mean				17 827				2.19
Solute: Met; $K_{mc} = 940$; $K_{mz} = 15$; p$K_a = 2.28$								
0.05	51.9	39.7	27.4	1603	1.67	0.00	−1.61	0.00
0.10	24.6	21.3	17.9	1861	2.60	0.35	−1.91	0.83
0.20	14.6	11.9	9.22	2158	2.27	0.38	−1.51	1.47
Mean				1874				0.77
Solute: PPA; $K_{mc} = 110$; $K_{mz} = 0.3$; p$K_a = 4.63$								
0.05	26.9	25.2	23.5	141	1.53	0.00	−0.52	0.00
0.10	14.9	14.5	14.1	161	0.52	0.16	−0.20	0.16
0.20	8.56	8.46	8.37	187	0.37	0.28	0.19	0.30
Mean				163				0.15

Phe, the values of k'_c are relatively precise, at 0.05 M the value is 58.2 ± 0.2. For Met the value is less precise, at 0.05 M the confidence intervals are k'_c ± 30%. However, the degree of variability tolerable for any one component in the separation of a test mixture, is dependent upon how important that particular component is to the overall quality of the separation. If methionine is not a component of the critical peak pair that determines the minimum resolution of the separation, then a greater latitude in precision is acceptable. For Phe, Trp and PPA, the respective standard errors of the mean (S.E.M.s) for the values of K'_{sc} are 2, 3 and 8% respectively. The mean K'_{sc} values are much greater than the mean K'_{sz} values, for Phe, Trp, Met and PPA, the mean K'_{sz} values are 0.026, 0.012, 0.041 and 0.092% of the respective K'_{sc} values. As these values are so small, then their overall impact on predicting retention at low pH will be small.

All five parameters are available and Fig. 3 shows the fitting of eqn. 14 to the experimental data for Phe. The figure shows that the apparent ionization constant in micellar mobile phase is 1.92–2.60 pH units greater than the aqueous ionization constant. This means that the limiting retention, k'_c, is approached at the low pH limit of a silica-based column (pH 2.5) especially at the higher micelle concentrations. Note that at high pH the solute is barely retained (limiting capacity factors k'_z 0.74–1.59) and therefore it is reasonable to neglect the contribution to retention of the anionic form of the solute and the second ionization constant.

The fit between the experimental and predicted data is not as good at [SDS] = 0.05 M. Inspection of Table II reveals that the calculated K'_{sc} of 5161 at this surfactant concentration is below the mean value of 5415 used in eqn. 12. However, a single mean value is required if eqn. 12 is to be used to predict retention at variable micelle concentration.

Optimization strategy

Fig. 3 shows that a change in the eluent strength of the mobile phase in MLC (changing the micelle concentration) also results in a change in the apparent ionization constant of the

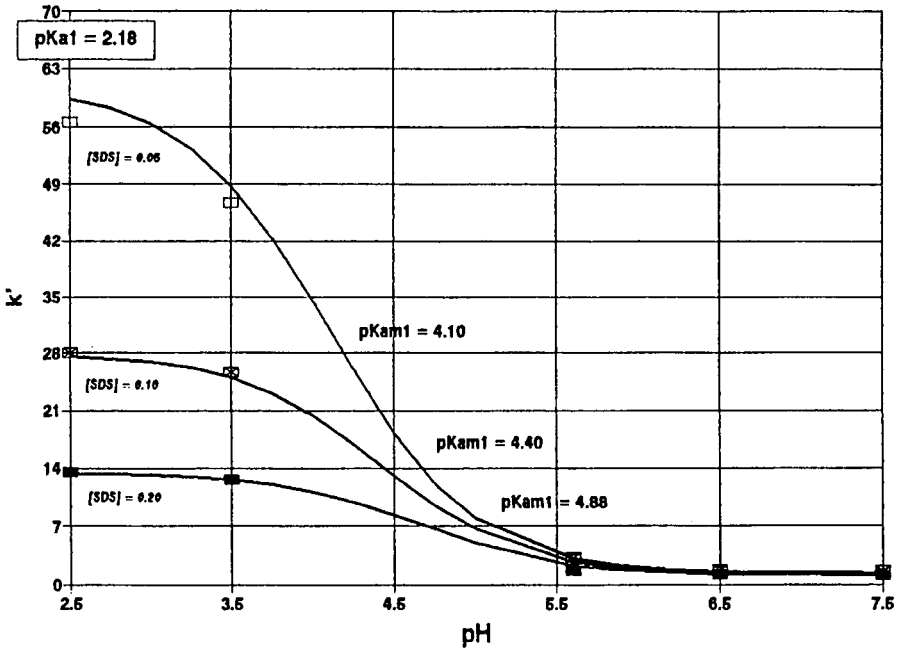


Fig. 3. Retention of Phe as a function of pH and micelle concentration with anionic micelles using eqn. 12 (bold lines). Surfactant concentrations: [SDS] = 0.05 M open squares, 0.10 M square with cross and 0.20 M solid square; [CMC] = 8.1×10^{-3} M. Also shown are apparent ionization constants at each surfactant concentration (pK_{am1} values calculated from the derivatives of the sigmoidal curves; not shown). Constants: $K'_{sc} = 5415$, $K'_{sz} = 1.4$, $pK_{a1} = 2.18$, $K_{mc} = 2100$, $K_{mz} = 1.8$.

solute as the optimum pH and micelle concentration in MLC are directly related. This constraint limits the flexibility of one parameter at a time optimization where the micelle concentration is defined first and then the pH optimized. It will not be possible to optimize by first optimizing pH and then varying the micelle concentration as the ionization constants of the solutes vary with micelle concentration. Therefore a simultaneous, two-parameter optimization of pH and SDS via eqn. 12 should prove an appropriate strategy.

Fig. 4 shows the retention of phenylalanine predicted by eqn. 12. The effect of increasing SDS concentration is shown on the left side of the figure as a parabolic decrease in k' and the sigmoidal effect of pH is shown on the right side of the figure.

Retention surfaces for the other three solutes can be constructed and all four superimposed and a grid search conducted to locate the optimum conditions. The criterion chosen was the maximum minimum resolution, *i.e.* at any location on the grid (in terms of [M] and pH) the resolution is calculated between the four peaks (a total of six computations [35]) and the minimum resolution plotted. The choice of minimum resolution insures adequate separation of the worst peak pair but this criterion does not consider the retention time of the last peak [36] and so the search was limited to [SDS] > 0.05 to ensure a relatively rapid elution of all the components. Fig. 5 shows the calculated minimum resolution for the four solutes as described by eqn. 12 with a maximum minimum resolution of 3.86 at pH 4.5 and [SDS] = 0.05 *M*.

The predicted optimum is investigated further in Fig. 6 which shows the retention as a function of pH at the optimum surfactant concentration (0.05 *M* SDS) in terms of the minimum resolution (line with triangles). The optimum at pH 4.5 and 0.05 *M* SDS is explainable for essentially two reasons. Firstly, at pH less than 3.5 the minimum resolution is less than one due to the similar retention of Trp and Phe but at pH 4.5 these two components will be well separated. It is important to note that the aqueous ionization constants of these solutes are similar (2.35 and 2.18) and below the normal operating pH of silica-based columns (2.5). Therefore the separa-

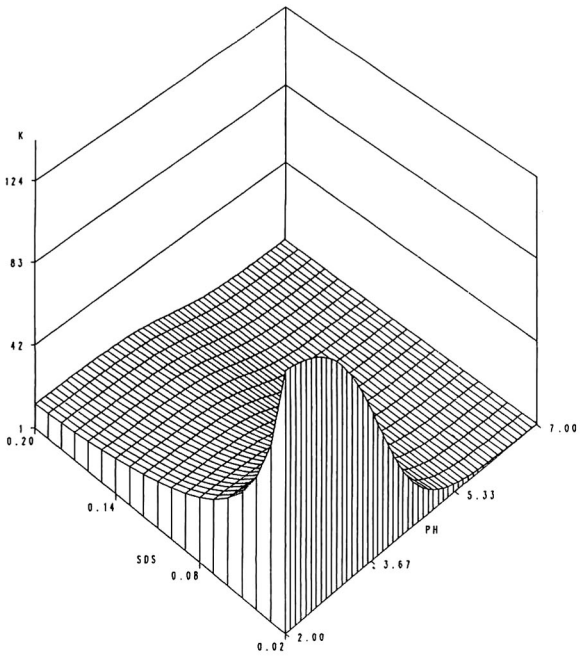


Fig. 4. Retention surface of Phe using eqn. 12, same constants as in Fig. 3.

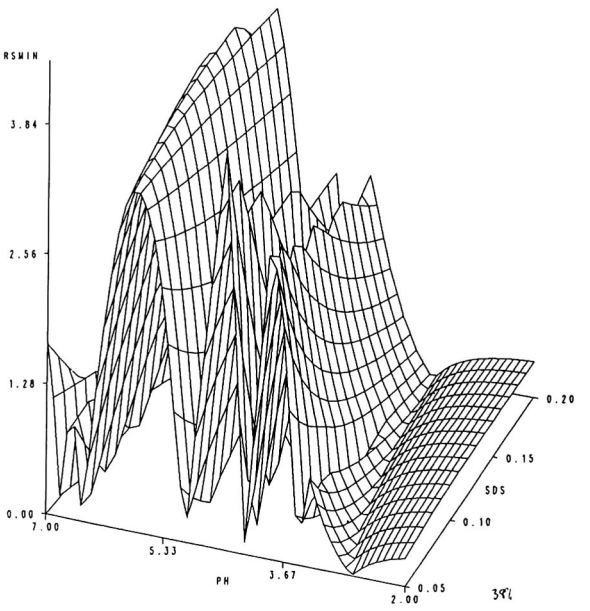


Fig. 5. Response surface in terms of the minimum resolution ($R_{s_{min}}$) for the four solutes as described by eqn. 12 using the constants from Table II.

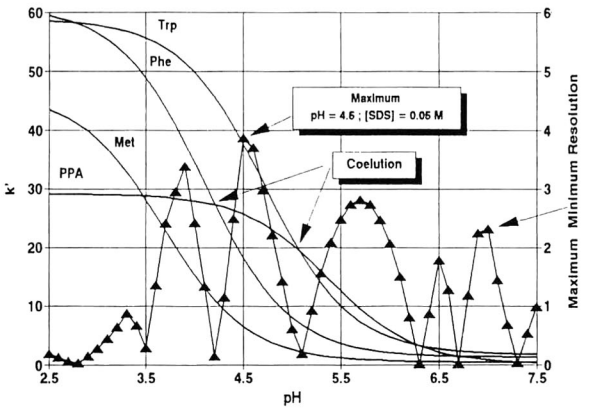


Fig. 6. Cross-section of Fig. 5 at [SDS] = 0.05 *M*. Solid lines, predicted retention of four test solutes. Solid line with triangles, minimum resolution. Also shown are the predicted pH values at which PPA coelutes with Phe and Trp.

tion of these two components at pH 4.5 illustrates the utility of SDS micellar mobile phases which shift the apparent ionization constants of "acidic" solutes to milder pH conditions within the operable limits of silica-based columns.

Secondly, at pH > 3.5 the minimum resolution is dictated by the retention behavior of PPA. Fig. 6 shows that between pH 4.2 and 4.3 the minimum resolution reaches a minimum as Phe and PPA coelute. A similar minimum is found at pH 5.1 where Trp and PPA coelute. Therefore, the model must be sufficiently accurate to predict the behavior of these three solutes between pH 4.3 and 4.8 or PPA may coelute with Phe or Trp.

Fig. 7a shows a simulation of the predicted optimum assuming $N = 2000$. Fig. 7b shows the experimental chromatogram where it is apparent that the first peak (methionine) has a poorer efficiency than the 2000 plates in the simulation. This is probably due to extra-column band broadening which more strongly affects earlier eluting peaks, however for the other three peaks, 2000 plates is a reasonable and typical value for MLC. A comparison of the two figures shows that the predicted and experimental retention are in good agreement with all the peaks well resolved.

Table III shows the predicted retention at the optimum condition compared with the actual retention. The results are consistent in that the predicted retention in all cases exceeds the experimental retention. The errors range from 4.1 to 10.5%. If the results shown in Fig. 7b did not yield the desired separation, then the data from this run would be reentered into the model

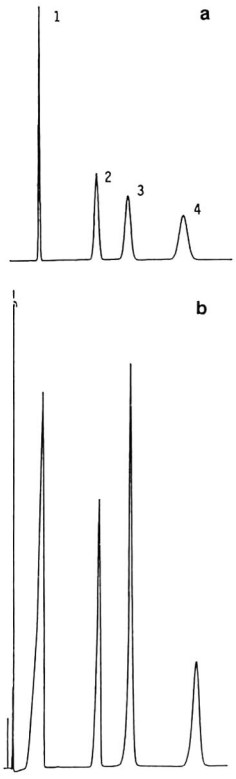


Fig. 7. (a) Predicted optimum using the results from Fig. 5. (b) Experimental verification of (a). 0.05 *M* SDS; 0.05 *M* NaH_2PO_4; pH 4.5. Peaks: 1 = Met; 2 = Phe; 3 = PPA; 4 = Trp.

TABLE III

PREDICTED AND EXPERIMENTAL RETENTION AT 0.05 *M* SDS, pH 4.5

Solute	Predicted		Experimental		% Error	
	k'	α	k'	α	k'	α
Met	6.43		5.95		8.1	
		2.83		2.88		−1.8
Phe	18.2		17.1		6.1	
		1.41		1.35		4.1
PPA	25.6		23.2		10.5	
		1.46		1.55		−5.8
Trp	37.5		36.0		4.1	

(*i.e.* refit eqn. 14 with this additional data point to calculate revised values for K'_{sc} and K'_{sz}) in an iterative procedure until the precision reached an acceptable level [37]. This option may be necessary as normally a precision of 1% or better is required to optimize retention [38]. However, this stringent condition is broadened when systematic as opposed to random error is involved. The results in Table III show that the error results in $k'_{experimental}$ being less than $k'_{predicted}$. What is important is not the absolute error but the relative differences in the errors as all the observed retention are less than the predicted. This is shown in the tabulated % error in α values where a negative value of % error α indicates an increase in peak selectivity while a positive value is indicative of a decrease. Using this criterion, the absolute error of 10.5% in k' for PPA replaced by the more meaningful % error in α of 4.1%.

When interpreting the % error in k' between predicted and experimental values it is important to assess the degree of parameter variability that will produce an equivalent error. This is shown in Table IV where the predicted retention at pH 4.54, [SDS] = 0.05 *M* is compared to the measured retention at pH 4.50, [SDS] = 0.05 *M*. A variation of pH of 0.04 pH units reduces the % error for three of the solutes (Met, Phe and Trp) to less than 1%. This means that when optimizing with pH, a large degree of error in predicting retention is to be expected as a result of small errors in pH measurement. However, if this error is systematic then the selectivity is less likely to be unduly compromised.

TABLE IV

PREDICTED AND EXPERIMENTAL RETENTION AT 0.05 *M* SDS, pH 4.5

Solute	Predicted		Experimental		% Error	
	k'	α	k'	α	k'	α
Met	5.98		5.95		0.5	
		2.86		2.88		−0.7
Phe	17.1		17.1		−0.2	
		1.48		1.35		9.4
PPA	25.4		23.2		9.3	
		1.43		1.55		−7.7
Trp	36.3		36.0		0.8	

TABLE V

PREDICTED AND EXPEIRMENTAL RETENTION AT 0.0566 *M* SDS, pH 4.5

Solute	Predicted		Experimental		% Error	
	k'	α	k'	α	k'	α
Met	6.01		5.95		1.0	
		2.88		2.88		−0.0
Phe	17.3		17.1		0.9	
		1.33		1.35		−1.8
PPA	23.0		23.2		−0.9	
		1.47		1.55		−5.4
Trp	33.8		36.0		−6.2	

Table V shows a similar variation where the influence of errors in surfactant concentration are assessed at the predicted optimum condition. By increasing the surfactant concentration to 0.0566 *M* the % error in retention for three of the solutes are reduced to 1% or less.

CONCLUSIONS

Eqn. 12 predicts that the apparent ionization constants of solutes in micellar solution are displaced from the values measured in purely aqueous media. The pK_{a1} of the carboxylic acid group are shown to increase with anionic SDS micelles.

Due to the dependence of the apparent ionization constants on micelle concentration in MLC, it is demonstrated that a simultaneous optimization of surfactant concentration and pH is the appropriate strategy for the prediction of the optimum condition with a limited number of experiments. In order to demonstrate the validity of the model it is necessary to optimize retention with respect to pH. However, it is difficult to predict and reproduce retention to a high degree of precision with this parameter as retention is strongly dependent on this variable when the pH is within (±) 1 pH unit of the solutes apparent ionization constants. However this does not detract from the utility of the presented approach; the above separation shows

that if the errors are systematic then the separation is not significantly compromised. Also, the multi-parameter approach enables the selection of the optimum condition and allows for the evaluation of the robustness of the optimum. If necessary, an educated decision can be made where selectivity (separation) can be sacrificed for a more robust analysis.

ACKNOWLEDGEMENT

This work was supported by a research grant from the National Institutes of Health (FIRST Award, GM 38738) to North Carolina State University.

REFERENCES

1 C.M. Kirkman, C. Zu-Ben, P.C. Uden, W.J. Stratton and D.E. Henderson, *J. Chromatogr.*, 317 (1984) 569.
2 J.H. Knox and R.A. Hartwick, *J. Chromatogr.*, 204 (1981) 3.
3 H.A.H. Billiet, J. Vuik, J.K. Strasters and L. de Galan, *J. Chromatogr.*, 384 (1987) 153.
4 D. Westerlund, *Chromatographia*, 24 (1987) 155.
5 J. Haginaka, J. Wakai, H. Yasuda and T. Nakagawa, *Anal. Chem.*, 59 (1987) 2732.
6 F.J. De Luccia, M. Arunyanart and L.J. Cline Love, *Anal. Chem.*, 57 (1985) 1564.
7 J.G. Dorsey, J.F. Clos and K.B. Sentell, *BioChromatography*, 4 (1989) 35.
8 Y.-N. Kim and P.R. Brown, *J. Chromatogr.*, 384 (1987) 209.
9 L.P. Stratton, J.B. Hynes, D.G. Priest, M.T. Doig, D.A. Barrow and G.L. Asleson, *J. Chromatogr.*, 357 (1987) 183.
10 F. Palmisano, A. Guerrieri, P.G Zambonin and T.R.I. Cataldi, *Anal. Chem.*, 61 (1989) 946.
11 W.L. Hinze and D.W. Armstrong (Editors), *Ordered Media in Chemical Separation* (*ACS Symposium Series*, Vol. 342), American Chemical Society, Washington, DC, 1987.
12 M. Arunyanart and L.J. Cline Love, *J. Chromatogr.*, 342 (1985) 293.
13 W.L. Hinze, H.N. Singh, Y. Bamba and N.G. Harvey, *Trends Anal. Chem.*, 3 (1984) 193.
14 M.S. Abdel-Latif and G.G. Guilbault, *Anal. Chim. Acta*, 221 (1989) 11.
15 F.N. Albahadily and H.A. Mottola, *Anal. Chem.*, 59 (1987) 958.
16 A. Berthod and J.G. Dorsey, *Analusis*, 16 (1988) 75.
17 C. Gonnet, A. Berthod and I. Girard, *Anal. Chem.*, 58 (1986) 1356.
18 J.G. Dorsey, M.G. Khaledi, J.S. Landy and J.-L. Lin, *J. Chromatogr.*, 316 (1984) 183.
19 L.S. Madamba, *M.Sc. Thesis*, North Carolina State University, Raleigh, NC, 1991.
20 M.G. Khaledi, J.K. Strasters, A.H. Rodgers and E.D. Breyer, *Anal. Chem.*, 62 (1990) 130.
21 J.P. Foley and W.E. May, *Anal. Chem.*, 59 (1987) 102.
22 B.L. Karger, J.N. Le Page and N. Tanaka, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography —Advances and Perspectives*, Vol. 1, Academic Press, New York, 1980, p. 113.
23 L.J. Cline Love and M. Arunyanart, *Anal. Chem.*, 57 (1985) 2837.
24 E. Pelizzetti and E. Pramauro, *J. Phys. Chem.*, 88 (1984) 990.
25 M. Arunyanaryt and L.J. Cline Love, *Anal. Chem.*, 56 (1984) 1557.
26 M.G. Khaledi and A.H. Rodgers, *Anal. Chim. Acta*, 239 (1990) 121.
27 A.H. Rodgers, *Ph.D. Dissertation*, University of New Orleans, New Orleans, LA, 1990.
28 A.H. Rodgers and M.G. Khaledi, *Anal. Chem.*, submitted for publication.
29 M.G. Khaledi, S.C. Smith and J.K. Strasters, *Anal. Chem.*, 63 (1991) 1820.
30 S.C. Smith and M.G. Khaledi, *J. Chromatogr.*, 432 (1993) 177.
31 O.A. El Seoud, *Adv. Colloid. Interface Sci.*, 30 (1989) 1.
32 E. Pelizzetti and E. Pramauro, *J. Phys. Chem.*, 88 (1988) 990.
33 J.P. Foley, *Anal. Chim. Acta*, 231 (1990) 237.
34 D.W. Armstrong and F. Nome, *Anal. Chem.*, 53 (1981) 1662.
35 J.L. Glajch, J.J. Kirkland, K.M. Squire and J.M. Minor, *J. Chromatogr.*, 199 (1980) 57.
36 A.C.H.J. Drouen, P.J. Schoenmakers, H.A.H. Billiet and L. de Galan, *Chromatographia*, 16 (1982) 48.
37 P.J. Schoenmakers, *Optimization of Chromatographic Selectivity* (*Journal of Chromatography Library*, Vol. 35), Elsevier, Amsterdam, 1986.
38 P.J. Schoenmakers and T. Blaffert, *J. Chromatogr.*, 384 (1987) 117.

Journal of Chromatography, 636 (1993) 213–220
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 915

Evaluation of reversed-phase columns for the analysis of very basic compounds by high-performance liquid chromatography

Application to the determination of the tobacco alkaloids

David Victor McCalley

Department of Chemical and Physical Sciences, University of the West of England, Frenchay, Bristol BS16 1QY (UK)

(First received November 10th, 1992; revised manuscript received January 14th, 1993)

ABSTRACT

A rapid separation of the major tobacco alkaloids with high efficiency and acceptable peak tailing is demonstrated on a RP-HPLC column containing a high-purity silica substrate, without use of silanol masking agents in the eluent. A test procedure based on that of Engelhardt and co-workers, with pyridine as an additional probe compound, was used to aid the selection of a suitable stationary phase from a number of the best commercially available columns for the separation of strongly basic compounds.

INTRODUCTION

The production and characterisation of packing materials suitable for the analysis of basic compounds by HPLC using RP columns has been an area of much recent work due to important applications in pharmaceutical analysis and in the separation of proteins [1–3]. The poor chromatography of these compounds has been largely attributed to undesirable interactions with residual silanol groups on the silica substrate. Engelhardt and co-workers [4,5], after careful review of a number of procedures, have proposed a test for the evaluation of the properties of RP columns, which includes assessment of their suitability for the analysis of basic compounds.

The quantitation of the tobacco alkaloids can be of importance in the development of breeding programmes designed to influence the levels of these compounds in the final product. Furthermore, much interest has been shown in biotechnological studies in determining these alkaloids in cell suspension and other plant cultures [6]. Although analysis of tobacco alkaloids can be performed by GC using deactivated columns [7], the ease of direct injection of aqueous extracts of culture materials by HPLC is a distinct advantage, especially when considering the potential volatility of nicotine and its possible loss in sample preparation procedures. These alkaloids include strong bases; the reported pK_a value of the pyrrolidine nitrogen atom in nicotine is 8.02 [8]. A recent study has indicated that peak tailing in RP-HPLC generally increases with increasing pK_a of the analyte [9]. For the analysis of the tobacco alkaloids, column performance characteristics have not been formally reported

0021-9673/93/$06.00

but a popular HPLC procedure shows rather low column efficiency and tailing peaks, even though the silanol masking agent triethylamine, which is widely recommended to improve peak symmetry, was incorporated in the mobile phase (pH 7.25) [10]. In addition, the μBondapak C_{18} column used is considered good for the analysis of basic compounds, amongst the long-established commercially available phases [9,11]. A more recent study [12] in which a triethylamine containing buffer at pH 3.5 was used seemed to show little improvement in column performance. Dolan and Snyder [13] have noted that the addition of triethylamine fails to yield symmetrical peaks for some compounds. Several authors, including Kimata *et al.* [14] have shown that metal impurities in alkylsilated silica packing materials may contribute detrimental interactions for some types of compound; this might explain why use of silanol masking agents has not given better results in the analysis of these alkaloids. Thus, in the present study we have investigated the performance of columns without use of amine additives. Such a study should also give an idea of the inherent overall activity of the column towards basic compounds; as noted by Vervoort *et al.* [9] the use of silanol blockers may conceal differences between columns. Some disadvantages are also inherent when amine additives are used: for instance, they may be difficult to remove from the column when changing mobile phases [13]. Some additives may be aggressive towards the stationary phase [9] or contribute to the complexity and detector background of the mobile phase (for instance when LC–mass spectrometry is used). Finally, although not important for the compounds of the present study, some analytes containing carbonyl groups may chemically react with the amine additive in the mobile phase [15]. Nevertheless, with triethylamine and other basic masking agents, good column efficiency and peak shape have been obtained with basic drugs and similar compounds using a variety of columns [9,16].

A test procedure based on that of Engelhardt and co-workers was investigated for its ability to select a suitable RP column for this application. The columns were chosen to be representative of some different approaches that have been taken in column manufacture in order to give good performance for basic compounds. Some of the columns tested here have been recommended as the best in previous comparative studies. The columns tested included a packing based on high-purity silica with low metal content, an "electrostatically shielded" reversed phase, a silica column with a high (polymeric) ODS loading, a porous graphitic carbon column and a polymer-coated pH-stable alumina column.

EXPERIMENTAL

The HPLC system consisted of a SP8800 pump, a Spectra 100 variable-wavelength UV detector with time constant 0.1 s and a 9-μl flow cell (all from Spectra-Physics, San Jose, CA, USA) and a valve injector equipped with a 5-μl loop (Rheodyne, Cotati, CA, USA). Efforts were made to keep instrumental dead volume to a minimum; in these comparative studies of column performance we did not wish to use mobile phases of differing organic solvent composition. This could affect both the wetting of the stationary phase (see below) and the degree of ionisation of ionisable analytes. Column efficiency values (N) were determined from peak widths at half height. Asymmetry factors (A_s) were calculated at 10% of the peak height from the ratio of the widths of the rear and front sides of the peak, using a Model 2000 data station (Trivector, Bedford, UK) in conjunction with a BASIC program. Peak identities were verified in biological samples using a photodiode array detector system (Integral 4000, Perkin-Elmer, Beaconsfield, UK). The new columns used included LiChrospher RP-8 Select B 5 μm, 25 × 0.4 cm I.D. (Merck, Darmstadt, Germany), Hypercarb 7 μm, 10 × 0.32 cm I.D. (Shandon, Runcorn, UK), Inertsil ODS 5 μm, 25 × 0.46 cm I.D. (GL Sciences, Tokyo, Japan), Nucleosil C_{18} AB 5 μm, 25 × 0.4 cm (Macherey–Nagel, Düren, Germany), pH-stable alumina–C_{18} 3 μm, 10 × 0.46 cm (Phase Sep, Deeside, UK) and Suplex pK_b-100 5 μm, 15 × 0.46 cm (Supelco, Bellefonte, PA, USA). All columns were operated at 20°C. Buffers were prepared by dissolving 6.803 g of KH_2PO_4 in 1 l of pure

water, and adjusting the pH (before addition of organic modifier) with either concentrated H_3PO_4 or 0.05 *M* KOH. The test mixture for use with unbuffered mobile phases consisted of uracil (*ca.* 1 mg l^{-1}), pyridine (*ca.* 5 mg l^{-1}), aniline (*ca.* 10 mg l^{-1}), phenol (*ca.* 30 mg l^{-1}), *o*-toluidine (*ca.* 5 mg l^{-1}), *m*-toluidine (*ca.* 5 mg l^{-1}), *p*-toluidine (*ca.* 5 mg l^{-1}) and benzene (*ca.* 150 mg l^{-1}). Standard solutions of the tobacco alkaloids contained 5–50 mg l^{-1} of each alkaloid. All column performance results are the average of duplicate determinations.

RESULTS AND DISCUSSION

The test mixture proposed by Engelhardt and co-workers contains toluene and ethylbenzene as probes of hydrophobic interaction, phenol and ethyl benzoate as neutral polar probes, aniline, N,N-dimethylaniline and the isomeric *o*-, *m*- and *p*-toluidines as basic probes, and thiourea as an inert probe. Methanol–water (55:45, v/v) without addition of buffer or salt solutions is used as the mobile phase. Under these conditions the RP is totally wetted (less than 60% water) giving the greatest influence of silanol groups on solute retention. According to Engelhardt *et al.* [5], at higher water concentrations, the interactions of the alkyl groups become stronger, and they can collapse to a film, shielding and covering the surface silanols. A good column for analysis of bases is signified by the elution of aniline before phenol with the ratio of A_s for aniline/phenol being 1.3 or less, and by the co-elution of the isomeric toluidines (or ratio of k' values being below 1.3). We have found this test procedure extremely useful for the characterisation of different columns. For the present study which concentrates on the evaluation of the properties of columns with regard to basic solutes, we have made some modifications to the test. We have preferred to use uracil as a void volume marker after the work of Bidlingmeyer *et al.* [17]. We omitted ethyl benzoate which can be used to distinguish between C_8 and C_{18} phases. Furthermore we have substituted benzene as a hydrophobic probe because when using the specified mobile phase, the retention of compounds such as toluene and ethyl benzene was excessive on some of the more heavily loaded phases utilised in this work. Finally, we have substituted pyridine, which has been used as a probe by some authors and commercial companies [18] for N,N-dimethylaniline (N,N-DMA). Engelhardt and co-workers state that N,N-DMA is not a particularly sensitive tracer for silanophilic interactions. In our own work we have found that aniline, despite having a lower pK_a value than N,N-DMA, usually gives more asymmetric peaks than N,N-DMA. Pyridine is a stronger base than either aniline or N,N-DMA according to the pK_a values (5.25, 4.63 and 5.15, respectively [8]); however, the ability of less sterically hindered amines to penetrate the phase and interact with active sites is also important [9,18].

We did not wish to evaluate a large number of well established ODS phases; much information is available from previous studies and from our own work [4,9,11,18]. We chose LiChrospher RP-8 Select B as a "benchmark" phase since it was stated as one of the best by Engelhardt and co-workers; we have also used this material successfully in the separation of the basic cinchona alkaloids [15]. The other columns chosen for further study here were more recently available materials all designed specifically for the separation of basic (and in some cases also acidic compounds). Nucleosil-type silicas were first identified by Köhler *et al.* [3] as giving low adsorption of basic solutes. The new Nucleosil C_{18} AB column, is according to the manufacturers, a cross-linked C_{18} phase with 25% carbon content, giving increased shielding of the silica matrix. The Suplex pK_b-100 column incorporates anion-exchange sites in the stationary phase which repel positively charged amines and thus give electrostatic shielding of the surface. This column gave superior performance for bases in two comparative surveys of deactivated columns [9,18]. Inertsil ODS is, according to the manufacturers, a material with 14% carbon content based on a spherical silica of 99.9% purity. Metal impurities are known to participate in the retention of some solutes (see above). So-called pH-stable silica columns have enhanced stability at high pH which allows work with buffers in which basic analytes may not be protonated,

eliminating undesirable ion-exchange interactions. They are generally prepared by encapsulation of the substrate with a polymeric layer such as polybutadiene [1]. Alumina columns of this type are also available. Finally, porous graphitic carbon phases are not designed especially for basic solutes, but being based on a different substrate, might not be expected to show the same problems as silica phases.

The silica-based columns other than Nucleosil C_{18} AB yielded 60–80 000 plates m^{-1} for benzene under the normal test conditions. The 3-μm alumina column showed slightly higher efficiencies for this test in terms of plates m^{-1}. However, such figures for short columns exaggerate their potential because the back pressure required for the operation of longer columns may not allow this performance to be realised in practice. Nucleosil C_{18} AB gave a somewhat lower plate number ($N = 50\,000$ plates m^{-1}) which we attribute to the high loading of polymeric ODS [19]. All of these columns gave A_s for benzene of between 1.00 and 1.35. Table I shows the other results obtained using the modified test of Engelhardt and co-workers for four of the above columns. All would be considered "good" for the analysis of basic compounds based on the criteria of Engelhardt and co-workers: all show coelution of the isomeric toluidines (ratio of $k' < 1.3$), all elute aniline before phenol (*i.e.* $k'_{phenol}/k'_{aniline} > 1$) and all show a ratio of the asymmetry of aniline/phenol peaks <1.3. However, it does not seem possible to distinguish "excellent" from merely "good" columns using this method because the test results are similar in each case. The ratio of the capacity factors of phenol and aniline (Table I) indicates by how much aniline is eluted before phenol. However, it does not seem possible to use this figure in a quantitative sense. The column efficiency and asymmetry for pyridine (or the asymmetry ratio pyridine/phenol) does appear to reveal some differences between the columns. Furthermore, on the Inertsil and Suplex columns, pyridine eluted before aniline. On Nucleosil pyridine coeluted with aniline, and on Lichrospher it coeluted with phenol, making separate measurement of pyridine data necessary. The chromatogram of these test compounds obtained using Inertsil ODS is shown in Fig. 1.

