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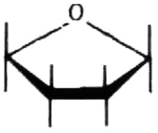

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PERFORMANCE AND PREPARATION OF IMMOBILIZED POLYSILOXANE STATIONARY PHASES IN 5–55 μm I.D. OPEN-TUBULAR FUSED-SILICA COLUMNS FOR LIQUID CHROMATOGRAPHY*

STAFFAN FOLESTAD, BJÖRN JOSEFSSON and MARITA LARSSON*
Department of Analytical and Marine Chemistry, Chalmers University of Technology and University of Göteborg, S-412 96 Göteborg (Sweden)
(First received October 7th, 1986; revised manuscript received November 24th, 1986)

SUMMARY

Cross-linked non-polar polysiloxanes were evaluated as stationary phases in open-tubular column reversed-phase liquid chromatography. Coating of 5–55 μm I.D. fused-silica capillaries with stationary phase films of a well defined thickness in the range 0.03–1.96 μm is described for the two polysiloxane gum phases PS-255 (methylvinyl silicone) and SE-54 (methylphenylvinyl silicone). The chromatographic properties of these columns were investigated using split injection and on-column laser-induced fluorescence detection. Gas chromatography was used complementarily in the evaluation of column stability, retention and inertness. A retentive layer thickness to column diameter ratio up to 1:27 could be prepared, and a linear relationship was observed between the retention and the stationary to mobile phase volume ratio. The selectivity was related to the polysiloxane structure and was constant for films thicker than 0.25 μm. The column band-broadening was studied regarding the contribution from stationary phase diffusion, and compared with theory. Depending on film thickness, the stationary phase diffusion coefficient D_s was in the range 10^{-8}–10^{-6} cm^2/s. The highest efficiency, 351 000 plates ($k' = 0.16$), was obtained with a 1.97 m × 11.7 μm I.D. open-tubular-column. An application to the gradient separation of fluorescence labelled amino acids is presented. Preliminary results are also reported on a new type of stationary phase, created by swelling the immobilized polysiloxane with *n*-heptane. A nearly ten-fold increase in retention and a change in selectivity were obtained.

INTRODUCTION

There is a great interest in liquid chromatography (LC) using open-tubular columns (OTC). In theoretical discussions, Knox and Gilbert[1], Knox[2] and Gui-

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ochon[3], have shown that OTC LC has inherent advantages compared with packed column LC. One of the main advantages is that OTCs have higher permeability and thus higher efficiencies can be obtained. The prerequisite is that the inner diameter is sufficiently small, *i.e.* in the range of a few micrometres.

In practice, many problems remain before complete success with OTC LC will be attained. Such columns have extremely small physical dimensions, which demand special instrumental arrangements. A lot of promising results have been published covering column preparation and equipment design[4–11]. However, very few applications that demonstrate the real potential of the technique have been made.

The main instrumental difficulty is to design and manage a detection system with such a small volume that the external contribution to peak broadening can be disregarded. On-column detection techniques are therefore of utmost importance. Although soft glass and borosilicate glass capillary columns have been used in OTC LC, fused-silica capillaries possess optical properties better suited to optimal spectroscopic detection conditions. Laser-induced fluorescence detection, where the laser beam can be focused tightly on the small diameter column, is then a practicable solution[12,13]. In addition, fused-silica capillaries have a smooth surface and the flexible material is convenient to handle.

In the literature there are different descriptions of ways to prepare non-polar stationary phases for reversed-phase (RP) OTC LC. An important aspect in OTC LC is to achieve useful capacity factors (k') with mobile phases of realistic compositions. The same degree of retention in an OTC, compared with a packed column, is not easily obtained owing to the high ratio of mobile phase to stationary phase volume[2]. Thus far, capacity factors obtained in OTC LC are only moderate compared with packed column LC.

Generally, the coating procedures used in OTC LC were first developed for gas chromatography (GC). There are three different approaches to applying non-polar stationary phases, based on mechanical deposition of liquids on the wall, chemical bonding of groups derived from monomers, and immobilization of polymers by cross-linking. Thus, the stationary phases are anchored to the wall with different mechanisms with consequently various chromatographic properties.

Mechanically coated viscous liquid phases, being attractive for the simplicity of column preparation, have been used in OTC LC[14,15], even with fused-silica columns[16]. Owing to a certain solubility of the stationary liquid in the mobile phase, the column stability is lower than that of bonded-phase columns. Furthermore, the exact film thickness is difficult to control.

In the preparation of chemically bonded stationary phases, *e.g.* octadecylsilane (ODS), the glass column material plays an important role. Columns have mainly been prepared from soda-lime glass or borosilicate glass with inner diameters down to 20 μm[17] and 8 μm[18], respectively. These columns have been subjected to various surface treatments, such as etching, in order to increase the inner surface area and the number of silanols available for bonding of the stationary phase via Si–O–Si–C bonds. Assuming that a certain stationary phase surface density can be prepared in different column dimensions, an increased retention can be obtained only with a reduced column inner diameter. Electro-etching of borosilicate columns, as described by Jorgenson and Guthrie[8], resulted in high k' values. Unfortunately, the electro-etching technique is limited to short columns owing to non-uniform etching in longer

capillaries[19]. The highest k' values have been reported on etched soda-lime glass capillaries, although these are difficult to prepare with small diameters owing to a tendency to clog[20]. Fused-silica columns in the range 30–100 μm have also been coated with ODS phases[21]. However, these columns gave low retention owing to insufficient surface coverage.

In principle, two types of siloxane stationary phase can be produced by bonding to surface silanols, *viz.* monomeric and polymeric bonded phases from monofunctional and polyfunctional reagents, respectively. The degree of cross-linking in polymeric bonded phases will depend on the type of silane used. If the trifunctional silanes react effectively they give a more dense network than does a difunctional silane. Extensive cross-linking will result in a phase of resinous character[22]. The rigid nature of such a phase results in restricted diffusion of the solute[23]. Another drawback of these polymeric phases is the difficulty in predicting the exact film thickness.

The third alternative to apply stationary phases in open tubular columns is to perform *in situ* cross-linking of silicone gums (linear high-molecular-weight polysiloxanes). By this technique, it is possible to create stationary phases of variable and well-defined thicknesses obtaining different degrees of retention, even in OTC LC[24]. The cross-linking process, which can be initiated by organic peroxides for example, results in carbon–carbon bonds between methyl and/or vinyl groups attached to silicon atoms (Si–C–C–Si, Si–C–C–C–Si). Only a small degree of cross-linking (0.1–1.0%) is required to change high-molecular-weight siloxane polymers to insoluble rubbers[25].

Silicones possess several advantageous properties[26]. The high flexibility of the Si–O bond explains the liquid-like behaviour of these polymers even at low temperatures[22], which is essential for efficient mass transfer in the stationary phase. It has been shown in GC that the solute diffusivity in gum phases is not altered by this type of cross-linking[27]. However, diffusion rates are lower than in low-molecular-weight solvents. The immobilized silicones are hydrolytically very stable and resistant to various solvents in terms of solubility[28]. Yerrick and Beck[29] reported that methanol and acetonitrile, typically used in reversed-phase LC, caused a minor (*ca.* 3%) swelling of dimethyl siloxane rubbers. The swelling effects on the chromatographic behaviour in RPLC have not been investigated. Recently, swelling of non-polar siloxanes was observed to increase the efficiency in supercritical fluid chromatography (SFC) when carbon dioxide and butane were used as mobile phases[30].

The cross-linking technique was introduced for capillary GC by Grob *et al.*[31,32] and has gained wide popularity. The columns have also been extensively used in SFC[33,34], where the stationary phases must resist strains under supercritical conditions that may be considerably more severe than in GC. For use in SFC, Fields *et al.*[35,36] have prepared immobilized phases with film thicknesses from 0.25 to 1 μm on untreated fused-silica capillaries down to 25 μm I.D. Polyorganosiloxane phases have also been applied in packed-column LC[37]. Silica and alumina particles were coated with immobilized organosiloxanes prepared from oligomers. With thin polymer films (0.27–1.52 nm), capacity factors increased with increasing film thickness.

Takeuchi *et al.*[38] introduced the cross-linking technique for OTC LC using etched soda-lime glass capillaries in the range 30–40 μm I.D. They cross-linked *in situ* different apolar polysiloxane phases typical for GC. The film thickness was not calculated since the roughened surface area of the capillary was not defined. Jorgen-

son *et al.*[39] applied the cross-linking technique to immobilize a vinyl-modified OV-17 polysiloxane in an etched 16 μm I.D. borosilicate capillary column. They stated that polymer-coated columns offer better stability than ODS bonded columns for LC. Recently Farbrot *et al.*[24] cross-linked non-polar siloxane gums of well-defined film thickness for use in reversed-phase OTC LC. Columns in the range 12–50 μm I.D. were prepared from untreated fused silica. The film thickness was varied from 25 nm to 625 nm, giving larger k' values with thicker films. The reported k' values were at least 10 times larger than values reported for ODS-modified fused silica.

The aim of the work reported here was to prepare non-extractable organosiloxane phases of various film thicknesses in 5–50 μm open tubular columns and to characterize in detail the chromatographic properties. The column development was made to establish practical OTC LC reversed-phase systems with high separation efficiencies. GC was used complementarily to evaluate the stationary phases, *e.g.* regarding retention and stability. The potential of these columns for use in routine LC work was preliminarily investigated.

THEORETICAL CONSIDERATIONS

The distribution of a solute between the mobile and the stationary phase can be described by the capacity factor k', which in partition chromatography is related to the distribution constant K and the phase volume ratio according to

$$k' = K\,V_s/V_m = K\,1/\beta \tag{1}$$

where s and m denote the stationary and the mobile phase, respectively. An increased retention in OTCs can thus be achieved by decreasing the phase ratio β, *i.e.* by increasing the film thickness for a given column inner diameter. However, the influence of different film thicknesses on column performance must also be considered.

Band-broadening in OTC chromatography is generally described by the mathematical model derived by Golay[40], where the plate height H is expressed as a function of the mobile phase linear velocity u by

$$\begin{aligned} H &= \frac{B}{u} + C_m u + C_s u \\ &= \frac{2\,D_m}{u} + \frac{1 + 6k' + 11\,k'^2}{96\,(1 + k')^2}\,\frac{d_c^2}{D_m}\,u + \frac{2k'}{3\,(1 + k')^2}\,\frac{d_f^2}{D_s}\,u \end{aligned} \tag{2}$$

where B, C_m and C_s are constants, depending on axial molecular diffusion and on resistance to mass transfer in the mobile phase and in the stationary phase, respectively. By this equation, the column performance is related to the tube inner diameter d_c, the stationary phase film thickness d_f, the solute diffusion in the mobile phase D_m and the solute diffusion in the stationary phase D_s. It is assumed that the stationary phase is a uniform film, coated on the inner wall of the tube.

In order to optimize the column dimensions, *i.e.* the phase ratio and the inner diameter, knowledge of the stationary phase diffusion rate is vital. Assuming a stationary phase diffusion coefficient of 10^{-5}–10^{-6} cm^2/s, Knox[2] and Poppe[41] have theo-

retically shown that a film thickness of 1/25 or 1/14 of the column internal diameter can be allowed for without sacrificing efficiency. Unfortunately, the literature lacks experimental data on stationary phase diffusion rates in OTC LC, especially regarding different immobilized polysiloxane phases. However, a batch measurement of the diffusion coefficient for anthracene in cross-linked OV-101 gave a D_s value of $5 \cdot 10^{-8}$ cm^2/s[42], indicating a relatively slow stationary phase mass transfer.

Fig. 1 shows a plot of plate heights, calculated from eqn. 2, for a 10-μm I.D. column. The chromatographic conditions resemble those used in the experiments of this paper (column no. 14, Table I). These curves demonstrate the effect that different stationary phase diffusion rates and film thicknesses will have on the column performance. With stationary phases where the diffusion rate is low, the maximum permissible film thickness will be smaller than that for films with higher diffusion rates. Considering a stationary phase diffusion coefficient of 10^{-8} cm^2/s, a ratio of film thickness to column inner diameter of *ca.* 1/70 will be more optimal, with respect to efficiency. However, with higher diffusion rates ($D_s = 10^{-6}$ cm^2/s), a very thick film will simultaneously yield a high efficiency as well as an adequate retention and sample capacity. For the thickest film in Fig. 1, a minimum plate height value by a factor of 3.5 is obtained if D_s has a value of 10^{-6} cm^2/s instead of 10^{-8} cm^2/s. Furthermore, the optimal flow-rate is affected. If eqn. 2 is an accurate model for band-broadening in 5–10 μm I.D. columns with immobilized polysiloxanes as stationary phases, then

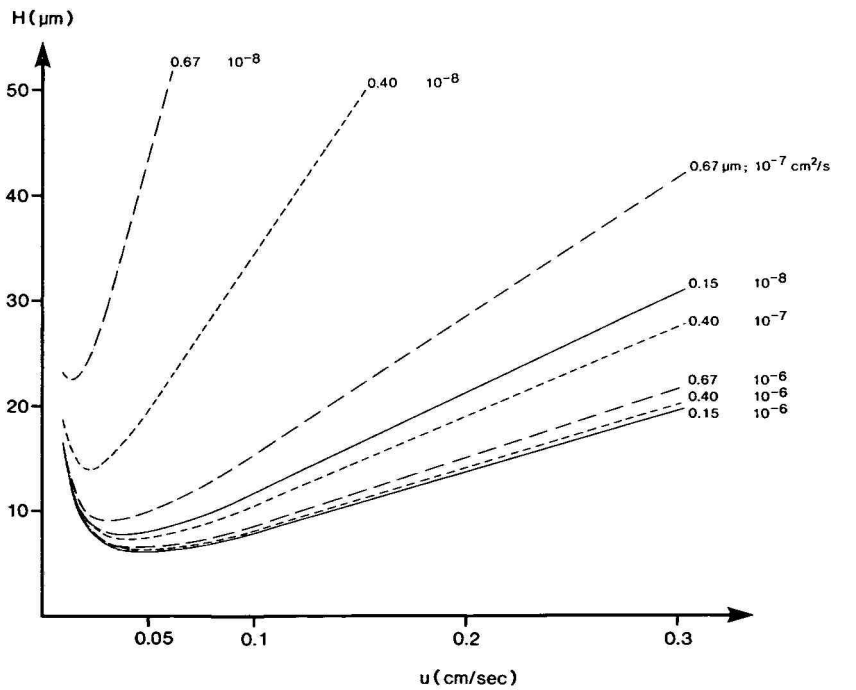

Fig. 1. Dependence of performance of a 10-μm OTC on different stationary diffusion coefficients and film thicknesses. Plate heights calculated from the Golay equation. Stationary phase film thickness and corresponding phase ratio d_f/d_C: (——) 0.15 μm (1/67); (– – –) 0.40 μm (1/25); (— — —) 0.67 μm (1/15). $D_m = 0.75 \cdot 10^{-5}$ cm^2/s; $k' = 0.5$.

TABLE I

DIMENSIONS OF FUSED-SILICA OPEN TUBULAR COLUMNS, COATED WITH IMMOBILIZED POLYSILOXANE LIQUID PHASES

Column	*I.D. (μm)**	*Length (cm)*	*Stationary phase*	*Concentration, C (% v/v)*	*β* (V_m/V_s)**	d_f *(μm)***
1	55.8	204	PS-255	0.2	499	0.028
2	55.4	204	PS-255	2.0	49	0.28
3	55.2	196	PS-255	2.0	49	0.28
4	54.7	218	PS-255	4.1	24	0.57
5	49.4	203	PS-255	4.8	20	0.62
6	53.1	209	PS-255	7.6	12	1.06
7	52.6	213	PS-255	10.9	8.2	1.56
8	51.6	187	PS-255	13.6	6.3	1.96
9	49.5	200	SE-54	2.0	49	0.25
10	53.8	212	SE-54	3.9	24	0.54
11	54.5	216	SE-54	3.9	24	0.55
12	49.4	225	SE-54	5.8	16	0.74
13	28.4	90	PS-255	2.0	49	0.14
14	11.6	208	PS-255	4.8	20	0.15
15	12.2	43	PS-255	2.0	49	0.062
16	5.3	45	PS-255	4.1	24	0.056

* After coating.
** Before solvent rinsing.

high performance at high separation speeds can only be achieved if the stationary phase diffusion coefficient is sufficiently high.

EXPERIMENTAL

Column preparation

The dimensions of the investigated columns are presented in Table I. The internal diameters of the coated columns were calculated from the column length and the resistance over the mercury-filled capillary, as described by Guthrie *et al.*[43]. A density of 0.98 g/ml for SE-54[44] was used for the calculation of the volumetric concentrations (v/v%), *C*, of the stationary phase solutions in Table I. Since data for PS-255 were not available, the value for a similar methylvinyl silicone gum (SE-33) of 0.98 g/ml was used.

The OTCs were prepared from 5–50 μm I.D. fused-silica capillaries (Scientific Glass Engineering, North Melbourne, Australia) of 0.5–2 m length by a static coating technique[45]. The coating solutions of polysiloxane phases were made in pentane at least 1 day before use. The concentrations were 2–13.6% (v/v) for PS-255 (methyl, 0.5–1.5% vinyl silicone, Petrarch Systems, Bristol, PA, U.S.A.) and 2–5.8% (v/v) for SE-54 (methyl, 5% phenyl, 1% vinyl silicone from General Electric, Applied Science, State College, PA, U.S.A.). Diisopropyl benzene peroxide (dicumyl peroxide, kindly supplied by H.-B. Larson, Unifos Kemi, Sweden) was added just before filling, and the mixture was then treated ultrasonically for 5 min. The concentration of the peroxide was 1% (w/w) of that of the polymer[46]. In order to remove acidic traces present

on the fused-silica surface[47], the untreated fused-silica capillaries were purged with helium for 30 min. The capillaries were then slowly filled with the stationary phase solutions by applying an appropriate helium pressure, which was gradually increased from 10 to 40 atm for 50 μm columns and to 80 atm for 5–10 μm capillaries. The device for column filling as well as sealing of the column end has been previously described[24]. After filling, the system was slowly depressurized. Static coating was performed with the capillary immersed in a water-bath at ambient temperature by applying vacuum to the column end. After coating, traces of pentane were removed by purging the capillaries with helium gas for 1 h. After flame-sealing, the silicone gum phases were immobilized by heating at 15°C/min to 175°C, then 175°C for 5 min[48]. The columns were then conditioned in a gas chromatograph at 2°C/min to 240°C and maintained at this temperature overnight before GC testing.

After testing, the columns were gently rinsed with acetone followed by dichloromethane and pentane, and subsequently dried in a gas stream before LC use. The columns were prepared for on-column fluorescence detection after the coating procedure. The polyimide outer coating was removed from 1 cm of the capillary by heat from a small butane flame. In order to avoid carbonization of the stationary phase, the column was purged with oxygen during flame-heating.

Gas chromatography

GC separations on 50-μm columns were performed in a Carlo Erba Fractovap 2101 gas chromatograph, modified for carrier gas pressures up to 15 atm and equipped with a flame ionization detector. The carrier gas was hydrogen at an average linear flow-rate of *ca.* 45 cm/s. Split injections (split ratio 1:2000) of 2-μl samples were made with a 10-μl gastight syringe (Hamilton, Bonaduz, Switzerland). The injected amount of each solute was *ca.* 40 pg.

Liquid chromatography

The LC system is presented in Fig. 2. Low volumetric flow-rates were created using a simple split-flow arrangement with an LDC Constametric I pump or a Varian 5000 pump[49]. The volumetric flow-rate was calculated from the measured column dimensions and the retention time of an unretained solute. Injections were made by static splitting or by heartcutting[50], using an electrically actuated Valco valve equipped with an external 20-μl loop. With static splitting, the split-tee was connected directly to the injector outlet (split-tee in position D) and the pump directly to the injector. Split ratios between 1:1000 and 1:25 000 were used to minimize band-broadening from the injection system, as well as to allow the use of pump flow-rates of 0.5–4 ml/min. In particular, the largest ratio was used when determining and comparing the HETP curves for different columns. The relative standard deviation of the retention times was typically 0.3–0.5%. With the heartcutting technique (outlined in Fig. 2), a narrow-bore fused-silica capillary was inserted between the split-tee coupling and the valve injector (between B and C). This was made to lower the volumetric flow-rate during the period of time when the on-off valve (D) was opened. Thereby, the injection times could be varied between 10 and 60 s.

On-column fluorescence detection was performed with a Shimadzu Model RF-530 fluorescence detector, which was modified and equipped with liquid filters to reduce background emission, caused by scattered light from the on-column cell[49].

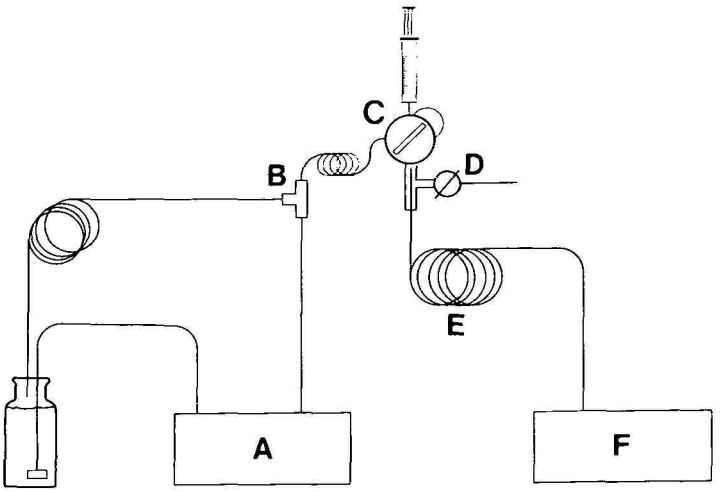

Fig. 2. Instrumental set-up for open tubular column LC. (A) Conventional LC pump; (B) split tee; (C) conventional LC loop injector; (D) on-off valve for "heart-cutting" or restrictor for static splitting; (E) open tubular column; (F) fluorescence detector. For details, see text.

The detection system was appropriate for columns down to 25 μm I.D. For high sensitivity detection, and with columns down to 5 μm I.D., the conventional fluorescence detector was replaced with a laser-induced fluorescence detection system[12,13]. A He–Cd laser, Model 4210NB from Liconix, yielded an output power of 1.5 mW at the UV line 325 nm. The layout of the optical system has been previously described[13,49].

Chemicals

A GC test solution was made in *n*-hexane (p.a.) and the solutes were the *n*-alkanes (reference substances for GC), C_{10}, C_{11}, C_{12} and C_{13}, *n*-octanol and naphthalene, all at *ca.* 40 mg/l. These chemicals as well as *n*-pentane and *n*-heptane (p.a. grade) were from Merck (Darmstadt, F.R.G.).

Different anthracene and fluorene derivatives, suitable for the fluorescence excitation wavelengths available, were selected as model compounds for LC. 9-Fluorenylmethanol was purchased from Fluka (Buchs, Switzerland), 9-phenylfluorene from Sigma (St. Louis, MO, U.S.A.) and 2-aminoanthracene from EGA (F.R.G.). The other anthracene derivatives were synthesized at the Department of Organic Chemistry, University of Göteborg. The solutes were dissolved in the mobile phase prior to injection. For the separation of amino acids, Vaminolac (KabiVitrum), an intraveneous solution containing seventeen of the common protein amino acids was used. The OPA–mercaptoethanol reagent and buffers were prepared according to ref. 51. Acetonitrile and methanol of HPLC grade was from Rathburn Chemicals (Walkerburn, U.K.) and the water was doubly distilled.

Ancillary equipment

Chromatograms were recorded on a Perkin-Elmer 56 strip chart recorder and a Spectra Physics 4270 integrator. Peak widths and retention times were measured manually from the chromatograms as well as calculated from the data recorded by the integrator.

Procedures

Film thickness. For low stationary phase concentrations, the stationary film thickness, d_f, is generally calculated from the column radius r and the stationary phase concentration, C (v/v%):

$$d_f = r \cdot C/200 \tag{3}$$

With increasing concentrations, this expression is no longer valid. For this reason, an exact expression was derived. The film thickness was calculated from the column radius measured after coating, r', and the concentration of the coating, r', solution, according to:

$$d_f = r' \left[1 + \frac{1}{100/C - 1} \right]^{1/2} - r' \tag{4}$$

The mobile to stationary phase volume ratio was obtained from

$$\beta = V_m/V_s = 100/C - 1 \tag{5}$$

Retention. Capacity factors were calculated in LC and GC from $k' = (t_r - t_0)/t_0$, where t_r and t_0 are the retention times of a retained and an unretained peak, respectively. In LC the capacity factors were determined at ambient temperature. With the 50 μm I.D. columns, nitromethane was tested for indicating the column void volume. A weak negative peak was obtained, owing to absorption of the excitation light. The retention time was found to be the same for the fluorene–methanol derivative, with acetonitrile–water (40:60) as mobile phase, except for the thickest films, where fluorene–methanol was slightly retained. With the anthracene derivatives, the 9-anthracenecarboxylic acid was used as an unretained peak.

The 50-μm columns were evaluated for GC by measuring capacity factors of *n*-dodecane and *n*-tridecane at 90°C. Owing to the high load of the stationary phase, the gas hold-up time (t_0) marker, butane, was retarded at the separation temperature, 90°C. Thus, t_0 was measured at 220°C and extrapolated from hydrogen viscosity data to the temperature actually used[52], according to:

$$t_0(90) = t_0(220)\ 0.812 \tag{6}$$

The gas hold-up time in a 2-m column is very short and can therefore not be accurately measured by simply injecting a non-retained component. Therefore, an alternative procedure was used, namely recording the time for emptying the column. Butane was injected into a split/splitless injector in the splitless mode (preset split flow 120 ml/min). The injector and column were filled with a dilute gas sample, which was detected by the flame ionization detector. When a signal decrease, due to sample dilution was observed, the split valve was opened. The time from this event to the distinct decrease in the detector signal, which was caused by the replacement of sample in the column by pure carrier gas, determined the gas hold-up time.

Selectivity. The selectivity in LC was calculated from $\alpha = k'_2/k'_1$. In GC,

measurements of Kovats' retention indices (RI)[53] for *n*-octanol and naphthalene were made at 90°C and calculated according to:

$$RI = 100 \cdot \frac{\log t'_{R,x} - \log t'_{R,n}}{\log t'_{R,n+1} - \log t'_{R,n}} + 100n \quad (7)$$

where the compound x is eluted between two adjacent *n*-alkanes with carbon numbers n and $n + 1$, each with the adjusted retention time t'_R.

Efficiency. The theoretical plate height was calculated from:

$$H = \frac{L\, w_{0.5}^2}{5.54 t_R^2} \quad (8)$$

where $w_{0.5}$ is the peak width at half the peak height. For each column the plate heights were determined in LC at five different flow-rates and for at least three injections at each flow-rate. The experimentally obtained plate heights were compared with the plate heights calculated from eqn. 2. The stationary film thickness and the column internal diameter were obtained as described above. The diffusion coefficients for the test solutes in the mobile phase were calculated according to ref. 54: 9-anthracenecarboxylic acid, $D_m = 0.68 \cdot 10^{-5}$ cm²/s; 9-cyanoanthracene, $0.72 \cdot 10^{-5}$ cm²/s; anthracene, $0.78 \cdot 10^{-5}$ cm²/s. The slopes of the experimentally determined HETP curves were obtained from linear regression. This allowed the subsequent calculation of the stationary phase diffusion coefficient D_s by rearranging eqn. 2.

RESULTS AND DISCUSSION

Column preparation

In the present study, all columns were prepared from untreated fused-silica tubing, which is known to possess sufficient wettability for coating with non-polar polysiloxanes[55]. The methylvinyl silicone gum PS-255, suggested for the preparation of thick films in GC by Grob and Grob[46], and the methylphenylvinyl silicone gum SE-54, were selected for comparison of retention, selectivity and efficiency. The general structure of these gum phases is shown in Fig. 3. The vinyl substitution makes these siloxanes easy to cross-link. This is important in the fabrication of thick films, since a decreasing degree of immobilization with increasing film thickness was reported by Grob and Grob[46], who obtained an extractability of more than 50% for a 5-μm SE-54 film, whereas a 0.2-μm film was virtually non-extractable.

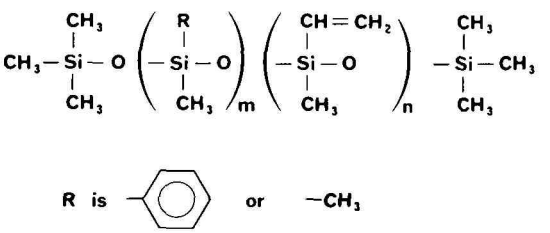

Fig. 3. General structure of the polysiloxane gum phases.

Preparation of small-bore columns with the static coating technique is facilitated by the use of coating solutions of relatively low viscosity. In this respect, PS-255 is to be preferred to SE-54 as well as to the methyl silicone gum phase OV-1[46]. The low viscosity methyl silicone oil phases, such as OV-101, produce coating solutions that yield a faster column filling. These phases are, however, more difficult to cross-link and require a considerably higher concentration of organic peroxide to produce the same degree of cross-linking than do the corresponding gum phases[32,56]. Unfortunately, there are indications from previous work in GC of a reduced column inertness from a high peroxide concentration of the radical initiator[31,56,57]. Various initiators for cross-linking non-polar polysiloxanes have been used in capillary GC. Wright *et al.* obtained highly inert columns when cross-linking non-polar siloxanes by repeated treatment with azo-*tert.*-butane[56]. The use of reactive organic peroxides, such as benzoyl peroxide, resulted in increased column activity[31,56,57]. In contrast, the column inertness was not significantly changed when low concentrations of the less reactive dicumyl peroxide (DCUP) were used to cure SE-54[56]. Despite this, a small increase in activity was observed for a polydimethyl siloxane gum, SE-30[57]. A reduced inertness was reported at higher concentrations (more than 1%) of DCUP[58]. Furthermore, residues of DCUP were incorporated in the polymer structure by covalent bonding when it was used to cure a methylvinyl silicone (SE-33)[59]. Thus the amount of peroxide should be minimized, adjusted only to give a sufficient degree of phase insolubility.

A series of 50-μm columns was prepared for evaluation of the maximum amount of stationary phase obtainable (Table I). The static coating technique allows control of the volume ratio of mobile to stationary phase as well as of the film thickness, and columns were successfully prepared from solutions with up to 13.6% PS-255 gum. With the SE-54 gum phase, it was difficult to prepare homogeneous solutions over 6% (v/v). The highest phase concentration (13.6%, column 8, Table I) corresponds to a phase ratio, $\beta = V_m/V_s$, of 6.3. The dimensions of these columns may be compared with the capillary columns generally used in GC, which are typically made from 0.2–0.5% solutions, corresponding to a phase ratio, $\beta = V_m/V_s$, of 200–500. Grob and Grob[46] prepared thick film GC columns with 8-μm PS-255 films in 0.32 mm I.D. capillaries, corresponding to a stationary phase concentration of 9.8% v/v and a β value of 9.3. The even lower phase ratios presented in Table I for columns 7 ($\beta = 8.2$) and 8 ($\beta = 6.3$) show that it is possible to produce capillary columns with a higher load of stationary phase, although the absolute film thickness in the 50-μm columns is smaller.