None of the columns gave satisfactory elution of benzylamine when using methanol–water mixtures with *no* additional components. Benzylamine is technically an aliphatic amine, with pK_a 9.3 [8]. Table I also indicates that nicotine gave similar results with distorted peaks and very low efficiency in these unbuffered

TABLE I

ANALYSIS OF TEST COMPOUNDS ON DEACTIVATED SILICA ODS COLUMNS

an = Aniline; phen = phenol; pyr = pyridine; bzylam = benzylamine. Mobile phase methanol–water (55:45, v/v), flow-rate 1 ml min^{-1}. Detection UV at 254 nm. Column temperature 20°C. Asymmetry factors are not reported for peaks with $N < 1000$ plates m^{-1}.

Column	k' ratio toluidines	an/phen A_s ratio	k' phen/an	pyr		pyr/phen A_s ratio	bzylam		nicotine	
				$N(m^{-1})$	A_s		$N(m^{-1})$	A_s	$N(m^{-1})$	A_s
Nucleosil C_{18} AB	1.0	1.16	1.4	5 320	3.80	2.66	<1000		<1000	
Suplex pKb-100	1.0	1.01	2.0	33 000	1.57	1.18	2700	0.58	2890	0.40
Inertsil ODS	1.0	1.05	1.5	44 400	1.68	1.36	<1000		1300	2.55
LiChrospher RP-8 Select B	1.1	1.10	1.3	26 500	2.30	1.56	retained		5760	4.55

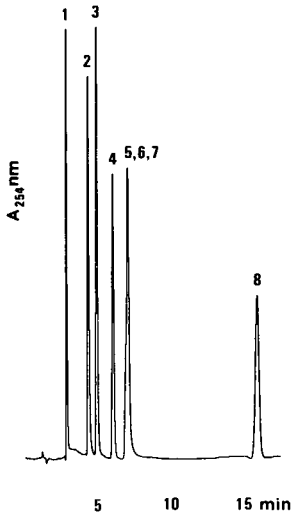


Fig. 1. Separation of test compounds using Inertsil ODS. Mobile phase methanol–water (55:45, v/v), flow-rate 1 ml min^{-1}. Detection UV at 254 nm. Column temperature 20°C. Peaks: 1 = uracil; 2 = pyridine; 3 = aniline; 4 = phenol; 5 = *o*-toluidine; 6 = *m*-toluidine; 7 = *p*-toluidine; 8 = benzene.

mobile phases. Aliphatic amines are much more difficult to analyse than aromatic amines. Thus it seems they cannot as yet be included in test mixtures for silica based columns designed for use with simple organic solvent–water mobile phases that do not contain buffer components. Buffer solutions not only stabilise the pH environment for chromatography of ionisable compounds but also can provide cations which can counter the ion exchange interaction of basic compounds with dissociated silanol groups in the packing. Table II shows the column performance for pyridine, benzylamine, nicotine and quinine using methanol–0.05 *M* phosphate buffer pH 6.25 (55:45, v/v). This pH was not necessarily optimum for analysis of the compounds; indeed it may highlight ion exchange with dissociated silanols. Silanol group dissociation is suppressed at lower pH; thus, nicotine and quinine, another basic alkaloid, gave improved results on the Lichrospher column using a pH 3 buffer [15]. In general, basic compounds tend to give sharper, more symmetrical peaks at pH 3 [20]; however, a recent study advocates the use of a higher pH (7.4) for compounds with moderate pK_a which, unlike nicotine, would not be protonated at this pH [9]. Column performance for pyridine seemed little changed by use of the pH 6.25 buffer rather than water in admixture with methanol. However, substantial improvements for the chromatography of benzylamine and nicotine were noted, presumably due to the competitive effect of buffer K^+ ions. Examination of Tables I and II shows that the performance for pyridine in unbuffered mobile phases does appear to give a reasonable indication of the likelihood of success for analysis of basic alkaloids in buffered mobile phases. This suggests that pyridine is a useful simple test

TABLE II

PERFORMANCE DATA FOR NICOTINE AND OTHER BASIC COMPOUNDS ON DEACTIVATED SILICA COLUMNS

Mobile phase methanol–0.05 *M* phosphate pH 6.25 (55:45, v/v), flow-rate 1 ml min^{-1}. Detection UV at 254 nm. Column temperature 20°C.

Column	Pyridine		Benzylamine		Nicotine		Quinine	
	$N(m^{-1})$	A_s	$N(m^{-1})$	A_s	$N(m^{-1})$	A_s	$N(m^{-1})$	A_s
Nucleosil C_{18} AB	4 920	3.81	1 990	6.47	5 100	4.87	7 660	3.64
Suplex pKb-100	36 200	1.64	9 130	1.60	22 300	2.47	21 700	1.72
Inertsil ODS	40 700	1.62	31 800	1.73	42 300	1.77	29 500	1.91
Lichrospher RP-8 Select B	24 300	2.10	10 600	2.04	8 400	4.62	6 720	4.60

compound which can be incorporated in the system of Engelhardt and co-workers. Benzylamine appears to be too severe a test compound, giving poor results even with the buffered mobile phase on most columns. Furthermore, this compound showed little retention on some columns, even when low concentrations of organic modifier were used.

The pH-stable alumina column gave poor results for the chromatography of basic compounds using the methanol–water mobile phase, and did not pass the asymmetry ratio test for aniline and phenol. This indicates a high activity of the underlying material. Nevertheless, these columns can be used at high pH without apparent ill effect. Excellent results were obtained for the chromatography of pyridine (40 000 plates m^{-1}, A_s 1.53) using acetonitrile–ammonium nitrate buffer (pH 10). Surprisingly, however, nicotine still gave some peak tailing even when a pH 12 sodium hydroxide buffer was used (best results about 30 000 plates m^{-1} with $A_s = 2.4$). This indicates the presence of undesirable interactions even when the compound is uncharged. A new pH-stable alumina RP material for basic compounds (Aluspher RP-select B) has recently become commercially available [21]. Manufacturers' literature would suggest that this material is more inert, but gives somewhat lower efficiency than the material tested here.

The porous graphitic carbon column tested showed low efficiency for phenol and benzene ($N < 18\,000$ plates m^{-1}) with considerable peak asymmetry ($A_s > 2$) when using organic solvent/water mixtures. Nevertheless, similar results were shown for basic compounds and nicotine gave $N = 17\,000$ plates m^{-1} with A_s 2.3 using an acetonitrile–water mixture without addition of salts, a result which is better than any of the other columns. However, we were not able to improve on these results by modification of the mobile phase, and the column was not considered further. Further development of such columns may yield improved performance for all types of compound.

Of the columns investigated in our study, the high-purity silica ODS column seems to give the best performance for the analysis of the tobacco alkaloids. However, in agreement with other studies [9,18], the Suplex pK_b-100 column also seems to give very good general performance for basic compounds. In addition to the detrimental effects of silanol groups, metals can provide surface acidity, ion-exchange sites or promote interactions with analytes that have chelating properties; it is possible that the alkaloids, having two basic nitrogen atoms, could possess some chelating properties, in addition to Lewis base properties. However, the stereochemistry appears unfavourable. We were unable to improve significantly the performance of the LiChrospher and Nucleosil columns for the analysis of nicotine by further addition of EDTA to the eluent used to obtain the results for Table II [13]. The Inertsil column also had the advantage of greater retention of nicotine than many of the other columns allowing greater flexibility of choice of the mobile phase composition and pH. Finally, it is in other respects a conventional ODS phase which can be used in the normal way. Fig. 2a shows the separation of the four major tobacco alkaloids nicotine, nornicotine, anabasine and anatabine together with the minor

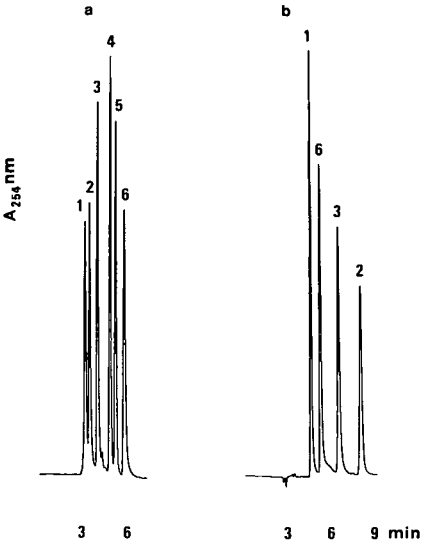


Fig. 2. Separation of tobacco alkaloids using Inertsil ODS. Detection UV at 254 nm. Flow-rate 1 ml min^{-1}. Column temperature 20°C. Mobile phase (a) methanol–0.05 *M* potassium phosphate pH 6.25 (60:40, v/v); (b) methanol–0.05 *M* potassium phosphate pH 3.00 (10:90, v/v) Peaks: 1 = nornicotine; 2 = anabasine; 3 = anatabine; 4 = myosmine; 5 = 2,3-dipyridyl; 6 = nicotine.

compounds myosmine and 2,3-dipyridyl on the Inertsil column using a similar mobile phase to that in Table II. In all cases, we injected small amounts of the alkaloids (see Experimental section). The injection of large amounts can contribute to peak tailing, presumably due to saturation of column active sites [9]. Doubling the concentration of the buffer solution to 0.1 *M* did not give significant improvements in the peak shape of these compounds, although interesting selectivity effects could be obtained. The retention of nicotine relative to the other alkaloids was noticeably affected by buffer strength; ion exchange still seems to give some contribution to the retention of these compounds at pH 6.25 even with this column. Reduction of the mobile phase pH increases the degree of protonation of the alkaloids and thus reduces hydrophobic retention of the compounds; furthermore ion-exchange retention of the alkaloids should be reduced due to suppression of silanol dissociation. On some of the columns, a reduction in pH produced very low capacity factors for the tobacco alkaloids even when using low concentrations of organic modifier in the mobile phase. However, the relatively high phase loading of the Inertsil ODS column gave reasonable retention of the alkaloids when using a pH 3.0 phosphate buffer containing 10% methanol. For routine analysis of the four major tobacco alkaloids we would recommend use of the pH 3.0 buffer system (Fig. 2b); column performance data are shown in Table III. Some improvement in peak shape, particularly for nornicotine, was noted at the lower pH. Furthermore, the first peak is eluted further from the void volume of the column, reducing the possibility of co-elution of the analytes with matrix compounds when biological samples are analysed. Finally, column deterioration may occur more rapidly at the higher pH [9]. On the other hand, improved results for the analysis of the minor alkaloids 2,3-dipyridyl and myosmine were obtained using the pH 6.25 eluent. 2,3-Dipyridyl gave excessive retention with the methanol–pH 3 buffer (10:90) mobile phase necessitating a gradient elution separation with increasing methanol concentration and the peak shape for myosmine was poorer using this eluent. Nevertheless, analysis of the major alkaloids is possible using either eluent and since the order of elution of the analytes (and potentially matrix constituents) is different in each, the possibility of peak overlap can be ascertained by comparison of results. This procedure can be used in addition to peak purity checks by diode array spectrophotometry. However, for samples containing low levels of alkaloids, considerable care must be taken in sample preparation procedures used to purify the

TABLE III

PERFORMANCE DATA FOR TOBACCO ALKALOIDS ON INERTSIL ODS

Mobile phase methanol–0.05 *M* phosphate buffer pH 3.0, flow-rate 1 ml min^{-1}. Detection UV at 254 nm. Column temperature 20°C.

	Nornicotine	Nicotine	Anatabine	Anabasine
$N(m^{-1})$	47 600	43 200	46 000	48 400
A_s	1.51	1.72	1.60	1.46

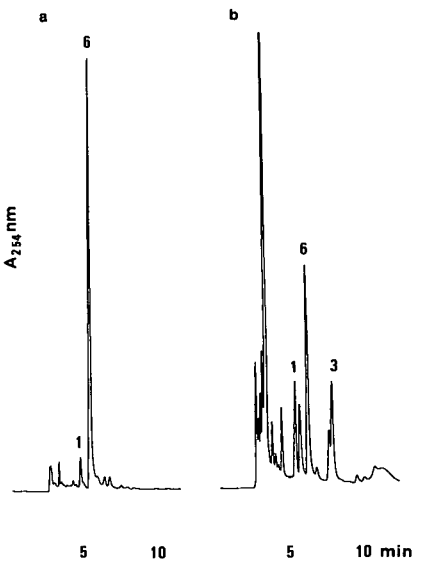


Fig. 3. Analysis of alkaloids in (a) commercial cigarette tobacco [mobile phase methanol–0.05 *M* potassium phosphate pH 3.00 (10:90, v/v)]. (b) hairy root culture of Wisconsin 38 [mobile phase methanol–0.05 *M* potassium phosphate pH 3.00 (8:92, v/v)]. Flow-rate 1 ml min^{-1}. Column temperature 20°C. Detection UV at 254 nm. Peaks as in Fig. 2.

extracts prior to HPLC; we are presently reviewing alternative procedures.

Fig. 3 shows the application of the method to the determination of the tobacco alkaloids in a phosphate buffer extract [10] of (a) a sample of commercial cigarette tobacco and (b) a hairy root culture of the tobacco plant Wisconsin 38. The chromatograms indicate an improvement on previously published methods for the HPLC analysis of these compounds.

CONCLUSIONS

Some of the best available stationary phases for RP-HPLC of basic substances were tested, with the objective of establishing an improved method for the analysis of the tobacco alkaloids. Superior results were obtained using a high purity silica-ODS phase. Pyridine is a useful additional test compound for evaluation of these phases. Rapid analysis of the tobacco alkaloids with symmetrical peaks was achieved without use of silanol masking agents. The HPLC method is especially suitable for applications where injection of aqueous extracts is desired. Moreover, the high performance of the method may make the method competitive with GC in some other applications.

ACKNOWLEDGEMENTS

The author thanks M.M. Yeoman, University of Edinburgh (UK) and S.J. Stotesbury, BAT, Southampton (UK) for generous gifts of alkaloid standards.

REFERENCES

1 M.J. Hetem, J.W. de Haan, H.A. Claessens, C.A. Cramers, A. Deege and G. Schomburg, *J. Chromatogr.*, 540 (1991) 53.
2 J.J. Kirkland, J.L. Glajch and R.D. Farlee, *Anal. Chem.*, 61 (1989) 2.
3 J. Köhler, D.B. Chase, R.D. Farlee, A.J. Vega and J.J. Kirkland, *J. Chromatogr.*, 352 (1986) 275.
4 H. Engelhardt and M. Jungheim, *Chromatographia*, 29 (1990) 59.
5 H. Engelhardt, H. Löw and W. Götzinger, *J. Chromatogr.*, 544 (1991) 371.
6 M.C. Hobbs and M.M. Yeoman, *J. Exp. Botany*, 42 (1991) 1371.
7 R.F. Severson, K.L. McDuffie, R.F. Arrendale, G.R. Gwynn, J.F. Chaplin and A.W. Johnson, *J. Chromatogr.*, 211 (1981) 111.
8 R.C. Weast (Editor), *CRC Handbook of Chemistry and Physics*, CRC Press, Boca Raton, FL, 1st student ed., 1988.
9 R.J. Vervoort, F.A. Maris and H. Hindriks, *J. Chromatogr.*, 623 (1992) 207.
10 J.A. Saunders and D.E. Blume, *J. Chromatogr.*, 205 (1981) 147.
11 D.V. McCalley, *J. Chromatogr.*, 357 (1986) 221.
12 F. Manceau, M.A. Fliniaux and A. Jacquin-Dubreuil, *Phytochem. Anal.*, 3 (1992) 65.
13 J.W. Dolan and L.R. Snyder, *Troubleshooting LC Systems*, Humana Press, Clifton, NJ, 1989, Ch. 14.
14 K. Kimata, N. Tanaka and T. Araki, *J. Chromatogr.*, 594 (1992) 87.
15 D.V. McCalley, *Analyst*, 115 (1990) 1355.
16 D.V. McCalley, *Chromatographia*, 17 (1983) 264.
17 B.A. Bidlingmeyer, F.V. Warren, A. Weston, C. Nugent and P.M. Froehlich, *J. Chromatogr. Sci.*, 29 (1991) 275.
18 T.L. Ascah and B. Feibush, *J. Chromatogr.*, 506 (1990) 357.
19 C.F. Poole and S.K. Poole, *Chromatography Today*, Elsevier, Amsterdam, 1991, Ch. 4, p. 326.
20 L.R. Snyder, J.L. Glajch and J.J. Kirkland, *Practical HPLC Method Development*, Wiley, New York, 1988, Ch. 4, 5.
21 K. Cabrera, D. Lubda, G. Jung, *Kontakte (Darmstadt)*, 1 (1992) 32.

Journal of Chromatography, 636 (1993) 221–229
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 879

Separation and analysis of amino alcohol-containing diacylglycerophospholipids and their hydrolytic metabolites

H. Kunze* and E. Bohn

Max-Planck-Institut für Experimentelle Medizin, Hermann-Rein-Strasse 3, W-3400 Göttingen (Germany)

(First received October 29th, 1992; revised manuscript received January 13th, 1993)

ABSTRACT

A simple radiometric procedure is presented for the separation and determination of amino alcohol-labelled zwitterionic phospholipids (phosphatidylcholine and phosphatidylethanolamine) and their hydrolytic metabolites. The protocol allows the determination of all potential amino alcohol-containing metabolites, in a range of at least 0.2–500 nmol, in the absence or presence of non-ionic (Triton X-100) and ionic (sodium deoxycholate, hexadecyltrimethylammonium bromide) detergents. The discontinuous assay is based on the determination of both water-soluble and lipid-soluble metabolites, *i.e.*, on lipid extraction and thin-layer and ion-exchange chromatography. In addition, simplified and less time-consuming modifications of the procedure have been developed for specifically monitoring phospholipase C and D activities in the course of enzyme purification. The validity of the methods is documented by employing various model phospholipases.

INTRODUCTION

The zwitterionic phospholipids PC and PE are major constituents of mammalian biomembrane diacylphosphoglycerides. As hydrolytic cleavage of these phospholipids has been shown to be implicated in signal transduction [1], there is now growing interest in methods for the determination of their metabolites. In general, phospholipid metabolism in membrane turnover and in particular its involvement in the regulation of various biological processes can be studied by stereospecifically labelling different moieties of these membrane constituents. Investigating the metabolism of a specific phospholipid species, however, requires labelling of the polar head group. Hydrolysis of amino alcohol-labelled phosphatidylcholine (PC) or phosphatidylethanolamine (PE), for example, may lead to radioactive [lysophosphatidylcholine (LPC), glycerophosphonylcholine (GPC), choline phosphate (CP) and choline (C) or lysophosphatidylethanolamine (LPE), glycerophosphorylethanolamine (GPE), ethanolamine phosphate (EP) and ethanolamine (E)] and non-radioactive metabolites (glycero-3-phosphate, free fatty acids (FFA), diacylglycerol, monoacylglycerol and glycerol). In this study, we developed simple methods for separating these radioactively labelled compounds from each other and for their determination by liquid scintillation spectrometry. The protocols were applied to amounts of up to 0.5 μmol of each compound and are easily performed with standard laboratory equipment.

EXPERIMENTAL

Chemicals and biological materials

All reagents and solvents were of analytical-reagent grade and, with the exception of those named below, were obtained from Sigma

* Corresponding author.

0021-9673/93/$06.00

(Deisenhofen, Germany). Organic solvents and precoated silica gel 60 thin-layer chromatographic plates (20 × 5 × 0.025 cm^3) were obtained from Merck (Darmstadt, Germany). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoryl[N-methyl-^{14}C]choline (52 mCi/mmol), 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine (55 mCi/mmol)1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine (56 mCi/mmol), 1,2-dioleoyl-*sn*-glycero-3-phosphoryl[2-^{14}C]ethanolamine (55 mCi/mmol), phosphoryl[methyl-^{14}C]-choline (55 mCi/mmol), [methyl-^{14}C]choline chloride (55 mCi/mmol) and [2-^{14}C]ethanolamine hydrochloride (54.1 mCi/mmol), were purchased from Amersham Buchler (Braunschweig, Germany). Glycero-3-phosphoryl[N-methyl-^{14}C]-choline and glycero-3-phosphoryl[2-^{14}C]ethanolamine were prepared by mild alkaline hydrolysis [2] of the parent diacylphosphoglycerides and purified by ion-exchange chromatography [3]. Phosphoryl[2-^{14}C]ethanolamine was prepared by incubating 1,2-dioleoyl-*sn*-glycero-3-phosphoryl[2-^{14}C]ethanolamine with *Bacillus cereus* phospholipase C (pH 8.0, 5 m*M* $CaCl_2$) and the water-soluble phosphorylethanolamine product purified by ion-exchange chromatography [4]. For enzyme assays, the radioactive phospholipids were adjusted to the specific radioactivity required (*ca.* 2000 dpm/nmol for standard assays), by adding the corresponding unlabelled phospholipid and subsequently measuring the radioactivity and phosphorus. Lipase (EC 3.1.1.32; *Rhizopus arrhizus*; 14 000 U/mg) and phospholipases C (EC 3.1.4.3; *Bacillus cereus*; 2000 U/mg) and D (EC 3.1.4.4; cabbage; 200 U/mg) were purchased from Boehringer (Mannheim, Germany). Porcine pancreatic phospholipase A_2 purified to homogeneity (1300 U/mg) was kindly provided by Professor Dr. G.H. de Haas (Department of Enzymology and Protein Engineering, Free University, Utrecht, Netherlands). The ion-exchange resin Dowex 1-X4 (200–400 mesh) was obtained from Serva (Heidelberg, Germany) and Bio-Rex 63 (100–200 mesh) from Bio-Rad (Munich, Germany).

Ion-exchange chromatography

Anion-exchange chromatography was performed with Dowex 1-X4 in Pasteur pipettes containing a small plug of glass-wool in the tips just below the shank. The resin was used in either the acetate or in the hydroxide form. The acetate form, in columns filled to a height of 2 cm (bed volume *ca.* 0.7 ml), was prepared with 3 ml of 1 *M* acetic acid followed by 3 ml of water. The hydroxide form, in columns filled to a height of 4 cm (bed volume *ca.* 1.4 ml), was prepared with 5 ml of 200 m*M* glycine–NaOH (pH 12) followed by 3 ml of 20 m*M* glycine–NaOH (pH 12).

Cation-exchange chromatography was performed in Pasteur pipettes filled with Bio-Rex 63 to a height of 4 cm (bed volume *ca.* 1.4 ml). This resin was converted into the H^+ form with 3 ml of 1 *M* HCl followed by 3 ml of water.

The columns can be reused indefinitely. To regenerate them, they are washed with 3 ml of methanol–1 *M* HCl (9:1, v/v) followed by the washing procedures described above for the respective resins.

Chemical analysis

Inorganic phosphorus was determined after ashing [5] as described by Chen *et al.* [6].

Polyacrylamide gel electrophoresis

Protein purity was assessed by sodium dodecyl sulphate (SDS) polyacrylamide slab gel electrophoresis (PAGE) according to the method of Laemmli [7], using Bio-Rad Mini-PROTEAN II system slab gradient gels (0.75 mm thick resolving gels with a linear gradient of 4–20% polyacrylamide and stacking gels of 2.6% polyacrylamide). The Pharmacia electrophoresis calibration kit was used as a molecular mass reference. The gels were stained with Coomassie Brilliant Blue G-250 [8] and silver [9]. The purity of the lipolytic enzymes used was established by applying 2 μg of purified enzyme. With the silver stain 0.5 ng of bovine serum albumin (corresponding to contamination of 0.25%) was still detectable.

Assays of phospholipases

Phospholipase A_1, A_2, C and D activities were determined as described in detail in Tables III and IV.

Recommended procedure for analysing phosphatidylcholine- and hydrolytic choline-containing products

Extraction. Incubation of samples (250 μl) is terminated by adding 1.5 ml of chloroform–methanol (1:2, v/v), 0.5 ml of chloroform, 0.5 ml of dilutte HCl (the concentration depends on the buffer used in the assay mixture and is previously assessed for acidification to *ca.* pH 3). After vortex mixing and centrifugation (2 min at 500 *g* in a laboratory centrifuge) the chloroform phase is quantitatively recovered and the aqueous phase is washed three times with 0.5-ml portions of chloroform. The combined chloroform phases are washed with 0.5 ml of water and all the aqueous phases are combined. Aliquots of the combined and washed chloroform phase are used for counting and for thin-layer chromatography. The combined aqueous phase is neutralized (with NaOH) and aliquots are taken for counting and for anion- and cation-exchange chromatography.

Ion-exchange chromatography (for separating GPC, CP and C). An aliquot of the aqueous phase is applied to Dowex 1-X4 (acetate form); the column is washed three times with 0.5-ml portions of water. The flow-through and washes contain GPC + C. CP is subsequently eluted with 3 ml of 1 *M* acetic acid and collected in a counting vial. After addition of 7 ml of Pico-Fluor 30 (Packard), CP is counted in a liquid scintillation spectrometer. The flow-through containing GPC + C is divided into two portions of equal volume (one for counting and the other for cation-exchange chromatography). For the latter, the flow-through of the Dowex column is applied to a Bio-Rex column and this column washed with 2 ml of water. This flow-through contains GPC and is counted after addition of 7 ml of Pico-Fluor 30 in a liquid scintillation spectrometer. C is eluted with two 1-ml portions of methanol–1 *M* HCl (9:1, v/v) into a counting vial and counted in a liquid scintillation spectrometer after adding 7 ml of Pico-Fluor 30. Contamination of the ion-exchange columns by PC and LPC can be removed by washing the resins three times with 1-ml portions of methanol–1 *M* HCl (9:1, v/v).

Thin-layer chromatography (for separation of PC and LPC). An aliquot of the combined chloroform phases is dried under vacuum. The residue is taken up in 100 μl of chloroform–methanol (2:1, v/v) and applied to silica gel G thin-layer plates for thin-layer chromatography, together with reference substances (PC and LPC). The thin-layer chromatographic plates are developed in chloroform–methanol–acetic acid–water (50:30:8:2, v/v) [10]. After scanning the radioactivity, the areas corresponding to PC and LPC are transferred to counting vials and counted for radioactivity, after addition of 2 ml of water and 7 ml of Pico-Fluor 30.

Recommended procedure for analysing phosphatidylethanolamine- and hydrolytic ethanolamine-containing products

Extraction. The reaction is terminated and the sample extracted as described for the PC substrate.

Anion-exchange chromatography (for separating GPE, EP and E). An aliquot of the aqueous phase is applied to Dowex 1-X4 (acetate form) and washed three times with 0.5-ml portions of water (the eluent contains GPE + E). EP bound to Dowex is eluted with 3 ml of 1 *M* acetic acid into a counting vial, and after addition of 7 ml of Pico-Fluor 30 is counted in a liquid scintillation spectrometer. The effluent containing GPE + E is made alkaline by adding 0.5 ml of 200 m*M* glycine–NaOH (pH 12) (buffer A) and aliquots are taken for counting and the second anion-exchange chromatography. The latter is applied to Dowex (OH^- form) and washed with 2 ml of 20 m*M* glycine–NaOH (pH 12) (buffer B). The flow-through contains E and is counted in a liquid scintillation spectrometer (after adding 7 ml of Pico-Fluor 30). GPE adsorbed to Dowex under these conditions is eluted with three 1-ml portions of 1 *M* acetic acid (into a counting vial) and counted for radioactivity (after adding 7 ml of Pico-Fluor 30) in a liquid scintillation spectrometer. Contamination of the Dowex columns by PE and LPE may be removed by washing the resins three-times with 1-ml portions of methanol–1 *M* HCl (9:1, v/v).

Thin-layer chromatography (for separation of PE and LPE). An aliquot of the combined chloroform phases is dried under vacuum. The residue is dissolved in 100 μl of chloroform–methanol (2:1, v/v) and applied to silica gel G

thin-layer plates for thin-layer chromatography, together with reference substances (PE and LPE). The thin-layer chromatographic plates are developed in chloroform–methanol–water (100:30:3, v/v/v). Areas corresponding to PE and LPE are transferred into counting vials and the radioactivity is counted, after addition of 2 ml of water and 7 ml of Pico-Fluor 30.

Truncated modifications for analysing for choline or ethanolamine

Time-saving modifications of the methods developed for PC and PE metabolites have been developed for specifically determining choline or ethanolamine in order to monitor, for example, phospholipase C and D activities during enzyme purification. Phospholipase C may be assessed with PC and phospholipase D with either PE or PC substrates. Each enzyme reaction is terminated by adding 1.5 ml of chloroform–methanol (1:2, v/v), followed by vortex mixing vigorously and adding 0.5 ml of chloroform.

For phospholipase C determination (assay PLC-I), 0.45 ml of NaOH or HCl (concentrations as required for neutralization) and 50 μl of 0.5 *M* glycylglycine–NaOH buffer (pH 7.5; to stabilize pH) are added. After phase separation, an aliquot (*e.g.*, 1 ml = 60%) of the aqueous layer (total volume 1.66 ml) is subjected to ion-exchange chromatography on Dowex 1-X4 (acetate form). After sample application, the resin is washed with water (2 ml), to remove potentially contaminating hydrolytic PC products such as GPC and C. Subsequently, the specific phospholipase C metabolite (CP) applied to the column (60%) is quantitatively eluted under acidic conditions (3 ml of 1 *M* acetic acid).

For monitoring phospholipase D activity with PE as a substrate (PLD-I), 0.5 ml of 200 m*M* glycine–NaOH (pH 12) is added. After phase separation, an aliquot (*e.g.*, 1 ml = 60%) of the aqueous phase (total volume 1.66 ml) is subjected to ion-exchange chromatography on Dowex (OH^- form). After sample application, the resin is washed with 20 m*M* glycine–NaOH (pH 12) (2 ml). Under these conditions, the specific phospholipase D metabolite (E) applied to the column (60%) is eluted quantitatively, whereas PE, LPE, GPE and EP are not eluted at all. This latter assay (PLD-I), however, does not work with PC substrates because CP under these conditions does not bind to Dowex in the OH^- form. Alternatively, employing PC as a substrate (assay PLD-II), 0.45 ml of NaOH or HCl (concentrations required for neutralization) and 50 μl of 0.5 *M* glycylglycine–NaOH buffer (pH 7.5; to stabilize pH) are added. After phase separation, an aliquot (*e.g.*, 1 ml = 60%) of the aqueous layer is subjected to ion-exchange chromatography on Bio-Rex (H^+ form). The resin is washed with water (2 ml) and the phospholipase D-specific metabolite (C) is eluted under acidic conditions [2 ml of methanol–1 *M* HCl (9:1, v/v)].

RESULTS AND DISCUSSION

For dissolving PC, PE and their metabolites, various buffers have been used, such as glycine–NaOH (pH 8.0), Tris–HCl (pH 8.0) and sodium acetate (pH 5.0), at final concentrations of 50 m*M* and with final volumes of up to 0.5 ml, without or with (acyl-containing compounds) ultrasonication, and without or with detergent, including up to 2 μmol of non-ionic (Triton X-100) or ionic (sodium deoxycholate or hexadecyltrimethylammonium bromide) detergents. The separation of the amino alcohol-containing phospholipids and their respective amino alcohol-containing metabolites has been accomplished with both non-radioactive and radioactively labelled compounds, up to 500 nmol, and is based on well proven methods.

First, the lipid-soluble diacylphosphoglycerides and monoacylphosphoglycerides are quantitatively separated from their water-soluble metabolites by lipid extraction, as outlined below. These lipids were separated according to standard procedures by thin-layer chromatography, the appropriate solvent mixture depending on the nature of the polar head group.

The water-soluble products were quantitatively recovered in the aqueous phase. After neutralizing the aqueous phase, they were separated from each other by ion-exchange chromatography.

For separating choline-containing metabolites, the neutralized aqueous phase was applied first

to an anion-exchange resin (Dowex 1-X4, acetate form) and the flow-through subsequently to a cation-exchange resin (Bio-Rex, H^+ form). Whereas GPC under these conditions is not bound to either of the exchangers, CP is quantitatively bound to the anion exchanger and C to the cation exchanger. Both are eluted under acidic conditions. For separating ethanolamine-containing metabolites, the neutralized aqueous phase was subjected first to an anion-exchange resin (Dowex 1-X4) in the acetate form and the flow-through, under alkaline conditions, subsequently to an anion-exchange resin (Dowex 1-X4) in the OH^- form. Whereas E under these conditions is not bound to either form, EP is quantitatively bound to the acetate and GPE to the OH^- form, respectively. Both are eluted under acidic conditions.

As assessed by phosphorus determination (data not shown) and/or radiometry (Tables I and II), each of the radioactive or non-radioactive amino alcohol-containing standards (applied in the range 0.5–500 nmol) was completely recovered in its corresponding chromatographic fraction. As a rule, quantitative recoveries in ion-exchange chromatography were obtained within the first 75% of the elution volumes of the respective fractions (data not shown). Hence the minor contaminations of standards in other fractions (Tables I and II) were undoubtedly due to hydrolytic impurities in the commercially available substances. Non-ionic and ionic detergents, of up to 2 μmol, did not interfere with the assays (data not shown).

As already mentioned above, the protocols are based on and combine proven standard chromatographic methods which have been modified for optimum separation and quantitative recovery of the amino alcohol-labelled PC, PE and their respective metabolites. Whereas standard procedures have been modified slightly for lipid extraction into chloroform phases [11] and lipid separation (diacylphosphoglycerides from monoacylphosphoglycerides) by thin-layer chromatography [10,12], separation of the water-soluble PC and PE metabolites represents simplified but specific applications of the general principles of ion-exchange chromatography. Ion exchangers have already been applied for separating (although not all) water-soluble hydrolytic products of PC, specifically for the assessment of phospholipases C and D (reviewed in refs. 13–16) or

TABLE I

DISTRIBUTION PATTERNS OF STANDARDS OF CHOLINE-LABELLED PC AND ITS METABOLITIES IN THIN-LAYER AND ION-EXCHANGE CHROMATOGRAPHY

PC and hydrolytic PC metabolites were separated from each other and determined as described in the text. Data from triplicate determinations are given as means ± S.D.

Standard applied		Amount recovered in fraction (%)				
Species	Amount (nmol)	PC	LPC	GPC	CP	C
PC	5	97.5 ± 2.2	2.2 ± 1.1	0.2 ± 0.2	0.3 ± 0.1	0.4 ± 0.1
	500	96.4 ± 2.4	3.5 ± 0.9	0.1 ± 0.3	0.4 ± 0.2	0.5 ± 0.2
LPC	5	0.4 ± 0.4	98.1 ± 1.9	0.2 ± 0.2	0.8 ± 0.2	0.4 ± 0.1
	500	0.2 ± 0.1	97.8 ± 2.4	0.2 ± 0.2	0.4 ± 0.2	0.4 ± 0.1
GPC	5	0 ± 0	0 ± 0	98.6 ± 0.5	0.3 ± 0.2	1.4 ± 0.1
	500	0 ± 0	0 ± 0	98.8 ± 0.9	0.6 ± 0.2	1.8 ± 1.1
CP	5	0 ± 0	0 ± 0	0.2 ± 0.1	98.7 ± 1.5	1.0 ± 0.3
	500	0 ± 0	0 ± 0	0.2 ± 0.2	98.4 ± 1.9	1.4 ± 0.6
C	5	0 ± 0	0 ± 0	0.5 ± 0.5	0.4 ± 0.3	98.8 ± 2.1
	500	0 ± 0	0 ± 0	0.2 ± 0.3	0.3 ± 0.2	99.1 ± 1.4

TABLE II

DISTRIBUTION PATTERNS OF STANDARDS OF ETHANOLAMINE-LABELLED PE AND ITS METABOLITIES IN THIN-LAYER AND ION-EXCHANGE CHROMATOGRAPHY

PE and hydrolytic PE metabolites were separated from each other and determined as described in the text. Data from triplicate determinations are given as means ± S.D.

Standard applied		Amount recovered in fraction (%)				
Species	Amount (nmol)	PE	LPE	GPE	EP	E
PE	5	98.5 ± 1.2	1.6 ± 0.4	0.1 ± 0.2	0.2 ± 0.1	0.3 ± 0.1
	500	97.9 ± 1.6	2.0 ± 0.7	0.1 ± 0.2	0.2 ± 0.2	0.3 ± 0.2
LPE	5	0.2 ± 0.6	97.9 ± 2.6	0.3 ± 0.2	0.4 ± 0.3	0.3 ± 0.2
	500	0.3 ± 0.4	97.3 ± 2.9	0.2 ± 0.1	0.3 ± 0.2	0.2 ± 0.1
GPE	5	0 ± 0	0 ± 0	98.2 ± 2.4	0.4 ± 0.3	1.2 ± 0.4
	500	0 ± 0	0 ± 0	97.8 ± 2.9	0.7 ± 0.4	1.3 ± 1.1
EP	5	0 ± 0	0 ± 0	0.4 ± 0.3	97.6 ± 2.0	2.0 ± 1.1
	500	0 ± 0	0 ± 0	0.5 ± 0.3	98.0 ± 2.3	1.2 ± 0.5
E	5	0 ± 0	0 ± 0	0.7 ± 0.6	0.2 ± 0.1	98.1 ± 1.9
	500	0 ± 0	0 ± 0	0.4 ± 0.3	0.4 ± 0.2	97.8 ± 1.5

TABLE III

PC METABOLITES FORMED IN THE PRESENCE OF VARIOUS PHOSPHOLIPASES

PC with a specific radioactivity of 1991 dpm/nmol was used. After incubation, radioactive PC and radioactive products were measured as described in the text. All reaction products determined in these experiments with PC substrate formed linearly with time up to the time periods employed. Data from triplicate determinations (corrected for non-enzymic hydrolysis which did not exceed 3.6%) are given in per cent of radioactivity, as means ± S.D., recovered in the respective fractions.

Enzyme	Substrate remaining (PC)	Metabolite			
		LPC	GPC	CP	C
Phospholipase A_1[a]:					
Without detergent	59.8 ± 2.1	26.8 ± 2.1	11.8 ± 0.6	0.2 ± 0.2	0.4 ± 0.1
With detergent	73.2 ± 3.9	20.2 ± 2.0	4.9 ± 0.1	0.3 ± 0.2	0.4 ± 0.2
Phospholipase A_2[b]	64.1 ± 2.5	26.7 ± 1.9	4.0 ± 0.8	0.2 ± 0.2	0.8 ± 0.2
Phospholipase C[c]	0 ± 1.9	0 ± 0	0.1 ± 0.2	98.8 ± 4.1	0.1 ± 0.2
Phospholipase D[d]	58.2 ± 3.1	0 ± 0	0.1 ± 0.1	0.2 ± 0.1	41.2 ± 3.3

[a] For assays of phospholipase A_1 activity, the reaction mixture (total volume 250 μl), containing 25 m*M* Tris–HCl (pH 8.0), 10 m*M* $CaCl_2$, 1.5 μg of *Rhizopus arrhizus* lipase and 127 μ*M* PC, without or with 0.1% (w/v) Triton X-100, was incubated at 37°C for 60 min.

[b] For assays of phospholipase A_2 activity, the reaction mixture (total volume 250 μl), containing 25 m*M* Tris–HCl (pH 9.0), 10 m*M* $CaCl_2$, 0.1 μg of porcine pancreatic phospholipase A_2 and 52 μ*M* PC, was incubated at 37°C for 30 min.

[c] For assays of phospholipase C activity, the reaction mixture (total volume 250 μl), containing 20 m*M* glycine–NaOH (pH 8.0), 5 m*M* $CaCl_2$, 2 μg of *Bacillus cereus* phospholipase C and 46.5 μ*M* PC, was incubated at 37°C for 30 min.

[d] For assays of phospholipase D activity, the reaction mixture (total volume 250 μl), containing 20 m*M* sodium acetate (pH 5.0), 10 m*M* $CaCl_2$, 10 μg of cabbage phospholipase D and 57.4 μ*M* PC, was incubated at 37°C for 15 min.

glycerophosphocholine phosphodiesterase [17] activities.

Different modes of elution have been used to determine specific PC or PE metabolites. Gradient elution of water-soluble hydrolytic PC metabolites from ion exchangers [3], although more accurate, is far more laborious, time consuming and not well suited for analysing large numbers of samples. Similarly, the stepwise group elution of water-soluble C-containing metabolites from an ion-exchange resin followed by volume reduction and further separation on charcoal [18] is not appropriate for multi-sample experiments. As only two compounds are involved, a simple and rapid method using stepwise elution from ion-exchange resins has been reported for the separation of ^{32}P-labelled choline phosphate from AT^{32}P [19]. This method, however, was not applicable to the separation of all the water-soluble PC metabolites used in our studies. Thin-layer chromatography as an alternative procedure for the separation of water-soluble C-containing compounds [3,18,20], is simple and rapid and has multi-sample capacity. Nevertheless, our method appears to be superior to thin-layer chromatography, simply because the latter requires more time for volume reduction and sample application to the plates. The same considerations also hold true for the separation of water-soluble PE metabolites by thin-layer chromatography [4]. Our procedures allow direct quantitative analysis of at least twelve aqueous samples within 30 min. Hence the protocols presented here for the separation of the water-soluble PC and PE metabolites avoid volume reduction and are extremely easy to perform with, excellent quality of separation, recovery, speed of analysis and cost of materials.

The validity of the procedures presented was demonstrated by incubating various purified and electrophoretically homogeneous (except for cabbage phospholipase D) lipolytic enzymes with PC (Table III) and PE substrates (Table IV) and determining the metabolites formed. The enzyme assays were designed so as to lead to a high accumulation of metabolites rather than to stay in the appropriate kinetic range of substrate hydrolysis. Phospholipases C and D and their expected positional specificities are correctly assessed with respect to the metabolites obtained (Tables III and IV). Likewise, phospholipases A were shown to catalyse the deacylation of

TABLE IV

PE METABOLITES FORMED IN THE PRESENCE OF VARIOUS PHOSPHOLIPASES

PE with a specific radioactivity of 1972 dpm/nmol was used. After incubation, radioactive PE and radioactive products were measured as described in the text. Formation of reaction products in these experiments with PE substrate was not linear with time. Data from triplicate determinations (corrected for non-enzymic hydrolysis) are given in per cent, as means ± S.D., recovered in the respective fractions.

Enzyme	Substrate remaining (PE)	Metabolite			
		LPE	GPE	EP	E
Phospholipase A_1[a]	55.3 ± 4.3	32.1 ± 2.3	13.9 ± 2.2	0.3 ± 0.3	0.2 ± 0.2
Phospholipase A_2[b]	2.1 ± 3.4	67.3 ± 0.4	24.1 ± 4.0	1.9 ± 1.0	1.5 ± 0.4
Phospholipase C[c]	10.2 ± 1.8	0 ± 0	0 ± 0.1	80.9 ± 3.6	0.2 ± 0.3
Phospholipase D[d]	42.0 ± 3.3	0 ± 0	0.2 ± 0.2	0 ± 0.2	58.8 ± 3.4

[a] For assays of phospholipase A_1 activity, the reaction mixture (total volume 250 μl), containing 25 m*M* Tris–HCl (pH 8.0), 10 m*M* $CaCl_2$, 7 μg of *Rhizopus arrhizus* lipase and 27 μ*M* PE, was incubated at 37°C for 60 min.

[b] For assays of phospholipase A_2 activity, the reaction mixture (total volume 250 μl), containing 25 m*M* Tris–HCl (pH 9.0), 10 m*M* $CaCl_2$, 0.1 μg of porcine pancreatic phospholipase A_2 and 31 μ*M* PE, was incubated at 37°C for 30 min.

[c] For assays of phospholipase C activity, the reaction mixture (total volume 250 μl), containing 20 m*M* glycine–NaOH (pH 8.0), 5 m*M* $CaCl_2$, 2 μg of *Bacillus cereus* phospholipase C and 28 μ*M* PE, was incubated at 37°C for 30 min.

[d] For assays of phospholipase D activity, the reaction mixture (total volume 250 μl), containing 20 m*M* sodium acetate (pH 5.0), 10 m*M* $CaCl_2$, 10 μg of cabbage phospholipase D and 29 μ*M* PE, was incubated at 37°C for 30 min.

TABLE V

COMPARISON OF STANDARD ASSAYS AND RAPID TESTS FOR PHOSPHOLIPASE C AND D ACTIVITIES

Reactions catalysed by phospholipases C (substrate 50.3 μM PC) and D (substrate 43.2 μM PE) were performed as described in Tables III and IV, respectively. The corresponding metabolites were analysed by the standard assays (SA) and rapid tests (PLC-I and PLD-I) as described in the text. Data from triplicate determination (corrected for non-enzymic hydrolysis) are given in dpm, as means ± S.D. recovered in the respective fractions.

Enzyme	Test	Metabolite			
		CP	(%)	E	(%)
Phospholipase C	SA	12110 ± 697	(100 ± 6)	ND[a]	
	PLC-I	7182 ± 341	(59 ± 3)	ND	
Phospholipase D	SA	ND		6008 ± 311	(100 ± 5)
	PLD-I	ND		3542 ± 199	(59 ± 3)

[a] ND = Not determined.

diacylphospholipids with the expected positional specificity, but all preparations employed here were shown also to catalyse the deacylation of lysophospholipids. *Rhizopus arrhizus* lipase analyses showed GPC and GPE formation from PC (Table III) and PE (Table IV), respectively, confirming earlier reports on an intrinsic lysophospholipase activity of a lipase catalysing phospholipid hydrolysis [10]. As seen from the disappearance of GPC relative to LPC formation (Table III), this lysophospholipase activity was almost abolished by Triton X-100, whereas phospholipase A_1 activity (based on the sum of LPC and GPC formation) was only reduced (by *ca.* 47%). Partial inhibition of phospholipase A_1 and complete inhibition of lysophospholipase activities of the *R. arrhizus* enzyme was confirmed by using identical assay conditions but substrates with a different stereospecific label. For example, hydrolysis of PC (112 μM) fatty acid-labelled in the *sn*-1-position was inhibited by *ca.* 44%, and hydrolysis of LPC (98 μM) fatty acid-labelled in the *sn*-1-position by about 98%. It may well be possible that hydrolysis of the *sn*-2-acyl-lysophospholipids generated during phospholipase A_1-catalysed hydrolysis of diacylphospholipids proceeds in two steps, *i.e.*, via acyl migration to the *sn*-1-position and subsequent hydrolysis of the *sn*-1-acyl ester bond. This possibility remains to be clarified.

Apparently *sn*-1-directed lysophospholipase activity (GPC and GPE formation from PC,1-acyl-LPC and PE, respectively) was also observed in the electrophoretically pure (contaminations of more than 0.25% would have been detectable by SDS-PAGE) phospholipase A_2 used here (Tables III and IV). Pancreatic phospholipase A_2-associated lysophospholipase activity is extremely low ($v_{max} \approx 0.08$ U/mg [21]) and detectable in purified bee venom phospholipase A_2 as well. Phospholipase A_2-associated lysophospholipase activity is also shown with 1-acyl-lysophospholipid susbstrates, and presumably due to acyl migration followed by enzymatically catalysed deacylation from the *sn*-2-position [21].

In their modified forms (see truncated modifications), our methods are particularly suitable for monitoring phospholipase C or D activities during purification of these enzymes. The specific phospholipase C metabolite (CP) and the specific phospholipase D metabolite (C or E) are quantitatively eluted (Table V).

ACKNOWLEDGEMENTS

The authors are grateful to Drs. Henk van den Bosch and Kay Nakamaye for critical discussions and advice.

REFERENCES

1 J.H. Exton, *J. Biol. Chem.*, 265 (1990) 1–4.
2 R.M.C. Dawson and N. Hemington, *Biochem. J.*, 162 (1977) 241–245.
3 S. Tokmakjian, D.S.M. Haines and F. Possmayer, *Biochim. Biophys. Acta*, 663 (1981) 557–568.
4 R. Sundler, *Biochim. Biophys. Acta*, 306 (1973) 218–226.
5 B.N. Ames and D.T. Dubin, *J. Biol. Chem.*, 235 (1960) 769–775.
6 P.S. Chen, T.Y. Toribara and H. Warner, *Anal. Chem.*, 28 (1956) 1756–1758.
7 U.K. Laemmli, *Nature*, 227 (1970) 680–685.
8 V. Neuhoff, R. Damm and H. Eibl, *Electrophoresis*, 6 (1985) 427–448.
9 H.M. Poehling and V. Neuhoff, *Electrophoresis*, 2 (1981) 135–141.
10 E. Bohn, V. Gerke, H. Kresse, B.-M. Löffler and H. Kunze, *FEBS Lett.*, 296 (1987) 237–240.
11 E.G. Bligh and K.J. Dyer, *Can. J. Biochem. Physiol.*, 37 (1959) 911–917.
12 H. Kunze, B. Hesse and E. Bohn, *Biochim. Biophys. Acta*, 711 (1982) 10–18.
13 H. van den Bosch, in J.N. Hawthorne and G.B. Ansell (Editors), *Phospholipids*, Elsevier Biomedical Press, Amsterdam, 1982, pp. 313–357.
14 L.J. Reynolds, W.N. Washburn, R.A. Deems and E.A. Dennis, *Methods Enzymol.*, 197 (1991) 3–23.
15 K.Y. Hostetler, M.F. Gardner and K.A. Aldern, *Methods Enzymol.*, 197 (1991) 125–134.
16 D.A. Kennerly, *Methods Enzymol.*, 197 (1991) 191–197.
17 S. Spanner and G.B. Ansell, *Neurochem. Res.*, 12 (1987) 203–206.
18 D.E. Vance, E.M. Trip and H.B. Paddon, *J. Biol. Chem.*, 255 (1980) 1064–1069.
19 R.E. McCaman and J. Stetzler, *J. Neurochem.*, 28 (1977) 669–671.
20 E. Yavin, *J. Biol. Chem.*, 251 (1976) 1392–1397.
21 H. Kunze and E. Bohn, in preparation.

Journal of Chromatography, 636 (1993) 231–241
Elsevier Science Publishers B.V., Amsterdam

CHROM 24 857

Chiral separations of β-blocking drug substances using derivatization with chiral reagents and normal-phase high-performance liquid chromatography

Lars Olsen*, Kirsten Brønnum-Hansen, Per Helboe, Gorm Herlev Jørgensen and Sonja Kryger

Chemical Laboratory, National Board of Health, 378 Frederikssundsvej, DK-2700 Brønshøj (Denmark)

(First received June 19th, 1992; revised manuscript received December 8th, 1992)

ABSTRACT

Eighteen β-blockers currently on sale in Denmark were investigated for the possible separation of the enantiomers by use of normal-phase HPLC following derivatization with three different chiral derivatization agents: (−)-camphanic acid chloride (*S*)-(−)-1-phenylethyl isocyanate and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate. Chromatography was performed using a 5-μm silica column (120 × 4.6 mm I.D.) with mixtures of dichloromethane, *n*-heptane and methanol as eluents. The results showed that all the β-blockers available in both enantiomeric forms (seventeen) can be baseline separated by at least one of the three procedures. The purpose of the investigations was an evaluation of the various approaches concerning the possibilities of method standardization.

INTRODUCTION

In recent years, the possibilities of resolving enantiomers by high-performance liquid chromatography (HPLC) have greatly improved. Compared with the traditional determination of optical rotation, these techniques offer an ability for the direct and accurate measurement of enantiomeric purity.

There are three possible approaches to enantiomeric resolution by use of HPLC: (1) separation on a chiral stationary phase and using an achiral eluent, (2) derivatization with a chiral reagent and separation of the resulting diastereomers using an achiral stationary phase and eluent and (3) separation on an achiral stationary phase by use of a chiral eluent.

A prerequisite for using HPLC methods for the evaluation of the optical purity, *e.g.*, in national and international pharmacopoeias, is the possibility of standardizing the analytical methods. Therefore, investigations to evaluate the above three possible approaches in that context were considered necessary.

The therapeutic group of β-adrenergic blocking agents was chosen as a model class. This group includes a series of chemically closely related substances, as can be seen from Fig 1. Further, the β-blocking agents exhibit enantiospecific therapeutic activity, usually with the *S*-form possessing the β-blocking activity (an exception being SOT), even though most β-blockers until now have been used as the racemic mixture. This specificity could suggest an increasing demand for standardized methods for determining the enantiomeric purity of these compounds.

The three different approaches mentioned above have been used previously on β-blocking agents. (1) The most feasible choice among the

* Corresponding author.

0021-9673/93/$06.00

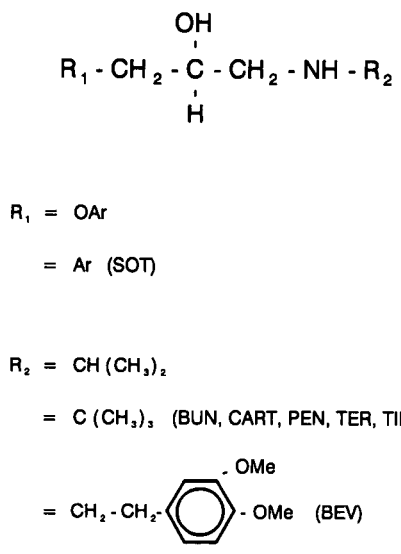


Fig. 1. Molecular structures of the eighteen β-blocking agents investigated.

chiral stationary phases appears to be cellulose tris(3,5-dimethylphenylcarbamate) [1–8], but the use of other stationary phases has been reported, such as the Pirkle type [9–11], immobilised α_1-acid glycoprotein [12–14] and another type of immobilized acid glycoprotein, ovomucoid [15]. The use of β-cyclodextrin [16] and immobilized bovine serum albumin (BSA) [17,18] has also been investigated but the results obtained seem less promising owing to low separation factors or considerable peak broadening. (2) Several examples of separating β-blockers following chiral derivatization have been described. In most instances the separation of the resulting diastereomers has been performed using reversed-phase HPLC: (*S*)-(−)- or (*R*)-(+)-1-phenylethyl isocyanate [(*S*)- or (*R*)-PEI] [19–26], (*R*)-(−)-1-(1-naphthyl)ethyl isocyanate (R-NEI) [27,28], 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (D-GITC) [29–32] and 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate [29], N-trifluoroacetyl-1-prolyl chloride [33], (*R*,*R*)-O,O′-diacetyltartaric acid anhydride [34,35], *tert.*-butoxycarbonyl-L-leucine anhydride [36,37], and different chloroformates [38–42]. A limited number of publications describing the use of normal-phase HPLC for the separation of the diastereomeric derivatives can be found with (*S*)-PEI [43] and (*S*)-NEI [44,45]. (3) The third approach, the use of chiral eluents, has also been attempted on β-blocking agents, *e.g.*, by adding to the eluent (+)-10-camphorsulphonate [46] and N-benzoxycarbonyl-glycyl-L-proline [47,48].

Further, a comprehensive review on the chromatography in general of β-blocking agents, including a section on chiral separation also dealing with the various approaches, has recently been published [49].

No publications so far have addressed the problem of standardization of methods and only a few have discussed the suitability of determining optical purity. The main object of most investigations has been to demonstrate the separation power of the approach investigated. In this work, eighteen β-blockers currently on sale in Denmark were investigated for the possible enantiomeric separation and purity testing using an easily standardizable method. For this purpose we used normal-phase HPLC following derivatization with three different chiral derivatization reagents, (−)-camphanic acid chloride [(−)-CACl], (*S*)-PEI and D-GITC. The use of (−)-CACl for the chiral separation of β-blockers has not previously been reported. With the proper set of reaction conditions, both isocyanates and thioisocyanates are known to react selectively with the amines of β-blockers owing to the relatively slow reaction of the hydroxy group, whereas the acid chloride was likely to show comparable reactivity towards the hydroxy group of the β-blockers, so that difficulties regarding selectivity of the reactions could be expected. However, this reagent is comparatively inexpensive and was included in the investigations mainly for this reason.

The number of investigations published so far using normal-phase chromatography following diastereomeric derivatization are limited compared with the number of papers using reversed-phase chromatography.

EXPERIMENTAL

Chemicals

In Table I are listed the compounds investigated and the companies from which they were obtained. Other drug substances, including

TABLE I

INVESTIGATED β-BLOCKING AGENTS AND THEIR SOURCES

Compound	Abbreviation	*R*-	*S*-	Rac.	Company
Acebutolol	ACE	+	+	+	Rhône-Poulenc (Dagenham, UK)
Alprenolol	ALP	+	+	+	Hässle (Mölndal, Sweden)
Atenolol	ATE			+	ICI (Runcorn, UK)
Atenolol				+	Benzon Pharma (Hvidovre, Denmark)
Betaxolol	BET	+	+	+	MEDA (Herlev, Denmark)
Betaxolol		+	+	+	Searle (Mölndal, Sweden)
Bevantolol	BEV	+	+	+	Parke-Davis (Ann Arbor, MI, USA)
Bevantolol		+	+	+	Benzon Pharma (Hvidovre, Denmark)
Bisoprolol	BIS			+	Merck (Darmstadt, Germany)
Bunolol	BUN	+	+		Allergan (Irvine, CA, USA)
Carazolol	CARA			+	Upjohn (Crawley, UK)
Carteolol	CART	+	+	+	Ercopharm (Vedbæk, Denmark)
Metipranolol	METI	+	+	+	Ciba-Geigy (Basle, Switzerland)
Metoprolol	METO		+	+	Hässle
Oxprenolol	OXP	+	+	+	Ciba-Geigy
Penbutolol	PEN		+		Hoechst (Frankfurt, Germany)
Pindolol	PIN			+	Durascan (Odense, Denmark)
Pindolol				+	Benzon Pharma (Hvidovre, Denmark)
Pindolol				+	NM Pharma (Sundbyberg, Sweden)
Pindolol				+	Dumex (Copenhagen, Denmark)
Propranolol	PRO	+	+		Sigma (St. Louis, MO, USA)
Sotalol	SOT	+	+	+	Bristol-Myers (Evansville, IN, USA)
Tertatolol	TER			+	Servier (Orleans, France)
Timolol	TIM	+	+	+	Merck, Sharp & Dohme (Rahway, NJ, USA)

racemic propranolol (*rac*-PRO), were of pharmacopoeial quality. (*R*)-PEI, (*S*)-PEI and (−)-CACl were of ChiraPur quality and obtained from Fluka (Buchs, Switzerland) and D-GITC from Polysciences (Warrington, PA, USA). All other chemicals were of analytical-reagent grade from Merck (Darmstadt, Germany).

Apparatus

The chromatographic systems consisted of a Kontron Model 410 or Model T-414 LC pump, a Rheodyne Model 7125 injection valve and a Kontron Model 735 LC, Cecil Model CE 2012 or CE 212 or LDC Spectromonitor II 1202 UV detector. Chromatograms were recorded on a Kipp & Zonen Model BD-8 recorder. Retention data were collected on a Hewlett-Packard Model 3359A laboratory data system.

Chromatography

All normal-phase experiments were performed on 120 × 4.6 mm I.D. columns from Knauer (Berlin, Germany) packed by the dilute slurry technique with 5-μm LiChrosorb Si 60, silica (Merck).

The eluents for normal-phase chromatography were mixtures of methanol, dichloromethane and heptane (see Table III). Prior to use the columns were activated by elution with a series of solvents, *viz.*, water, methanol, acetone, ethyl acetate, dichloromethane and heptane.

Reversed-phase chromatography was performed on 120 × 4.6 mm I.D column from Knauer packed by the dilute slurry technique with 5-μm LiChrosorb RP-18. The eluent was 2 m*M* sodium 1-dodecylsulphonate in triethylamine–glacial acetic acid–methanol–water (0.2:0.2:75:25)

Derivatization procedure

(R)- and (S)-PEI. The compounds to be derivatized, either as a salt or free base, were dissolved in dichloromethane–triethylamine (10:1), typically at a concentration of 5 mg/ml.

To 200 μl (or a volume containing a sample amount of about 1 mg) were added 50 μl of a solution containing 3 mg of the derivatization agent in dichloromethane. The mixture was allowed to react for 5 min at room temperature and evaporated to dryness using a stream of nitrogen. The residue was dissolved in 800 μl of dichloromethane–heptane (1:1).

D-GITC. The procedure was as described for PEI, with the exception of a reaction time of 15 min. The residue was dissolved in 1 ml of heptane–methanol (40:1).

(−)-CACl. The procedure was as described for PEI, with the exception of the addition of 10 mg of derivatization agent (in 50 μl of solvent) and reaction times of 30–60 min. The residue was dissolved in 1.5 ml of dichloromethane–heptane (2:1).

RESULTS AND DISCUSSION

Chromatography

Preliminary investigations showed that the selectivity towards the diastereomeric derivatives was strongly influenced by the relative composition of the eluent. This effect when using mixtures of methanol, dichloromethane and heptane is illustrated in Table II with the results obtained during initial experiments with (*S*)-PEI derivatives of *rac*-PRO. It appears that the chromatographic behaviour of the diastereomeric derivatives varies from complete co-elution to baseline separation. Other alcoholic modifiers were investigated such as 2-propanol and higher alcohols, but the combination of methanol, dichloromethane and heptane was found to be the most suitable for the separation of all three types of derivatives investigated with respect to selectivity and peak shape.

Fig. 2 shows chromatograms of *rac*-PRO derivatized with the three different reagents. A detailed presentation, giving capacity factors (k'), separation factors (α), resolution (R_s) and asymmetry factors (A_s) of the chromatographic results obtained by the above-mentioned procedures is shown in Table III. It appears that with the exception of PEN, which was available as the *S*-form only, all the racemic β-blockers could be baseline separated as (*S*)-PEI and D-GITC derivatives. Using (*S*)-PEI, resolutions of not less than 2.9 were obtained (except for ATE and BEV, with R_s values of 1.6 and 1.8, respectively) with k' below 6 and acceptable values of A_s. Correspondingly, when using D-GITC, resolutions of not less than *ca.* 1.5 were found in all instances with asymmetry at an acceptably low level. Even though the retentions, with k' values up to *ca.* 15, were higher than for the (*S*)-PEI derivatives, the D-GITC derivatives were in most instances separated within an acceptable time of analysis. For both reagents, the order of elution of the derivatized β-blocking agents was identified. For both (*S*)-PEI and D-GITC, the *R*-forms of the β-blocking agents eluted first [or the *S*-forms, when using (*R*)-PEI]. The only exception to this behaviour was found with SOT,

TABLE II

RETENTION (CAPACITY FACTORS, k'), SEPARATION FACTORS, α, RESOLUTION, R_s, AND ASYMMETRY, A_s, OF (*S*)-PEI-DERIVATIZED *rac*-PRO USING NORMAL-PHASE HPLC WITH SIX DIFFERENT ELUENTS

Derivatization procedure as described under Experimental. Detection wavelength, 290 nm.

Eluent composition			$k'(1)$	$k'(2)$	α	R_s	A_s
Methanol	Dichloromethane	Heptane					
1.0	85.0	14.0	2.55	3.54	1.39	4.9	3.0
1.2	70.0	18.8	2.00	2.85	1.43	5.4	1.9
1.5	50.0	48.5	1.77	2.50	1.41	5.3	1.5
1.5	30.0	68.5	2.77	3.61	1.30	4.9	1.6
2.0	20.0	78.0	2.93	3.45	1.18	3.1	1.2
4.0	0	96.0	8.00	8.00	1.00	0.0	1.5

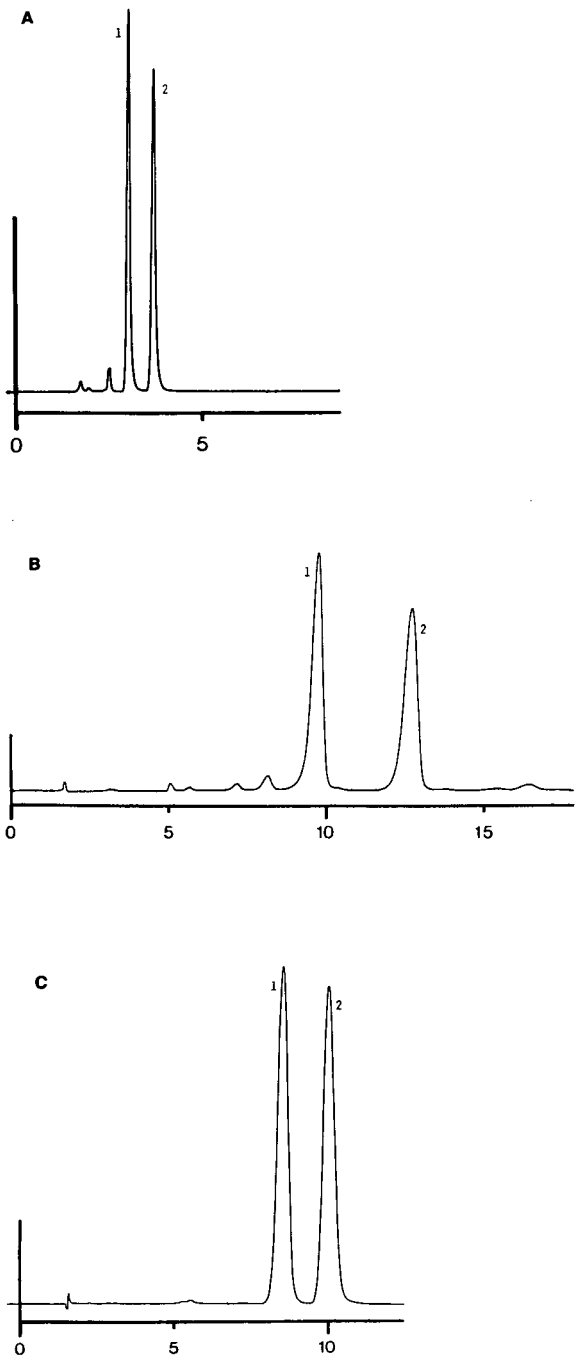


Fig. 2. Chromatograms of *rac*-PRO derivatized with (A) (*S*)-PEI, (B) D-GITC and (C) (−)-CACl as described under Experimental. Column: 5-μm LiChrosorb Si 60 (120 × 4.6 mm I.D.). Eluent: methanol–dichloromethane–heptane, (A) (1.5:50:48.5), (B) (3:10:90) and (C) (0.35:60:40). Detection at 290 nm. Peaks: 1 = (*R*)-PRO; 2 = (*S*)-PR. Time scales in Figs. 2–5 in min.

which for both reagents showed an opposite order of elution. This is caused by the sequence rules for naming the configurations as *R* or *S* because, as can be seen from Fig. 1, the aryloxymethyl ligand otherwise present in the molecular structures of β-blockers is replaced with aryl in this compound.