When very concentrated solutions are handled for coating capillary columns by the static technique, the risk of bubble formation is enhanced, since the coating solution is saturated with gas when it is forced into the capillary by high pressure gas. When nitrogen was used, bubbles were released at the column end during filling. This was prevented by the use of the less soluble helium gas. Occasionally, at low flow-rates of coating solution, the final millimetres of the capillary were not filled unless evaporation of the solvent was hindered by temporarily sealing the end.

The time needed for solvent evaporation in the static coating procedure depends on column dimensions such as length and inner diameter as well as on the viscosity of the coating solution. Accordingly, the 50 μm I.D. columns, all of *ca.* 2 m length, required coating times ranging from 65 min for the less concentrated so-

lutions to 130 min for the most concentrated polysiloxane solutions. In contrast, a column of the same length but with 11.6 μm I.D. (column 14, Table I) required 4 h for coating. The risk of breakthrough (*i.e.* bubble formation in the capillary) was minimized by performing the coating at ambient temperature.

The preparation of 5–10 μm columns, as compared with wider bore columns, means longer times for filling and coating. Consequently, an increased number of failures such as blocking and breakthrough was observed. Despite these difficulties, the presented method allows a successful preparation of OTCs down to 5 μm I.D. However, more work on these small capillaries is needed for coating longer columns with thick stationary phase films.

Using laser-induced fluorescence detection, a considerable increase in fluorescence background was obtained from thick film coatings. The fluorescence emanates from products formed in the capillary during the flame destruction of the polyimide coating at the detection point. This artefact can be avoided by coating the capillary after removal of the polyimide film.

Column stability

The stability in terms of phase immobilization was examined by GC. Capacity factors were measured before and after solvent rinsing. The loss of stationary phase was typically 8–10%, irrespective of film thickness. The effect of a 9% loss in retention on the phase ratio and film thickness is exemplified in Table II. In general, a higher efficiency was measured by GC after column rinsing.

A certain mechanical instability was indicated for the thicker films (columns 7 and 8, Table I) during the LC experiments. A variation in retention times was observed, owing to fluctuations in the flow resistance, especially at low flow-rates. This effect was partly caused by an incomplete decomposition of the stationary phase at the ends of the detection zone during removal of the column outer protective polyimide coating. The stationary phase is not attached to the column wall by chemical bonding since it was coated directly on the untreated fused-silica surface. A deactivation layer, containing organic groups that are amenable to covalent linkage to the stationary phase, is expected to increase the mechanical stability of the phase[60].

The effect of aqueous mobile phases on column inertness was monitored by using *n*-octanol as a GC test substance before and after LC use. Symmetrical peaks were observed in all cases for the *n*-alkanes. Thick films exhibit a shielding effect on the fused-silica surface and show symmetric peaks of *n*-octanol. Furthermore, thick film columns show no significant change in peak symmetry after LC use. The situa-

TABLE II

CHANGES IN PHASE RATIO, $\beta = V_m/V_s$, AND FILM THICKNESS, d_f, MEASURED BY GC AFTER SOLVENT RINSING

Column	*Before rinsing*		*After rinsing*	
	β	d_f (μm)	β	d_f (μm)
3	49	0.28	54	0.25
7	8.2	1.56	9.0	1.43

TABLE III

EFFECT OF AQUEOUS MOBILE PHASES ON FUSED-SILICA COLUMN INERTNESS AT VARIOUS FILM THICKNESSES

Kovats' retention indices for *n*-octanol measured by GC at 90°C.

Column	*Stationary phase*	*RI before LC*	*RI after LC*
4	PS-255, 0.57 μm	1057	1060
7	PS-255, 1.56 μm	1054	1054
10	SE-54, 0.54 μm	1074	1080

tion for thin film columns is somewhat different. Firstly, tailing is observed on the freshly prepared columns. Secondly, the retention index of *n*-octanol increases markedly after extensive washing with aqueous solvents (Table III), reflecting a higher silica surface activity.

An attempt was made to examine the presence of acidic sites in the LC column by injection of a basic solute, 2-aminoanthracene, on untreated 50-μm capillaries, as well as on columns with various film thicknesses. This test did not reveal any adsorption, since symmetrical peaks were observed for the amine on all columns tested.

Retention

Farbrot *et al.*[24] reported an increased retention for 0.03–0.6 μm thick films of non-polar polysiloxanes. This range has in the present study been extended to cover film thicknesses up to 2 μm (Table I). Fig. 4a presents LC data on the dependence of k' on the initial phase ratio, representing a film thickness of 0.28–2 μm for PS-255 and of 0.25–0.74 μm for SE-54 in 50-μm columns. A linear increase in retention is observed for these thicker films. It should be noted that the true β values are slightly higher than those presented in Table I (see Table II), owing to removal of the soluble part of the polymer by solvent rinsing before LC testing.

The LC retention data are supported by the data from the GC evaluation of the same columns. The capacity factors in GC (measured on non-rinsed columns) for *n*-dodecane and *n*-tridecane as a function of phase ratio are presented in Fig. 4b. Compared with the LC data in Fig. 4a, for fluorene derivatives, the difference in k' values between SE-54 and PS-255 in GC is less significant. This is reasonable since the phenyl substitution in SE-54 affects the distribution constant to a lesser extent for an alkane than for an aromatic compound.

Retention was also investigated in columns with approximately the same phase ratio, but in different inner diameters (Table IV). A rough agreement was obtained between the 54.7 and 11.6 μm I.D. columns. The 5.3 μm I.D. column shows higher capacity factors, indicating a higher load of stationary phase than expected. Note the change in selectivity with decreasing inner diameter.

In Table V, the retention behaviour for columns 7, 8 and 12 are compared with data from the literature. From the data obtained by Takeuchi *et al.*[38] on cross-linked polysiloxane phases in OTC LC, it can be seen that the capacity factors for the thickest films in the present work are roughly 3–9 times higher. The retention on the thickest PS-255 phase is also higher, by a factor of *ca.* 3, even when compared with their data on a chemically bonded ODS phase. Recently Vargo[19] reported the

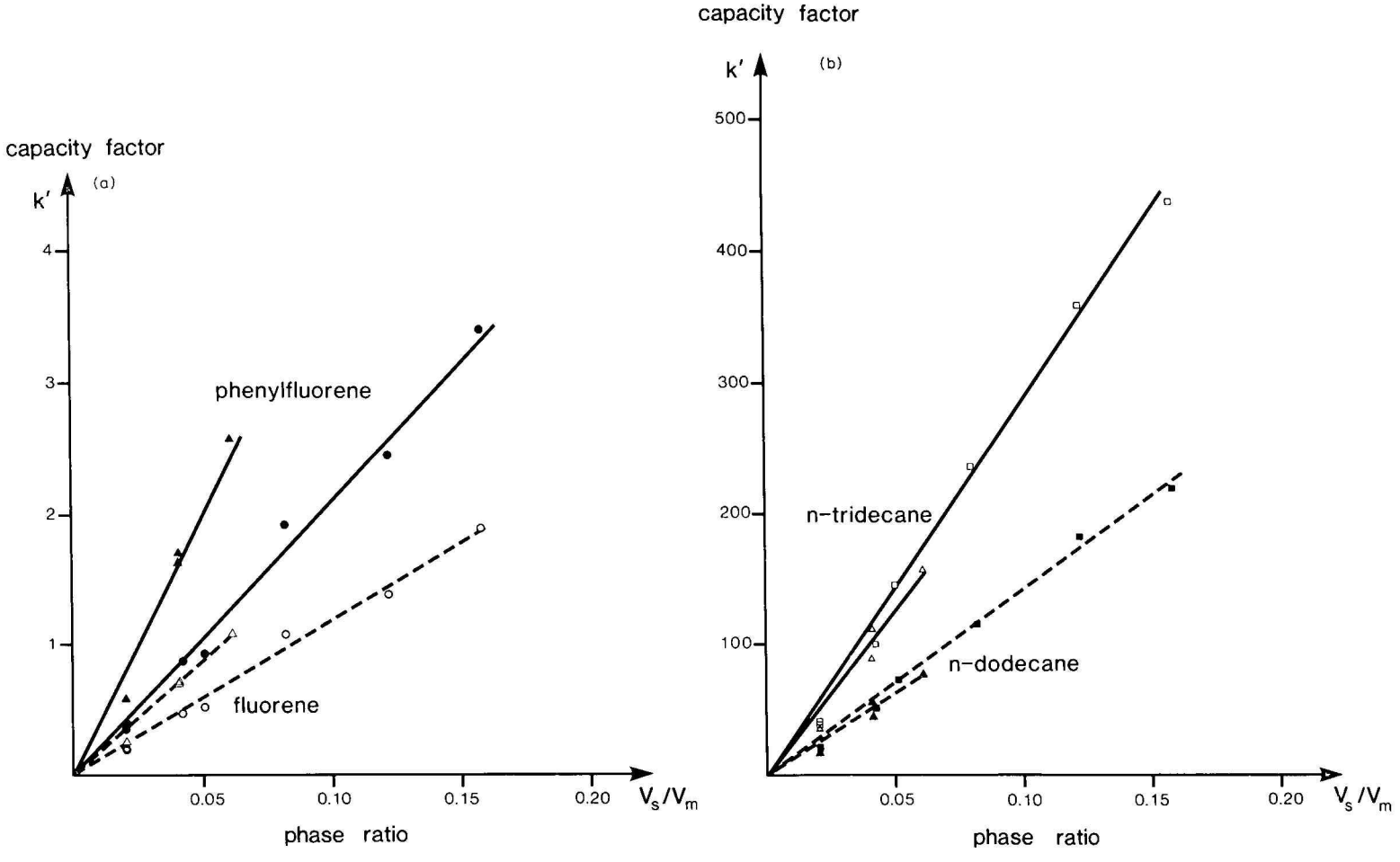

Fig. 4. (a) Dependence of capacity factors on phase ratio in OTC LC for two immobilized polysiloxane stationary phases. Columns, No. 2–12, 2 m × 50 μm I.D.; mobile phase, acetonitrile–water (40:60); solutes, fluorene and phenylfluorene. (b) Dependence of capacity factors on phase ratio in OTC GC for two immobilized polysiloxane stationary phases. k' values were measured at 90°C before column rinsing. Columns as in (a). Solutes, *n*-dodecane and *n*-tridecane. Data points: (a) circles, PS-255; triangles, SE-54; (b) squares, PS-255; triangles, SE-54.

TABLE IV

COMPARISON OF LC RETENTION IN COLUMNS OF DIFFERENT INTERNAL DIAMETERS

Mobile phase, acetonitrile–water (40:60).

Column	*I.D. (μm)*	d_f *(μm)*	*Capacity factor (k')**		*Selectivity (α)*
			Cyano-anthracene	*Anthracene*	
4	54.7	0.57	0.12	0.52	4.3
14	11.6	0.15	0.14	0.46	3.3
16	5.3	0.056	0.39	1.28	3.3

* Normalized to the β-value of columns 4 and 16.

highest capacity factors obtained thus far in OTC LC, using an ODS phase on etched soda-lime columns. Compared with these ODS phase values, a slightly higher retention was obtained for the thickest PS-255 phase (column 8) in the present work. In addition, considering the effect of swelling (see Corollary), a further enhancement in capacity factors of up to 10 times is possible on the cross-linked polysiloxane phases. The retention will then be considerably higher than that obtainable on chemically bonded ODS phases, even in highly etched soft glass columns.

Selectivity

Selectivity factors in LC for fluorene and phenylfluorene are presented for 50 μm I.D. columns in Table VI. A difference in selectivity is found between the siloxanes studied. The highest α values, obtained on the phenyl-substituted phase SE-54 (methyl, 5% phenyl, 1% vinyl silicone), reflect the higher affinity for the solute phenyl group with this phase.

The results from GC measurements of Kovats' retention indices of *n*-octanol and naphthalene are displayed in Table VII. The difference in stationary phase composition is obvious from the higher selectivity obtained for an aromatic substance,

TABLE V

COMPARISON OF LC RETENTION ON IMMOBILIZED POLYSILOXANE LIQUID PHASES WITH OTC LC DATA REPORTED IN THE LITERATURE

Reference	*Column*	*Stationary phase*	*k' in acetonitrile–water**	
			30:70	*50:50*
This work	7	PS-255	7.0	0.51
This work	8	PS-255	9.5	0.71
This work	12	SE-54	5.2	0.38
38	Soda-lime	OV-1	0.78	–
38	Soda-lime	SE-54	1.6	–
38	Soda-lime	ODS	3.31	–
20	Soda-lime	ODS	–	0.65

* Solute anthracene.

TABLE VI

SELECTIVITY FACTORS FOR STATIONARY FILMS LARGER THAN 0.25 μm OF PS-255 AND SE-54 IN OTC LC

Solutes, fluorene and phenylfluorene; mobile phase, acetonitrile–water (40:60); columns, 50 μm I.D.

Column	*Phase*	*α*
2	PS-255	1.83
3	PS-255	1.82
4	PS-255	1.81
5	PS-255	1.79
6	PS-255	1.78
7	PS-255	1.77
8	PS-255	1.80
9	SE-54	2.36
10	SE-54	2.38
11	SE-54	2.33
12	SE-54	2.37

naphthalene, on the SE-54 columns 9–12 than on the PS-255 columns 4–8. The significantly higher retention index values for *n*-octanol on columns 5 and 9 probably originate from a higher surface activity for one of the batches of fused-silica material. Batch-to-batch variations of the fused silica can be handled by deactivation of the capillaries.

As can be seen from LC data in Table VI, the selectivity is constant for film thicknesses larger than 0.25 μm. In contrast, an unexpected deviation in selectivity is observed for thin film columns. The data in Table IV, for the solute pair anthracene–cyanoanthracene, show significantly lower α values for the 5- and 11.6-μm columns compared with a 50-μm column of the same phase ratio. Furthermore, selectivity changes are also observed for 50 μm columns with thin stationary phase films (Table VIII). The α value for the solutes phenylfluorene–fluorene with the 28-nm film (column 1) is significantly higher than with the 280-nm film (column 2), and an even higher increase in α is observed for a narrow-bore column (column 15). Thus,

TABLE VII

KOVATS' RETENTION INDICES FOR *n*-OCTANOL AND NAPHTHALENE AT 90°C

Sample amount, 40 pg.

Column	*Phase*	*RI (n-octanol)*	*RI (naphthalene)*
4	PS-255	1057	1154
5	PS-255	1064	1155
6	PS-255	1056	1154
7	PS-255	1054	1154
8	PS-255	1053	1154
9	SE-54	1093	1187
10	SE-54	1074	1186
11	SE-54	1074	1186
12	SE-54	1076	1187

TABLE VIII

SELECTIVITY FACTORS FOR STATIONARY FILMS UP TO 0.28 μm OF PS-255

Solutes, fluorene and phenylfluorene; mobile phase, acetonitrile–water (30:70).

Column	*I.D. (μm)*	d_f *(nm)*	β	α
2	55.4	280	49	2.9
1	55.8	28	499	3.5
15	12.2	62	49	4.1

the retention and selectivity behaviour of these polymer films are related not only to the phase ratio: the absolute film thickness and the column inner diameter must also be considered.

The different selectivities obtained may have several origins. The thin films discussed have thicknesses ranging from 25 nm to 150 nm. Assuming a uniform film and using 126 Å^3 (calculated from density data) as the volume of a $Si(CH_3)_2O$ group, the corresponding number of dimethylsiloxy units is 50–300. As shown in GC, adsorption of the solute on the fused-silica surface, are diffusion through these thin layers, was observed for *n*-octanol on column 1. This effect is less likely to occur in the LC experiments since no adsorption was observed on uncoated fused-silica capillaries for the test solutes employed in this study. Still, the special behaviour of thin films may be an effect of the fused-silica surface. It is known that a methylsilicone can be adsorbed on a silica surface, possibly via interactions with the silanol groups[61]. A local effect on the polymer structure, such as a certain polymer chain orientation, seems likely. It may be speculated that the helical conformation of dimethylsiloxane chains[62], where the methyl groups are pointing outwards while the siloxane bonds are shielded, may be disrupted close to the silica surface. The apparent selectivity of the phase could then be changed. Whether adsorptive interactions in the stationary phase to mobile phase interphase contribute to the total retention and selectivity behaviour, as has been indicated in packed column LC[63], is not clear. More investigations are needed to reveal the detailed selectivity behaviour of thin polysiloxane films.

Efficiency

The performance of OTCs with different immobilized silicone gum phases is shown as HETP plots in Figs. 5 and 6. For an unretained solute on the 50-μm columns with the PS-255 phase (Figs. 5a and 5b), a close agreement was found between experimental data and values calculated from eqn. 2. This indicates the validity of eqn. 2 for unretained solutes for these column dimensions, and that any extra-column band-broadening, attributed to the on-column detector and to the split injector, was negligible in the present study. This condition was further verified by measuring the band-broadening in untreated 50 μm I.D. tubes. Despite this, column 10 with a SE-54 phase gave higher plate heights for the unretained solute than expected (Fig. 5c), although the same LC system was used. Whether this is due to the unretained solute being in fact slightly retained on that particular column or to some other effect is not clear.

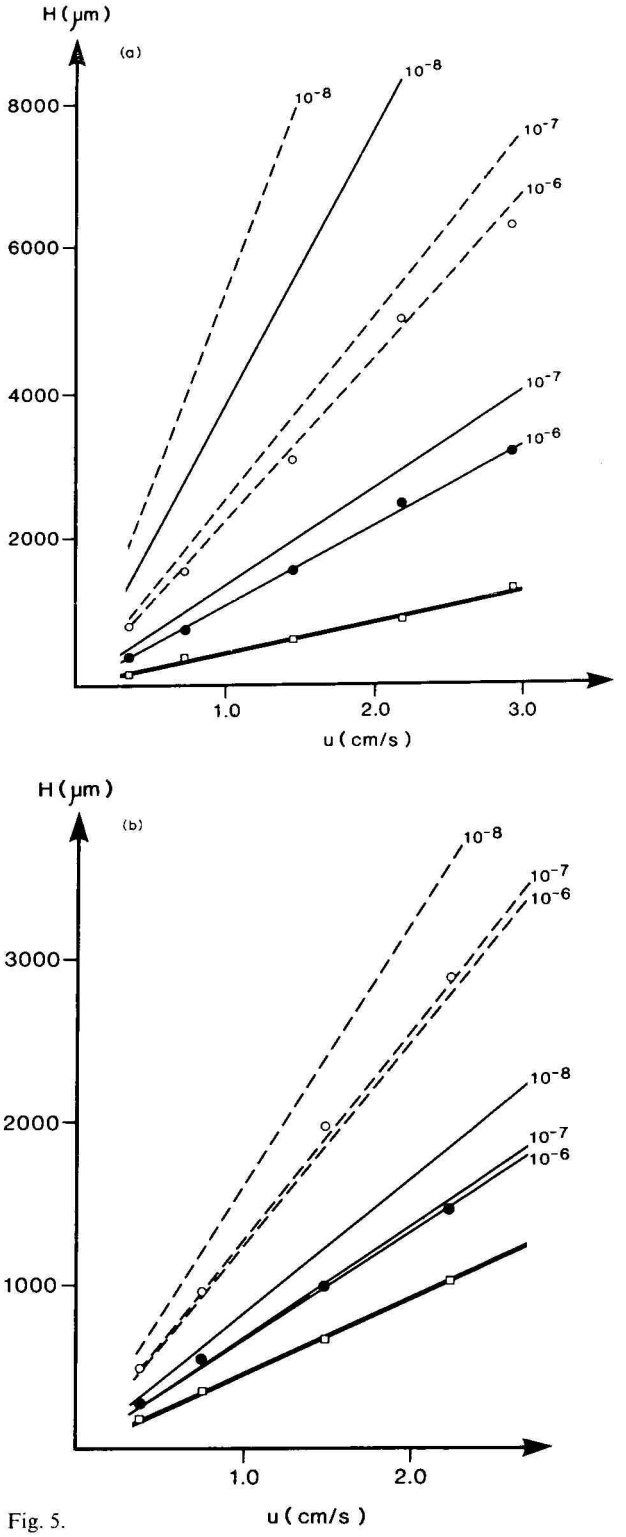
H (μm)
(a)
8000
6000
4000
2000
10^-8
10^-8
10^-7
10^-6
10^-7
10^-6
1.0
2.0
3.0
u (cm/s)
H (μm)
(b)
3000
2000
1000
10^-8
10^-7
10^-6
10^-8
10^-7
10^-6
1.0
2.0
u (cm/s)

Fig. 5.

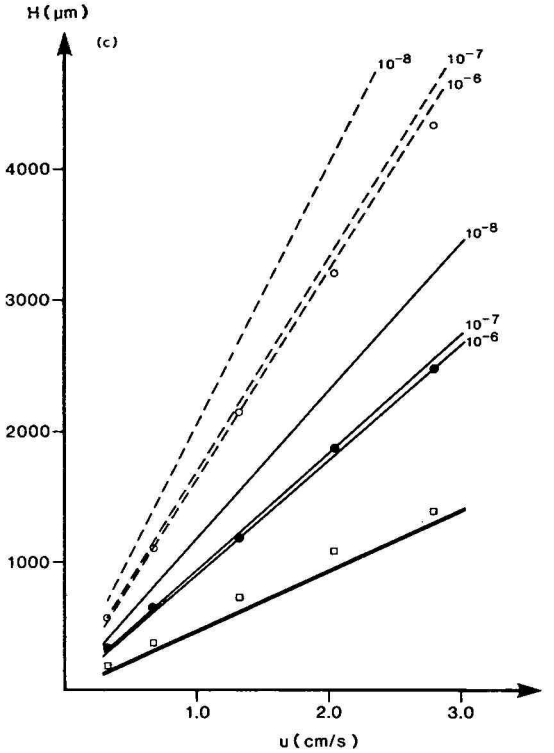

Fig. 5. Plots of experimental and calculated plate heights *versus* linear velocity for different polymer phases and film thicknesses in 50 μm I.D. columns. Calculated plate heights from the Golay equation using D_s values of 10^{-6}, 10^{-7} and 10^{-8} cm²/s. (a) Column 7, PS-255; d_f = 1.56 μm; k'_{CN} = 0.40; k'_{ANT} = 1.70; (b) column 4, PS-255; d_f = 0.57 μm; k'_{CN} = 0.12; k'_{ANT} = 0.52; (c) column 11, SE-54; d_f = 0.55 μm; k'_{CN} = 0.25; k'_{ANT} = 0.85. Experimental plate heights: □ = 9-anthracenecarboxylic acid; ● = 9-cyanoanthracene; ○ = anthracene. Calculated plate heights: ———, ——— and – – – for solute □, ● and ○, respectively. Mobile phase, acetonitrile–water (40:60); temperature, 23°C. Further details in text.

Using eqn. 2 and the experimental values of the tube inner diameter and the film thickness, plate heights were calculated for different D_s values and plotted together with the experimental data in Figs. 5 and 6. These results show that the apparent D_s value is 0.7–2.5 · 10^{-6} cm²/s for column 7 with a 1.56-μm thick PS-255 film, as well as for column 11 with a 0.55-μm SE-54 film. For column 4, with a thinner film of PS-255, 0.57 μm, a lower D_s value, ≃ 10^{-7} cm²/s, was obtained. For column 14, with the PS-255 phase and 11.6 μm I.D., the stationary phase diffusion rate is even lower, 0.9–2.2 · 10^{-8} cm²/s.

The somewhat surprising tendency of achieving lower plate heights for thicker polymer films (compare column 4 and 7 above) have in fact been observed earlier. Both Cramers *et al.*[27], in capillary GC, and Kirkland[63], in LC with polymer-coated particles, observed the same effect. The benefits from this, high performance at high separation speeds, should be clear in the light of the discussion in the theory section. With very thick polymer films on particles, a slow equilibration between the mobile and stationary phase was reported to lead to an increase in peak asymmetry at higher

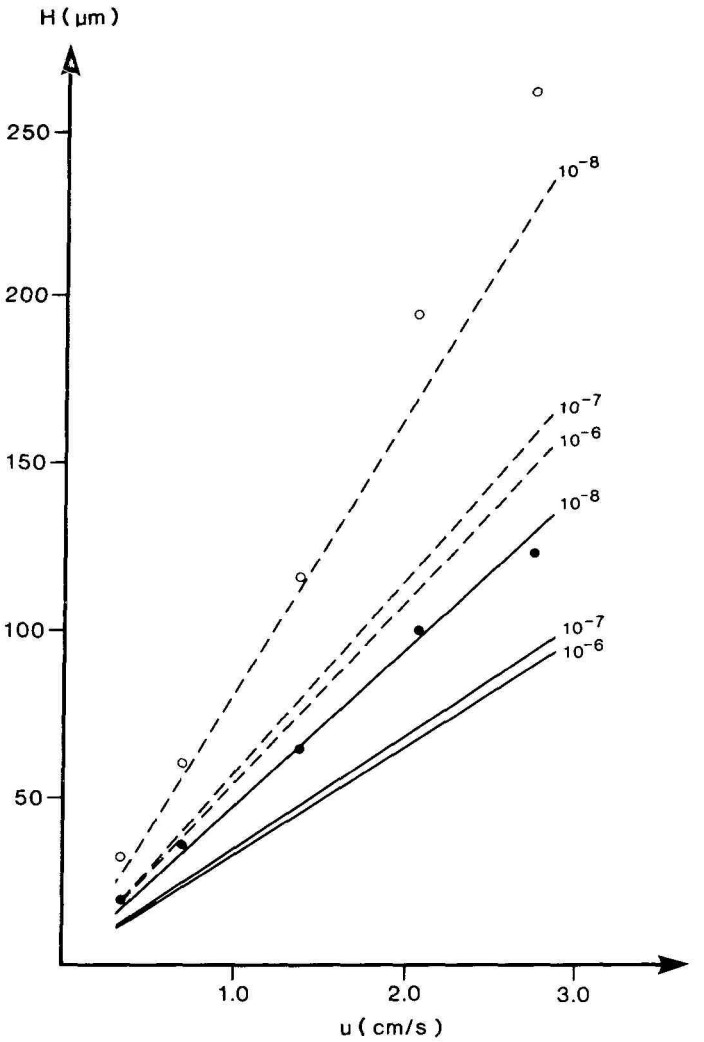

Fig. 6. Plot of experimental and calculated plate heights *versus* linear velocity for column 14, PS-255; d_f = 0.15 μm; 197 cm × 11.7 μm I.D.; k'_{CN} = 0.16; k'_{ANT} = 0.50. Other details as in Fig. 5.

velocities[62]. It should, however, be noted that symmetrical peaks were obtained throughout these investigations, even for the thickest films and at high mobile phase velocities.

The differences in column performance, even with the same type of polymer but in different film thicknesses, as well as in different column inner diameters, may have various origins. The differences in the final polymer film structure, when varying the absolute film thickness, as discussed above (see Selectivity and Tables IV and VIII) may also affect the apparent stationary phase diffusion rates. However, existence of irregularities in the stationary phase, due to drop formation, seems unlikely since in GC no corresponding difference in efficiency was observed between these columns.

Slow interfacial mass transfer[64] may also be the cause of differences in performance, an effect that should increase in importance with smaller column inner diameters. The lower D_s value obtained for the 11.6-μm column, compared with the 54.7-μm column, which has approximately the same phase ratio, supports the occurrence of this effect. A slow interfacial mass transfer should also cause a larger effect for solutes with larger k' values. However, the experimental uncertainty in the plate heights does not permit any observation of significant variations in D_s with k' for these columns.

The highest efficiency, in number of plates, was obtained for column 14 (1.97 m × 11.7 μm I.D.). A standard separation of anthracene derivatives on this column is shown in Fig. 7, as well as a comparison of the performance of the OTC with that of a packed column. The linear velocity during the OTC LC separation was 0.35 cm/s, which is a factor of *ca.* 10 higher than the assumed optimal flow-rate, whereas for the packed column, the rate was near the optimal. Operating column 14 close to optimal conditions (flow-rate 0.042 cm/s) yielded very high plate numbers: 351 000 ($k' = 0.16$) and 266 000 ($k' = 0.5$) plates for peak 1 and 3, respectively. Furthermore, for the corresponding plate heights at this low flow-rate an excellent agreement was obtained between the experimental values, H_{exp}, and those calculated from the Golay equation, H_{Gol}, using the experimentally determined D_s values obtained at high flow-rates. For peak 1, $H_{exp} = 5.6$ and $H_{Gol} = 5.2$, and for peak 3, $H_{exp} = 7.4$ and $H_{Gol} = 7.4$.

The relative performance of different column types can be compared by using the Knox separation impedance parameter[2], $E = h^2\varphi$, where h is the reduced plate height and φ the flow-resistance parameter. The optimal value for a packed column should ideally be 2000, whereas the value for an OTC operated under optimal conditions is substantially lower, *e.g.* as low as *ca.* 13 for column 14 ($k' = 0.5$). The E values for peak 1 in Fig. 7 are 1800 and 3300 for the OTC and the packed column, respectively. A comparison of the performance of OTCs and packed columns based solely on the separation impedance can, however, be somewhat misleading. For packed columns the efficiency, expressed as the plate number, is roughly the same for all k' values in a chromatogram, whereas for OTCs there is a decrease in efficiency at higher k' values, and thereby only a narrow capacity factor range gives optimal conditions in an OTC separation. In addition, the hold-up time of an OTC, with a bore larger than the particle size in the packed column, is long compared with that of a packed column of similar efficiency. A straightforward approach is to run the same separation on the two column types. In Fig. 7 no thorough optimization was made of the respective separation conditions. Nevertheless, the elution order of the anthracene derivatives is the same on the OTC with the PS-255 phase as on the packed column with the ODS phase. It is also shown that with the same total separation time the hold-up time for the OTC is much longer. Furthermore, owing to the different selectivity, an enhanced resolution was obtained for peaks 1–3. It should be noted that as a result of the different retention characteristics, the acetonitrile content of the mobile phase was lowered for the OTC. Still, the k' value of the last eluting peak is smaller on the OTC.