In Table III are also listed results obtained using (−)-CACl as the chiral derivatization agent. It can be seen that the polarity of the eluent used for nine of the β-blocking agents was much lower than for the eluents used in connection with the other reagents and the remaining compounds derivatized with (−)-CACl. A possible explanation to the apparent difference in the lipophility of the derivatives is further discussed below.

Derivatization

(S)-PEI and D-*GITC.* The molecules of the β-blockers investigated exhibit two groups available for derivatization, a secondary hydroxy and a secondary amino group.

It has been shown previously for PRO that only the amino group is derivatized, resulting in urea derivatives, when (*R*)-PEI is used as reagent [19]. Correspondingly, the isothiocyanate group of D-GITC is known to react selectively with the amino group [29,50–52]. As the derivatization procedures used in this investigation are slightly modified compared with previously published methods, the reactivity towards alcohols and amino groups was examined.

During an earlier study of (*S*)-PEI as a potential chiral derivatization agent for separation of norgestrel enantiomers, the reactivity towards the secondary alcohol was found to be considerably higher in dichloromethane than in other more polar solvents such as acetonitrile [53], although a high temperature and long reaction time were required. Therefore, in order to obtain a full turnover of the compounds in the synthesis, it was assumed that dichloromethane should be preferred as the solvent for both reagents. This was supported by derivatization experiments on *rac*-PRO. By using the reversed-phase HPLC system described under Experimental, it was possible to determine the amount of underivatized *rac*-PRO, with a detection limit of

TABLE III

RETENTION (CAPACITY FACTOR, k'), SELECTIVITY, α, RESOLUTION, R_s, AND ASYMMETRY, A_s, OF EIGHTEEN β-BLOCKING AGENTS DERIVATIZED WITH THREE DIFFERENT CHIRAL REAGENTS

Derivatization as described under Experimental. Eluents: methanol–dichloromethane–heptane, (A) (1.5:50:48.5); (B) (2.5:49.5:48); (C) (3.5:49:47.5); (D) (3:10:90); (E) (3:20:80); (F) (3:25:75); (G) (3:30:70); (H) (3:50:50); (I) (0.35:60:40); (J) (1.5:60:40); (K) (2:60:40).

Compound	λ (nm)	(*S*)-PEI						D-GITC						λ (nm)	(−)-CSCl					
		Eluent	$k'(1)$	$k'(2)$	α	R_s	A_s	Eluent	$k'(1)$	$k'(2)$	α	R_s	A_s		Eluent	$k'(1)$	$k'(2)$	α	R_s	A_s
Acebutolol	320	B	4.10	4.81	1.17	4.0	1.5	G	6.99	7.80	1.12	1.4	1.0							
Alprenolol	275	A	1.10	1.57	1.43	4.8	1.0	D	4.96	6.85	1.38	4.7	0.7	275	I	5.94	8.29	1.40	4.8	1.4
Atenolol	275	C	6.44	7.05	1.09	1.6	1.5	H	15.7	17.2	1.10	1.7	1.4							
Betaxolol	275	A	1.70	2.34	1.37	4.4	1.6	D	9.15	12.09	1.32	4.4	0.9	275	I	10.01	11.82	1.18	2.0	1.3
Bevantolol	275	A	1.55	1.79	1.15	1.8	1.0	F	2.10	2.49	1.19	1.9	1.6	275	I	7.19	8.75	1.22	2.5	1.2
Bisoprolol	275	A	1.90	2.51	1.32	3.8	1.6	D	9.40	11.88	1.26	3.6	0.7							
Bunolol	267	A	2.80	4.23	1.51	6.0	0.9	E	4.70	5.45	1.16	1.4	0.9	267	K	1.23	1.63	1.33	2.4	1.7
Carazolol	285	A	1.83	2.40	1.31	3.8	1.6	F	5.21	6.34	1.22	2.9	1.2	253	I	6.35	7.39	1.16	2.2	1.2
Carteolol	275	B	2.63	3.23	1.23	3.2	1.4	F	12.20	13.60	1.11	1.4	1.0	275	K	5.25	6.38	1.21	1.9	1.5
Metipranolol	275	A	1.63	2.36	1.44	5.1	1.4	D	7.09	9.40	1.33	4.5	0.7	275	I	7.65	8.90	1.16	1.9	1.9
Metoprolol	275	A	2.06	2.78	1.35	4.2	1.2	D	8.85	11.64	1.32	4.1	0.9	275	I	13.65	15.80	1.15	1.9	1.3
Oxprenolol	275	A	1.45	1.81	1.25	2.9	1.0	D	6.47	7.86	1.21	2.7	0.8	275	I	5.92	7.40	1.25	2.8	1.3
Penbutolol	270	A	1.06				1.1	D	3.18				1.2	270	K	0.78				0.9
Pindolol	265	A	2.45	3.39	1.38	5.1	1.1	F	6.76	7.58	1.12	1.8	0.9	265	I	9.75	11.88	1.22	2.9	1.1
Propranolol	290	A	1.08	1.56	1.44	4.3	1.3	D	5.86	7.99	1.36	4.2	0.8	290	I	4.60	5.60	1.23	2.5	1.1
Sotalol	254	B	3.31	3.97	1.20	3.4	1.7	F	15.8	17.7	1.12	1.4	0.8							
Tertatolol	290	A	0.78	1.06	1.36	3.3	1.2	D	5.65	6.52	1.15	2.0	1.1	290	I	1.94	2.53	1.30	1.7	1.8
Timolol	300	A	1.49	2.27	1.52	5.5	1.3	E	2.79	3.59	1.29	2.4	1.2	300	J	2.43	3.39	1.39	3.3	1.4

0.1% of the amount employed in the reaction. By using the suggested procedures for (*S*)-PEI and D-GITC, no trace of underivatized *rac*-PRO could be detected, indicating complete turnover in the synthesis. When using acetonitrile as the solvent up to 15–20% of underivatized *rac*-PRO was observed when (*S*)-PEI was used as the reagent.

There was no indication that the reactions of the two reagents were not selective towards the amino group. However, for (*S*)-PEI, on increasing the reaction time to several hours it was possible to detect an extra set of peaks as the result of a subsequent carbamate reaction of the hydroxy group in significant amounts. Under the conditions described here less than 0.1% of these double derivatives, eluted early in the chromatograms, was seen.

To investigate the selectivity of (*S*)-PEI further, *rac*-PRO was derivatized with this reagent and subsequently treated with (−)-CACl. The resulting chromatogram was compared with that obtained after (*S*)-PEI treatment alone. As can be seen from Fig. 3, the peaks from PRO–urea derivatives are completely removed after (−)-CACl treatment, which may indicate that the hydroxy group was fully accessible to the acid chloride reagent.

(−)-CACl. A preliminary investigation with *rac*-PRO as the model substance for (−)-CACl derivatization showed that relative to (*R*)- and (*S*)-PEI and D-GITC, a larger excess of reagent and longer reaction time were necessary in order to obtain a complete reaction with a single set of peaks in the chromatograms. This procedure, however, resulted in highly lipophilic derivatives compared with the ureas and thioureas of the other reagents. This was probably caused by the formation bis-derivatives as a result of acylation of both the amino and hydroxy group of PRO. As shown in Table III, nine of the eighteen β-blockers investigated could be separated by using eluent F. For these compounds, the *R*-form is eluted first.

For the remainder of the substances successfully separated, a stronger eluent had to be employed to elute the derivatives. A possible explanation of this could be that only mono derivatives were formed for these substances,

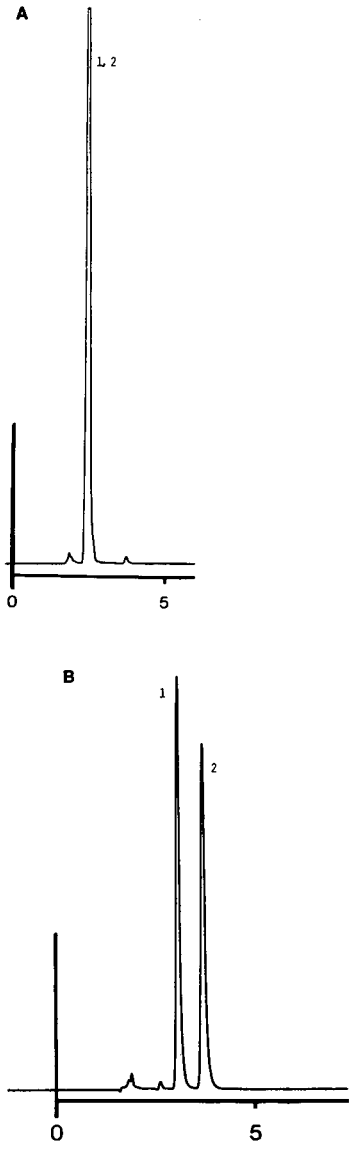


Fig. 3. Chromatograms of *rac*-PRO derivatized with (*S*)-PEI and subsequently with (A) (−)-CACl or (B) (*S*)-PEI alone. Derivatization procedures as described under Experimental. Column, 5-μm LiChrosorb Si 60 (120 × 4.6 mm I.D.); eluent, methanol–dichloromethane–heptane (1.5:50:48.5); detection at 290 nm. Peaks: 1 = (*R*)-PRO; 2 = (*S*)-PRO.

resulting in derivatives of higher polarity. For TIM, PEN, LEV and TER it appears from Fig. 1 that they all have *tert.*-butyl radicals attached to the amino function instead of the typical isopropyl group. The difference in the bulkiness of the moieties offers the possibility that only the

amine is accessible to the acid chloride, with a subsequent reaction of the hydroxy group sterically hindered. To investigate this assumption, *rac*-TIM (bearing a *tert.*-butyl radical) was treated with (*S*)-PEI and subsequently with (−)-CACl by the same procedure as described above for *rac*-PRO. As can be seen from Fig. 4, the addition of (−)-CACl resulted only in a partial disappearance of the *rac*-TIM urea peaks. Compared with the complete turnover of the *rac*-PRO ureas (*cf.*, Fig. 3), this indicates steric hindrance due to the bulkiness of the *tert.*-butyl radical compared with an isopropyl group. The *S*-form of the four compounds containing *tert.*-butyl eluted first, whereas the *R*-form of the isopropyl-containing compounds was eluted first.

From another group of β-blockers which all included an amide (ACE, ATE, CAR) or a sulphonamide moiety (SOT), it was only possible to obtain a successful separation of CAR, for which eluent G had to be employed. In this instance the *S*-form was eluted first.

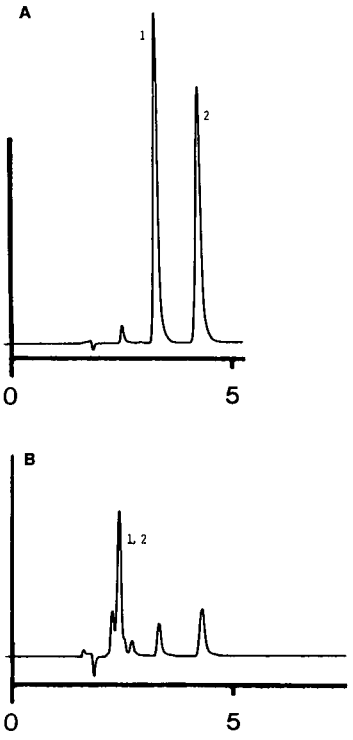


Fig. 4. Chromatograms of racemic timolol (TIM) derivatized with (*S*)-PEI and subsequently with (A) (−)-CACl or (B) (*S*)-PEI alone. Derivatization procedures as described under Experimental. Column, 5-μm LiChrosorb Si 60 (120 × 4.6 mm I.D.); eluent, methanol–dichloromethane–heptane (1.5:50:48.5); detection at 300 nm. Peaks: 1 = (*R*)-TIM; 2 = (*S*)-TIM.

The remaining β-blockers, including BIS, an isopropyl-containing compound without an amide group, could not be successfully separated using (−)-CACl as a reagent. No significant peaks were detected on testing a series of eluents with gradually increasing eluting strength with eluent G as the strongest. As the other two reagents investigated offered significant separations, no further investigations were performed.

Choice of chiral reagent

With a view to performing a further study of the applicability of normal-phase chromatography following chiral derivatization, a validation study was devised. The experience gained from the work so far performed with the different reagents enabled us to suggest the use of (*S*)-PEI as the most feasible choice. As criteria for this decision were considered parameters such as stability of the chromatography, including selectivity and asymmetry, but also stability of the derivatives and selectivity in the reaction of the reagent. During the following work the EEC guideline on analytical validation [54] was used.

Optical purity during synthesis

In order to use the approach of chiral derivatization for the determination of the optical purity of a substance, the enantiomeric purity of the reagent itself has to be of high optical purity, compared with the level of the substance being investigated. The optical purities of the reagents used in this investigation were guaranteed to be ≥99.5% for both (*S*)-PEI and (−)-CACl, according to the suppliers, but no information was available from the supplier of D-GITC. It is nevertheless likely that D-GITC was also of high purity, given the natural origin of the substance.

To examine the chiral purity of (*S*)- and (*R*)-PEI further, (*R*)- and (*S*)-PRO of very high purity were derivatized. By the method of standard additions only 0.1–0.2% of enantiomeric impurity was found for both enantiomers using the above-mentioned reagents. This indicates an optical purity of the reagents of at least 99.8%. The stability of the solutions used in the derivati-

zation procedure, (*S*)-PEI 60 mg/ml in dichloromethane, (*S*)-PRO 5 mg/ml in dichloromethane–triethylamine (10:1), and the solutions of the (*S*)-PRO derivatives of (*S*)-PEI were investigated. Using freshly prepared solutions of reagent and substrate, only about 0.1% corresponding to the peak of the (*R*)-PRO derivative was found with (*S*)-PRO derivatized with (*S*)-PEI. When stored for 14 days in a refrigerator the level was found to increase to about 0.5%. (*S*)-PRO solutions stored for 7, 45 and 105 days and derivatized with freshly prepared (*S*)-PEI showed enantiomeric impurity levels of 0.1%, 0.15% and 0.5%, respectively. Finally, a freshly prepared solution of (*S*)-PRO was derivatized with a solution of (*S*)-PEI that had been stored for 14 days in a refrigerator, and in this instance the enantiomeric impurity level reached about 1.0%. Overall the results indicate that the solution containing the β-blocker could be stored in refrigerator for up to 14 days, whereas the (*S*)-PEI solution and the solution of the derivative should be freshly prepared and analysed within hours.

Stability of the chromatographic systems used for the (S)-PEI derivatives

To investigate the stability of the chromatographic systems used for the (*S*)-PEI derivatives, the changes in the retention times of (*S*)-PEI propranolol derivatives were examined during a 3-week period. Each day a freshly prepared eluent was prepared. In 8 h, during a working day, the capacity factors usually increased by *ca.* 3%. This may be explained by a small evaporation primarily of dichloromethane, causing a slight decrease in the polarity of the eluent. Also an increase in temperature during the day was seen, which in normal-phase chromatography is expected to reduce retention.

On a day-to-day basis, using a freshly prepared eluent each day, the capacity factors were found to decrease by about 12% in 3 weeks. Throughout the period, a large number of samples were injected on to the column and, with the lipophilic nature of the system in mind, it could be expected that triethylammonium salts from the derivatization procedure would be adsorbed on the silica. This would result in a gradual deactivation of the solid phase, leading to lower retentions. By reactivating the column (see Experimental), the characteristics of the silica were found to be fully restored.

Validation of the methods

The possible use of the different reagents to monitor the optical purity in two β-blockers currently marketed as pure enantiomeric forms, (*S*)-TIM and (*S*)-BUN, was validated according to the EEC guideline [54] regarding specificity, reproducibility, accuracy, linearity and limit of quantification. For both compounds the chromatographic system given in Table III was used, with the exception that 310 nm was used as detection wavelength to reduce interference from reagent peaks. Fig. 5 depicts the results for (*S*)-BUN with the addition of 1% of the *R*-form. Linearity of the detector response for the *R*-form was ensured up to a content of 8%. The regression equations (area counts *versus* percentage) were $y = 5627x + 882$ ($r = 0.9996$) and $y =$

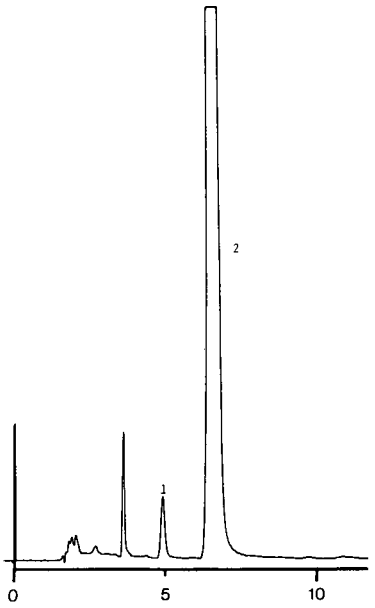


Fig. 5. Chromatogram of (*S*)-BUN (levobunolol), with 1% of (*R*)-BUN added, derivatized with (*S*)-PEI as described under Experimental. Column, 5-μm LiChrosorb Si 60 (120 × 4.6 mm I.D.); eluent, methanol–dichloromethane–heptane (1.5:50:48.5); detection at 310 nm. Peaks: 1 = (*R*)-BUN; 2 = (*S*)-BUN.

$9673x + 1466$ ($r = 0.9996$) for TIM and BUN, respectively, and the limits of detection were *ca.* 0.1 and 0.05 μg, respectively (corresponding to 0.1% and 0.05% of the amount in the main peak). By use of standard additions, the content of the *R*-form in the samples investigated was determined to *ca.* 0.15% for both (*S*)-TIM and (*S*)-BUN corresponding to the level resulting from the reagent. The reproducibility of the methods was examined for both TIM and BUN by use of a series of ten samples of the *S*-form to which were added 1% of the *R*-form and injecting each sample four times; $\bar{x}_{10} = 6333$, R.S.D. = 6.6% and $\bar{x}_{10} = 10\,605$, R.S.D. = 5.0% were found for TIM and BUN, respectively.

CONCLUSIONS

A number of HPLC methods based on normal-phase chromatography following chiral derivatization were investigated with a view to separating the enantiomers of eighteen different β-blocking agents currently on sale in Denmark. All β-blockers for which both enantiomeric forms were available were separated by at least one of the procedures described. One of the reagents, (*S*)-PEI, was selected as the most suitable choice for the separation of two β-blocking agents marketed as the enantiomeric forms (in both instances the *S*-form) with the intention of monitoring the optical purity. In both instances it was established that the procedures apply to the requirements described in the EEC guideline [54]. The limits of detection were determined to be *ca.* 0.1% and 0.05% for the *R*-form in (*S*)-TIM and (*S*)-BUN, respectively, with actual amounts found to be *ca.* 0.15% in the available samples for both compounds. In addition, the chromatographic system was found to exhibit satisfactory stability, this parameter being investigated with *rac*-PRO as a model substance. It is therefore concluded that the procedure of normal-phase HPLC following derivatization with (*S*)-PEI is well suited as a standardized method for the determination of the enantiomeric purity of β-blocking agents, *e.g.*, TIM and BUN.

REFERENCES

1 Y. Okamoto, M. Kawashima, R. Aburatani, K. Hatada, T. Nishiyama and M. Masuda, *Chem. Lett.*, (1986) 1237.
2 H. Takahashi, S. Kanno, H. Ogata, K. Kashiwada, M. Ohira and K. Someya, *J. Pharm. Sci.*, 77 (1988) 993.
3 A.M. Krstulovic and M.H. Fouchet, *J. Chromatogr.*, 452 (1988) 477.
4 C. Hartmann, D. Krauss, H. Spahn and E. Mutschler, *J. Chromatogr.*, 496 (1989) 387.
5 D.R. Rutledge and C. Garrik, *J. Chromatogr.*, 497 (1989) 181.
6 M.S. Ching, M.S. Lennard, A. Gregory and G.T. Tucker, *J. Chromatogr.*, 497 (1989) 313.
7 H.Y. Aboul-Enein and M.R. Islam, *Chirality*, 1 (1989) 301.
8 H.Y. Aboul-Enein and M.R. Islam, *J. Chromatogr.*, 511 (1990) 109.
9 I.W. Wainer and T.D. Doyle, *J. Chromatogr.*, 306 (1984) 405.
10 A.M. Dyas, M.L. Robinson and A.F. Fell, *Chromatographia*, 30 (1990) 73.
11 M. Ohwa, M. Akiyoshi and S. Mitamura, *J. Chromatogr.*, 521 (1990) 122.
12 J. Hermansson, *J. Chromatogr.*, 325 (1985) 379.
13 K. Balmer and B. Persson, *J. Chromatogr.*, 477 (1989) 107.
14 M. Enquist and J. Hermansson, *J. Chromatogr.*, 519 (1990) 285.
15 J. Haginaka, J. Wakai, K. Takahashi, H. Yasuda and T. Katagi, *Chromatographia*, 29 (1990) 587.
16 D.W. Armstrong, T.J. Ward, R.D. Armstrong and T.E. Beesley, *Science*, 232 (1986) 1132.
17 E. Kusters and D. Giron, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 531.
18 E. Delee, L. Le Garrec, I. Jullien, S. Berranger, J.C. Pascal and H. Pinhas, *Chromatographia*, 24 (1987) 357.
19 J.A. Thompson, J.L. Holtzman, M. Tsuru, C.L. Lerman and J.L. Holtzman, *J. Chromatogr.*, 238 (1982) 470.
20 M.J. Wilson and T. Walle, *J. Chromatogr.*, 310 (1984) 424.
21 J. Gal and A.J. Sedman, *J. Chromatogr.*, 314 (1984) 275.
22 A.A. Gulaid, G.W. Houghton and A.R. Boobis, *J. Chromatogr.*, 318 (1985) 393.
23 P. Hsyu and K.M. Giacomni, *J. Pharm. Sci.*, 75 (1986) 601.
24 G. Pflugmann, H. Spahn and E. Mutschler, *J. Chromatogr.*, 421 (1987) 161.
25 S.K. Chin, A.C. Hui and K.M. Giacomini, *J. Chromatogr.*, 489 (1989) 438.
26 H.G. Schaefer, H. Spahn, L.M. Lopez and H. Derendorf, *J. Chromatogr.*, 527 (1990) 351.
27 G. Gübitz and S. Mihellyes, *J. Chromatogr.*, 314 (1984) 462.
28 A. Darmon and J.P. Thenot, *J. Chromatogr.*, 374 (1986) 321.
29 A.J. Sedman and J. Gal, *J. Chromatogr.*, 278 (1983) 199.

30 D. Schuster, M. Woodruff Modi, D. Lalka and F.M. Gengo, *J. Chromatogr.*, 433 (1988) 318.
31 E.J. Eisenberg, W.R. Patterson and G.C. Kahn, *J. Chromatogr.*, 493 (1989) 105.
32 R. Hasegawa, M. Murai-Kushiya, T. Komuro and T. Kimura, *J. Chromatogr.*, 494 (1989) 381.
33 J. Hermansson and C. von Bahr, *J. Chromatogr.*, 221 (1980) 109.
34 W. Lindner, C. Leitner and G. Uray, *J. Chromatogr.*, 316 (1984) 605.
35 W. Lindner, M. Rath, K. Stoschitzky and G. Uray, *J. Chromatogr.*, 487 (1989) 375.
36 J. Hermansson and C. von Bahr, *J. Chromatogr.*, 227 (1982) 113.
37 R.J. Guttendorf, H.B. Kostenbauer and P.J. Wedlund, *J. Chromatogr.*, 489 (1989) 333.
38 H.F. Schitthenner, M. Fedorchuk and D.J. Walter, *J. Chromatogr.*, 487 (1989) 197.
39 R. Mehvar, *J. Chromatogr.*, 493 (1989) 402.
40 R. Mehvar, *J. Pharm. Sci.*, 78 (1989) 1035.
41 S. Einarsson and B. Josefsson, *Anal. Chem.*, 59 (1987) 1191.
42 M. Ahnoff, S. Chen, A. Green and I. Grundevik, *J. Chromatogr.*, 506 (1990) 593.
43 W. Dieterle and J.W. Faigle, *J. Chromatogr.*, 259 (1983) 311.
44 M. Piquette-Miller, R.T. Foster, F.M. Pasutto and F. Jamali, *J. Chromatogr.*, 526 (1990) 129.
45 M. Piquette-Miller and R.T. Foster, *J. Chromatogr.*, 533 (1990) 300.
46 C. Petterson and G. Schill, *J. Chromatogr.*, 204 (1981) 179.
47 C. Petterson and M. Josefsson, *Chromatographia*, 21 (1986) 321.
48 A. Karlsson, C. Pettersson and B. Björkman, *J. Chromatogr.*, 494 (1989) 157.
49 C.L. Davies, *J. Chromatogr.*, 531 (1990) 131.
50 N. Nimura, H. Ogura and T. Kinoshita, *J. Chromatogr.*, 202 (1980) 375.
51 T. Kinoshita, Y Kasahara and N. Nimura, *J. Chromatogr.*, 210 (1981) 77.
52 N. Nimura, Y. Kasahara and T. Kinoshita, *J. Chromatogr.*, 213 (1981) 327.
53 P. Helboe, unpublished results.
54 *Analytical Validation. The rules governing medicinal products in the European Community*, Vol. III, Addendum 1, Brussels, 1990.

Journal of Chromatography, 636 (1993) 243–247
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 812

Isotope effects in liquid chromatography of imipramine and desmethylimipramine

B.K. Kudelin*, L.V. Gavrilina and Yu.L. Kaminski

V.G. Khlopin Radium Institute, 194021 St. Petersburg (Russian Federation)

(First received July 7th, 1992; revised manuscript received November 30th, 1992)

ABSTRACT

The effects of tritium substitution in imipramine (IMI) and N-desmethylimipramine (DMI) on their liquid chromatography are described. Increased elution times for tritiated analogues of IMI and DMI were observed using isocratic reversed-phase HPLC techniques. It was shown that the retention of labelled molecules depends on number and position of the tritium atoms. An explanation of the observed phenomena is offered.

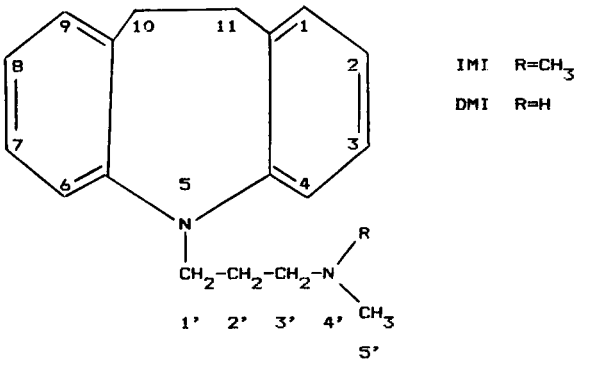
INTRODUCTION

Isotope effects in the chromatography of labelled compounds have repeatedly been observed and a labelled compound can demonstrate more or less mobility in comparison with the corresponding unlabelled analogue. Most commonly the substitution of protium atoms by heavier isotopes resulted in a decrease in chromatographic retention [1–4].

However, for some preparations, in particular for tricyclic and tetracyclic antidepressants, different trends have been observed [5,6]. Deuterated mianserin and Org GC 94 (1,3,4,14b-tetrahydro - 2,7 - dimethyl - 2*H* - dibenzo[*b*,*f*]pyrazino [1,2-*d*][1,4]oxazepine maleate) on a normal-phase column were eluted after the corresponding protium analogues. The behaviour of deuterated imipramine (IMI) on thin layers of silica, alumina and cellulose has been investigated in detail [7]. A decrease in IMI mobility was observed when protium atoms in N-methyl groups were replaced with deuterium.

Tritium-labelled analogues of such compounds have been used as radioligands in serotonin receptor investigations and in the screening of potential antidepressants [8,9].

This paper is devoted to a discussion of the observed isotope effects on the chromatographic behaviour of tritiated IMI and desmethylimipramine (DMI) and the corresponding unlabelled compounds on normal- and reversed-phase columns.



EXPERIMENTAL

The chromatographic separations were performed on columns with a reversed stationary phase (LiChrosorb RP-18; Merck, Darmstadt,

* Corresponding author.

0021-9673/93/$06.00

Germany) (5 μm, 250 × 4 mm I.D.) and a normal stationary phase (TSK NH_2-60; Toyo Soda, Japan) (5 μm, 250 × 4.6 mm I.D.). An LKB (Bromma, Sweden) liquid chromatograph was used with UV detection at 254 nm. The radioactivity of the fractions obtained after chromatographic separation was measured by liquid scintillation counting with a Beckman LS 9800 instrument.

The HPLC conditions were as follows: temperature, 22°C; eluents, ethanol–water (95:5, v/v) containing 0.1–0.9% of triethylamine (TEA) for the reversed-phase column and heptane–chloroform–2-propanol (83:10:7, v/v/v) for the normal-phase column; and flow-rate, 0.2–0.5 ml/min. All chemicals were obtained from Sojuzkhimreaktiv (Moscow, Russian Federation). The eluents were degassed with helium before use.

TLC separations were performed on commercially precoated plates (Silufol UV254, 15 × 15 cm; Kavalier, Sklárny, Czechoslovakia). The solvent was ethyl acetate–2-propanol–ammonia solution ($d = 0.904$ g/ml) (40:30:3, v/v/v).

Tritiated IMI and DMI were obtained in our laboratory by solid-state catalytic exchange with gaseous tritium. Chemical homogeneity and identity with the unlabelled substances were tested by UV spectrophotometry and thin-layer radiochromatography. The locations of the labels were determined by ^3H NMR spectroscopy (Bruker AC250 instrument, 266.8 MHz).

RESULTS AND DISCUSSION

There have been many reports on the HPLC of tricyclic antidepressants (for a review, see ref. 10). In reversed-phase chromatography, buffer eluents are usually used. We tried to find eluents without any salts for easier subsequent isolation of the labelled compounds from the column effluent. Suitable results were obtained with ethanol–water (95:5, v/v) containing 0.1–0.5% of triethylamine (TEA) for reversed-phase and heptane–chloroform–2-propanol (83:10:7, v/v/v) for normal-phase HPLC. In both instances considerable fractionation of the chromatographic zones of tritiated IMI and DMI, depending on their molar activity, was observed.

In the solid-state catalytic exchange reaction with gaseous tritium, a set of products with different extents of protium–tritium substitution were formed. On HPLC, such samples give a set of unresolved peaks corresponding to products with different numbers of tritium atoms in the molecule. Fig. 1 shows typical curves. Separate fractions of the effluent were analysed by ^3H NMR, UV spectrophotometry and thin-layer radiochromatography to determine their chemical homogeneity and identity with unlabelled IMI and DMI. All the analyses confirmed that the separate peak fractions of the labelled compounds differ only in molar activity, *i.e.*, in the number of tritium atoms. The molar activity data are given in Table I. The volume of the fractions was random.

In most reports in the literature, hydrogen isotope effects lead to a decrease in the retention volumes of labelled compounds in comparison with the unlabelled analogues [3,4]. However, in this work, for both reversed and normal phases the labelled compounds had longer retention times than the unlabelled analogues. The observed order of elution allows some conclusions to be drawn about the retention mechanism.