Operating column 14 near the optimal flow-rate, 0.042 cm/s, a drastic increase in separation efficiency was obtained (from 22 500 to 351 000 plates for peak 1), but at the cost of a much longer separation time (a factor of *ca.* 8.3). For the packed

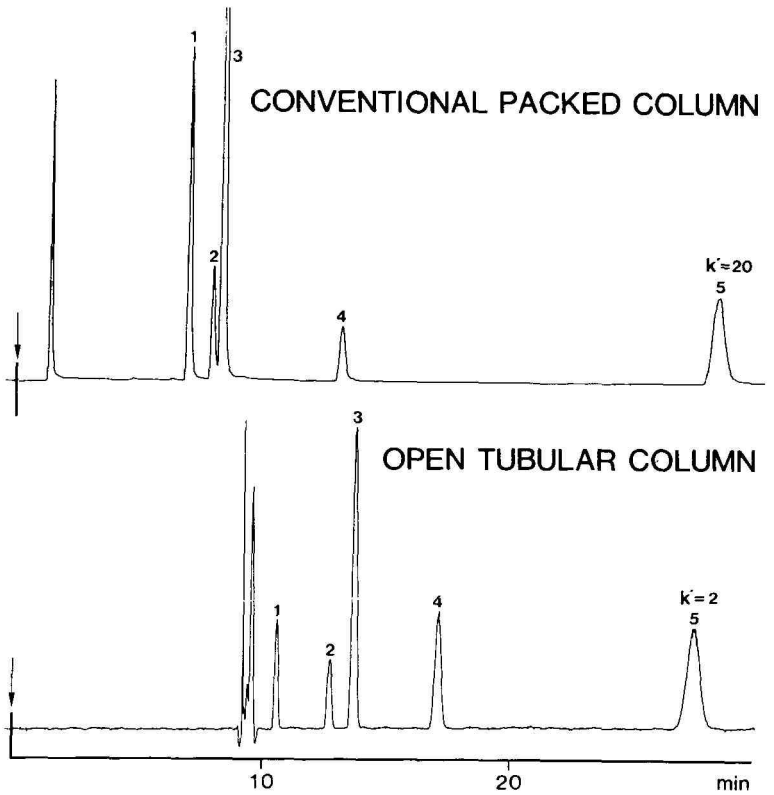

Fig. 7. Comparison of performance of an open tubular column with that of a packed column. Column 14, 197 cm × 11.7 μm I.D.; acetonitrile–water (40:60) at 22 nl/min; split, 1:24 600. Packed column (150 × 4.6 mm I.D.) Spherisorb ODS2, 5 μm; acetonitrile–water (75:25) at 0.7 ml/min. Solutes: 1 = 9-cyanoanthracene; 2 = 9-methoxyanthracene; 3 = anthracene; 4 = 9,10-dimethylanthracene; 5 = 9-phenylacetyleneanthracene.

column, the only way to improve the resolution under isocratic conditions is to increase the column length, which also will increase the separation time. Alternatively, a decrease in the particle size will improve the resolution but, simultaneously, increase the pressure drop. It is interesting to note that with an 8.3 times longer packed column, yielding a corresponding 8.3 times increase in the plate number and the same separation time as with the OTC, the increase in number of plates is only about half of that for the OTC (a factor of 15.6). Concurrently, the pressure drop will increase for the packed column but decrease for the OTC.

Application

A preliminary attempt was made to evaluate the potential of OTC LC with polysiloxane phases for use in routine LC work. An intraveneous solution containing 17 of the common protein amino acids was treated with the *o*-phthaldialdehyde (OPA)–mercaptoethanol precolumn reagent and separated on the OTC No. 14 (1.97 m × 11.6 μm I.D.). The separation was performed at two different flow-rates by gradient elution as shown in Fig. 8a and b. The mobile phase conditions are similar to those described by Jones and Gilligan[51] for conventional LC except for the simple

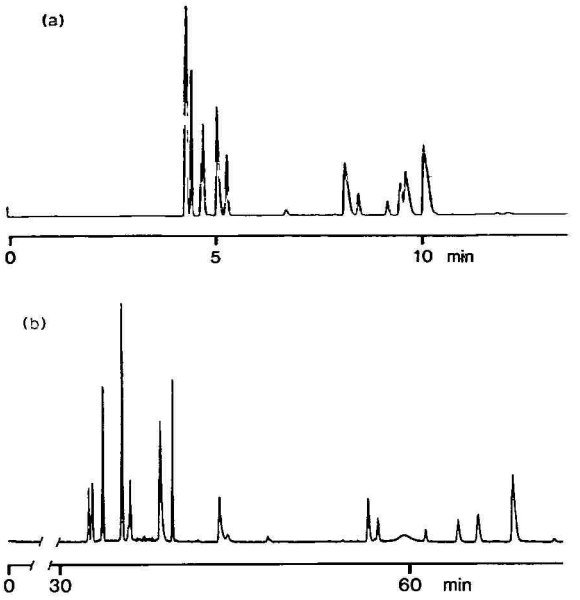

Fig. 8. Separation of OPA–mercaptoethanol derivatized amino acids. Column 14, 197 cm × 11.7 μm I.D.; mobile phase, methanol–buffer (0.1 *M* sodium acetate, pH 7.2); sample, 13–30 μM of each amino acid. (a) Flow-rate, 24 nl/min; linear gradient, 0–40% methanol from 1.0 to 8.0 min; injection volume, 0.3 nl. (b) Flow-rate, 6.5 nl/min; linear gradient, 0–40% methanol from 5.0 to 60 min; injection volume, 0.1 nl.

linear gradient employed for the present OTC separations. Several of the seventeen amino acids could be baseline-separated, although a 100% mobile phase buffer concentration was needed to increase the resolution of the most hydrophilic amino acid derivatives. However, to resolve all the amino acids in the first part of the chromatogram, an increase in column retention is desirable. A change in phase ratio by increasing the film thickness, or alternatively increasing the retention by swelling the stationary phase with non-polar solvents (see below) may both improve the separation conditions. Still, it is clearly demonstrated that it is possible to separate and detect the majority of the common protein amino acids efficiently, even in the concentrations commonly found in physiological fluids. Furthermore, as can be seen in Fig. 8, and in contrast to the properties of packed columns, the same OTC can be used for both fast separations with moderate resolution (Fig. 8a) and for high resolution separations (Fig. 8b).

COROLLARY

The effect of stationary phase swelling on retention

It is well known that organosiloxanes swell in various solvents. The solvent is trapped in the polymer matrix, yielding a considerably larger stationary phase volume than in the unswollen state[29]. The degree of swelling depends on the type of solvent, the polymer composition and the degree of cross-linking. Methyl silicones show the highest degree of swelling in hydrocarbons and in chlorinated solvents. A dimethyl silicone rubber is able to swell to more than three times its initial volume in these

TABLE IX

EFFECT OF SWELLING OF AN IMMOBILIZED METHYLPHENYLVINYL SILICONE GUM PHASE WITH *n*-HEPTANE

Stationary phase, SE-54, column 11.

	Acetonitrile–water (40:60)		*Acetonitrile–water (40:60) saturated with n-heptane*	
	Fluorene	*Phenylfluorene*	*Fluorene*	*Phenylfluorene*
k'	0.70	1.64	6.3	15.8
α	2.33		2.51	

solvents[29]. In chromatography, only a few observations have been reported on this phenomenon. Swelling of the stationary phase has been observed at sample injection in capillary GC[32,65]. Recently, the effects of swelling of non-polar siloxanes in SFC were discussed[30,66]. In straight-phase LC with polar siloxanes, coated on particles, the mobile phase caused swelling of the stationary phase[63].

A stationary phase, consisting of a non-polar solvent trapped in an immobilized layer of a non-polar silicone, have properties that are attractive for use in OTC reversed-phase LC. The main advantage of this approach is the convenient preparation of stationary phases with very large film thicknesses. In addition, swelling with low molecular weight solvents is likely to affect the stationary phase diffusion. The D_s value can be expected to increase, improving the mass transfer in the stationary phase and thereby the column efficiency.

Preliminary results demonstrate that cross-linked siloxane phases swollen by non-polar solvents can be used for reversed-phase LC. In Table IX, k' data (column 10) obtained after the saturation of both the stationary and mobile phases with *n*-heptane, demonstrate a nearly ten-fold increase in retention and a parallel change in selectivity. The characteristics and the utility of this type of OTC liquid–liquid chromatographic system are presently under investigation[67].

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POLARITY OF AMINOETHER ALCOHOLS AND THEIR ETHERS MEASURED BY REVERSED-PHASE GAS CHROMATOGRAPHY

ADAM VOELKEL and JAN SZYMANOWSKI*
Technical University of Poznań, Institute of Chemical Technology and Engineering, Pl. M. Skłodowskiej-Curie, Poznań (Poland)
and
JÖRG BEGER and KERSTIN EBERT
Mining Academy at Freiberg, Freiberg (G.D.R.)
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SUMMARY

Gas–liquid chromatography was used to determine the polarity of pure model aminoether alcohols and their ether analogues. Relationships between the polarity parameters are discussed. The polarity parameters are correlated with the compounds' structures and increments for characteristic groups have been determined. They can be used to estimate the polarity of compounds using only their formulae.

INTRODUCTION

The polarity parameters, as measured by reversed-phase gas chromatography (RP-GC), are of importance in determining the hydrophilic–lipophilic balance of non-ionic surfactants and their application has ben the subject of several reviews[1,2]. Recently, we demonstrated that the polarity is also an important parameter in the field of metal extraction[3,4]. Depending upon the extractant polarity, the extraction can proceed according to different mechanisms, *i.e.*, at the interface or in the bulk of the aqueous phase, and with different rates.

The aim of this work was (1) to determine the polarity of new model pure aminoether alcohols and their ethers which have found application as extractants[5], phase-transfer catalysts, surfactants[6] and therapeuticals[7]; (2) to discuss relationships between different polarity parameters and to estimate appropriate increments for characteristic groups present in the molecules, which can be used to predict the compounds' polarities using only their formulae. Thus, this work is a continuation of our previous studies concerning poly(oxyethylene glycol) dialkyl ethers and their sulphur analogues[8,9].

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TABLE I

STRUCTURES AND ANALYTICAL DATA OF THE INVESTIGATED COMPOUNDS

Compound No.	*R*	*n*	*B.p. (°C/Pa)*	n_D^{20}	*Molecular weight*	*Elemental analysis, calc. (found)*			*Yield (%)*	*Complexation**
						C	*H*	*N*		
3	n-C_4H_9**	1	104–105/20	1.4470	261.1	64.39 (64.43)	11.87 (11.57)	5.36 (5.31)	50	1
4	n-C_4H_9**	2	151–154/27	1.4519	349.2	61.91 (62.03)	11.17 (11.09)	4.01 (3.88)	59	2
5	n-C_6H_{13}	2	175–177/13	1.4541	405.2	65.20 (65.17)	11.60 (11.63)	3.45 (3.35)	54	2
6	n-C_8H_{17}	2	200–205/13	1.4558	461.3	67.70 (67.41)	11.92 (11.80)	3.03 (2.80)	50	2
7	n-C_4H_9**	3	218–222/7	1.4557	438.8	60.29 (60.22)	10.95 (10.80)	3.20 (2.96)	59	3
8	n-C_4H_9	1	134–235/11	1.4429	361.2	66.50 (66.49)	11.90 (11.79)	3.88 (3.52)	36	1
9	n-C_4H_9	2	196–206/20	1.4499	493.3	63.30 (63.55)	11.15 (11.15)	2.84 (2.73)	34	3
10	n-C_6H_{13}	2	215–220/7	1.4525	577.3	66.57 (66.53)	11.60 (11.66)	2.42 (2.3)	30	3
11	n-C_8H_{17}	2	242–245/13	1.4548	661.4	69.00 (69.02)	11.94 (11.83)	2.12 (2.00)	31	3

* Ability to dissolve solid potassium permanganate in chloroform: 1, no coloration; 2, a weak violet colour; 3, a strong violet colour.

** Compounds previously described[6].

EXPERIMENTAL

Reagents

Nine pure model compounds (Table I) were used. They were synthesized according to the folowing reaction schemes:

$$\underset{1}{H_2NCH_2CH_2OH} + \underset{2}{R(OCH_2CH_2)_nCl} \xrightarrow{Na_2CO_3} \underset{3\text{–}7}{[R(OCH_2CH_2)_n]_2NCH_2CH_2OH}$$

$$\underset{1}{H_2NCH_2CH_2OH} + \underset{2}{R(OCH_2CH_2)_nCl} \xrightarrow{NaOH} \underset{8\text{–}11}{[R(OCH_2CH_2)_n]_2N(CH_2CH_2O)_{n+1}R}$$

The aminoethanol (1) and the solid base (sodium carbonate or sodiumhydroxide) were placed in a four-neck round-bottom flask (500 ml) equipped with a stirrer, dropping funnel, thermometer and a reflux condenser. The flask was then heated to 80°C with vigorous stirring, and the alkylglycol chloride (2) was slowly added. The mixture was heated under reflux to 120–140°C for 15–20 h, then cooled to room temperature and the liquid was separated from the solids by suction. The precipitate was washed with acetone, and the cooled organic phases were dried over sodium sulphate. After removing the acetone, the residue was repeatedly distilled under reduced presure. The molar ratio of the reagents for aminoether alcohol (3–7) synthesis was 1:2:Na_2CO_3 = 1:2:1.2 (yields 50–60%) and for the aminoethers (8–11) was 1:2:NaOH = 1:3:3.2 (yields 30–40%).

The purity of the compound was demonstrated by thin-layer chromatography (TLC) and/or GC. Silufol plates (UV 254; Kavalier, Czechoslovakia) were used. The products were developed with toluene–acetone–methanol–aqueous ammonia (31:21:5:1.5, v/v) and detected by spraying with a solution of cobalt (II) thiocyanate or Dragendorffs reagent. For GC, the columns (1 m × 3 mm I.D. or 2 m × 3 mm I.D.) were filled with SE-30 as a liquid phase. The column temperature was 230–310°C.

Chromatographic measurements

Chromatographic measurements were carried out using a Chrom 5 gas chromatograph (Kovo, Czechoslovakia) equipped with a flame ionization detector. The conditions were as follows: column, 1 m × 3 mm I.D.; column temperature, 70 and 90°C; column packing, 25% (w/w) extractant on Porolith (mesh size 0.2–0.5 mm); carrier gas (helium), 40 ml/min; solutes, methanol, ethanol, butanol, 2-pentanone, benzene, pyridine, nitropropane and C_5–C_{11} *n*-alkanes; time for column stabilization, 10 h.

For each surfactant, five different measurements were made and the average values of the polarity parameters were calculated. The following polarity parameters were considered: retention index of methanol and ethanol; polarity index of methanol and ethanol, PI = 100 log $(C-4.7)$ + 60, where C is the apparent carbon number of a standard *n*-alkane having the same retention time as the alcohol; coefficient ρ, defined as the ratio of the retention times of the alcohol and *n*-hexane; partial molal free energies of solution of hydroxyl, $\Delta G_s^m(OH)$, and carbonyl groups, $\Delta G_s^m(>C=O)$; McReynolds constants.

Partial molal Gibbs free energies of solution were calculated as described by Risby[10,11] and used in our previous work[12]. McReynolds constants were calculated in the standard way using the retention indices of benzene, butanol, 2-pentanone, pyridine and nitropropane as determined on the surfactant considered and on squalane, respectively.

RESULTS AND DISCUSSION

The values of the polarity parameters obtained are given in Tables II and III (the compound numbering is as in Table I). The precision of the determination of I_R, PI and ρ is good and similar to that in our previous work[8,9]. The confidence limits at the significance level of 0.05 amount to about 1, 0.1 and 0.01 for I_R, PI and ρ, respectively.

For ethanol (EtOH), higher values of I_R, PI, ρ and $\Delta G_s^m(OH)$ were obtained in comparison to methanol (MeOH). The two sets of parameters can be correlated according to the following linear equations:

$$I_R^{EtOH} = 1.0044 \cdot I_R^{MeOH} + 40.7 \quad , R = 0.9980$$

$$PI^{EtOH} = 0.8162 \cdot PI^{MeOH} + 25.22 \quad , R = 0.995$$

$$\rho^{EtOH} = 1.2509 \cdot \rho^{MeOH} + 0.322 \quad , R = 0.9890$$

$$\Delta G_s^m(OH)^{EtOH} = 1.0731 \cdot \Delta G_s^m(OH)^{MeOH} + 2.12 \quad , R = 0.9700$$

The values of the regression coefficient, R, are high and demonstrate the statistical significance of these equations.

The relationships between first three polarity parameters are similar to these found previously for other groups of compounds (Fig. 1). As the coefficient ρ increases the retention index and the polarity index also increase, both for methanol and ethanol.

The partial molal Gibbs free energies of solution of the hydroxyl and carbonyl groups, and the McReynolds constants, change in a similar order to that of the three previous parameters. However, the influence of the compounds' structures is different for each parameter considered. The coefficient ρ is the most sensitive and the quantity

TABLE II

POLARITY PARAMETERS

Compound No.	I_R		*PI*		ρ	
	MeOH	*EtOH*	*MeOH*	*EtOH*	*MeOH*	*EtOH*
3	695	740	95.4	103.0	2.15	3.40
4	720	765	99.7	107.5	2.70	3.86
5	666	706	89.5	97.4	1.73	2.40
6	638	683	82.8	93.1	1.37	1.99
7	736	785	102.0	109.3	3.16	4.28
8	644	687	84.4	94.0	1.45	2.10
9	761	800	107.6	111.9	3.99	5.15
10	660	705	87.3	97.2	1.95	2.63
11	624	666	79.6	89.7	1.23	1.77

TABLE III

POLARITY PARAMETERS

Compound No.	$\Delta G_s^m(OH)$ (kJ/mol)		$\Delta G_s^m(>C=O)$ (kJ/mol)	$\sum_{i=1}^{5} \Delta I$
	MeOH	EtOH		
3	−10.4	− 9.1	− 9.8	1071
4	−11.0	− 9.8	−10.0	1308
5	−10.2	− 8.7	− 9.3	1030
6	− 9.8	− 8.5	− 8.7	935
7	−11.6	−10.4	−10.3	1341
8	−10.1	− 8.7	− 8.8	864
9	−10.8	− 9.2	−10.0	1351
10	− 9.7	− 8.3	− 9.0	882
11	− 9.7	− 8.3	− 8.9	833

$(\rho_{max} - \rho_{min}/\rho_{min}) \cdot 100\%$, changes by about 200%. The McReynolds constants change by about 60%, but all other parameters change by only about 20%. Thus, the use of ρ and $\Sigma\Delta I$ to characterize the compounds' polarities should be favoured.

However, when the relationships between $\Sigma\Delta I$ and I_R, PI, ρ, ΔG_s^m(OH) and $\Delta G_s^m(>C=O)$ are considered, the parameters discussed usually increase as the $\Sigma\Delta I$ increases. However, the experimental points are scattered (Figs. 2 and 3), and the derivation of a simple and accurate relationship is impossible. Therefore linear relationships were derived as a first approximation, and their regression and correlation coefficients are given in Table IV. Although the correlation coefficients are relatively low, *i.e.*, in the range of 0.91–0.96, these approximately linear relationships between the polarity parameters calculated only from the retention times of ethanol and methanol demonstrate that proton donor–proton acceptor interactions are the most important for the group of compounds considered and can well characterize their polarities.

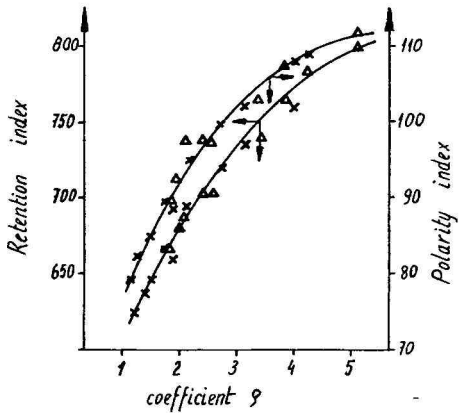

Fig. 1. Relationships between the retention index, the polarity index and the coefficient ρ for methanol (×) and ethanol (△).

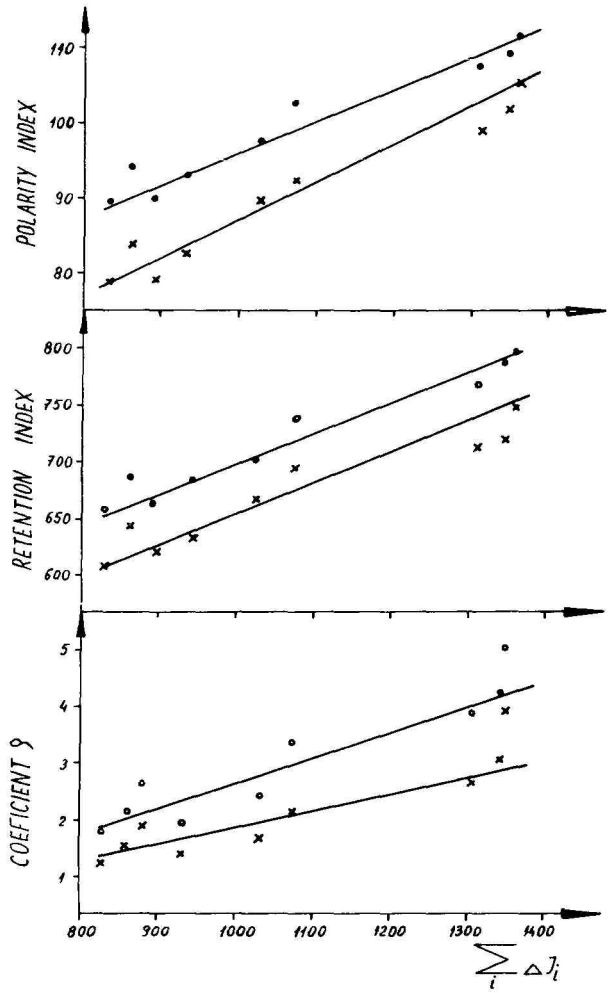

Fig. 2. Relationships between the polarity parameters determined from the retention times of alcohols (×, methanol; ○, ethanol) and the McReynolds constants.

The influence of the compounds' structures upon their polarity parameters is shown in Fig. 4. The values of all the parameters considered increase almost linearly as the number of the oxyethylene units increases. Thus, the slopes of the straight lines give the increments in the polarity parameters per oxyethylene unit for each homologous series of compounds. The values of the polarity parameters decrease as the alkyl chain length increases. However, deviations from the straight lines are significant and the influence of the alkyl chain length decreases for compounds having longer chains.

The values of the parameters considered can be used to estimate the average increments for the characteristic groups present in the compounds. Assuming additivity of the polarity parameters, the polarity of a compound A_i (P_{A_i}), can be expressed as

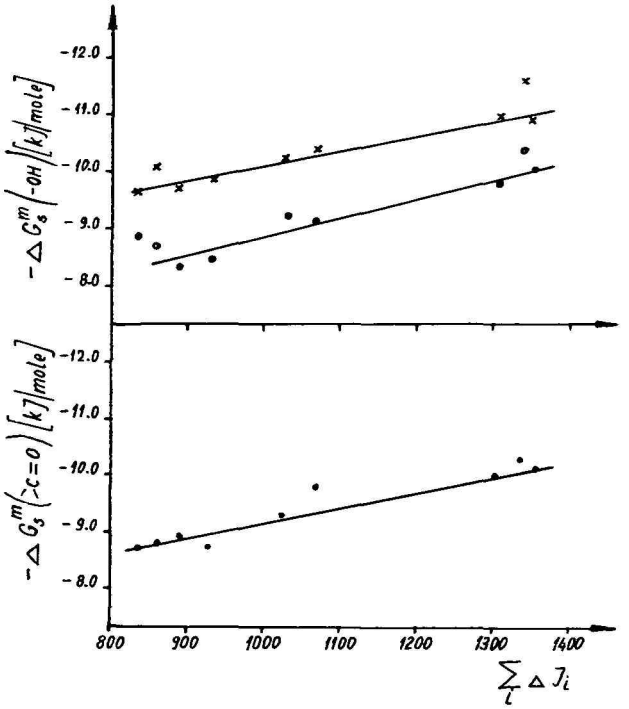

Fig. 3. Relationships between the partial molal Gibbs free energies of solution and the McReynolds constants for methanol (×) and ethanol (○).

TABLE IV

REGRESSION AND CORRELATION COEFFICIENTS FOR THE RELATIONSHIP

$P_i = a + b\Sigma\Delta I$

Polarity parameter, P_i	*Alcohol*	*a*	*b*	*Correlation coefficient*
I_R	MeOH	452.4	0.2155	0.9609
	EtOH	494.0	0.2174	0.9633
PI	MeOH	45.92	0.0432	0.9566
	EtOH	62.37	0.0355	0.9608
ρ	MeOH	−2.06	$3.981 \cdot 10^{-3}$	0.9159
	EtOH	−2.42	$5.137 \cdot 10^{-3}$	0.9346
ΔG_s^m(OH)	MeOH	−7.34	$-2.834 \cdot 10^{-3}$	0.9231
	EtOH	−5.86	$-2.935 \cdot 10^{-3}$	0.9346
ΔG_s^m(>C=O)		−6.56	$-2.679 \cdot 10^{-3}$	0.9385

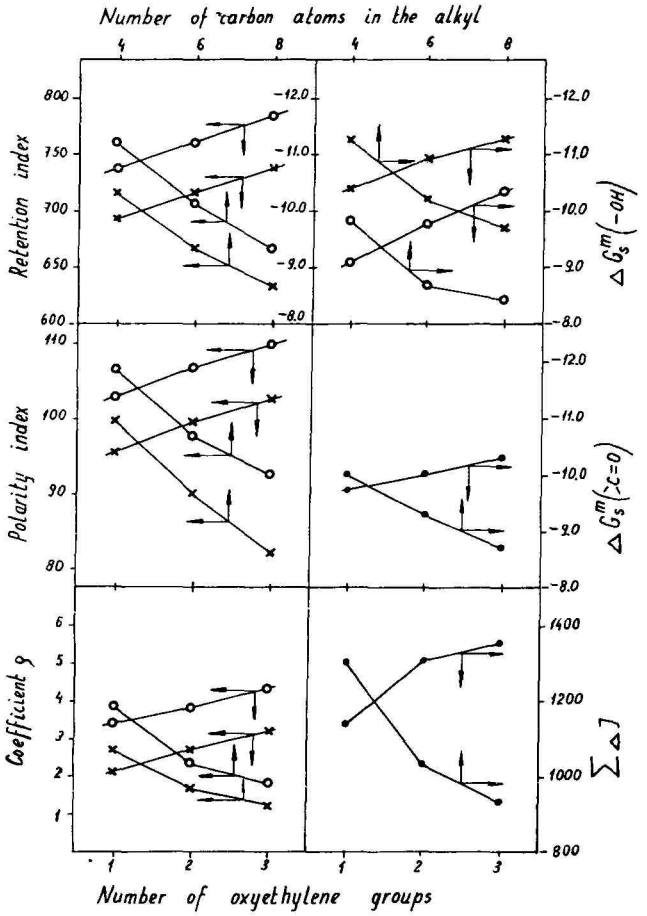

Fig. 4. Effect of the oligooxyethylene and alkyl chains upon the polarity parameters for methanol (×) and ethanol (○).

$$P_{A_i} = \sum_{j=1}^{m} a_{ji} \Delta P_{G_j} + \text{constant}$$

where it is assumed that the increment, ΔP, for a group G_j is constant for all compounds present in the system. The coefficient a_{ji} denotes the number of G_j groups in compound A_i, whose chemical formula can be expressed as

$$A_i = (G_1)_{a_{1i}} (G_2)_{a_{2i}} \ldots (G_m)_{a_{mi}}$$

where j denotes the characteristic group considered and $j = 1,2,\ldots, m$.

When the set of polarity parameters obtained for all compounds present in the system is considered (n being the number of compounds present) the following set of linear equations is obtained

TABLE V

INCREMENTS OF THE POLARITY PARAMETERS

Polarity parameter		*CH_2, CH_3*	*–O–*	*>NOH*	*>N–*	*Constant*	*Error*	
							Absolute	*Relative*
I_R	MeOH	−9.40	44.57	2838	2867	−3533	12	1.7
	EtOH	−9.47	45.34	4333	4300	−3594	11	1.5
PI	MeOH	−1.91	8.71	1055	1049	−959	2.4	2.5
	EtOH	−1.57	7.23	250.8	256	−354	1.8	1.7
ρ	MeOH	−0.374	0.905	23.62	23.2	−21.67	0.26	11.6
	EtOH	−0.288	1.107	49.8	50.4	−53.03	0.34	11.2
ΔG_s^m(OH)	MeOH	0.114	−0.546	−71	60.4	0.2	2.0	
	EtOH	0.114	−0.550	−95.1	−96.0	104	0.2	3.0
ΔG_s^m(>C=O)		0.108	−0.545	−40.9	−41.6	50.5	0.2	1.8
$\Sigma\Delta I$		−38.16	172	6357	6144	−5129	70	6.6

$$P = A \cdot \Delta P$$

where

$$P = [P_{A_1}, P_{A_2}, \ldots, P_{A_n}]^T$$

$$\Delta P = [\Delta P_{A_1}, \Delta P_{A_2}, \ldots, \Delta P_{A_n}]^T$$

$$A = (a_1, a_2, \ldots, a_n)$$

$$a_i = [a_{1i}, a_{2i}, \ldots, a_{mi}]^T$$

By solving these equations in the same way as in our previous work[13] the values of the increments were obtained (Table V). They can be used to predict the polarity parameters for the compounds considered and their homologues only from their formulae. The accuracies of such predictions are good and the errors amount to only 1.3–3% for I_R, PI, ΔG_s^m(OH) and ΔG_s^m(>C=O) and 7% for $\Sigma\Delta I$. The coefficient ρ is determined with lower precision, the error exceeding 10%.

CONCLUSIONS

The polarity parameters calculated only from the retention times of the alcohols (methanol or ethanol) and standard alkanes characterize well the polarity of the compounds considered. They are approximately linearly correlated with the McReynolds constants. The polarity increases as the number of oxyethylene units increases and as the length of the alkyl chain decreases. The increments determined for characteristic fragments of the compounds can be used to estimate the polarity parameters only from the compounds' formulae. The errors of such predictions are 1.3–3% for I_R, PI, ΔG_s^m(OH), ΔG_s^m(>C=O), 7% for $\Sigma\Delta I$ and above 10% for the coefficient ρ.

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HIGH-SPEED AND HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY OF PROTEINS ON A NEW HYDROPHILIC POLYSTYRENE-BASED RESIN

YAN-BO YANG and M. VERZELE*
Laboratory of Organic Chemistry, State University of Ghent, Krijgslaan 281 (S4), 9000 Ghent (Belgium)
(Received November 19th, 1986)

SUMMARY

High-performance size-exclusion chromatography of some standard proteins, peptides and amino acids on a new hydrophilic packing material obtained by chemical transformation of a cross-linked polystyrene–divinylbenzene copolymer was studied. Columns filled with 4 and 7 μm particles were compared. The influence of the concentration of acetonitrile, isopropanol and trifluoroacetic acid in the mobile phase on the chromatographic performance was investigated. A good linear calibration graph covering the molecular weight range from 200 to 700 000, was obtained under the optimal conditions. The packing material can be used for separations, for molecular weight determinations and for the pre-fractionation of proteins. The high rigidity of the packing material allows relatively high pressures to be used and therefore fast separations to be achieved. The packing material was applied to the chromatography of proteins from beer, bones and milk.

INTRODUCTION

Size-exclusion chromatography (SEC), an important method for protein separations[1], is based on the differences in the molecular weights of the solutes. There should be no chemical or adsorptive interaction between the stationary phase and the solutes. The larger molecules are eluted earlier than the smaller molecules, and all molecules should elute ahead of the solvent peak[2]. This is clearly different from adsorption, reversed-phase and ion-exchange chromatography. SEC therefore has advantages for protein separations with regard to denaturation, mostly caused by strong adsorption of proteins on the stationary phase[3]. SEC is recognized as an indispensable tool for pre-fractionations, for molecular weight determinations and for desalting proteins, even though it has a limited peak capacity[4,5].