It has been reported that the main role in the retention of basic compounds on reversed phases is played by residual polar silanol groups [11]. Our data confirm this: the order of elution of the labelled and unlabelled compounds on the normal- and reversed-phase columns is the same, indicating an identical retention mechanism on both columns. Small amounts of strongly polar modifiers such as triethylamine play a specific role. Usually such additives are used for the suppression of undesirable interactions of the components to be separated with the residual silanol groups of reversed-phase sorbents. We observe a strong dependence extent of fractionation of the labelled compounds on the amount of triethylamine used in the mobile phase. In Table II the capacity factors are given both for unlabelled IMI (standard) and for the beginning and the end of the unresolved peak sequence of the different isotopic forms of labelled IMI. It is obvious that such a suppression of the interaction of the chromatographed compound with the silanol groups of the reversed-phase sorbent

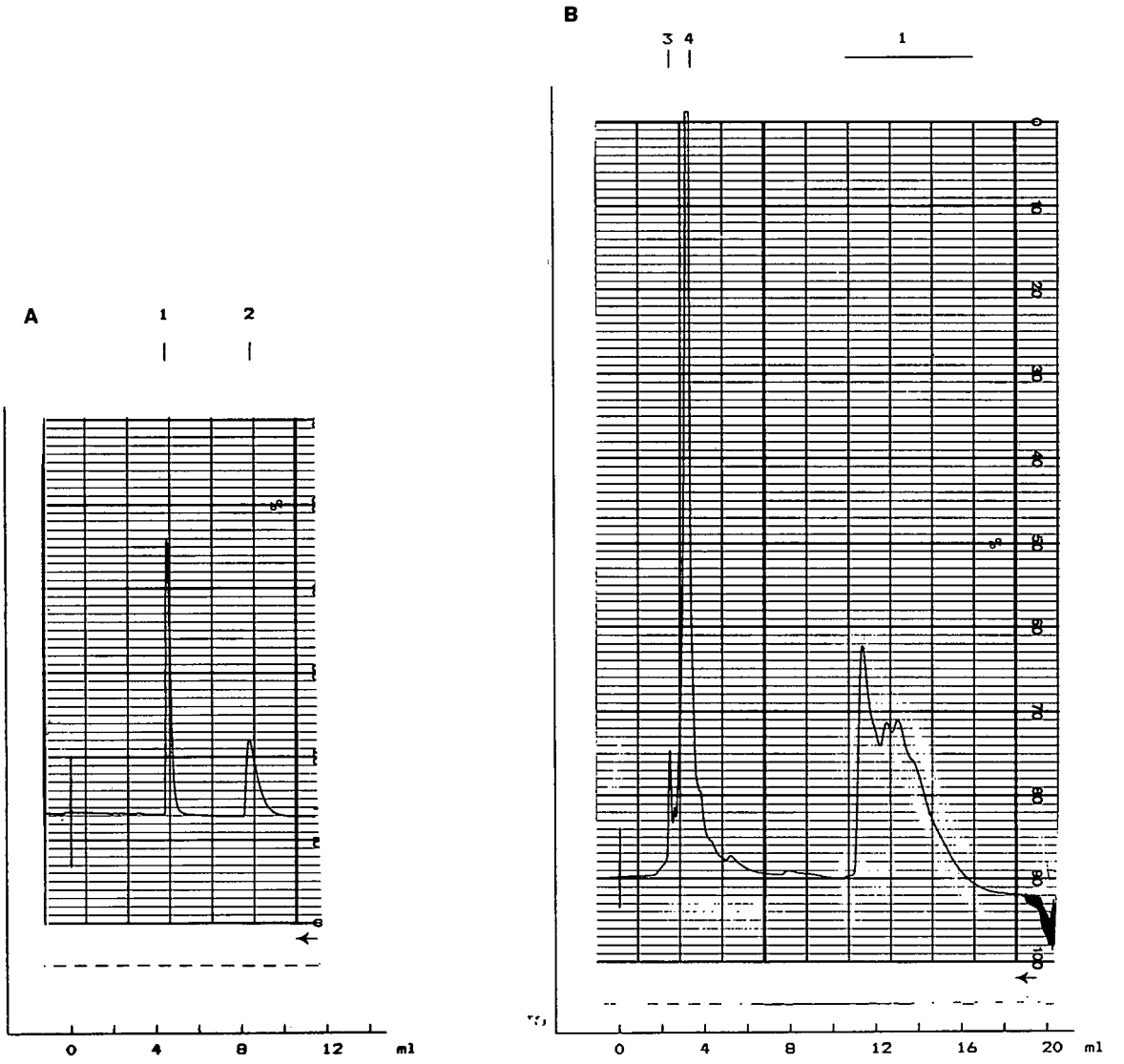


Fig. 1. Typical chromatogram for (A) standard mixture of unlabelled IMI and DMI and (B) sample of tritiated IMI. Chromatographic conditions: column, LiChrosorb RP-18; eluent, ethanol–water (95:5, v/v); flow-rate, 0.3 ml/min; temperature, 22°C. Addition of TEA to the eluent: (A) 0.9% and (B) 0.15%. Peaks: 1 = IMI; 2 = DMI; 3,4 = unidentified compounds from the reaction mixture.

results in a decrease in the fractionation of labelled IMI.

The participation of the polar groups of the sorbent in retention in this work may be due to the formation of hydrogen bonds between the nitrogen atoms of IMI or DMI and the protons of the surface OH groups of the sorbent. Such an interaction is very significant, as evidenced by the stronger retention of DMI than IMI [on the reversed-phase column using ethanol–water (95:5, v/v) containing 0.9% of TEA, the capacity factors are 2.27 and 0.81, respectively (see Fig. 1A)], although usually the substitution of the methyl group by a hydrogen atom leads to a decrease in retention on a reversed-phase column owing to the increase in the polarity of the compound. In IMI and DMI, the substitution of the methyl group by a hydrogen atom and the

TABLE I

MOLAR ACTIVITY OF THE SEPARATE FRACTIONS OF UNRESOLVED PEAKS OF TRITIATED IMI AND DMI

Run No.	Sample	Eluent[a] and sorbent	Fraction No.	Molar activity, Ci/mmol	Activity output[b] (%)
1	[^{3}H]IMI	A, reversed phase	1	75	34
			2[c]	158	25
2	[^{3}H]IMI (second fraction from run 1)	A, reversed phase	1	55	19
			2	162	28
			3[c]	192	10
3	[^{3}H]DMI	B, reversed phase	1	25	7
			2	71	39
			3	102	22
			4	137	20
			5[c]	170	6
4	[^{3}H]DMI	B, reversed phase	1	27	7
			2	43	10
			3	92	34
			4	113	19
			5[c]	126	20
5	[^{3}H]IMI	A, reversed phase	1	–	–
			2	67	4
			3	211	31
			4[c]	208	20
6	[^{3}H]IMI	C, normal phase	1	246	8
			2	322	29
			3[c]	340	22

[a] Eluents: A = ethanol–water (95:5, v/v) + 0.15% TEA; B = ethanol–water (95:5, v/v) + 0.5% TEA; C = heptane–chloroform–2-propanol (83:10:7, v/v/v).
[b] Proportion of the total peak activity in the fraction.
[c] Subsequent fractions were not analysed.

TABLE II

DEPENDENCE OF THE CAPACITY FACTORS OF IMI ON CONCENTRATION OF TRIETHYLAMINE IN THE ELUENT

Eluent, ethanol–water (95:5, v/v); reversed-phase column.

TEA concentration (%)	Capacity factor	
	Unlabelled compound	Tritiated compound
0.02	8.9	9.3–16.6
0.06	4.9	5.1–8.7
0.10	4.0	4.2–7.2
0.20	3.0	3.0–5.4
0.30	2.5	2.5–4.6

corresponding increase in polarity produced suitable conditions for the formation of stronger hydrogen bonds between the 4′-nitrogen atom and surface OH groups of the sorbent. The important role of this nitrogen atom in the retention of the molecule was noted by Heck *et al.* [7]. They showed that under the conditions of thin-layer chromatography, replacement of the protons on the benzene ring in the IMI molecule by deuterium has no influence on retention, whereas incorporation of deuterium in the N-methyl groups decreases the chromatographic mobility. Our data confirm this conclusion.

The results of the ^{3}H NMR analysis of the distribution of tritium atoms for tritiated DMI in the three fractions with different molar activities

TABLE III

^{3}H NMR DATA ON TRITIUM ATOM DISTRIBUTION IN DMI MOLECULES FOR DIFFERENT FRACTIONS OF PEAK (RUN NO. 7)

Fraction No.	Molar activity (Ci/mmol)	Proportion of activity for different positions of tritium atoms in the molecule (%)			
		Benzene ring	10- and 11-positions	3′-Position	5′-Position
1	82	31	30	8	31
2	108	27	23	14	36
3	101	23	22	17	38

are given in Table III. The fractions were collected during elution of the series of unresolved peaks. It is seen that the retention is largest for molecules in which tritium atoms are concentrated in the 3′- and 5′-positions.

Analysis of the results obtained allows the following conclusion to be drawn about the mechanism of the isotope effect in the present instance. The N-4′ atom plays a significant role in the retention of the molecule owing to the formation of hydrogen bonds with surface OH groups of the sorbent. Replacement of protons at C-3′ and C-5′ by tritium make these carbons more electronegative. In turn, this leads to an increased electronegativity of the N-4′ atom and promotes the formation of stronger hydrogen bonds with the surface OH groups of the sorbent. The latter decreases the chromatographic mobility of the labelled molecules.

This behaviour of labelled compounds of the given class and difference in the capacity factors of the labelled and unlabelled compounds may be employed to obtain preparations with desired molar activities by chromatographic procedures. Such preparations can be used as tracer compounds in pharmacokinetic and other studies.

ACKNOWLEDGEMENT

We are grateful to Dr. S.G. Rosenberg for obtaining the ^{3}H NMR spectra of the labelled compounds.

REFERENCES

1 N. Tanaka and E.R. Thornton, *J. Am. Chem. Soc.*, 98 (1976) 1617.
2 R. Baweja, *J. Liq. Chromatogr.*, 9 (1986) 2609.
3 R. Baweja, *Anal. Chim. Acta*, 192 (1987) 345.
4 W.J.S. Lockley, *J. Chromatogr.*, 483 (1989) 413.
5 J.J. De Ridder and H.J.M. Van Hal, *J.Chromatogr.*, 121 (1976) 96.
6 C.N. Filer, R. Fazio and D.G. Ahern, *J. Org. Chem.*, 46 (1981) 3344.
7 H.A. Heck, R.L. Simon and M. Anbar, *J. Chromatogr.*, 133 (1977) 281.
8 J.J. Clark and J.D. Jauaice (Editors), *Principles of Psychopharmacology*, Academic Press, New York and London, 1978.
9 M.D. Mashkovskii, *Pharmacology of Antidepressants* (in Russian), Meditsina, Moscow, 1983.
10 R. Fazio, E. Spina and F. Pisani, *J. Liq. Chromatogr.*, 10 (1987) 223.
11 B. Pfleiderer and E. Bayer, *J. Chromatogr.*, 468 (1989) 67.

Journal of Chromatography, 636 (1993) 249–254
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 896

Rapid method for the enrichment of very long-chain fatty acids from microorganisms

T. Řezanka*

Institute of Microbiology, Vídeňská 1083, 14220 Prague (Czech Republic)

M.Yu. Sokolov☆

Institute of Microbiology, Prosp. 60-letia Oktiabrya 7/2, 117811 Moscow (Russian Federation)

(First received November 20th, 1992; revised manuscript received January 13th, 1993)

ABSTRACT

A method for the enrichment very long-chain fatty acids (VLCFAs) from total fatty acid-containing samples is described. The method is based on the use of reversed-phase high-performance liquid chromatography and/or solid-phase extraction cartridges with subsequent identification of VLCFAs by capillary gas chromatography–mass spectrometry. By combining the two techniques, unusual VLCFAs (higher than C_{22}) were identified in newly isolated oligotrophic bacteria. VLCFAs have already been described in sulphate-reducing bacteria and in some wood-decaying fungi.

INTRODUCTION

Except for mycobacteria, in which very long-chain fatty acids (VLCFAs) were detected a long time ago [1], we have very little knowledge about these unusual acids. Therefore, this topic has attracted much attention during recent years [2,3].

One of the ways of identifying VLCFAs (having more than 22 carbon atoms in the molecule) in microorganisms, in which their content is very low and highly variable, is to enrich the sample. Our task was to develop such an enrichment method. This point has been neglected very often, and few papers deal with a complex study of enrichment of VLCFA-containing samples.

One of the two following methods may be chosen. The first one involves separation of individual lipid classes and identification of VLCFAs in particulate molecular species (*e.g.* sphingomyelin [4] or phosphatidylcholine [5]). The second method includes selective enrichment of VLCFA-containing mixtures of total fatty acids (FAs) by chromatography. The latter procedure is preferable for the analysis of microbial lipids as some of them are unusual. In addition, this method is less labour-intensive and allows the isolation of as much as milligrams of VLCFAs within several hours.

Good results were obtained by Takayama *et al.* [6], who studied mycobacterial VLCFAs. The method was based on classical column chromatography with lipophilic Sephadex LH-20. Identification was performed by further TLC and RP-HPLC with MS.

Excellent resolutions of standards was obtained by TLC on silica gel, but separation of a natural mixture resulted in total failure [7] (*i.e.* degradation of unsaturated fatty acids, insuffici-

* Corresponding author.
☆ Present address: GBF, Bereich Mikrobiologie, Mascheroder Weg 1, W-3300 Braunschweig, Germany.

0021-9673/93/$06.00

ent plate numbers and very difficult detection with some problems with non-destructive stains on the Ag^+ layer). Similar results were reported by Quereshi *et al.* [8], who succeeded in TLC separation of VLCFAs from mycobacteria as *p*-bromophenacyl esters and obtained four fractions. The subsequent RP-HPLC showed that almost all peaks were present in all four TLC fractions, even if at different concentrations.

The best results were obtained if the reversed phase was used, either in TLC or in HPLC [7]. Nevertheless, some disadvantages of TLC were observed, *e.g.* capacity of the plate, partial degradation of unsaturated FAs and, also, non-separation of homologues in a natural mixture that differed by one CH_2 group. The best method of all turned out to be RP-HPLC, which could be used not only for separation of the individual compounds but also for enrichment of the total FAs with VLCFAs.

As early as 1984 a method [9] was developed enabling the enrichment of total FAs with VLCFAs by using HPLC (RP-1) at the semi-preparative scale. The same method was successfully used in another study, with the difference that the authors used a C_{18} reversed-phase column [10].

Having already faced the problem of identification of VLCFAs in microbial cells several times [11–13], we developed a simple and rapid technique of enrichment of VLCFA-containing samples. This method is based on separation of the total FAs, by using either RP-HPLC or solid-phase extraction (SPE) cartridges packed with an octadecyl-bonded silica.

EXPERIMENTAL

Preparations of standards

Stearic (1704 mg, 6 mmol) and cerotic (1188 mg, 3 mmol) (Sigma, St. Louis, MO, USA) acids were derivatized by reacting the free acids with boron fluoride–methanol [13]. Methyl esters of behenic acid were obtained in the same way. Two standard mixtures of fatty acid methyl esters (FAMEs) from 18:0 and 26:0 FAMEs were prepared in chloroform (standard 1 = 908 mg; molar ratio 1:1; standard 2 = 888 mg, molar ratio 99:1).

The mixture of natural FAMEs was obtained from:

(1) Soil oligotrophic bacteria *Renobacter vacuolatum* NP-300-G [11].

(2) Fungi (*Ganoderma applanatum*) of the Basidiomycetes family [12].

(3) Sulphate-reducing bacterium *Desulfotomaculum* sp. str. 43 [13].

Derivatization and GC–MS identification

The oxazolines were prepared using a modified method, as described before [14]: 5 mg of dicyclohexylcarbodiimide were added to a solution of 5 mg of FAs in 1 ml of dichloromethane. After stirring for 10 min, 5 mg of 2-amino-2-methylpropanol were added (20°C, 4 h). Evaporated mixture was dissolved in 1 ml of diethyl ether and treated with 0.5 ml of thionyl-chloride ($SOCl_2$) (20°C, 1 h), then washed with ice-cold water and eluted through a column with anhydrous sodium sulphate and silica gel.

High-performance liquid chromatography

HPLC was performed in the Gradient LC System G-I (Shimadzu, Kyoto, Japan) with two LC-6A pumps (0.5 ml/min), an SCL-6A system controller, an SPD ultraviolet detector (207 nm), an SIL-1A sample injector and a C-R3A data processor. A semipreparative column Separon SGX C_{18} (250 mm × 8 mm I.D.) (Tessek, Prague, Czechoslovakia) with 7-μm particles were used.

The column was first eluted with methanol for 60 min, then with diethyl ether for 15 min and finally with methanol for 15 min. The fractions collected within more than 9 min were used for further analysis (for 22:0 retention time is 8.2 min).

Solid-phase extraction

The Separon SGX C_{18} silica-cart plastic cartridge system (1-ml tubes) (Tessek) was used. A total amount of 1–2 mg of FAMEs was applied to the column in a small volume of dichlormethane. Methanol–isopropanol (98:2) was allowed to flow under slight syringe pressure at a rate of approximately 0.5 ml/min; 0.1-ml fractions were collected manually. Better results are obtained with detectors but the price of detec-

tor(s) is higher. The solvent mixture was then evaporated in a vacuum and each fraction was analysed by GC–MS. The retention volume for FAME 22:0 was 2.1 ml.

For identification of FAMEs we used a Shimadzu QP-1000 GC–MS system (Shimadzu) with a 60 m × 0.32 mm I.D. (0.25 μm particle size)/SPB-1 fused-silica capillary column (Supelco, Gland, Switzerland), a split/splitless injector and helium as a carrier gas. The oven temperature was programmed from 100 to 320°C at the rate of 4°C/min. Ionization energy was 70 eV and electron multiplier voltage was 2.5 kV.

Oxazolines were identified under similar conditions as methyl esters, with the only difference being in the column temperature. In this case the oven was programmed from 150 to 330°C at the rate of 5°C/min.

RESULTS AND DISCUSSION

The capacity of RP-HPLC was between 100 and 200 mg of the injected mixture (standard No. 1) as shown in Table I. By using SPE, the injected amount decreases to about 10 mg. In both cases, a ratio (18:0 to 26:0) of 98–99:2–1 was taken as a satisfactory enrichment. The proportion of the injected mixture to the weight of the sorbent remained constant (1:100). Based on these facts, the two methods RP-HPLC and SPE are considered to be adequate.

In this paper we used methyl esters, as they are more suitable for GC–MS. UV-absorbing or fluorescent derivatives cannot be used for GC–MS as they are usually not sufficiently volatile. In general, lower organisms (microorganisms) contain very unusual types of complex lipids and their identification is very difficult.

Unfortunately, when the molar ratio of 18:0 to 26:0 was 99:1 (standard No. 2), which was quite a common value in the natural material, the results changed significantly. In particular, a decrease in the capacities of the column and cartridge of as much as one order of magnitude was observed. More important was the mass proportion of 18:0 and 26:0 in the eluate. For example, if the injection was 1 mg per SPE column, the enriched eluate contained 5.74 μg of 18:0 FAME and 16.44 μg of 26:0 FAME. So, if a natural mixture with C_{18} content a hundred times more than that of C_{26} acids was analysed, the resulting content of the two compounds reached almost 1:3. Excluding this limitation, the method seemed to be suitable for a preseparation at the VLCFA enrichment step.

To find out how far the theoretical assumptions derived from the analysis of two simple component mixtures could be extrapolated to the natural mixture, three sources were used: standards 1 and 2 (mentioned above) and the natural mixture.

In our experiments the natural mixture revealed almost the same patterns as standard 2. Some complications could be observed only during the elution of monoenoic FAMEs, where the saturated homologue shorter by two carbon

TABLE I

DEPENDENCE OF THE SAMPLE ENRICHMENT WITH VLCFAs ON THE TYPE OF SEPARATION (RP-HPLC, SOLID-PHASE EXTRACTION), COLUMN CAPACITY AND THE FAME MOLAR RATIO

FAME	Molar ratio	Type of separation (amount of injected mixture, mg)											
		LC–SPE (1)		LC–SPE (10)		LC–SPE (50)		LC–SPE (100)		LC–SPE (200)		LC–SPE (500)	
18:0	50:50[a]	0.1[b]	0.3	0.2	1.4	0.4	12.1	0.8	45.7	3.8	49.9	25.7	50.0
26:0		99.9	99.7	99.8	98.6	99.6	87.9	99.2	54.3	96.2	50.1	74.3	50.0
18:0	99:1	0.5	1.4	1.3	19.6	7.8	44.2	32.1	99.0	99.0	99.0	99.0	99.0
26:0		95.5	98.6	98.7	80.4	92.2	55.8	67.9	50.0	1.0	1.0	1.0	1.0

[a] Molar ratio of 18:0 and 26:0 FAMEs in injected mixture.
[b] Molar ratio of 18:0 and 26:0 FAMEs in eluted mixture.

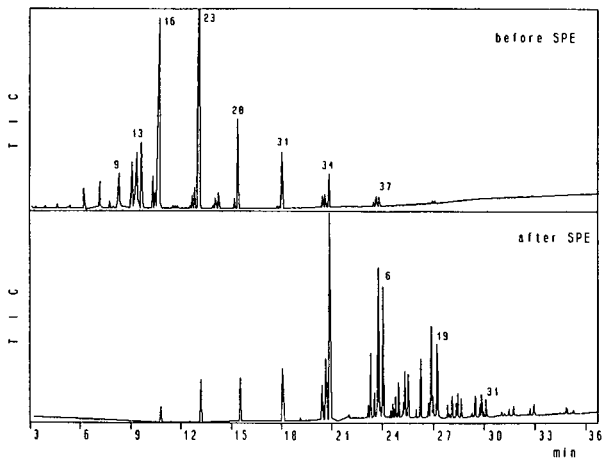


Fig. 1. Gas chromatography of FAMEs from soil oligotrophic bacteria *Renobacter vacuolatum* NP-300-G. Upper trace, total FAME = before SPE. For peak numbers, see Table II. Lower trace, methyl esters of VLCFAs after SPE (fraction above 2.2 ml was collected; for details, see Experimental section). For peak numbers, see Table III.

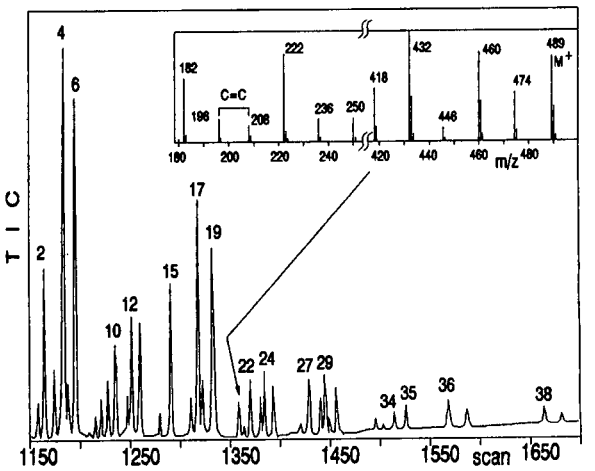


Fig. 2. GC–MS of oxazolines, prepared from VLCFA enrichment. The identities of peaks are listed in Table III. In the upper right corner the mass spectrum of ai-20–29:1 oxazoline is presented.

TABLE II

COMPOSITION OF FATTY ACIDS FROM OLIGOTROPHIC BACTERIA *RENOBACTER VACUOLATUM* NP-300-G (BEFORE ENRICHMENT)

i = Iso; ai = *ante*-iso.

Peak number	Fatty acid	Percentage	Peak number	Fatty acid	Percentage
1	8:0	0.18	20	9–18:1	0.28
2	9:0	0.25	21	i-18:0	0.95
3	10:0	0.37	22	11–18:1	1.47
4	11:0	0.18	23	18:0	22.15
5	12:0	1.69	24	i-19:0	0.16
6	13:0	2.17	25	ai-19:0	0.95
7	i-14:0	0.66	26	19:0	1.43
8	7–14:1	0.18	27	11–20:1	0.77
9	14:0	2.75	28	20:0	9.23
10	i-15:0	3.24	29	i-22:0	0.57
11	ai-15:0	4.56	30	13–22:1	0.34
12	8–15:1	0.44	31	22:0	6.70
13	15:0	5.37	32	i-24:0	0.95
14	i-16:0	3.61	33	15–24:1	1.19
15	7–16:1	1.47	34	24:0	3.29
16	16:0	19.49	35	i-26:0	0.38
17	i-17:0	0.32	36	26:1	0.89
18	10–17:1	0.41	37	26:0	0.79
19	17:0	0.18			

atoms was co-eluted. This rule of critical pairs is well known [15].

In addition to the four originally identified peaks (24:1, 24:0, 26:1 and 26:0) and another two tentative ones (28:1 and 28:0), we identified a total of 33 new peaks in the natural mixture (Fig. 1). The content of 18:0 and 26:0 became almost 1:3 compared with the standard mixtures. Fig. 1 reveals enrichment in other acids as well. C_{24} acids were found to be greatly enriched and, thus, 24:0 turned out to be a major acid. Fig. 2 depicts a chromatogram of oxazolines starting from C_{26} acids. If Figs. 1 (bottom) and 2 are compared, no significant differences in either separation or proportion of the compounds are observed. So, we could suggest that methyl esters and oxazolines of VLCFAs behave in a similar way.

When FAMEs from a fungus and a sulphate-reducing bacterium were analysed, similar results were obtained.

Fig. 2 shows a mass spectrum of ai-20–29:1 oxazoline. The mass spectra of VLCFA methylesters have been described previously [12,13]. Methyl esters have the best chromatographic properties but, unfortunately, the information obtained from their mass spectra is not satisfactory. It is impossible to determine the position of double bond(s) and very difficult to identify the substitution of the main chain with a methyl group. Using these derivatives, first described by Yu *et al.* [14,16], we were able to identify the monoenoic and branched acids even in such a complex mixture as was found in oligotrophic bacteria. The elution temperature of oxazolines was higher than that of methyl esters by 5°C only.

The mass spectrum of ai-20–29:1 oxazoline possesses two interesting regions. The first of them comprises the values of m/z in the neighbourhood of the molecular ion and, thus, enabled us to identify the branching of the chain easily [14]. In oxazolines, the splitting of the individual fragments was more evident than in methyl esters. Therefore, the ions m/z 489 [M^+], 474 [$M - CH_3$], 460 [$M - C_2H_5$] and 432 [$M - C_4H_9$] were more abundant in the mass spectrum. In contrast, the ion m/z 446 [$M - C_3H_7$],

TABLE III

VLCFAs FROM SOIL OLIGOTROPHIC BACTERIA *RENOBACTER VACUOLATUM* NP-300-G (AFTER ENRICHMENT BY SPE)

Peak number	Fatty acids	Percentage	Peak number	Fatty acids	Percentage
1	i-17–26:1	0.83	21	i-29:0	0.45
2	i-26:0	6.50	22	18–29:1	1.85
3	15–16:1	1.97	23	ai-29:0	1.15
4	17–26:1	14.97	24	20–29:1	1.97
5	19–26:1	1.59	25	29:0	1.59
6	26:0	14.14	26	i-21–30:1	0.51
7	i-19–27:1	0.64	27	i-30:0	1.53
8	ai-19–27:1	0.96	28	19–30:1	1.15
9	i-27:0	1.72	29	21–30:1	1.85
10	15–27:1	3.18	30	23–30:1	0.76
11	ai-27:0	1.59	31	30:0	1.46
12	19–27:1	3.89	32	ai-22–31:1	0.45
13	27:0	3.57	33	ai-31:0	0.32
14	i-19–28:1	0.96	34	22–31:1	0.57
15	i-28:0	4.59	35	31:0	0.76
16	17–28:1	1.72	36	23–32:1	0.89
17	19–28:1	9.11	37	32:0	0.51
18	21–28:1	2.17	38	25–34:1	0.45
19	28:0	6.37	39	34:0	0.25
20	ai-20–29:1	1.08			

corresponding to the structure of the iso-acid, was almost absent. The ions having lower m/z values, *e.g.* 418 $[M - C_5H_{11}]$, 404 $[M - C_6H_{13}]$, etc., exhibited normal abundances and their intensity was similar to that of saturated acids. The other region important for the determination of the molecular structure was the region of the ions that originate from the double bonds. The major ions in this region were the peaks of m/z 182 and 222, which were formed by the allylic cleavage on both sides of the double bond. Naturally, the difference of 12 a.m.u. between the ions m/z 196 and 208 totally confirmed this fact. Therefore, from the MS and chromatographic behaviour observed, we suggest the structure of ai-20–29:1 to be a novel one, never described before.

In conclusion, we would like to mention that a simple calculation could be carried out, showing that SPE is much cheaper than RP-HPLC. The price of one cartridge is about US$ 3, whereas that of a C_{18} semipreparative column is about US$ 800. In these calculations we do not include the prices of instruments, but only the customer products.

REFERENCES

1 K. Takayama, N. Quereshi and H.K. Schnoes, *Lipids*, 13 (1975) 575.
2 T. Řezanka, J. Cudlín and M. Podojil, *Folia Microbiol.*, 32 (1987) 149.
3 T. Řezanka, *Progr. Lipid Res.*, 28 (1989) 147.
4 A. Poulos, D.W. Johnson, K. Beckman, I.G. White and C. Easton, *Biochem. J.*, 248 (1987) 961.
5 A. Poulos, P. Sharp, D. Johnson and C. Easton, *Biochem. J.*, 253 (1988) 645.
6 K. Takayama, N. Quereshi, H.C. Jordi and H.K. Schnoes, in G.L. Hawk (Editor), *Biological Biomedical Applications of Liquid Chromatography*, Vol. 12, Marcel Dekker, New York, 1979, p. 375.
7 T. Řezanka, T. Koza, R. Kysilka, I. Víden and M. Wurst, *J. Chromatogr.*, 472 (1989) 290.
8 N. Quereshi, K. Takayama and H.K. Schnoes, *J. Biol. Chem.*, 255 (1980) 182.
9 T. Řezanka and M. Podojil, *Lipids*, 19 (1984) 472.
10 T. Řezanka and M.Yu. Sokolov, *J. Chromatogr.*, 508 (1990) 275.
11 T. Řezanka, I.V. Zlatkin, I. Víden, O.I. Slabova and D.I. Nikitin, *J. Chromator.*, 558 (1991) 215.
12 T. Řezanka and P. Mareš, *J. Chromatogr.*, 409 (1987) 390.
13 T. Řezanka, M.Yu. Sokolov and I. Víden, *FEMS Microbiol. Ecol.*, 73 (1990) 231.
14 Q.T. Yu, B.N. Liu, J.Y. Zhang and Z.H. Huang, *Lipids*, 23 (1988) 804.
15 P.R. Brown, J.M. Beebe and J. Turcotte, *Anal. Chem.*, 21 (1989) 193.
16 Q.T. Yu, B.N. Liu, J.Y. Zhang and Z.H. Huang, *Lipids*, 24 (1989) 79.

Journal of Chromatography, 636 (1993) 255–261
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 900

Glass fibre prefilter–XAD-2 sampling and gas chromatographic determination of airborne chlorophenols

Helena Kontsas and Christina Rosenberg*
Institute of Occupational Health, Topeliuksenkatu 41 a A, SF-00250 Helsinki (Finland)

Paavo Jäppinen
Enso-Gutzeit Oy, SF-55800 Imatra (Finland)

Marja-Liisa Riekkola
Department of Analytical Chemistry, University of Helsinki, Vuorikatu 20, SF-00100 Helsinki (Finland)

(First received November 24th, 1992; revised manuscript received January 22nd, 1993)

ABSTRACT

A procedure for air sampling and analysis of polychlorinated phenols was developed and used for field measurements at two pulp-bleaching plants. The sampler consisted of an XAD-2 resin tube with a glass fibre prefilter. The filter and the resin were separately eluted with toluene, followed by acetylation of the chlorophenols with a mixture (5:2) of acetic acid anhydride and pyridine and determination by capillary gas chromatography using electron-capture and mass-selective detection. The sampler was tested in the $\mu g/m^3$ range for desorption and collection efficiency. The collection efficiency of the sampling device was not affected by humidity (40–80%) or sampling rate (200–750 ml/min) for the tested compounds. Stability tests at +20, +4 and −20°C showed no degradation of the chlorophenols studied during 4 weeks. The detection limit was below the $\mu g/m^3$ range for 180-l air samples.

INTRODUCTION

Chlorophenols have been found in spent bleaching liquors and in waters and water organisms downstream of pulp-bleaching plants [1,2]. During the production of pulp, lignin is broken down into a variety of monomeric phenols, which are chlorinated in the bleaching process [3]. The total amount of chlorinated phenolics produced in the course of chlorine bleaching depends on the pH, temperature and the amount of chlorine and chlorine dioxide used in the process [4]. In all, over 250 chlorinated compounds have been identified in effluents from pulp mills [5].

Chlorophenols show moderate bioaccumulation, and their toxicity increases with increasing degree of chlorination. In humans, exposure to chlorophenols can cause nausea, headache and irritation of the eyes and respiratory tract [6].

A number of different sampling procedures have been used for airborne chlorophenols, including freezing and condensing by drawing air

* Corresponding author.

0021-9673/93/$06.00

through cooling traps [7], absorption in liquids and adsorption on silica gel or porous polymers [8–13]. The compounds are then desorbed with an appropriate organic solvent or by thermal desorption and determined using gas or liquid chromatography.

Chlorophenols can be analysed directly [14] or as derivatives. At low concentrations, however, direct chromatographic separation is hindered by adsorption problems characteristic of the analysis of polar compounds. To overcome such difficulties and, furthermore, to improve analytical sensitivity, *e.g.* to mono- and dichlorophenols [15], the compounds of interest are often converted into less polar derivatives prior to gas chromatographic analysis. The most common derivatization methods are alkylation, silylation and acylation. Of these, acylation produces considerably less interference than the other methods [16].

Although chlorophenols have been extensively studied in environmental matrices, little is known about the possible release of these compounds into the work environment, *e.g.* during bleaching of pulp. We therefore developed a simple sampling procedure and method of analysis for airborne di-, tri-, tetra- and pentachlorinated phenols. The systems were used for field measurements at a hardwood and a softwood bleaching plant using chlorine-containing bleaching agents. The chlorophenols monitored were 2,4- and 2,6-dichlorophenol (2,4-DCP and 2,6-DCP), 2,4,5- and 2,4,6-trichlorophenol (2,4,5-TCP and 2,4,6-TCP), 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP) and pentachlorophenol (PCP). These congeners were chosen on account of their occurrence in mill effluents [4,17–19].

EXPERIMENTAL

Apparatus

Gas chromatograph. A Hewlett-Packard (HP) 5890A gas chromatograph equipped with an electron-capture detector (^{63}Ni) operating at 350°C or an HP-5970A mass-selective detector with electron impact (EI) ionization (70 eV) was used. The temperatures of the transfer line and ion source were 250°C and 180°C, respectively. The emission current was 0.3 mA. The mass-selective detector was operated in the selective-ion monitoring (SIM) mode. The following ion pairs were monitored: dichlorophenols m/z 162/164, trichlorophenols m/z 196/198, tetrachlorophenol m/z 230/232 and pentachlorophenol m/z 266/268. A fused-silica capillary column (HP-5), 25 m × 0.32 mm I.D., coated with diphenyl–dimethyl (5:95) polysiloxane (0.17 μm film thickness) was used with electron-capture detection (ECD). An equivalent but 50-m-long column directly coupled to the ion source was used with mass-selective detection (MS). The column temperature programme, identical for both columns, was as follows: 0.5 min at 75°C, heating to 200°C at 7°C/min, 1 min at 200°C, heating to 250°C at 15°C/min, 1 min at 250°C. Helium was used as carrier gas at flow-rates of 2 ml/min (ECD) and 1.55 ml/min (MS).

An autoinjector was used to introduce 1 μl samples into the gas chromatograph. The injector was operated in splitless mode with an inlet temperature of 220°C and a splitless time of 0.5 min (ECD) or with an inlet temperature of 230°C and a splitless time of 0.75 min (MS).

Materials

Chemicals. All solvents were of analytical grade and purchased from Merck (Darmstadt, Germany), except methyl-*tert.*-butyl ether, which was HPLC grade and purchased from Rathburn (Walkerburn, UK). Borax, potassium hydrogenphosphate, potassium carbonate, sodium hydroxide, sodium sulphate and acetic acid anhydride were of analytical grade (Merck). Pyridine was obtained from Pierce (Rockford, IL, USA). XAD-2 resins (Servachrom XAD-2 cat. No. 42821, Serdolit AD-2 cat. No. 42821, Serdolit AD-2 cat. No. 42420) were purchased from Serva (Heidelberg, Germany), commercial XAD-2 tubes (cat. No. 226-30) from SKC (Eighty Four, PA, USA) and silica and C_{18} adsorbent tubes (Bond-Elut, 1 cm^3) from Analytichem (Harbor City, CA, USA). 2,4-DCP was obtained from Ega-Chemie (Steinheim, Germany) and 2,6-DCP, 2,4,5-, 2,4,6-TCP, 2,3,4,6-TeCP and PCP from Fluka (Buchs, Switzerland). Glass fibre filters (binderfree, 13 mm) were from Gelman (Ann Arbor, MI, USA), filter holders (cat. No. SX001300) from Millipore

(Molsheim, France) and silanized glass wool from Ohio Valley Speciality Chemicals (Marietta, OH, USA).

Acetylation reagent. The acetylation reagents were prepared immediately before use by mixing acetic acid anhydride and pyridine in the following ratios: 2:5, 5:2 and 1:1.

Buffer solutions. Solutions of 0.1 *M* Borax, 1 *M* potassium hydrogenphosphate (pH adjusted to 7 with sodium hydroxide) [20] and 0.1 *M* potassium carbonate were prepared in distilled water.

Purification of XAD resins. The XAD resins were subjected to intensive cleanup before use as follows: 100 g of resin were washed twice with 300 ml of distilled water and twice with 300 ml of methanol and extracted for 6 h with diethyl ether in a Soxhlet apparatus. The resin was dried at 60°C for 12 h and stored in a desiccator [21].

Procedure

Preparation of chlorophenol standard solutions. Stock solutions of each of the chlorophenols were prepared in hexane. From these solutions, working standard mixtures of four different concentration levels, 25, 50, 100 and 250 pg of each chlorophenol per μl, were prepared in each of the following solvents: ethyl acetate, hexane, toluene, dichloromethane and methyl-*tert.*-butyl ether. 2,3,6-TCP was used as an internal standard at a concentration of 46.5 pg/μl.

Acetylation reaction. Optimum reaction conditions were studied by adding 100 μl of acetylation reagent (2:5, 5:2 or 1:1) to 1 ml of standard in either ethyl acetate or hexane and agitating for 2 min. Buffer (2 ml) was added at the same time or after the acetylation reaction to remove excess acetylation reagent. Three buffers, borax, potassium hydrogenphosphate and potassium carbonate, were tested.

Regarding chlorophenols collected with commercial XAD-2 tubes, the acetylated and buffer-extracted organic phase was purified further by solid phase extraction in prepacked silica and reversed-phase (C_{18}) columns. Briefly, the silica column was wetted with 1 ml of hexane, after which the acetylated sample in 1 ml of hexane was added and the derivatives eluted with 1 ml of hexane. The total hexane fraction was recovered. The C_{18} column was wetted with 1 ml of hexane, the acetylated sample added in 1 ml of hexane and the derivatives eluted with 1 ml of ethyl acetate.

Sampling device. The sampling system consisted of a glass fibre filter mounted in a filter holder in front of a resin tube (70 × 4 mm I.D.) containing 150 mg of XAD-2. The resin was plugged with glass wool at both ends.

Desorption efficiency. The efficiency of desorption from the adsorbent was investigated using the phase equilibrium technique by applying known amounts of the chlorophenols to the resin. The resin (150 mg) was placed in a screw-capped test tube and 1 ml of standard solution containing 25, 100 or 250 pg/μl of each chlorophenol was added and allowed to stand for 1 h before treatment. The glass fibre filter and the commercial XAD-2 tube were tested in a similar way.

Collection efficiency. Collection efficiency and possible breakthrough were tested by evaporation of the chlorophenols to produce a test atmosphere. A standard solution (10 μl) in hexane containing 25 ng/μl of each chlorophenol was injected into glass wool packed in a glass tube (130 × 4 mm I.D.), which was then heated to 90°C. This tube, a sampling device and a back-up resin tube were connected in series and humidified air was drawn through the system for 4 h at 200, 500 or 750 ml/min. An air flow of known rate and relative humidity (40 or 80%) was obtained by humidifying the air in a bath of distilled water and diluting it with dry air in a mixing chamber (10 l) fitted with a relative humidity meter (Vaisala HMI 32, Helsinki, Finland).

Stability tests. For stability testing, glass fibre filters and XAD-2 tubes were spiked with 10 μl of a standard solution containing 25 ng/μl of each chlorophenol. The tubes were stoppered, placed in plastic bags and stored at +20, +4 or −20°C for 1, 2 or 4 weeks before analysis.

Field sampling and sample treatment. Sampling of workplace air was carried out at one hardwood and one softwood bleaching plant using chlorine-containing bleaching agents. Air was sampled for 4 h at a flow-rate of 750 ml/min.

Samples were collected at different bleaching stages near the washing drums at a height of 1.5 m from floor level. After sampling, the sampling devices were stoppered and placed in a plastic bag. In the laboratory, the samples were stored in a freezer (−20°C) until further treatment.

For analysis, the glass fibre filter and the resin plug were transferred to separate test tubes. Toluene (1 ml) containing the internal standard was added to each test tube, and the tubes were allowed to stand for 1 h. The solvent was then transferred to another test tube, mixed with 100 μl of the acetylation reagent (5:2) and agitated for 2 min. The mixture was allowed to stand for 10 min, after which 2 ml of 0.1 *M* potassium carbonate was added and the tube shaken for 2 min. After separation of the phases, the organic layer was transferred to another test tube and dried with sodium sulphate. The extract was then transferred to autosampler bottles for gas chromatography.

RESULTS AND DISCUSSION

Acetylation reaction

According to Ballesteros *et al.* [22], the optimum temperature for acetylation is over 40°C. Pekari *et al.* [23] determined 2,4,6-TCP from urine, using acetylation without heat treatment. They extracted the urine with hexane and shook the organic phase with borax buffer and acetylation reagent (acetic acid anhydride and pyridine, 2:5) for 5 min. In the present study, however, simultaneous buffer extraction and acetylation resulted in an incomplete reaction. We initially attempted acetylation with ethyl acetate or hexane as the solvent and borax as the extraction buffer. It was difficult to achieve reproducible results with ethyl acetate, because the water solubility of this solvent hampers its quantitative removal from the buffer solution. Hexane did not exhibit these problems and thus was used. In the following experiments, acetylation reagent containing two parts acetic acid anhydride and five parts pyridine was added simultaneously with borax buffer to the standard solution in hexane, and the mixture was agitated for 2 min. According to the analysis, 16–35% of the TCPs and 20% of the 2,3,4,6-TeCP remained unreacted, whereas the DCPs and the PCP were almost completely derivatized. The reagent blank showed an interference peak that co-eluted with the acetylated PCP. Hereafter 2,4,6-TCP was used as a test compound to study the acetylation reaction.

Changing the acetylation reagent to five parts acetic acid anhydride and two parts pyridine improved the reaction, but it was still incomplete (Table I). Increasing the reaction time from 2 to 10 or 20 min did not improve the result, nor did the use of a 1:1 reagent mixture. Acetic acid anhydride alone yielded over 90% derivatization, but at the same time there was a 16-fold increase in the interference peak co-migrating with PCP. When the buffer solution was added 2 min after the 2:5 acetylation reagent, almost complete derivatization was achieved. The 5:2 reagent gave a quantitative acetylation reaction, and the interference peak was clearly diminished. To establish optimum derivatization conditions, further experiments were done with potassium carbonate or potassium hydrogenphosphate as the extraction buffer. The use of these buffers produced reagent blank chromatograms almost devoid of interfering peaks, potassium carbonate being superior in this respect.

Desorption efficiency

The results of the desorption efficiency tests are presented in Table II. Initially, a batch of

TABLE I

FORMATION (%) OF AN ACETYL DERIVATIVE FROM 2,4,6-TRICHLOROPHENOL (25 pg/μl) DEPENDING ON ACETYLATION REAGENT COMPOSITION AND TIMING OF BORAX BUFFER EXTRACTION

Acetic acid anhydride: pyridine/time of buffer extraction	Acetylated form (%)	Unreacted form (%)
2:5/simultaneously	55	45
5:2/simultaneously	87	13
1:1/simultaneously	77	23
Acetic acid anhydride/ simultaneously	91	9
2:5/after acetylation	98	2
5:2/simultaneously	100	0

TABLE II

DESORPTION EFFICIENCY (%) OF CHLOROPHENOLS FROM XAD-2 RESIN AND GLASS FIBRE FILTER[a]

AG = Analytical grade; RG = research grade; MTBE = methyl-*tert.*-butyl ether

Chlorophenol	Servachrom XAD-2 (AG)/ECD, hexane[b]	Serdolit AD-2 (RG)/ECD, hexane[c]	Serdolit AD-2 (AG)/ECD		Serdolit AD-2 (AG)/MS, toluene[f]
			Hexane[d]	MTBE[e]	
2,4-DCP	81–86	14–37	36–46	116	87–97
2,6-DCP	80–89	26–60	49–67	115	97–110
2,4,5-TCP	84–104	9–10	14–32	101	78–97
2,4,6-TCP	93–100	12–26	36–57	100	98–113
2,3,4,6-TeCP	102–107	4	3–14	62	73–103
PCP	98–100	2–4	4–13	28	40–96 50–82[g]

[a] The chlorophenols were added at three levels (25, 100 and 250 ng of each chlorophenol) to 150 mg of resin, extracted with an organic solvent, acetylated with 5:2 acetylation reagent, purified with potassium carbonate buffer and determined by GC–ECD or GC–MSD. The values represent means of a minimum of two measurements at each level, with the exception of [c] and [e], where only two levels, 25 and 250 ng, and one level, 250 ng, respectively, were used.
[b] $n = 22$.
[c] $n = 4$.
[d] $n = 12$.
[e] $n = 1$.
[f] $n = 6$.
[g] Glass fibre prefilter.

analytical-grade resin, XAD-2 (Servachrom) dating from 1984, was used with hexane as the desorption solvent, resulting in excellent recovery (80–107%). Later, a new batch of analytical-grade resin purchased in 1990, AD-2 (Serdolit), yielded poor recoveries, especially of TeCP and PCP (3–14%). A new batch of research-grade resin, AD-2 (Serdolit), having the same catalogue number as the initial analytical-grade XAD-2 exhibited still poorer recovery (2–4%) with hexane. The reason for these inadequate recoveries could not be found. Commercial XAD-2 tubes were also used in desorption efficiency tests. These tubes produced immense amounts of impurities when desorbed with hexane. The impurities could not be removed even with an additional cleanup step by solid phase extraction with either silica or C_{18} columns.

Dichloromethane was examined as an alternative solvent to desorb the chlorophenols. Satisfactory recoveries were achieved with both new XAD-2 resins. However, this solvent caused severe degradation of the chromatographic column after only a few injections. This was avoided by replacing dichloromethane with hexane after the desorption step. As a drawback, there was a 20–30% loss of DCPs and TCPs and a 10% loss of TeCP. PCP was not affected when dichloromethane was evaporated with a gentle stream of nitrogen.

The use of methyl-*tert.*-butyl ether for desorption improved the recovery of DCP, TCP and TeCP (62–116%) from the analytical-grade Serdolit AD-2 resin, but the recovery of PCP was still low (28%, Table II). Good desorption of DCP, TCP and TeCP from the resin (73–113%) and PCP from the glass fibre filter (50–82%) was achieved with toluene, which was thus chosen as the desorption solvent.

Collection efficiency

Collection efficiency studies using only the adsorbent tube showed that PCP was not retained on the XAD-2 resin, even at air flow-rates as low as 200 ml/min. In subsequent experiments with a glass fibre prefilter, PCP was recovered from the prefilter. These results contradict previ-

ously reported studies [13] in which the XAD-2 resin alone was used for sampling of TCP, TeCP and PCP.

No breakthrough of DCP, TCP and TeCP was observed with the XAD-2 tube tested at sampling flow-rates ranging from 200 to 750 ml/min for 4 h in either 40% or 80% relative humidity test atmospheres. The collection efficiency in the 40% test atmosphere was 55–78% for DCP, 53–79% for TCP and 47–52% for TeCP ($n = 5$). The corresponding values for the 80% test atmosphere were 58–96%, 55–86% and 40–69% ($n = 5$). The collection efficiency of PCP with the glass fibre filter ranged from 88 to 108% and from 47 to 96% when the relative humidity was 40% ($n = 5$) and 80% ($n = 5$), respectively.

Stability tests

Storage of spiked resins (DCP, TCP and TeCP) and glass fibre filters (PCP) caused no degradation of the chlorophenols during 4 weeks at +20, +4 or −20°C.

Gas chromatography

The sensitivity of ECD for halogenated compounds makes its use rational for trace analysis of chlorophenols. Nevertheless, the inherent selectivity offered by the SIM technique renders MS more attractive. The fragmentation pattern and the ratio of the ions chosen add a second dimension to the identity of the compounds, in addition to retention time. In the EI fragmentation process, the acetyl group is lost from the acetylated chlorophenol, followed by hydrogen rearrangement. This corresponds to the loss of m/z 42 typical for acetates [24]. Monitoring of the resulting intense fragments, $M^+ = M_{deriv} - CH_2{=}C{=}O$, yielded characteristic ion clustering caused by the chlorine isotopes. The ions used were DCP m/z 162/164, TCP m/z 196/198, TeCP m/z 230/232 and PCP m/z 266/268. The relative abundances were 100/66, 100/99, 75/100 and 100/67, respectively. The ions represent $M^+/M^+ + 2$, for DCP, TCP and TeCP, and $M^+ + 2/M^+ + 4$ for PCP.

The instrumental limits of detection for the ECD and MSD were calculated by dividing the concentration of the lowest chlorophenol standard used in recovery studies by the signal-to-noise ratio and multiplying the quotient by 3, which was used as a safety factor. The instrumental detection limits were similar for the two instruments (Table III). Limits of determination calculated for 180-l air samples for GC–ECD and GC–MSD are presented in Table III.

TABLE III

INSTRUMENTAL LIMIT OF DETECTION AND LIMIT OF DETERMINATION OF THE CHLOROPHENOLS BY GC–ECD AND GC–MSD

Chlorophenol	Instrumental limit of detection (pg/μl)		Limit of determination (ng/m³)[a]	
	ECD	MS	ECD	MS
2,4-DCP	6.8	3.6	38	20
2,6-DCP	13.0	4.1	72	23
2,4,5-TCP	2.2	1.6	12	9
2,4,6-TCP	1.5	1.6	8	9
2,3,4,6-TeCP	2.0	3.4	11	19
PCP	1.6	3.1	9	17

[a] Calculated for a 180-l air sample.

Field sampling

Over 50 air samples were collected at the bleaching plants. At the softwood bleaching plant, use of chlorine and chlorine dioxide in a ratio of 1:1 at the first chlorination stage resulted in PCP being found in 20% of the samples. The concentrations ranged from 17 to 40 ng/m³. No other chlorophenols were found. When chlorine dioxide alone was used in the bleaching, no PCP was detected. The hardwood bleaching plant only utilized chlorine dioxide at the first chlorination stage. Here PCP was found in only 10% of the samples, the concentrations ranging from 40 to 115 ng/m³. The better selectivity of the MS-SIM technique compared with ECD was evident in the analysis of the field samples, with the latter yielding false-positive results for DCPs, TCPs and TeCP in several samples.

The formation of chlorophenols during the bleaching of pulp has been found to be dependent on various factors, especially on the chlorination agents used. In a Swedish survey [25], airborne 2,3,4,6-TeCP and PCP were detected at

a softwood bleaching plant using only chlorine at the first chlorination stage. Traces of 2,4,6-TCP were observed when a 1:1 mixture of chlorine and chlorine dioxide was used. Both 2,4,6-TCP and 2,3,4,6-TeCP were also detected at a hardwood bleaching plant using a 1:1 mixture. The concentrations reported in the Swedish study were of the same order of magnitude as in the present study.

CONCLUSIONS

A sampling device, consisting of an XAD-2 resin tube with a glass fibre prefilter, allowing simultaneous sampling of airborne di-, tri-, tetra- and pentachlorophenols was developed. Gas chromatographic determination of acetylated chlorophenols by MS with the selective-ion monitoring technique is similar in sensitivity but superior in selectivity to ECD.

ACKNOWLEDGEMENTS

We wish to thank Ms. Ritva Wirmoila for skilful technical assistance. Financial support from the Finnish Work Environment Fund is gratefully acknowledged.

REFERENCES

1 K. Lindström and J. Nordin, *J. Chromatogr.*, 128 (1976) 13.
2 J. Paasivirta, *Kemia-Kemi*, 17 (1990) 34.
3 A.B. McKague, *J. Chromatogr.*, 208 (1981) 287.
4 H.-B. Lee, R.L. Hong-You and P.J.A. Fowlie, *J. Assoc. Off. Anal. Chem.*, 72 (1989) 979.
5 L.R. Suntio, W.Y. Shiu and D. Mackay, *Chemosphere*, 17 (1988) 1249.
6 WHO, *Environmental Health Criteria*, 93 (1989).
7 I. Gebefügi, H. Parlar and F. Korte, *Ecotoxicol. Environ. Safety*, 3 (1979) 269.
8 A. Dahms and W. Metzner, *Holz. Roh-Werkstoff*, 17 (1979) 331.
9 G.D. Kleinman, S.W. Horstman, D.A. Kalman, J. McKenzie and D. Stanzel, *Am. Ind. Hyg. Assoc. J.*, 47 (1986) 731.
10 T.P. Kauppinen and L. Lindroos, *Am. Ind. Hyg. Assoc. J.*, 46 (1985) 34.
11 A.S. Todd and C.Y. Timbie, *DHHS (NIOSH) Publ. No. 83-106*, 1983.
12 J.S. Warren, L.L. Lamparski, R.L. Johnson and R.M. Gooch, *Bull. Environ. Contam. Toxicol.*, 29 (1982) 719.
13 J.-O. Levin, C.-A. Nilsson, M. Burström and L. Carleborg, *Natl. Board of Occup. Safety and Health Report*, 24, 1978.
14 B.G. Reigner, J.F. Rigod and T.N. Tozer, *J. Chromatogr.*, 533 (1990) 111.
15 J. Hajšlová, V. Kocourek, I. Zemanová, F. Pudil and J. Davídek, *J. Chromatogr.*, 439 (1988) 307.
16 L. Renberg, *Natl. Swedish Environ. Protection Board Report*, 1410 (1981).
17 B. Starck, P.O. Bethge, M. Gerlov and E. Tälka, *Pap. puu-papp. trä*, (1985) 745.
18 J. Paasivirta, K. Heinola, T. Humppi, A. Karjalainen, J. Knuutinen, K. Mäntykoski, R. Paukku, T. Piilola, K. Surma-Aho, J. Tarhanen, L. Welling and H. Vihonen, *Chemosphere*, 14 (1985) 469.
19 A.J. Sequeira and L.T. Taylor, *J. Chromatogr. Sci.*, 29 (1991) 351.
20 G. Scarping, C. Sangö and B.E.F. Smith, *J. Chromatogr.*, 208 (1981) 313.
21 K. Andersson, C. Hallgren, J.-O. Levin and C.A. Nilsson, *Anal. Chem.*, 56 (1984) 1730.
22 E. Ballesteros, M. Gallego and M. Valcárcel, *J. Chromatogr.*, 518 (1990) 59.
23 K. Pekari, C. Boudène and A. Aitio, *Arch. Toxicol.*, 59 (1986) 41.
24 K. Blau and G.S. King (Editors), *Handbook of Derivatives for Chromatography*, Heyden, Bristol, UK, 1977, p. 144.
25 A.-B. Antonsson, R. Bjurström, L. Rondahl and U. Rydevik, *Inst. för Vatten- och Luftvädsforskning Rapport, Swedish Environ. Res. Inst., ILV-Publ. B 966*, Stockholm, 1989.

Journal of Chromatography, 636 (1993) 263–270
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 893

Determination of phenolics from sediments of pulp mill origin by *in situ* supercritical carbon dioxide extraction and derivatization

Hing-Biu Lee* and Thomas E. Peart

Research and Applications Branch, National Water Research Institute, Environment Canada, 867 Lakeshore Road, Burlington, Ontario L7R 4A6 (Canada)

Robert L. Hong-You

Wastewater Technology Centre Laboratories, Burlington, Ontario L7R 4L7 (Canada)

(First received November 10th, 1992; revised manuscript received December 31st, 1992)

ABSTRACT

A method for the determination of extractable chlorinated phenolics in sediments collected downstream of chlorine-bleaching mills was developed by using a single-step *in situ* derivatization technique in conjunction with supercritical fluid extraction (SFE). Phenolics in air dried samples were extracted with carbon dioxide and simultaneously acetylated under static SFE conditions by acetic anhydride in the presence of triethylamine. The derivatives were then removed from the matrix in the dynamic extraction stage. Within an extraction chamber temperature range from 40 to 120°C, the best overall recovery for the phenolics was obtained at 110°C. A carbon dioxide density of 0.71 g/ml (pressure 37 MPa) was used for the extraction–derivatization experiments since lower CO_2 densities adversely affected the recovery of the catechols. Two extractions of the same sample were necessary for the quantitative recovery of extractable phenolics in weathered sediments. For sample size of 1 g, 120 μl of acetic anhydride and 30 μl of triethylamine were found to produce the optimal results. While the results obtained by this SFE–derivatization method were comparable to conventional technique such as Soxhlet extraction, the SFE approach required no solvent in the extraction steps and was extremely time-efficient (*ca.* 35 min).

INTRODUCTION

Of all the pulp and paper mills operating in Canada, 47 of them use chlorine for bleaching either entirely or in at least one of the multiple bleaching steps. In a 1991 report jointly published by Environment Canada and Health and Welfare Canada [1], it was estimated that Canadian mills used over $610 \cdot 10^6$ kg of chlorine annually to produce over $10 \cdot 10^9$ kg of bleached pulp and released over 10^9 kg of chlorinated organics to the aquatic environment. Hundreds of compounds were found in the final effluents of the bleached kraft mills, including the chlorinated dibenzofurans and dibenzo-*p*-dioxins, phenolics, resin and fatty acids, and a variety of low-molecular-mass aliphatic compounds [2,3]. Recent studies carried out by the Pulp and Paper Research Institute of Canada indicated that the undesirable production of the highly toxic furans and dioxins can be greatly minimized by the elimination of the non-chlorinated dibenzo-*p*-dioxin and dibenzofuran in defoamers used in chlorine bleaching mills [4]. Chlorinated phenolics such as catechols, guaiacols, vanillins and syringols in the bleachery effluents are derived from the degradation of lignin during the

* Corresponding author.

0021-9673/93/$06.00

bleaching process. Although substituting chlorine dioxide for chlorine in the bleaching steps reduces the formation of the total chlorinated phenolics [5], complete elimination of these compounds would require the use of non-chlorine bleaching techniques. Installation of secondary (biological) waste treatment facilities by the pulp mills also removes many toxic substances including the phenolics from the effluents before they are discharged into the receiving waters.

Many chlorinated phenolics are acutely toxic to fish and their 96-h LC_{50} values (concentration which kills 50% of a test population over a 96-h exposure) range from 0.3 to 3 mg/l [6]. The octanol–water partition coefficients (K_{ow}) of chlorinated guaiacols and catechols are similar to those of chlorophenols with the same level of chlorine substitution [7], thus, accumulation of the toxic phenolics in the sediments is predicted and has actually been observed [8,9]. Therefore, there is a need to monitor the level of phenolic contamination in sediments created by the bleaching process from the paper mills.

Different approaches to the extraction of phenolics from sediments have been used [10]. Nearly all of them are either time-consuming or use a lot of solvent or both. We have previously developed a method for the extraction of resin and fatty acids from sediments collected downstream of pulp mills using supercritical carbon dioxide [11]. This supercritical fluid extraction (SFE) method not only provided recovery of the acids equal to or better than the Soxhlet technique, but was also extremely time-efficient and used practically no solvent. Recently, a technique involving the *in situ* extraction and chemical derivatization of polar compounds under SFE conditions has been demonstrated. Some successful examples include silyation of acidic components in coffee beans, tea and marine sediments [12], methylation of herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and dicamba in soil [13], methylation of fatty acids from whole cells of *Escherichia coli* [13], methylation and trifluoroethylation of phenols in coal gasification wastewater and wood soot leachate [13], pentafluorobenzylation of resin and fatty acid in river sediments downstream of pulp mills [11], as well as acetylation of chlorophenols in soil from a wood treatment plant [14]. This approach further reduced sample preparation time and simultaneously enhanced the extractability of polar organic compounds. In this paper, we shall describe an efficient SFE method for the determination of extractable chlorinated phenolics commonly found in sediments downstream of chlorobleaching mills.

EXPERIMENTAL

Reagents and chemicals

All chlorinated phenolics were obtained from Helix-Biotech Scientific (Vancouver, Canada) and used without further purification. These included 4,5- and 4,6-dichloroguaiacols (45G and 46G), 3,4,5- and 4,5,6-trichloroguaiacols (345G and 456G), 3,4,5,6-tetrachloroguaiacol (3456G), 3,5- and 4,5-dichlorocatechols (35C and 45C), 3,4,5-trichlorocatechol (345C), 3,4,5,6-tetrachlorocatechol (3456C), 6-chlorovanillin (6V), 5,6-dichlorovanillin (56V), and 3,4,5-trichlorosyringol (345S). Stock solutions of each individual compound were prepared in acetone at 1000 μg/ml and kept at −20°C in crimped top vials. A mixture of the above 11 phenolics at 10 μg/ml was also prepared in acetone for spiking and preparation of the acetylated standards.

Triethylamine and acetic anhydride were purchased from Aldrich (Milwaukee, WI, USA). The anhydride was triple distilled before use. SFC-grade carbon dioxide without helium head pressure was obtained from Scott Specialty Gases (Troy, MI, USA) and Praxair (Oakville, Canada). Silica gel (GC grade 950, 60–200 mesh, Fisher Scientific) was activated overnight at 200°C and the 5% deactivated silica gel was prepared by adding 5 ml of water to 95 g of the activated adsorbent.

Grab sediment samples were collected downstream of several Ontario pulp mills using chlorine bleaching. These samples were air dried at room temperature, crushed, ground and sieved through a 60-mesh screen before they were used in the extraction experiments.

SFE of sediment samples

All supercritical fluid extractions were carried

out with carbon dioxide using the Hewlett-Packard 7680A or 7680T extractor module. The two modules have similar capabilities except that, in the case of the 7680T, a series of up to eight thimbles can be prepared and loaded into the extractor for unattended sequential extraction. Prior to the extraction, two layers of Whatman GFC filter paper cut to internal diameter of the extraction thimble were placed at the bottom of the thimble before it was filled with 200 mg of Celite. The filter paper and Celite kept the sediment fines from plugging the fritted thimble cap and also prevented the modifier from leaking out of the thimble. The thimble was then filled with 1 g of sediment, followed by spiking 30 μl of triethylamine to the sample. The thimble contents were mixed for 30 s on a vortex mixer before the addition of another 200 mg of Celite. The derivatization reagent, 120 μl of acetic anhydride, was added to the top Celite layer. The thimble was then mixed again for 30 s. In a typical extraction, the extractor was set at a temperature of 110°C and a constant pressure of 37 MPa. Sample extraction and derivatization were first performed in the static mode for 10 min, followed by a 5-min dynamic extraction with a flow-rate of 2 ml/min to remove the analytes. During the dynamic extraction stage, the acetylated phenolics were collected on a built-in octadecylsilane (ODS) trap connected to a variable diameter restrictor nozzle which was responsible for the depressurization of supercritical carbon dioxide. The trap temperature was set at 15°C for the extraction stages and 40°C during the rising stage. Finally, the derivatized extract was removed from the trap by two 1-ml rinses of dichloromethane.

Column cleanup

The above dichloromethane rinses were combined and solvent exchanged into 1 ml of isooctane. The extract was then cleaned up on a 5-cm 5% deactivated silica gel column prepared with a 23-cm Pasteur pipet. After the extract was applied, the column was eluted with 5 ml of 5% dichloromethane in light petroleum (b.p. 30–60°C) and the eluate was discarded. The acetyl derivatives of the phenolics were eluted from the column by 10 ml of 1% methanol in dichloromethane. This fraction was subsequently solvent exchanged into 1 ml of isooctane for final analysis.

Chromatographic analysis

Gas chromatographic analysis was performed with both electron-capture detection (ECD) and mass-selective detection (MS). ECD was used for the routine analysis of sediment extracts for all phenolics and MS was used for the confirmation of peak identity. Splitless injection (1 μl) was made by a HP7673 autosampler onto a 25 m × 0.2 mm I.D. HP-5 fused silica column. The initial oven temperature was 70°C (0.75 min hold) and it was programmed to 120°C at 30°C/min and then to 180°C at 2°C/min. Splitless time was 0.75 min. Constant carrier (hydrogen) flow at 1.5 ml/min was maintained by an electronic pressure controller. In the case of MS analysis, selected ion monitoring (SIM) of the characteristic $[M-42]^+$ and $[M-42-15]^+$ ions for each compound was performed [15].

A mixture of the acetyl derivatives was prepared by an aqueous acetylation of known amounts of the phenolics [15] and appropriate dilutions of this mixture were used as external standards for the quantitation of the samples.