However, the development and application of SEC in the high-performance liquid chromatography (HPLC) of proteins has been hampered by the lack of suitable packing materials[6] that are hydrophilic enough, chemically and physically stable, have an inert surface etc. Traditionally, SEC separations of proteins have used soft gels[7] such as dextran-based gels (*e.g.*, Sephadex), agarose gels (*e.g.*, Sepharose, Bio-

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Gel A) and polyacrylamide gels (*e.g.*, Bio-Gel P). These gels, in addition to sometimes specifically adsorbing some solutes[8,9], are too soft to withstand the high pressures required in HPLC. In addition, these gels are difficult to obtain with the small particle sizes necessary for high efficiency[10]. In recent years, many new packing materials for protein chromatography have been introduced[10–15]. Most are based on silica or controlled-pore glass (CPG), which means that they can only be used over a limited pH range (3–8)[16]. Silica is readily degraded by water and buffer solutions, causing deterioration of the column performance[17,18]. In addition, adsorption effects are observed[11,15].

Recently, cross-linking of agarose was reported to improve the rigidity[19,20]. However, the gels can withstand maximum pressures of only 15 and 30 bar for Superose 6 and Superose 12, respectively. Clearly, the need for supports for protein separations that are inert, rigid and stable is still evident. It is well known[21–23] that a spherical, porous, highly cross-linked polystyrene–divinylbenzene (PS) resins can meet the demands on rigidity and stability with the recent improvement of the techniques of synthesis. Unfortunately, PS packings cannot readily be applied in the SEC of proteins because of their hydrophobic properties[16,22,24].

In a previous paper[23], we reported on a new packing material, Rogel-P (RSL/Alltech Europe, Eke, Belgium), in which neutral hydrophilic functionalities are introduced chemically by ether linkages to PS. This material possesses sufficient hydrophilicity, yet remains physically and chemically stable, has a wide applicable pH range and is charge-free. The new material is even more rigid than the basic PS packing[23]. In this paper, we report on the application of this material in the HPSEC of proteins.

EXPERIMENTAL

Reagents

Proteins were obtained from Serva (Heidelberg, F.R.G.) or Sigma (St. Louis, MO, U.S.A.), all small and medium molecular weight peptides were kindly donated by Dr. J. Van Beeumen (Laboratium voor Microbiologie en Microbiele Genetica, RUG, Belgium). These proteins and peptides are listed in Table I. Trifluoroacetic acid (TFA) was purchased from Riedel-de Haën (Seelze, F.R.G.), and water, isopropanol and acetonitrile of HPLC/Spectro grade from Alltech (Deerfield, IL, U.S.A.). All chemicals were used as received.

Chromatographic systems

Chromatography was performed on a Varian Model 5000 LC system (Varian, Palo Alto, CA, U.S.A.) equipped with a Varian UV-50 variable-wavelength absorbance detector, a Varian CDS 401 (Vista Series) integrator and a Varian A-25 recorder. Samples were introduced on to the column using a Valco 7000 p.s.i., 10-μl manual loop injector and detected at 220 or 280 nm.

The columns were packed in 25 cm $\times$ 0.7 cm I.D. stainless-steel tubes closed with 3/8-in. Valco fittings and 2-μm stainless-steel frits.

Rogel-P has a pore size of 17 nm, a surface area of 228 m^2/g and a particle size of 4 or 7 μm.

A typical mobile phase used in this work was a mixture of water, acetonitrile

TABLE I

PROTEINS USED

Protein	*MW**	*pI**	*Weight (μg)***	*Source*
Thyroglobulin (porcine)	660000	4.5	20	Serva
Urease (jack bean)	485000	4.88	75	Serva
Ferritin (horse)	440000	4.40	6	Serva
Edestin (hemp)	300000	–	15	Serva
Catalase (bovine)	250000	5.4	–	Serva
γ-Globulin (bovine)	90000	5.17	15	Serva
Serum albumin (bovine)	67000	4.90	10	Sigma
Ovalbumin (egg)	45000	4.70	8	Serva
Pepsin (porcine)	34000	2.86	12	Serva
Chymotrypsinogen A (bovine)	25000	9.0	5	Serva
Myoglobin (equine)	17800	6.8	5	Serva
Cytochrome *c* (horse)	12300	9	7	Serva
Insulin (bovine)	11500	5.72	7	Serva
Cytochrome *c*-551 (Ps. perf.)***	7660	–	2	J.V.B.
Peptide 1	1807	–	0.4	J.V.B.
Bacitracin	1450	6.0	12	Serva
Peptide 3	1241	–	4	J.V.B.
Peptide 5	706	–	1.6	J.V.B.
Peptide 6	603	–	–	J.V.B.
Peptide 7	309	–	–	J.V.B.
Peptide 4	259	–	2.6	J.V.B.
Tryptophan	204	–	1.0	Sigma

* Mainly based on manufacturers data and refs. 12, 15 and 25.

** Amount of sample in each injection for constructing the calibration graph.

*** From *Pseudomonas perfectomarinus*. J.V.B. samples donated by Dr. J. Van Beeumen,
Peptide 1, A V T Y T E H A K R K T V T A M;
Peptide 3, N M V G P A L A G V V G R;
Peptide 4, L A G;
Peptide 5, V G A T K M T;
Peptide 6, L A D P N A;
Peptide 7, K Mes (Mes = methionine sulfone).

and isopropanol containing a small amount of TFA. This mixture is often used for the chromatography of proteins, because all the solvents and chemicals are volatile and can be removed from the proteins by evaporation. All solvents were degassed under vacuum with ultrasonic agitation or by boiling.

All chromatography was carried out at ambient temperature.

Column packing procedure

The particles were slurried in water–acetonitrile (70:30) and packed upwards into the column (25 cm × 0.7 cm I.D.) using a pre-column (50 cm × 0.7 cm I.D.) with distilled water as following solvent at a flow-rate of 6 ml/min. The presure was then about 210 bar and was applied using a Varian Model 5000 liquid chromatograph. The packing eluent (water) was pre-heated to 60°C.

Protein recovery assay

Protein recoveries were measured by comparing the peak area under UV detection after passing through the 25 cm × 0.7 cm I.D. column with that without passing through the column. A 10 cm × 0.7 cm I.D. column packed with the same material was used prior to the main analytical column in order to have some peak spreading when the analytical column was disconnected. Peak areas were integrated with the Varian CDS 401 (Vista Series) integrator.

RESULTS AND DISCUSSION

Calibration

In true SEC, the smallest molecules can penetrate all pores of the packing and elute with the total permeation volume ($V_t = V_o + V_i$). The larger molecules are excluded completely from the pores and elute with the exclusion volume (V_o). Molecules in between have elution volumes (V_E) defined by the equation

$$V_E = V_o + K_D V_i \quad (1)$$

where V_o is the liquid volume in the interstitial space between the particles, V_i is the internal pore volume of the column support and K_D is the size-exclusion distribution coefficient, ranging from 0 to 1. Plots of the logarithm of solute molecular weight

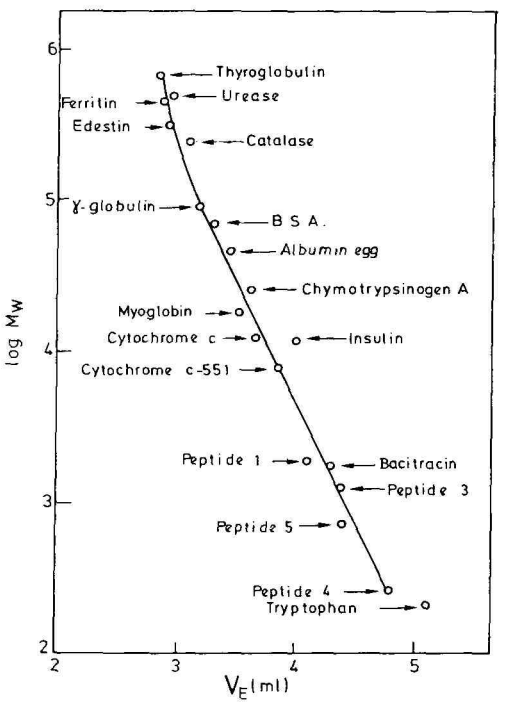


Fig. 1. Calibration graph for proteins on a 25 cm × 0.7 cm I.D. column (7 μm particle size). Eluent: 70% (95% water–5% isopropanol + 0.1% TFA)–30% (95% acetonitrile–5% isopropanol + 0.1% TFA) at a flow-rate of 1.0 ml/min at 70 atm. The samples were detected at 220 nm.

TABLE II

MASS RECOVERY OF PROTEINS

Column: 25 cm × 0.7 cm I.D. (7 μm particle size). Mobile phase: as in Fig. 1. Flow-rate: 1.0 ml/min. Temperature: ambient.

Sample	*MW*	*Weight (μg)*	*Detection (nm)*	*Recovery* (%)*
Tryptophan	204	5	280	115
Bacitracin	1715	36	280	102
Albumin (egg)	45000	20	220	101
BSA	67000	200	280	99
γ-Globulin	90000	20	220	99

* Recovery = (total peak area with column/total peak area without column) × 100.

versus elution volume (V_E) produce the well known calibration graph with a nearly linear relationship between K_D values of 0.15 and 0.80[1].

A typical calibration graph for a series of protein standards on the 7 μm particle size column is shown in Fig. 1. The graph is linear over a wide molecular weight range from 200 to 700000. There is a more linear and better separation range from molecular weights of 200 to 100000. This indicates that the pore size distribution of the material is very broad. The determination of the pore size by Halasz and Martin's method[26] using polystyrene standards and THF as the solvent indicated that the pore diameters of the material ranged from 1 to 71 nm. The column internal porosity, $\varepsilon_o = V_i/V_c$, is about 43% (V_c is the volume of the empty column). Most samples fall on the straight line, giving a good relationship. The deviation of tryptophan from the straight line can be explained by the hydrophobic interaction between the stationary phase and tryptophan, which has the highest hydrophobic fragmental constant ($\Sigma_f = 2.31$)[13] among the amino acids.

Mass recovery

The mass recovery was investigated for a number of proteins (Table II) using the method described. The results show a good recovery of proteins.

Influence of particle size on resolution

It is known that SEC has a limited peak capacity (n); the maximum number of peaks that can be resolved in the separation range of SEC is defined by the equation[27]

$$n = 1 + \sqrt{N}/4 \ln (V_t/V_o) \quad (2)$$

where N is the efficiency of the column (plate number). Eqn. 2 shows that the peak capacity can be increased by increasing the column efficiency (N). To do this, the particle size should be reduced as much as possible. This is even more important for proteins because they have low diffusion coefficients, as mentioned by some chromatographers[28–30]. This is confirmed by comparison of the separations of protein standards on the columns packed with 7 and 4 μm particles, shown in Fig. 2. Appar-

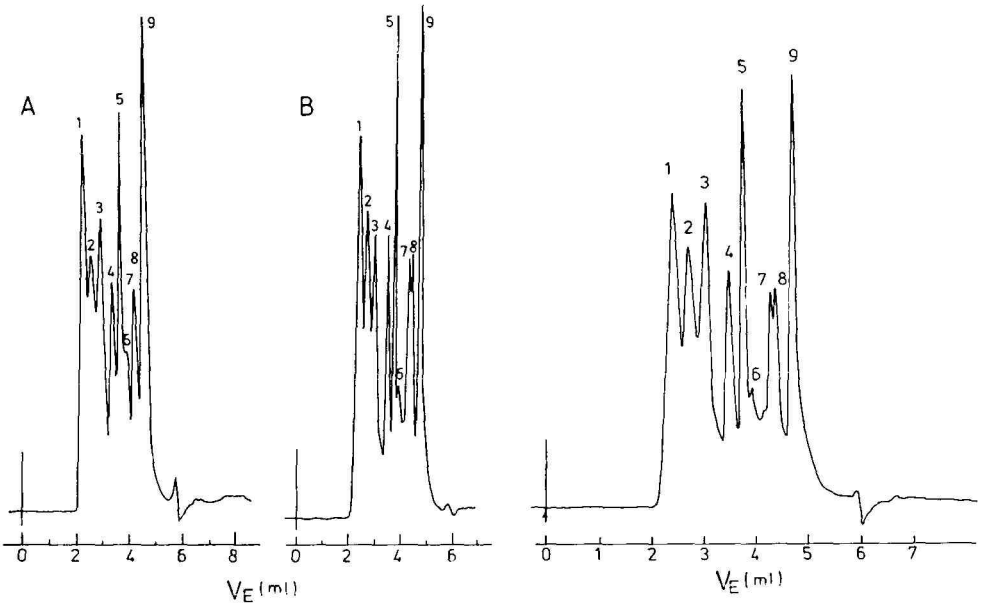


Fig. 2. Comparison of separations of protein standards on columns of 25 cm × 0.7 cm I.D. with particle sizes of (A) 7 μm and (B) 4 μm. Same eluent as in Fig. 1. Flow-rate: 0.5 ml/min. Detection at 220 nm with 0.2 a.u.f.s. Peak identification: 1 = thyroglobulin (5.3 μg); 2 = BSA (2.6 μg); 3 = cytochrome *c* (1.5 μg) and myoglobin (2.1 μg); 4 = insulin (2.1 μg); 5 = bacitracin (3.9 μm); 6 = impurity from bacitracin; 7 = peptide 6 (1.2 μg); 8 = peptide 7 (0.6 μg); 9 = tryptophan (0.3 μg).

Fig. 3. Separation of protein standards. Conditions as in Fig. 2B except with a higher chart speed.

ently, peaks 7 (peptide 6) and 8 (peptide 7) in Fig. 2B (4 μm) are eluted together in Fig. 2A (7 μm). The impurity in bacitracin giving a small peak (peak 6) in Fig. 2B, is only a shoulder in Fig. 2A. This can be seen better in Fig. 3, which shows the same

TABLE III

RELATIONSHIP BETWEEN THE ELUTION VOLUME (V_E) AND THE PERCENTAGE OF ORGANIC MODIFIER (SOLVENT B)

Column: 25 cm × 0.7 cm I.D. (7 μm particles). Flow-rate: 1.0 ml/min. Eluent: 95% water–5% isopropanol +0.1% TFA (solvent A); 95% acetonitrile–5% isopropanol + 0.1% TFA (solvent B). Temperature: Ambient. Detection: UV (220 nm).

Protein	V_E *(ml)*				
	10% B	*15% B*	*25% B*	*30% B*	*85% B*
Tryptophan	7.13	6.48	5.41	5.13	4.78
Bacitracin	5.13	4.74	4.32	4.30	–
Cytochrome *c*	–	Retained	3.63	3.66	–
BSA	Retained	3.32	3.28	3.32	3.18
γ-Globulin	Retained	3.33	3.21	3.19	–
Ferritin	–	Retained	2.93	2.87	–
Thyroglobulin	–	Retained	2.90	2.84	2.99

separation as in Fig. 2 on the 4 μm particle size column but with a faster paper speed. This is a good example of a rapid, high-efficiency SEC separation on only a 25 cm long column.

Influence of acetonitrile in the mobile phase

It was observed that the material still has hydrophobic properties, which give unexpected adsorption of proteins on the stationary phase. Therefore, a certain amount of organic modifier is needed. Table III lists the relationship between the elution volume of protein and the percentage of the organic modifier (solvent B), which is acetonitrile–isopropanol (95:5) containing 0.1% TFA, on the 7 μm particle size column. Cytochrome *c*, ferritin and thyroglobulin were retained on the column until the solvent B level reached 25%. BSA and γ-globulin also needed 15% B before elution started. These large proteins exhibit no significant change in retention once they start to elute, even though 85% solvent B is used. The smaller molecules, tryptophan (MW 204) and bacitracin (MW 1450) are different. They elute with all the solvent compositions but the retention decreases with increasing percentage of solvent B. This is consistent with phenomena described previously[28,31,32]. It seems, from this result, that at least 25% of solvent B in the column eluent is necessary.