RESULTS AND DISCUSSION

Conventional extraction of phenolics from sediments

Organics from sediments are usually extracted by a solvent or a mixture or solvents at an elevated temperature (*e.g.* the Soxhlet procedure) or at ambient temperature (*e.g.* by an ultrasonic or high-speed mixing technique). In many cases, acidic compounds are better recovered from the sediment if a strong acid is present with the solvent system. However, in the cases of sediments with high contents of humic substances such as those samples collected from pulp and paper mills, extraction under acidic conditions produces a large amount of coextractives which may precipitate when the solvent is being evaporated. The precipitate not only changes the homogeneity of the extract if it is to be subsampled but can also adversely affect the derivatization reaction which is often required

for the gas chromatographic analysis of the acidic compounds.

Another approach that has been applied to the determination of pentachlorophenol (PCP) in sediment was steam distillation [16]. In our work, we found that some free phenols such as the less chlorinated catechols could not be fully recovered by this technique, presumably due to their higher water solubilities than other chlorophenols. We have also attempted to acetylate the phenolics in the sediment suspended in a potassium carbonate slurry and subsequently steam distilled the acetyl derivatives from the mixture. This method worked well with all chlorinated phenols, guaiacols and syringols but did not work with the chlorinated vanillins and catechols. The latter compounds were not recovered since their acetyl derivatives were completely decomposed during the steam distillation stage. Thus, before the advent of the SFE technique, solvent extraction was the only way to recover all the phenolics from a sediment sample.

TABLE I

% RECOVERY OF CHLORINATED PHENOLICS FROM SPIKED SEDIMENT SAMPLES USING THE *IN SITU* EXTRACTION AND DERIVATIZATION TECHNIQUE

All extractions were done at 80°C and 37 MPa with 1-g samples.

Spiking level (ng/g)	500	500	500	50
Amount of Et_3N (μl)	30	30	30	30
Amount of Ac_2O (μl)	30	30	120	120
Static time (min)	5	10	10	10
Dynamic time (min)	5	5	5	5
No. of replicates (n)	3	3	6	6
Recovery (%)				
45G	80	89	97 ± 5	94 ± 7
45C	67	92	92 ± 4	93 ± 6
345G	78	95	100 ± 7	98 ± 4
56V	54	81	98 ± 5	89 ± 6
345C	50	89	96 ± 8	92 ± 6
3456G	56	87	89 ± 4	96 ± 5
345S	73	90	91 ± 5	87 ± 6
3456C	16	44	84 ± 8	92 ± 7

Development of a SFE method for chlorinated phenolics in sediment

In the beginning, we were using the *in situ* extraction and derivatization method developed for the determination of PCP and other chlorophenols [14]. Using sediment spiked at 500 ng/g of the phenolics, a 1-g aliquot was extracted for 5 min statically and then dynamically with 37 MPa supercritical carbon dioxide at a temperature of 80°C in the presence of 30 μl each of triethylamine and acetic anhydride. Although the above *in situ* derivatization condition was also feasible for the extraction of the catechols and guaiacols from sediment samples, the results (column 2, Table I) indicated the recovery of the phenolics was far from complete, particularly for 3456C. An increase in static extraction time from five to 10 min produced a significant improvement on the recovery of all compounds (column 3, Table I), yet longer dynamic extraction did not help since the derivatization occurred during the static extraction stage. While chlorophenols and chloroguaiacols were easily converted into their acetyl derivatives under SFE conditions, our previous work on the aqueous acetylation of phenolics indicated that complete derivatization of the chlorocatechols required an excess of acetic anhydride [15]. This principle again applied to our present work, since an increase of the amount of anhydride used from 30 to 120 μl produced a recovery better than 85% for each phenolic compound from spiked sediment samples using the SFE technique (columns 4 and 5, Table I).

Once we had a method that worked reasonably well with spiked samples, the next phase of development was to optimize this procedure by applying it to naturally contaminated samples. In the following work, a bulk sediment collected approximately two km downstream of a bleached kraft mill was used as a reference sample. Analysis of effluent samples collected in the same area indicated the site was contaminated by resin and fatty acids as well as the chlorinated phenolics. By following the procedure developed for the spiked samples, all the common phenolics were detected in this reference sample. However, we were also able to recover an additional 30% or more of these phenolics from a second extraction of the same sample, indicating that the extraction–derivatization conditions were still not optimized for natural samples.

Factors affecting the SFE recovery of phenolics

Among the many factors that can affect the SFE results, the effect of extraction chamber temperature was the first one to be studied. The temperature dependence on the recovery of six major phenolic components in the reference sample, namely, 45G, 45C, 345G, 56V, 345C, 3456G and 3456C, was examined in 10°C increments from 40 to 120°C. In these experiments, 1-g aliquots of the sample were extracted for 10 minutes in the static mode and for a further 5 min in the dynamic mode at 37 MPa using 30 μl of triethylamine and 120 μl of acetic anhydride for the acetylation reaction. In order to have an easy comparison of the results, percent recovery obtained at various temperatures relative to that at 110°C was calculated for each compound. At an extraction chamber temperature of 40°C, less than 15% of the phenolics were extracted from the sediment and acetylated. Although the recovery of the catechols was vastly improved when the extraction and derivatization was carried out at 60°C, the guaiacols and 56V were still poorly recovered ($\leq$40%) at this temperature. Continuous increase in recovery for all phenolics were observed when the extraction chamber temperature was increased to 100°C, where the recovery of catechols reached a maximum. While the recovery of the catechols began to drop at higher temperatures, highest recoveries for 56V and the guaiacols were obtained at 120°C. We were not able to study the recovery of these phenolics at even higher temperatures since 120°C is the maximum extraction chamber temperature that our extractor can reach. Since the optimal recovery of different phenolics were obtained at different temperatures, 110°C was chosen for the extraction and derivatization of sediments since it gave the best overall recovery of all compounds. A graphical summary of the temperature effect on the recovery of phenolics is depicted in Fig. 1.

The recovery of the chlorinated phenolics was also studied at four different extraction fluid densities, namely, 0.71, 0.64, 0.55, and 0.50 g/ml. No difference in the phenolics results was observed at the two highest fluid densities, suggesting that a further increase in density (or carbon dioxide pressure) would not result in better extraction efficiency. Although the chlorinated guaiacols and vanillins did not seem to be affected, the recovery of the catechols, particularly 3456C, dropped substantially at fluid densities of 0.55 and 0.50 g/ml and thus extraction with the lower-density fluid is not recommended. Extraction times of 10 (static) and 5 min (dynamic) were always used since shorter static time caused a reduction in the recovery while longer static and dynamic extractions did not improve the yield for the reference sample.

The amount of reagents used and the presence of solvents can also affect the derivatization and

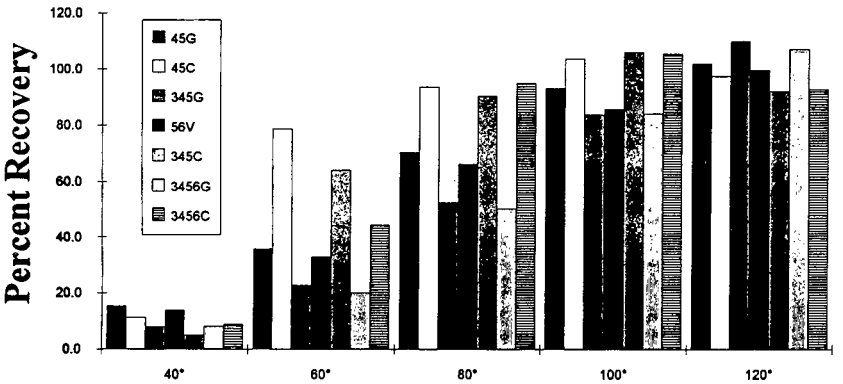


Fig. 1. SFE recovery of chlorinated phenolics from sediment at various extraction temperatures. All data are relative to those obtained at 110°C.

the recovery of the phenolics too. For example, the recoveries of guaiacols and catechols were *ca.* 60 and 15%, respectively, lower if triethylamine was not used in the derivatization. However, there was no significant change in the results when 60 instead of 30 μl of the base was used and there was a slight decrease in recovery when 240 instead of 120 μl of the anhydride was employed. We were also unable to improve the recovery of phenolics by the addition of a modifier such as dichloromethane to the sample. Yet, it was noted that the presence of either methanol or water was detrimental to the derivatization of all phenolics. Less than 25 or 50% of the phenolics could be recovered if 250 μl of methanol or water, respectively, were added to the sample prior to extraction. This result is not unexpected since both methanol and water react with the anhydride causing a deficiency in the reagent for derivatization. Therefore, the *in situ* SFE–acetylation technique should not be applied to a wet sediment sample.

Using the above optimized extraction and derivatization conditions, we were able to recover *ca.* 80% of the extractable phenolics from a natural sediment sample in the first extraction. An additional 10 to 20% of the phenolics could be recovered if a second extraction of the sample at 110°C with fresh reagents was performed. A third extraction, however, recovered less than 5% of the derivatized products. Therefore, two extractions of the same sample are required for the quantitative recovery of the extractable chlorinated phenolics from sediments.

Method evaluation and application

For further evaluation of this *in situ* extraction and acetylation technique, results for the reference sediment (sample A) obtained by SFE were compared with those acquired by conventional techniques such as steam distillation and Soxhlet extraction with acidified acetone (Table II). As mentioned earlier, only chloroguaiacols were recovered by our modified steam distillation procedure since the derivatives of chlorinated vanillins and catechols decomposed under such conditions. It is obvious from Table II that the SFE results, obtained by a single extraction, were very similar to the steam distillation results for chloroguaiacols and were slightly higher than all of the Soxhlet results. In the absence of a certified sediment reference material for total (free and bound) chlorinated phenolics, we were unable to ascertain how close were the SFE

TABLE II

LEVELS OF CHLORINATED PHENOLICS IN SEDIMENT SAMPLES DETERMINED BY VARIOUS TECHNIQUES AND FROM DIFFERENT LOCATIONS

All SFE results were based on a single extraction at 110°C and 37 MPa.

Compound	Concentration (ng/g)					
	Sample A, extraction by steam distillation[a]	Sample A, Soxhlet extraction[a]	Sample A, SFE[b]	Sample B, SFE	Sample C, SFE	Sample D, SFE
45G	410	381	396 ± 41	717	284	822
6V	N.D.	65	83 ± 6	505	222	303
45C	N.D.	305	325 ± 28	342	133	428
345G	123	126	131 ± 10	297	82	2258
56V	N.D.	40	44 ± 5	111	45	83
345C	N.D.	1205	1364 ± 75	1264	209	1416
3456G	13	11	15 ± 2	65	27	1502
3456C	N.D.	666	688 ± 42	982	113	2796

[a] Mean of two determinations. N.D. = None detected.

[b] Mean of six determinations and the uncertainty is 1 standard deviation.

results to the total phenolic contents in naturally contaminated sediments. However, our findings already indicated that the SFE technique was at least capable of producing precise and quantitative results for the free or extractable phenolics commonly found in sediments downstream of bleached kraft mill. Contrary to the procedures involving methanolic KOH hydrolysis [10], the SFE technique employed here will not convert catechols into guaiacols and produce biased results.

This SFE method has been applied to the determination of chlorinated phenolics in sediment samples of pulp mill origin and some of the results are tabulated in Table II. In all cases, the SFE extracts were subject to a silica gel column cleanup for the removal of polar coextractives such as acids and pigments. Failure to do so would cause interference in the subsequent GC–ECD analysis as well as a shortening of the life of the analytical column. Samples B and C were obtained from sites approximately 2 and 5 km, respectively, downstream of a chlorine-bleaching mill. A GC–ECD chromatogram of the acetylated SFE extract for sample B is shown in Fig. 2. Sample D came from the sedimentation basin of another bleached kraft mill and thus it is not surprising to find that its phenolic levels are higher than those in the river sediments. The predominant phenolics in these samples are 45G, 345G, 3456G, 45C, 345C and 3456C and their presence is consistent with previous findings [8–10]. Based on a 1-g sample and a final volume of 1 ml, the detection limit for these phenolics is *ca.* 10 ng/g. ECD gives a linear response over a range from 10 to 1000 pg/μl for all 11 acetyl derivatives [15].

CONCLUSIONS

An *in situ* extraction and acteylation procedure has been optimized for the determination of the extractable chlorinated phenolics in sediment samples. For the best recovery of all compounds involved in this work, the sample should be air dried prior to supercritical carbon dioxide extraction at 37 MPa and a temperature of 110°C. For 1 g of sediment, 30 μl of triethylamine and 120

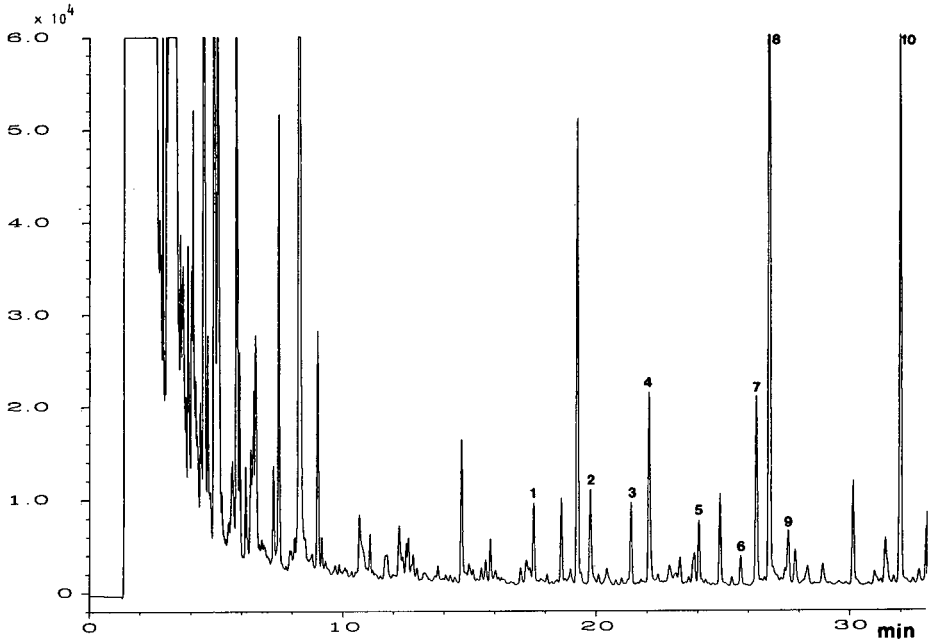


Fig. 2. A GC–ECD chromatogram of a SFE extract for a sediment (sample B) collected downstream of a chlorine bleaching mill. Peaks identified are acetyl derivatives of: 1 = 46G; 2 = 6V; 3 = 45C; 4 = 345G; 5 = 456G; 6 = 56V; 7 = PCP; 8 = 345C; 9 = 3456G; 10 = 3456C. y-Axis represents detector response.

μl of acetic anhydride were found to produce the best results for the acetylation of phenolics. A second extraction of the sample should be performed if quantitative recovery of the extractable phenolics in sediments is required. This procedure should not be applied to wet sediments since the presence of water in the sample is detrimental to the derivatization of the phenolics under SFE conditions.

ACKNOWLEDGEMENT

The authors are indebted to Hewlett-Packard (Canada) Ltd. for the generous loan of the HP 7680A and HP 7680T Supercritical Fluid Extraction Modules.

REFERENCES

1 *Priority Substances List Assessment Report No. 2, Effluents from Pulp Mills Using Bleaching*, Environment Canada and Health and Welfare Canada, Ottawa, 1991.
2 C.C. Walden and T.E. Howard, *Pulp Pap. Can.*, 82, No. 4 (1981) 115.
3 I. Rogers, H. Mahood, J. Servizi and R. Gordon, *Pulp Pap. Can.*, 80, No. 9 (1979) 94.
4 R.H. Voss, C.E. Luthe, B.I. Fleming, R.M. Berry and L.H. Allen, *Pulp Pap. Can.*, 89, No. 12 (1988) 151.
5 N. Liebergott, B. van Lierop, A. Nolin, M. Faubert and J. Laflamme, *Modifying the Bleaching Process to Decrease AOX Formation, Report*, Pulp and Paper Research Institute of Canada, Pointe Claire, 1989.
6 L.T.K. Chung, H.P. Meier and J.M. Leach, *Tappi*, 62, No. 12 (1979) 71.
7 T.-M. Xie, B. Hulthe and S. Folestad, *Chemosphere*, 13 (1984) 445.
8 T.-M. Xie, K. Abrahamsson, E. Fogelqvist and B. Josefsson, *Environ. Sci. Technol.*, 20 (1986) 457.
9 A.-S. Allard, M. Remberger, T. Viktor and A.H. Neilson, *Water Sci. Tech.*, 20 (1988) 131.
10 M. Remberger, P. Hynning and A.H. Neilson, *Environ. Toxicol. Chem.*, 7 (1988) 795.
11 H.B. Lee and T.E. Peart, *J. Chromatogr.*, 594 (1992) 309.
12 J.W. Hills and H.H. Hill, Jr., *Anal. Chem.*, 63 (1991) 2152.
13 S.B. Hawthorne, D.J. Miller, D.E. Nivens and D.C. White, *Anal. Chem.*, 64 (1992) 405.
14 H.B. Lee, T.E. Peart and R.L. Hong-You, *J. Chromatogr.*, 605 (1992) 109.
15 H.B. Lee, R.L. Hong-You and P.J.A. Fowlie, *J. Assoc. Off. Anal. Chem.*, 72 (1989) 979.
16 D.W. Kuehl and R.C. Dougherty, *Environ. Sci. Technol.*, 14 (1980) 447.

Journal of Chromatography, 636 (1993) 271–276
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 903

Capillary gas chromatographic determination of copper and nickel using microwave-induced plasma atomic emission detection

M.Y. Khuhawar[☆], A. Sarafraz-Yazdi[☆☆] and P.C. Uden*

Department of Chemistry, Lederle Graduate Research Tower A, University of Massachusetts at Amherst, Amherst, MA 01003 (USA)

(First received November 17th, 1992; revised manuscript received January 25th, 1993)

ABSTRACT

Four tetradentate β-ketoamine reagents were examined for derivatization and the separation of copper and nickel as their chelates, by capillary GC using flame ionization detection. Complete separation between copper and nickel complexes was obtained in each case. The chelates were also evaluated for simultaneous, selective and sensitive spectral detection of copper and nickel at 325 and 301 nm, respectively, using microwave-induced plasma atomic emission detection. The selectivity of copper at 325 nm with respect to nickel was high; some response of copper at the nickel emission line was observed, but it did not interfere with the determination of nickel. The reagent $H_2(pnAA_2)$ was used for the analysis of copper and nickel in an ore sample. The detection limits for both copper and nickel were 0.8 pg reaching the detector.

INTRODUCTION

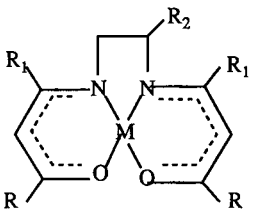
The effective gas chromatography (GC) of metal chelates is dictated by their volatility and thermal stability under instrumental conditions. Complexing reagents used successfully for the GC determination of metals include β-diketones, β-thioketones, bidentate and tetradentate β-ketoamines (ketoenamines), dialkyldithiocarbamates and dialkyldithiophosphates [1]. Tetradentate β-ketoamine ligands are notable because of their selective reaction with a number of divalent metal ions [2], these reactions being generally quantitative at all concentrations. The reagents [4,4′-(1,2-ethanediyl)dinitrilo]bis(2-pentanone), or bis(acetylacetone)-ethylenediimine, $H_2(enAA_2)$], [4,4′-(1-methyl-1,2-ethanediyl)dinitrilo]bis(2-pentanone) [or bis(acetylacetone)-propylenediimine, $H_2(pnAA_2)$], [4,4′-(1,2-ethanediyl)dinitrilo]bis(5,5′dimethyl-hexen-2-one), [or bis(acetylpivalylmethane)-ethylenediimine, $H_2(enAPM_2)$] and [4,4′-(1,2-ethanediyl)-dinitrilo]bis(6-methyl-hepten-2-one), [or bis(isovalerylactone)-ethylenediimine, $H_2(enIVA_2)$] (Fig. 1), have been used for GC determination of copper and nickel using packed columns and flame ionization detection (FID), but complete separation between copper and nickel complexes has remained a problem [2–7]. These complexes are now examined by capillary column GC with FID or microwave-induced plasma atomic emission detection (MIP-AED) for the quantitative separation and selective determination of these metals.

Interfacing high-resolution chromatography with element-selective detection can simplify complex analyses. GC has been effectively cou-

* Corresponding author.
☆ Permanent address: Institute of Chemistry, University of Sindh, Jamshoro, Pakistan.
☆☆ Permanent address: Department of Chemistry, Faculty of Science, University of Mashhad, Mashhad, Iran.

0021-9673/93/$06.00

Tetradentate β-Ketoaminates



M =Cu(II) & Ni(II)

(1) R = CH_3, R_1 = CH_3, R_2 = H
(2) R = CH_3, R_1 = CH_3, R_2 = CH_3
(3) R = $C(CH_3)_3$, R_1 = CH_3, R_2 = H
(4) R = $CH_2CH(CH_3)_2$, R_1 = CH_3, R_2 = H

Fig. 1. Structures of reagents and their metal chelates. 1 = Bis(acetylacetone)-ethylenediamine, $H_2(enAA_2)$; 2 = bis-(acetylacetone)-propylenediamine, $H_2(pnAA_2)$; 3 = bis(acetylpivalylmethane)-ethylenediamine, $H_2(enAPM_2)$; 4 = bis-(isovalerylacetone)-ethylenediamine, $H_2(enIVA_2)$.

pled with different atomic spectroscopic systems, atomic absorption spectroscopy (AAS) [8], flame emission spectroscopy (FES) [9] and nobel-gas plasma atomic emission spectroscopy (PAES) [10–16]. GC with atomic emission detection (AED) using a helium MIP was described by McCormack *et al.* [17] in 1965. The majority of AED systems for GC are now based on MIP [18].

A GC–AED instrument has been introduced [19,20] which utilizes a water-cooled reentrant cavity to maintain a helium plasma, modified as required by additional reagent gases. The spectrometer, based on a concave holographic grating, is purged with nitrogen and operates from 160 to 800 nm. The detector is a 12.5 mm long 211 pixel silicon photodiode array which can be positioned along the 350 mm length of the focal plane of the spectrometer. The sensitivity and element selectivity of this system are particularly effective for transition metal determinations.

EXPERIMENTAL

The copper and nickel complexes of the ligands $H_2(enAA_2)$, $H_2(pnAA_2)$, $H_2(enAPM_2)$ and $H_2(enIVA_2)$ were prepared as reported previously [3–8].

A Model 3700 gas chromatograph (Varian Instruments, Palo Alto, CA, USA) was used for FID. A Model HP 5890 II gas chromatograph (Hewlett-Packard, Avondale, PA, USA) was used with a manual and an autoinjector, and a split/splitless capillary injection port operated in the split mode; it was interfaced to a HP 5921 atomic emission detector and HP 330 computer with HP 35920 GC–AED software. High-purity helium was used as carrier and make up gas, and hydrogen and oxygen as reagent gases. The major features of the instrumentation are reported elsewhere [19,20].

Columns used were DB-5 (30 m × 0.25 mm I.D. with 0.25-μm layer film thickness and 30 m × 0.32 mm I.D. with 0.25-μm layer film thickness) (J&W Scientific, Folsom, CA, USA). Fresh solutions of complexes containing 1 mg/ml were prepared in acetone or cyclohexane and further solutions prepared by appropriate series dilution.

Solvent extraction procedure

A similar extraction procedure was used as reported earlier [5]. Aliquots (1 ml) of solutions containing 0.1–160 mg of copper and nickel were transferred to screw cap vials, followed by 4 ml of (1%) ketoamine reagent solution in aqueous 2% sodium carbonate. The mixture was warmed at 50–60°C for 10 min and was then allowed to cool at room temperature. Dichloromethane (2 ml) was added and the mixture was shaken for 30 s. The layers were allowed to separate and the organic layer transferred to a glass vial and sealed with a rubber seal and crimp top to prevent evaporation of the solvent. The reagent blank was also run with 1 ml of deionized water.

A matte Ore Sample (No. 1) (Falconbridge Nikkelverk, Kristiansands, Norway) (1.00 g) was dissolved in aqua regia (50 ml) and heated almost to dryness. Two 25-ml portions of concentrated hydrochloric acid were added, the mixture being heated each time gently nearly to dryness. Finally concentrated hydrochloric acid (8 ml) was added and the volume adjusted to 100 ml. The solution was further diluted 50 times and 1 ml was taken for analysis using the described extraction procedure. A sample blank was also prepared using 1 ml of water. Solutions were gas chromatographed in triplicate using the autoinjector, in a sequence from dilute to concentrated

solutions. Column temperature was 240°C and injection port and transfer line temperatures were 260°C. The carrier gas flow-rate was 1.8 ml/min and the split ratio was 1:58.

RESULTS AND DISCUSSION

The copper and nickel complexes were chromatographed on the 30-m DB-5 column using FID in a column temperature range 220–250°C, with injection port range 230–260°C and helium carrier flow-rate 1.3 ml/min. Complete separation of each of the four metal chelate pairs derived from the ligands $H_2(enAA_2)$, $H_2(pnAA_2)$, $H_2(enAPM_2)$ and $H_2(enIVA_2)$ was achieved within 15 min. Fig. 2 shows separations for the first two chelate pairs, the peaks showing only a small amount of tailing. The FID detection limits ($S/N = 3$) for different chelates under the optimum conditions of separation were from 1.47–7.35 ng of a complex, corresponding to 0.23–1.63 ng of copper or nickel. The copper and nickel complexes of the reagent $H_2(enAPM_2)$ showed maximum FID sensitivity with lowest detection limits corresponding to 0.25 ng copper and 0.24 ng of nickel, followed in decreasing sequence of detection sensitivity by $H_2(enIVA_2)$, $H_2(pnAA_2)$ and $H_2(enAA_2)$.

To examine the effect of different substituents on the relative retention of metal chelates, separate mixtures of the copper and the nickel complexes of the four ligands were chromatographed under optimal separation conditions. Complete separation was obtained for both copper and nickel series with elution of metal chelates in the sequence $H_2(pnAA_2)$, $H_2(enAA_2)$, $H_2(enAPM_2)$ and $H_2(enIVA_2)$ (Fig. 3). A mixture of saturated hydrocarbons between $C_{22}H_{44}$ and $C_{30}H_{62}$ was also chromatographed under the conditions used for the separation of copper or nickel complexes in order to calculate Kováts indices for the complexes. The values in Table I indicate that introduction of a methyl group into the parent ligand $H_2(enAA_2)$ at the bridge position, to form $H_2(pnAA_2)$, decreases the Kováts index for their chelates by 85–95 units, but introduction of tertiary butyl or

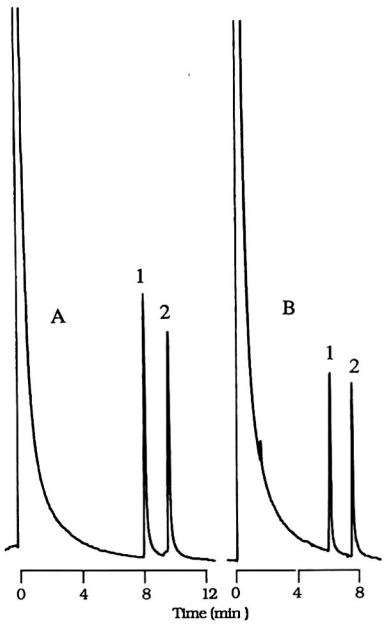


Fig. 2. GC separation of (1) copper(II) and (2) nickel(II) chelates of (A) $H_2(enAA_2)$ and (B) $H_2(pnAA_2)$. Column: DB-5 (30 m × 0.25 mm); temperatures; column 225°C, injection port and flame ionization detector 250°C. Helium carrier gas flow-rate 1.3 ml/min. Inlet split ratio 1:17.

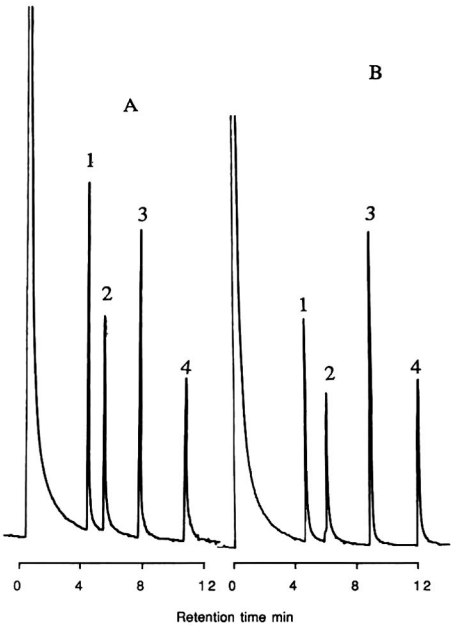


Fig. 3. Comparative GC elution of (A) copper(II) and (B) nickel(II) complexes of (1) $H_2(pnAA_2)$, (2) $H_2(enAA_2)$, (3) $H_2(enAPM_2)$ and (4) $H_2(enIVA_2)$. Column: DB-5 (30 m × 0.25 mm); temperatures: column 240°C, injection port and flame ionization detector 260°C. Helium carrier gas flow-rate 1.2 ml/min. Inlet split ratio 1:26.

TABLE I

KOVATS INDICES FOR COPPER AND NICKEL CHELATES

Complex	Kováts index	Complex	Kováts index
$Cu(pnAA_2)$	2360	$Cu(enAPM_2)$	2575
$Ni(pnAA_2)$	2410	$Ni(enAPM_2)$	2640
$Cu(enAA_2)$	2445	$Cu(enIVA_2)$	2690
$Ni(enAA_2)$	2505	$Ni(enIVA_2)$	2740

isobutyl groups adjacent to the carbonyl function in the ligands $H_2(enAPM_2)$ and $H_2(enIVA_2)$, increases the index for their metal chelates by 130–135 and 235–245 units, respectively, for both copper and nickel complexes.

The copper and nickel complexes were easily separated on the capillary column and showed predicted FID sensitivity, but to examine the selectivity of AED for copper and nickel and its use for their quantitative determination, capillary GC coupled with MIP detection was investigated. The emission wavelengths for copper and nickel at 325 and 301 nm, respectively, were selected for simultaneous monitoring of elemental response. A high flow-rate of helium make up gas (150 ml/min) was preferred along with hydrogen and oxygen as reagent gases at the pre-set flow conditions set by the instrument manufacturer. When a mixture of copper and nickel complexes was injected under the optimized GC conditions, some response of the copper complex at the nickel line was observed. The effects of copper emission on the nickel response and of nickel emission on the copper response were therefore examined. Solutions of copper and nickel complexes of $H_2(enAPM_2)$ with chelate ratios of 1:10 and 10:1 were prepared and injected at a column temperature of 250°C and helium carrier flow rate of 1.2 ml/min. No response for nickel at the 325-nm copper line was observed and selectivity of copper against nickel at that wavelength was greater than 10^3. In contrast, the selectivity of nickel at 301 nm with respect to copper was found to be *ca.* 125; the response of copper at 325 nm was coincidentally *ca.* 125 times greater than at 301 nm. In order to compare the spectral features of copper emission at 325 and 301 nm and of nickel emission at 301 nm, three-dimensional displays of emission signal output (snapshots) with respect to wavelength and time are shown in Fig. 4. These plots show changes in spectral peak shape with respect to retention time. Nickel and copper show well defined signal peaks at 301 and 325 nm, respectively (Fig. 4A and B), but the

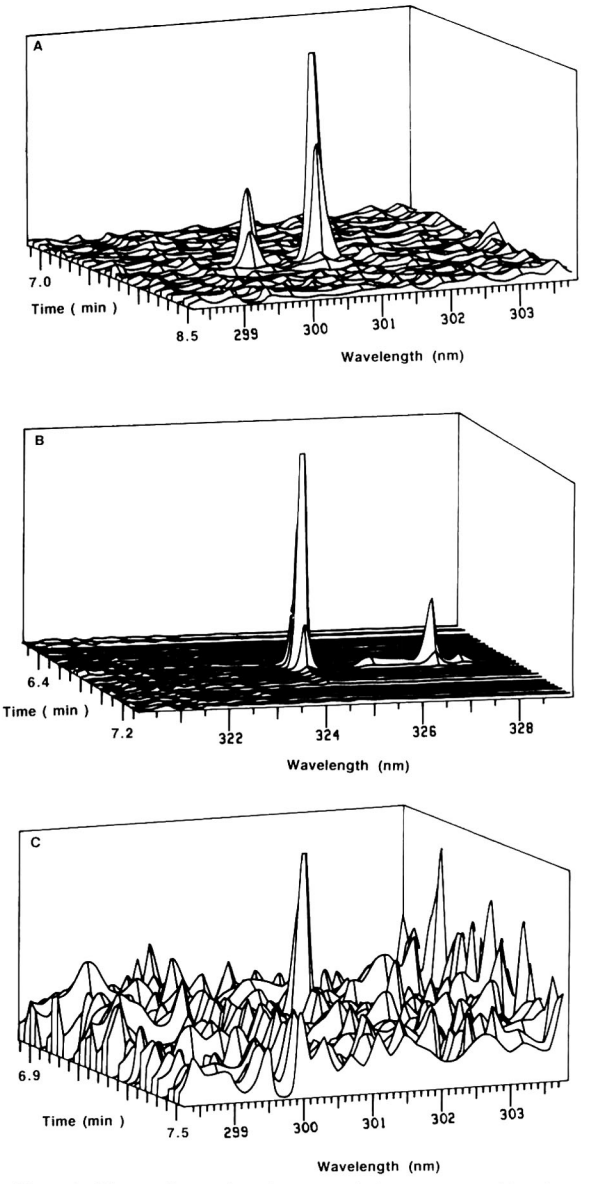


Fig. 4. Three-dimensional spectral-chromatographic detection display (snapshots) of (A) nickel complex at 301 nm, (B) copper complex at 325 nm and (C) copper complex at 301 nm.

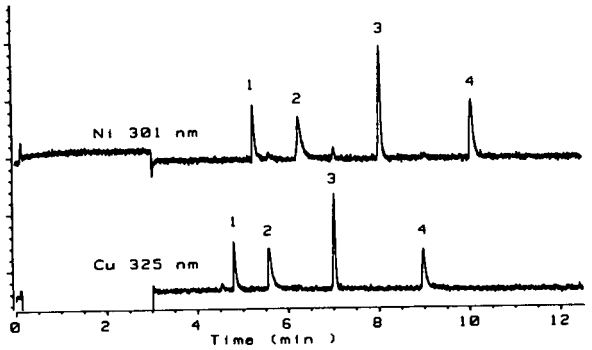


Fig. 5. Element-specific chromatograms for copper at 325 nm and nickel at 301 nm using MIP-AED detection. Copper and nickel chelates of (1) $H_2(pnAA_2)$, (2) $H_2(enAA_2)$, (3) $H_2(enAPM_2)$ and (4) $H_2(enIVA_2)$. Column: DB-5 (30 m × 0.25 mm); temperatures: column 250°C, injection port and transfer line 260°C. Helium flow-rate 1.2 ml/min. Inlet split ratio 1:23.

copper response at 301 nm (Fig. 4C) shows low intensity relative to background spectral structure. However since there was complete chromatographic separation of copper and nickel complexes, there was no experimental interference of nickel determination due to copper, and emission signals could be used for the simultaneous determination of copper and nickel. This was confirmed when a mixture of all eight chelates, four copper complexes and four nickel complexes, was chromatographed and their elution monitored at 301 and 325 nm. All peaks were observed independently, without any chromatographic or spectral interference (Fig. 5).

Any of the four reagents could be equally well used for the simultaneous determination of copper and nickel using solvent extraction. As an example, $H_2(pnAA_2)$ was chosen for the GC–MIP-AED analysis of a matte ore sample, to enable comparison with the results reported earlier using atomic absorption and packed-column GC–FID [5]. Calibration curves relating mean peak height to the amount of the copper and nickel injected showed good linearity over the necessary concentration range (Fig. 6). The results of analysis are in good agreement with those obtained from atomic absorption (Table II). The detection limit for copper and nickel in the 1-ml aliquot was *ca.* 100 ng for both copper and nickel. The extraction into 2 ml of dichloromethane, with 1 μl injected at a split ratio of 1:58 on the GC column, corresponds to about 0.8 pg of copper and nickel reaching the detector.

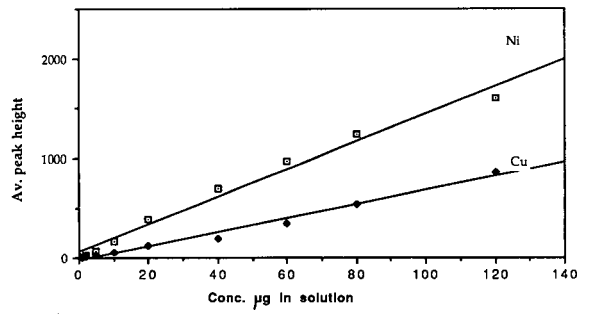


Fig. 6. Calibration graphs of extracted copper(II) and nickel(II) with $H_2(pnAA_2)$ as complexing agent. Equations: nickel, $y = 48.944 + 13.920x$, $R^2 = 0.983$; copper: $y = -21.547 + 6.9784x$, $R^2 = 0.989$.

TABLE II

ANALYSIS OF MATTE ORE SAMPLE

Atomic absorption [5] (μg/g)		Packed-column GC–FID [5] (μg/g)		Capillary GC–MIP-AID ± S.D. (μg/g) ($n = 4$)	
Cu	Ni	Cu	Ni	Cu	Ni
215	469	413	531	250 ± 18	425 ± 22

CONCLUSIONS

The use of capillary GC coupled with FID or MIP-AED for the effective separation and quantitative determinations of copper and nickel as metal chelate compounds has been demonstrated. MIP-AED improves the sensitivity of metal ion detection to pg levels. Tetradentate β-ketoamine ligands showed adequate thermal stability and volatility for the quantitative GC determination of copper and nickel simultaneously; their reactions are quantitative and the metal chelates are easily extractable into organic solvents.

ACKNOWLEDGEMENTS

The University of Meshhad, Iran, and the Ministry of Culture and Higher Education, Is-

lamic Republic of Iran, are acknowledged for the grant of sabbatical leave to one of us (A.S.-Y). M.Y.K. acknowledged the receipt of a Fulbright Research award. We wish to acknowledge support for this work from the Hewlett-Packard Company and the Mobil Research and Development Corporation. Thanks are also due to Bruce Quimby, James Sullivan and Frank DiSanzo for their advice and encouragement.

REFERENCES

1 P.C. Uden, *J. Chromatogr.*, 313 (1984) 3.
2 S. Dilli and E. Patsalides, *Anal. Chim. Acta*, 128 (1981) 109.
3 R. Belcher, R.J. Martin, W.I. Stephen, D.E. Henderson, A. Kamalizad and P.C. Uden, *Anal. Chem.*, 45 (1973) 1197.
4 R. Belcher, K. Blessel, T.J. Cardwell, M. Pravica, W.I. Stephen and P.C. Uden, *J. Inorg. Nucl. Chem.*, 35 (1973) 1127.
5 P.C. Uden and D.E. Henderson, *J. Chromatogr.*, 99 (1974) 309.
6 R. Belcher, A. Khalique and W.I. Stephen, *Anal. Chim. Acta*, 100 (1978) 503.
7 M.Y. Khuhawar, A.G. Vasandani and A.I. Soomro, *J. Chem. Soc. Pak.*, 12 (1990) 119.
8 L. Ebdon, S. Hill, A.P. Walton and R.W. Ward, *Analyst*, 113 (1988) 1159.
9 X.Y. Sun and W.A. Aue, *J. Chromatogr.*, 467 (1989) 75.
10 D.L. Windsor and M.B. Denton, *J. Chromatogr. Sci.*, 17 (1979) 492.
11 R.J. Lloyd, R.M. Barnes, P.C. Uden and W.G. Elliott, *Anal. Chem.*, 50 (1978) 2025.
12 B.D. Quimby, M.F. Delaney, P.C. Uden and R.M. Barnes, *Anal. Chem.*, 52 (1980) 259.
13 S.A. Estes, P.C. Uden and R.M. Barnes, *Anal. Chem.*, 53 (1981) 1829.
14 A.H. Mohammad, M. Zerezghi and J.A. Caruso, *Anal. Chem.*, 58 (1986) 469.
15 T.H. Risby and Y. Talmi, *CRC Crit. Rev. Anal. Chem.*, 14 (1983) 231.
16 P.C. Uden (Editor), *Element-Specific Chromatographic Detection by Atomic Emission Spectroscopy (ACS Symposium Series*, No. 479), American Chemical Society, Washington, DC, 1992.
17 A.J. McCormack, S.C. Tong and W.D. Cooke, *Anal. Chem.*, 57 (1965) 1470.
18 P.C. Uden, in R.M. Harrison and S. Rapsomanikis (Editors), *Environmental Analysis using Chromatography Interfaced with Atomic Spectroscopy*, Ellis Horwood, Chichester, 1989, Ch. 5.
19 B.D. Quimby and J.J. Sullivan, *Anal. Chem.*, 62 (1990) 1027.
20 J.J. Sullivan and B.D. Quimby, *Anal. Chem.*, 62 (1990) 1034.

Journal of Chromatography, 636 (1993) 277–283
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 895

Optimization of a flame photometric detector for supercritical fluid chromatography of organotin compounds

J. Dachs and J.M. Bayona*

Environmental Chemistry Department, C.I.D.–C.S.I.C., Jordi Girona Salgado 18, 08034-Barcelona (Spain)

(First received November 19th, 1992; revised manuscript received January 15th, 1993)

ABSTRACT

A single flame photometric detector has been optimized for capillary supercritical fluid chromatography (cSFC) of trialkyltin chlorides for the first time. Detection variables (temperature, hydrogen and air flow-rates) were optimized by a combination of factorial experimental design and simplex. Furthermore, the position of the restrictor in the flame and injection volumes were also considered. Optimum sensitivity was achieved using high hydrogen–air ratios (1.8). Using the optimum conditions, baseline was stable during pressure programming. The detection limit of tributyltin chloride ($S/N = 3$) was around 40 pg (as tin). Reproducibility was high (R.S.D. = 3.8%, $n = 5$) and dynamic range was measured over two orders of magnitude. Furthermore, dibutyl-, diphenyl- and triphenyltin chlorides were also successfully eluted for the first time using linear density programming (carbon dioxide, 50 °C).

INTRODUCTION

Organotin speciation is of paramount importance in environmental studies in order to distinguish the most toxic species from the less toxic degradation products. Until now most organotin speciation techniques have been based on the GC resolution of volatile derivatives (*i.e.* hydrides or alkyl) coupled to elemental detection techniques [atomic absorption spectrometry (AAS), flame photometric detection (FPD) and atomic emission detection (AED)]. Furthermore, the high toxicity of organotins requires analytical techniques with extremely high sensitivity and selectivity.

However, it is suspected that derivatization reactions for organotins in complex mixtures of compounds present in environmental samples are not quantitative and might modify the original composition of the sample, leading to significantly biased results [1]. Furthermore, derivatization procedures are time-consuming, and consequently their application to environmental monitoring programmes are of limited interest because they are difficult to automate.

On the other hand, the lack of UV-absorbing functional groups in alkyltin compounds restricts the application of LC to post-column derivatization techniques or linked systems (LC–MS, LC–inductively coupled plasma-MS) [2], which are not well suited to surveys dealing with a large number of samples.

At present, the use of supercritical fluid chromatography (SFC) to analyse organometallic compounds is still in its infancy [3–5], but it could be an alternative to classical speciation techniques. Its potential is associated to the versatility of detection systems, especially the capillary column versions, combined with the solvating properties of supercritical fluids, which allow the elution of analytes at low temperatures. Until now only the tetra-alkyl- and tetra-

* Corresponding author.

0021-9673/93/$06.00

aryltins have been analysed by SFC–ICP-MS [6]. Nevertheless, these analytes can also be determined by capillary GC (cGC), in contrast to the lower substituted alkyl and aryltins, which require volatile derivatives.

Although capillary SFC (cSFC) coupled to FPD has been previously evaluated for the determination of sulphur heterocycles [7,8] and high-molecular-weight organosulphur compounds [9], similar attempts have not been undertaken for organotins, despite the fact that cGC using tin-selective FPD is one of the most sensitive detection techniques for the determination of derivatized compounds [10].

In this work, the optimization of a tin-selective single-flame FPD system for cSFC has been carried out for the first time, with the aim of evaluating its applicability to organotin speciation of the corresponding chlorides, which cannot be successfully analysed by cGC–FPD. Organotin chlorides are one of the most important intermediate derivatives, which are generated in sample pretreatments in order to displace more ionic counterions (CO_3^{2-}, SO_4^{2-}, S^-) from ionic organotin species present in the aquatic environment.

Chloride derivatives of organotin compounds are easily obtained after a hydrochloric acid treatment during the extraction step [11]. Unfortunately, GC analysis of these compounds is not successful because the strongly polarized tin–chloride bonds are presumably thermolabile. It requires carrier gas saturation with hydrochloric acid and is obviously not suited for routine analysis [12].

EXPERIMENTAL

Apparatus

SFC-grade carbon dioxide was obtained from Air Products (Barcelona, Spain). Carbon dioxide was cooled at 4 °C in the syringe pump. SFC analyses were performed with an SFC-3000 chromatograph equipped with a dual-syringe pump system (Fisons, Milan, Italy) coupled to an FPD 250 series instrument (Fisons) using a broad band-pass filter with peak transmission at 610 nm (LOT, Darmstadt, Germany). Nitrogen was used as a make-up gas at a flow-rate of 55 ml/min. Data were acquired by a Nelson-PE interface with a sampling frequency of 100 Hz and handled by a PS computer. Standard solutions were injected at 30 °C dissolved in hexane into a timed-split rotary injection valve (Valco, Houston, TX, USA) using an internal loop of 200 nl. The analytical capillary column was a 10 m × 0.1 mm I.D. column coated with 0.4 μm film thickness of SE-52, and was fitted directly into the injection valve and to an integral restrictor (J & W, Folsom, USA) (2 ml/min, as gas), using a zero-dead-volume connector. Carbon dioxide pressure was programmed from 0.2 g/ml to 0.65 g/ml at 0.02 g/ml/min.

TABLE I

OPTIMIZATION OF SFC–FPD VARIABLES BY FACTORIAL DESIGN

Experiment No.	Hydrogen (ml/min)	Air (ml/min)	Temperature (°C)	Response[a] (%)
1	75	44	175	29
2	111	44	175	46
3	75	92	175	45
4	111	92	175	78
5	75	44	250	38
6	111	44	250	51
7	75	92	250	50
8	111	92	250	100

[a] Response normalized to the optimum value.

Reagents

Diphenyltin (DPhTCl) chloride and tetrabutyltin (TeBT) were obtained from E. Merck (Darmstadt, Germany). Dibutyltin (DBTCl), triphenyltin (TPhTCl), tributyltin (TBTCl) and tripropyltin (TPrTCl) chlorides were from Fluka (Buchs, Switzerland). Stock solutions were prepared with pesticide-grade hexane (Merck) and stored at 4°C in the dark.

Statistical optimization

An experimental design [13] focused on three variables was applied for FPD optimization, each one chosen at two levels (high and low). Variables and their values are presented in Table I. Response effects were calculated according to the Yates algorithm [14]. Optimum conditions for FPD were investigated by using two independent simplex runs, to ascertain that the maximum was reached [15].

RESULTS AND DISCUSSION

FPD response optimization

In order to evaluate the response of FPD variables to the organotin sensitivity, an experimental design was outlined and the TBTCl response was determined (Table I). Following the evaluation of response effects, significant variables were the FPD fuel gas composition, hydrogen and air. Conversely, the effect of temperature on sensitivity was negligible in the range 175–250°C, and it was kept constant in the subsequent experiments at 225°C.

In order to optimize the detector gases, simplex runs were carried out. Fig. 1 shows the response surface of TBT chloride as a function of hydrogen and air composition, where the maximum response was found at flow-rates of 220 and 120 ml/min, respectively (CO_2 at 76 μl/min). Background exhibited a moderate increase with hydrogen flow (0.2%), which is almost insignificant in comparison with the SnH response enhancement (30%). The high flow-rates of hydrogen required in the optimum conditions are in contrast to the moderate values used in cGC–FPD of alkyl-derivatized organotin compounds [10]. Similar results were also obtained in case of sulphur compounds and were tentatively attributed to a reaction between carbon dioxide and radicals of the flame gases [8] that are important to the formation of SnH, responsible for tin emission in FPD [16].

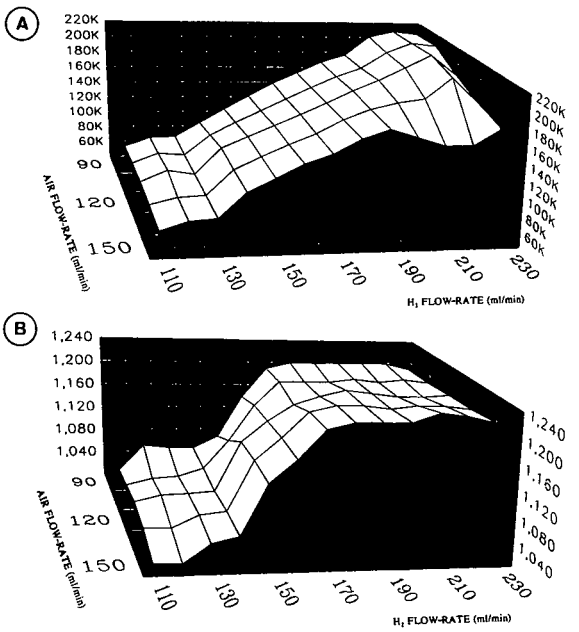


Fig. 1. (A) Variation of measured tin hydride emission (response $\times 10^3$) and (B) background emission as a function of hydrogen and air flow-rates. *y*-Axes are in arbitrary units.

Another variable optimized was the distance of the restrictor from the flame. Fig. 2 shows that the optimum position was 1.2 cm lower than the flame base, while background was almost constant over the range of column positioning evaluated. The highest decrease in sensitivity was obtained when the restrictor was further beneath the flame, probably associated to band-broadening effects and excessive mixing with fuel gases. On the other hand, positioning the restrictor close to the flame leads to a poor mixing effects between hydrogen and air, which are flowing coaxially with the column effluent.

Furthermore, under density programming conditions no baseline drift was apparent, probably because of reduced quenching effects at the wavelength monitored in the tin-selective FPD (610 nm). In fact, a great selectivity of tin over carbon is evident from the solvent peak in the

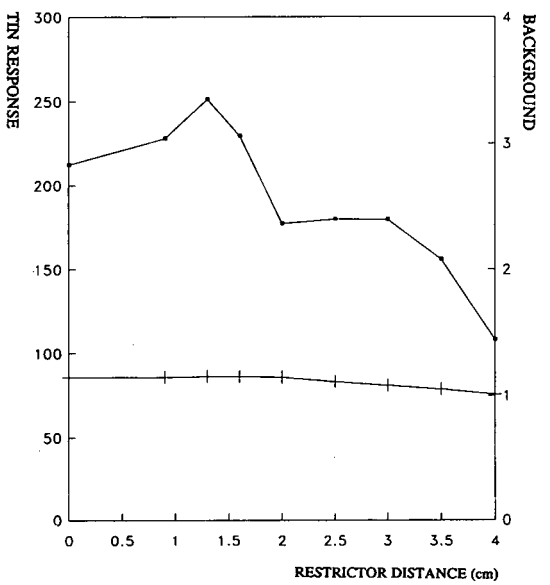


Fig. 2. Variation of measured tin hydride emission (□), and background emission as a function of position of restrictor from the flame base (+). *y*-Axes are in arbitrary units.

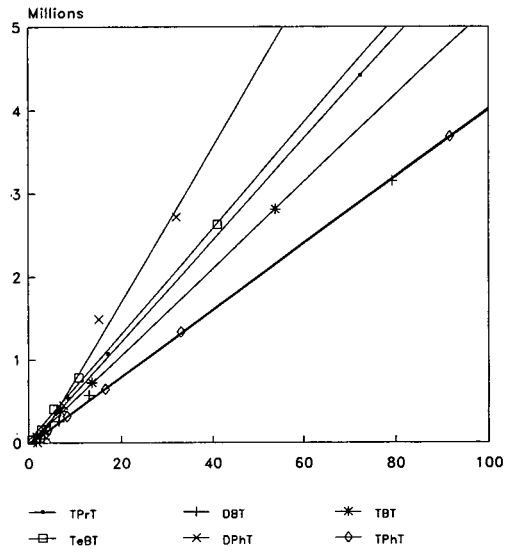


Fig. 3. Response in arbitrary units *vs.* injected amounts in ng (as organotin chlorides) under the optimum cSFC–FPD conditions.

optimum conditions. These results are clearly in contrast with those reported formerly for the cSFC–FPD of organosulphur and organophosphorus compounds, which exhibited a positive baseline drift under similar operating conditions, leading to a significant reduction in sensitivity [7].

Furthermore, in order to evaluate the optimum amount of sample to inject while maintaining column efficiency, injection time, and as a consequence injection volume, were also evaluated. The results show no significant band broadening when slow injections (2 s) were performed using the 200-nl loop in comparison with the fast injection technique (0.2 s). This could be because of the focusing effect in injection conditions involving low densities. Accordingly, further sensitivity evaluation was carried out using long injection times.

The dynamic range of the SnH response was

TABLE II

LIMIT OF DETECTION (LOD) OF ORGANOTIN COMPOUNDS (SIGNAL-TO-NOISE RATIO = 3)[a] AND REPRODUCIBILITY ($n = 5$) UNDER THE OPTIMUM CONDITIONS

Compound	LOD		R.S.D. (%)
	(pg as tin)	(pg as chloride)	
Tripropyltin chloride	122	291	–[b]
Dibutyltin chloride	334	1 099	14.6
Tributyltin chloride	40	110	3.8
Tetrabutyltin	50	147	5.7
Diphenyltin chloride	623	1 804	5.2
Triphenyltin chloride	123	400	5.4

[a] Noise was measured by peak-to-peak baseline.
[b] Injection amount at the picogram level. Tripropyltin chloride was used as internal standard.

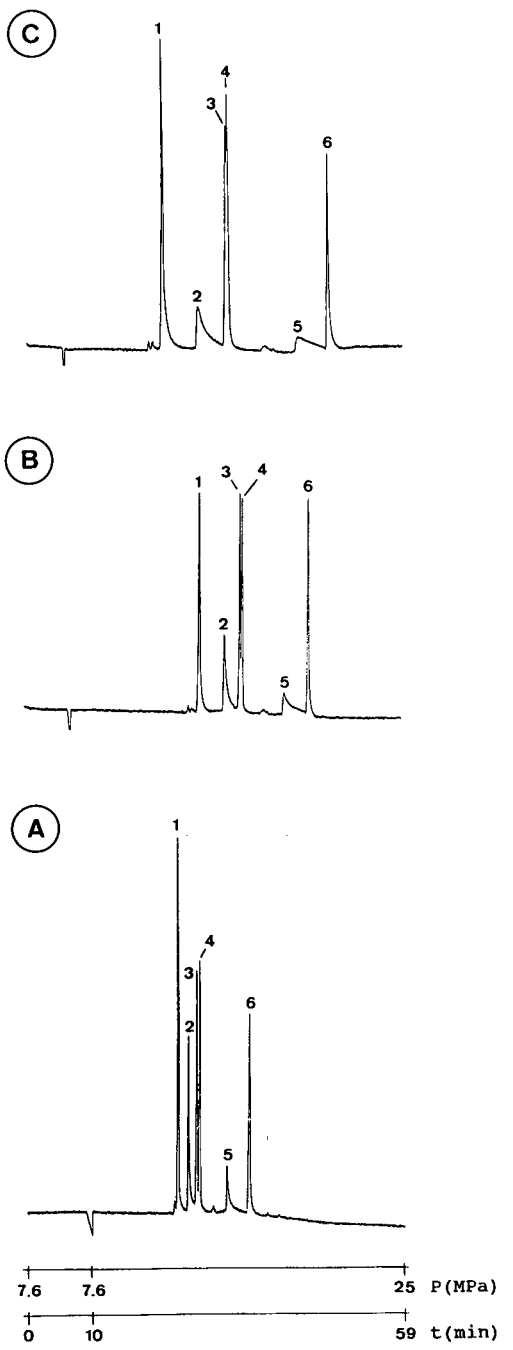


Fig. 4. cSFC–FPD chromatograms of organotin compounds obtained at different column temperatures: (A) 50°C, (B) 80°C and (C) 110°C. Injection was performed at 7.6 MPa and then programmed to 25 MPa at 0.35 MPa/min, after a 10-min isobaric period. Compounds are as follows: 1 = TPrTCl; 2 = DBTCl; 3 = TBTCl; 4 = TeBTCl; 5 = DPhTCl; and 6 = TPhTCl. Injected amount of organotin chlorides was in the low-ng range (0.5–5 ng).

evaluated from near the detection limits to injected amounts around 500 pg. In this range, a linear response was found for all the components ($r > 0.999$) (Fig. 3). This behaviour is similar to the cGC–FPD of alkyl derivatives of organotin compounds (tetraalkyltins), although response factors are slightly higher in the GC conditions.

The limit of detection (LOD) and reproducibility of butyltin and phenyltin chlorides under the optimum conditions are shown in Table II. Trialkyltins and tetraalkyltins exhibited lower LOD than triphenyltin, but the highest LOD values were obtained for dialkyl- and diaryltins, probably because of the lower stability of these compounds in the analysis conditions.

As far as precision is concerned, dibutyltin chloride exhibited the highest R.S.D. (14.6%), while the R.S.D. was considered acceptable for the rest of components (3.8–5.7%), taking into account the low volume injected (< 200 nl). It is interesting to note the difference in precision between diaryltin and dialkyltin. In this regard, the R.S.D. of DPhTCl was much lower than that of DBTCl, despite the fact that DPhTCl is more labile than DBTCl. Therefore, the poor precision of DBTCl seems not to be related to the compound instability. In spite of this, co-elution with another component seems a more reasonable explanation of the high R.S.D. for the latter compound.

Application to organotin speciation

In order to evaluate the feasibility of cSFC–FPD for organotin speciation in environmental samples, a standard mixture containing tributyltin and triphenyltin chlorides and the most toxic degradation products, dibutyltin and diphenyltin, was analysed at different temperatures under pressure programming conditions. As soon as column temperature increased, significant peak tailing of DBTCl and DPhTCl became apparent, probably due to compound decomposition during analysis. In this regard, DPhTCl is eluted very poorly at 110°C and co-elution of TeBT and TBTCl is apparent. As a consequence, temperatures as low as possible are preferred for the analysis of these thermolabile compounds. However, temperatures lower than 50°C were not evaluated in order to keep the

TABLE III

CORRELATION BETWEEN CAPACITY FACTOR (k') IN SFC AND CARBON (C) AND CHLORINE (Cl) NUMBERS OF SIX ORGANOTIN CHLORIDES: TRIPROPYLTIN, DIBUTYLTIN, TRIBUTYLTIN, DIPHENYLTIN, TRIPHENYLTIN CHLORIDES AND TETRABUTYLTIN

Temperature (°C)	a^a	b^a	c^a	r^a
50	−0.368	0.128	0.429	0.973
80	−0.641	0.287	1.010	0.984
110	−4.01	0.520	1.840	0.965

[a] a, b and c correspond to the constants in the correlation equation $k' = a + b\mathrm{C} + c\mathrm{Cl}$; r is the corresponding correlation coefficient.

analysis time as short as possible. At this temperature, the resolution of six organotin compounds exhibited reasonable peak shape and an acceptable analysis time (35 min) was obtained (Fig. 4).

In order to model the retention behaviour of the organotins analysed, the capacity factor (k') was correlated with chlorine and carbon number for every compound by applying a multiple linear regression at the three temperatures evaluated. Table III shows that a significant correlation was obtained at each temperature. Previous studies of ethyl derivatives of organotin compounds using cGC–FPD have shown a simple linear correlation between carbon number and k' [17].

The retention time was dependent on compound volatility and the column temperature. At intermediate temperatures, the retention time of all components increased as a result of a decrease in density. Conversely, at higher temperatures the more volatile components (*i.e.* trialkyltin chlorides and tetrabutyltin) showed a decrease in retention time owing to the predominance of partition mechanisms, in contrast to the low-volatility components (*i.e.* di- and triphenyltins).

CONCLUSIONS

The suitability of single-flame FPD coupled to cSFC for the determination of organotin chlorides is demonstrated for the first time. Although detection limits are slightly higher than in cGC, the possibility of analysing tributyltin and triphenyltin chlorides and their degradation products (*i.e.* dibutyltin and diphenyltin chlorides), which are the most toxic organotin compounds, is of great interest for the speciation of organotin compounds in environmental samples since tedious and time-consuming derivatization steps can be avoided. Further, retention of organotin chlorides can be modelled at every temperature under density programming conditions by applying a linear regression with two variables, carbon and chlorine number in the molecule.

Recent findings regarding the suitability of FPD for the detection of the main group of elements [18,19] could expand the applications of FPD to the less volatile organometallic compounds using modified carbon dioxide as mobile phase.

ACKNOWLEDGEMENTS

This work was supported by the National Plan for Research (C.I.C.Y.T.) (Grant AMB92-0218). One of us (J.D.) acknowledges a fellowship from the Spanish Research Council (C.S.I.C).

REFERENCES

1 X. Dauchy, A. Astruc, M. Borsier and M. Astruc, *Analusis*, 20 (1992) 41–48.
2 Ph. Quevauviller, Bureau Community of Reference (EC), Brussels, personal communication.
3 K. Jinno, H. Mae and C. Fujimoto, *J. High Resolut. Chromatogr.*, 13 (1990) 13–17.
4 K.E. Lainz, G.M. Shieh and C.M. Wai, *J. Chromatogr. Sci.*, 30 (1992) 120–123.
5 K.E. Lainz, J.-J. Yu and C.M. Wai, *Anal. Chem.*, 64 (1992) 311–315.
6 W.-L. Schen, N.P. Vela, B.S. Sheppard and J.A. Caruso, *Anal. Chem.*, 63 (1991) 1491–1496.
7 K.E. Markides, E.D. Lee, R. Bolick, and M.L. Lee, *Anal. Chem.*, 54 (1986) 740–743.
8 S.V. Olesik, L.A. Pekay and E.A. Paliwoda, *Anal. Chem.*, 61 (1989) 58–65.
9 L.A. Pekay and S.V. Olesik, *Anal. Chem.*, 61 (1989) 2616–2624.
10 I. Tolosa, J.M. Bayona, J. Albaigés, L.F. Alencastro and J. Tarradellas, *Fresenius' J. Anal. Chem.*, 339 (1991) 646–653.

11 H.A. Meinema, T. Burger-Wiersma, G. Verselius-de Haan and E.Ch. Gevers, *Environ. Sci. Technol.*, 12 (1978) 288–293.
12 K.W. Siu, P.S. Maxwell and S.S. Berman, *J. Chromatogr.*, 475 (1989) 373–379.
13 E.D. Morgan, *Chemometrics: Experimental Design*, Wiley, Chichester, 1991, Ch. 4 and 5 .
14 G.E.P. Box, W.G. Hunter and J.S. Hunter, *Statistics for Experimenters*, Wiley, New York, 1978.
15 S.N. Deming and S.L. Morgan. *Anal Chem.*, 45 (1973) 278A–283A.
16 R.M. Dagnall, K.C. Thompson and T.S. West, *Analyst*, 93 (1968) 518–521.
17 M. Müller, *Anal. Chem.*, 59 (1987) 617–623.
18 W.A. Aue, X.-Y. Sun and B. Millier, *J. Chromatogr.*, 606 (1992) 73–86.
19 W.A. Aue, B. Millier and X.-Y. Sun, *Anal. Chem.*, 63 (1991) 2951–2955.

Author Index

PUBLICATION SCHEDULE FOR THE 1993 SUBSCRIPTION

Journal of Chromatography and *Journal of Chromatography, Biomedical Applications*

MONTH	1992	J	F	M	A	M	J	
Journal of Chromatography	623–627	628/1 628/2 629/1 629/2	630/1 + 2 631/1 + 2 632/1 + 2 633/1 + 2	634/1 634/2	635/1 635/2 636/1 636/2	637/1 637/2 638/1 638/2	639/1 639/2 640/1 + 2	The publication schedule for further issues will be published later.
Cumulative Indexes, Vols. 601–650								
Bibliography Section				649/1			649/2	
Biomedical Applications		612/1	612/2	613/1	613/2 614/1	614/2 615/1	615/2 616/1	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 609, pp. 437–443. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, Netherlands.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Review articles, Short Communications and Discussions. Short Communications are usually descriptions of short investigations, or they can report minor technical improvements of previously published procedures; they reflect the same quality of research as Full-length papers, but should preferably not exceed five printed pages. Discussions (one or two pages) should explain, amplify, correct or otherwise comment substantively upon an article recently published in the journal. For Review articles, see inside front cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he/she is submitting the paper for publication in the *Journal of Chromatography.*

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

Abstract. All articles should have an abstract of 50–100 words which clearly and briefly indicates what is new, different and significant. No references should be given.

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

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

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

Dispatch. Before sending the manuscript to the Editor please check that the envelope contains four copies of the paper complete with references, legends and figures. One of the sets of figures must be the originals suitable for direct reproduction. Please also ensure that permission to publish has been obtained from your institute.

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

0021-9673(19930430)636:2;1-Z