Influence of TFA in the mobile phase

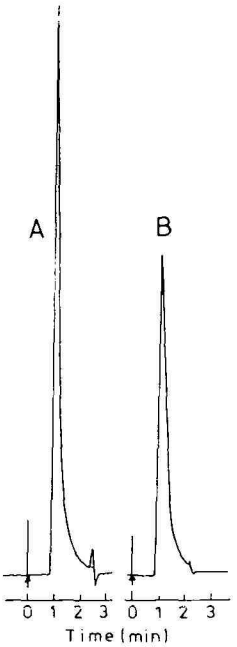
TFA is mostly used as an additive in the mobile phase for the chromatography of proteins, because not only is it volatile, but also it influences the chromatographic performance of proteins. Some results of TFA addition on retention are shown in Table IV. High-molecular-weight proteins (thyroglobulin, ferritin, BSA and cytochrome *c*) were completely retained without addition of TFA to the mobile phase, and only tryptophan (amino acid) and bacitracin (small molecule) were eluted. However, all compounds elute in the SEC mode when TFA is added, even though only at a concentration of 0.025%. This must be due to the effect of TFA on the conformation and protonation state of the proteins. The concentration of TFA can slightly influence the elution volume. This influence is not unique for all solutes studied; most

TABLE IV

INFLUENCE OF TFA IN THE ELUENT ON ELUTION VOLUMES (V_E) OF PROTEINS

Column: 25 cm × 0.17 cm I.D. (7 μm). Mobile phase: 30% (95% acetonitrile–5% isopropanol)–70% (95% water–5% isopropanol) with different percentages of TFA. Flow-rate: 1.0 ml/min. Detection: UV (220 nm).

Protein	V_E *(ml)*			
	0.000% TFA	*0.025% TFA*	*0.050% TFA*	*0.100% TFA*
Thyroglobulin	Retained	2.81	2.85	2.78
Ferritin	Retained	2.80	2.90	2.91
BSA	Retained	2.83	3.00	3.01
Cytochrome *c*	Retained	3.03	3.28	3.44
Bacitracin	3.28	3.69	3.97	4.23
Tryptophan	5.99	5.07	5.09	5.00



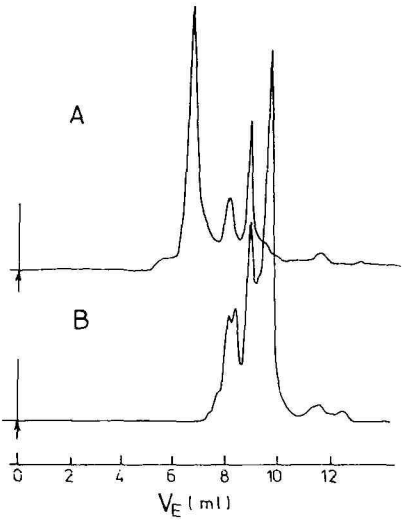


Fig. 4. Comparison of chromatograms of γ-globulin (A) with (B) without isopropanol in the mobile phase. Conditions: column, 10 cm × 0.7 cm I.D. (7 μm particle); eluents (A) 70% water–30% (75% acetonitrile–25% isopropanol) with 0.1% TFA and (B) 70% water–30% acetonitrile with 0.1% TFA at 1.0 ml/min; detection, 280 nm at 0.2 a.u.f.s.

Fig. 5. Separation of milk proteins, (A) as such and (B) hydrolysed. Conditions: two columns, 25 cm × 0.7 cm I.D. with particle size 7 μm; eluent, as in Fig. 1 at a flow-rate of 0.5 ml/min; sample, 10 μl of a 15 mg/ml solution in water; detection, 220 nm at 0.5 a.u.f.s.

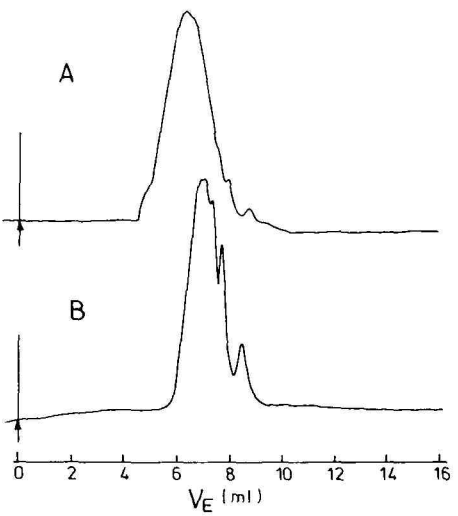


Fig. 6. Separation of cow bone proteins, (A) as such and (B) hydrolysed. Conditions as in Fig. 5 except injection volume, 1.2 μl of a 50 mg/ml solution in water; detection, 220 nm and 1.0 a.u.f.s.

of them (*e.g.*, ferritin, BSA, cytochrome *c* and bacitracin) showed a slight increase in elution volume when the concentration of TFA was increased. It is recommended that 0.025%–0.100% of TFA is added to the mobile phase.

Influence of isopropanol in the mobile phase

Fig. 4 shows two chromatograms for γ-globulin with (A) and without (B) isopropanol in the mobile phase. The peak in Fig. 4A is much sharper and higher than that in Fig. 4B. Too high a concentration of isopropanol is not recommended owing to its high viscosity, which often leads to a high column pressure.

Application

The systems discussed above have been applied to the separation of proteins in beer, bones and milk.

Fig. 5 shows the separation of milk proteins (A) and the corresponding hydrolysed product (B). The hydrolysis high-molecular-weight proteins (10000–60000) can be easily monitored in this way.

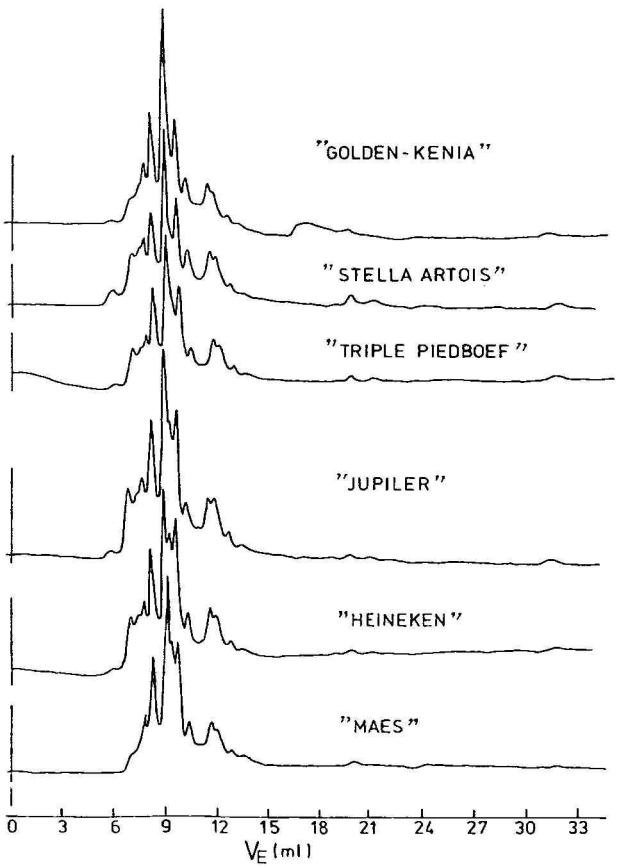


Fig. 7. Elution profiles of proteins in commercial beers. Conditions as in Fig. 5 except 10 μl of beer were injected directly.

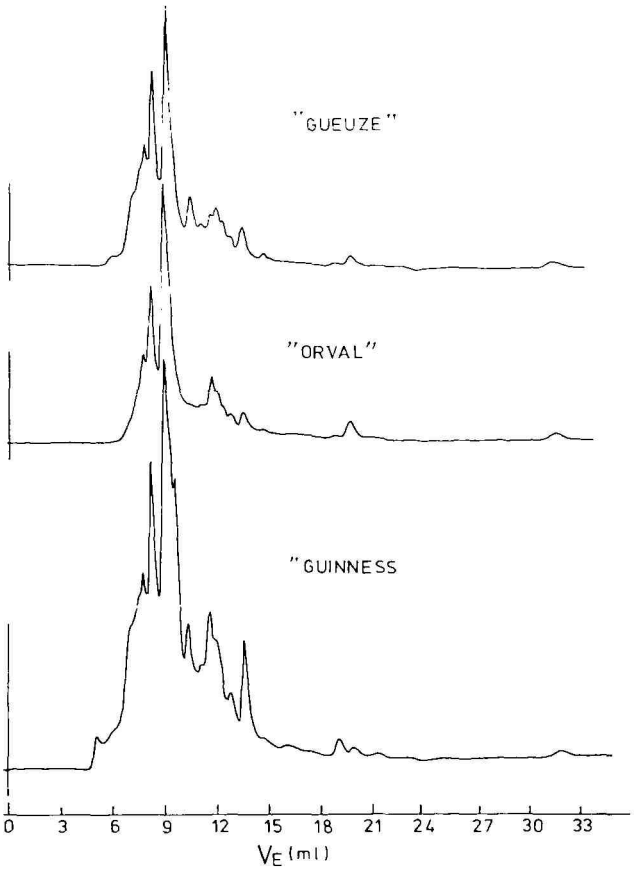


Fig. 8. Elution profiles of proteins in commercial beers. Conditions as in Fig. 7.

Proteins extracted from cow bone and the corresponding hydrolysed products were similarly chromatographed (Fig. 6). The molecular weights of the proteins in the hydrolysed products (B) are much lower than those in the unhydrolysed starting material (A).

Proteins in commercial beers were chromatographed on two coupled 25 cm × 0.7 cm I.D. columns. The elution profiles of proteins in lager beers of different origin are similar, as shown in Fig. 7. Only the concentrations (or relative proportions) of some fractions are slightly different. Their molecular weights range from hundreds to about 20000. We have not studied whether the differences are significant or irrelevant. Fig. 8 shows the protein profiles in some special beers of Belgian and Irish origin. The differences are now much larger.

It can be concluded that Rogel-P is a suitable packing material for fast HP-SEC and that very small particle sizes should be used.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MEMBRANE PROTEINS

YOSHIO KATO*, TAKASHI KITAMURA, KOJI NAKAMURA, AKANE MITSUI, YOSUKE YAMASAKI and TSUTOMU HASHIMOTO
Central Research Laboratory, Toyo Soda Mfg. Co., Ltd., Tonda, Shinnanyo, Yamaguchi 746 (Japan)
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SUMMARY

Various modes of high-performance liquid chromatography, gel filtration, ion-exchange chromatography, hydrophobic interaction chromatography, reversed-phase chromatography and metal chelate affinity chromatography, were investigated for the separation of membrane proteins. All were found applicable to membrane proteins, although the usefulness of each mode differed. For satisfactory results it was important to select appropriate elution conditions. The type and concentration of detergent was of special importance. The effects of other conditions, flow-rate, gradient steepness, type of buffer and salt, eluent pH, etc., were similar to those observed for soluble proteins.

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) for protein separation was first reported about 10 years ago. It is now one of the most important techniques for the purification of water-soluble proteins, and has also been applied to membrane proteins[1–54]. However, studies of the chromatographic conditions have been rather limited so far and the optimum conditions have not been established. Accordingly, we evaluated the chromatographic conditions in various modes of HPLC for membrane proteins. Gel filtration, ion-exchange chromatography, hydrophobic interaction chromatography, reversed-phase chromatography and metal chelate affinity chromatography, which are now applicable to soluble proteins, were examined.

EXPERIMENTAL

Rat liver microsomal membrane proteins and human placenta mitochondrial membrane proteins were used as model samples. Liver microsomes prepared by the usual method from phenobarbital-treated rats were suspended in 4 ml of 0.1 *M* sodium phosphate buffer (pH 7.5) containing 30% (v/v) glycerol. To this suspension, 0.5 ml of 10% sodium cholate and 0.5 ml of 10% Emulgen 911 were added with

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stirring. The mixture was vortexed for 30 min at room temperature and then centrifuged at 78000 *g* for 60 min. The resulting supernatant was used as a crude sample of rat liver microsomal membrane proteins. It contained 43 mg proteins/ml. This sample was a generous gift from Dr. Funae of Osaka City University. Human placental mitochondria prepared by the usual method was suspended in 100 ml of 10 m*M* Tris–HCl buffer (pH 7.4). To this suspension, 100 ml of 10 m*M* Tris–HCl buffer (pH 7.4) containing 1% octyl glucoside were added and the mixture was stirred overnight at 4°C. Then, it was centrifuged at 100000 *g* for 90 min. The sediment was resuspended in 100 ml of 10 m*M* Tris–HCl buffer (pH 7.4), and 100 ml of 10 m*M* Tris–HCl buffer (pH 7.4) containing 2% octyl glucoside were added. After stirring in an ice-bath for 3 h, the mixture was centrifuged at 100000 *g* for 90 min. The resulting supernatant was used as a crude sample of human placenta mitochondrial membrane proteins. It contained 4.8 mg proteins/ml. This sample was a generous gift from Dr. Naoi of Nagoya University.

Chromatography was performed at 25°C with a Model CCPM pump and two variable-wavelength UV–VIS detectors Model UV-8000 (Toyo Soda, Tokyo, Japan). The two detectors were used in series. One of them was usually operated at 280 nm to monitor total protein concentrations in the column effluents. It was operated at 220 nm in the case of reversed-phase chromatography or at 246 nm when the eluent contained Emulgen 911 or Triton X-100, which has rather strong UV absorption at 280 nm. The other detector was operated at 417 nm to monitor the concentration of haemoproteins, such as cytochrome P-450 and cytochrome b_5. Gel filtration was carried out on a TSK G3000SW column[55] (600 mm × 7.5 mm I.D.). The eluent was 50 m*M* phosphate buffer (pH 7.0) containing 0.2 *M* sodium chloride, detergent and 20% (v/v) glycerol. The flow-rate was 0.8 ml/min. Ion-exchange chromatography was performed on a TSK DEAE-5PW column[56] (75 mm × 7.5 mm I.D.) with a 60-min linear gradient from 0 to 1 *M* sodium chloride in 20 m*M* Tris–HCl buffer (pH 8.0) containing detergent and 20% (v/v) glycerol, at a flow-rate of 0.8 ml/min. Hydrophobic interaction chromatography was carried out on a TSK Ether-5PW column[57] (75 mm × 7.5 mm I.D.) with a 60-min linear gradient from 1–2.25 to 0 *M* ammonium sulphate in 0.1 *M* Tris–HCl buffer (pH 7.5) containing detergent and 20% (v/v) glycerol, at a flow-rate of 0.8 ml/min. Reversed-phase chromatography was carried out on a TSK Phenyl-5PW RP column[58] (75 mm × 4.6 mm I.D.) with a 2-min linear gradient from 5 to 20% acetonitrile followed by a 48-min linear gradient from 20 to 80% in 0.05 or 0.1% trifluoroacetic acid (TFA), at a flow-rate of 1 ml/min. Metal chelate affinity chromatography was carried out on a TSK Chelate-5PW column[59] (75 mm × 7.5 mm I.D.) with a 60-min linear gradient from 0 to 0.2 *M* glycine in 20 m*M* Tris–HCl buffer (pH 8.0) containing 0.5 *M* sodium chloride, detergent and 20% (v/v) glycerol, at a flow-rate of 0.8 ml/min.

The detergents examined were octyl glucoside, octaethylene glycol dodecyl ether ($C_{12}E_8$), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate) (CHAPS), Emulgen 911 and Triton X-100. They were included in eluents at various concentrations. Other chromatographic conditions were also varied in some experiments.

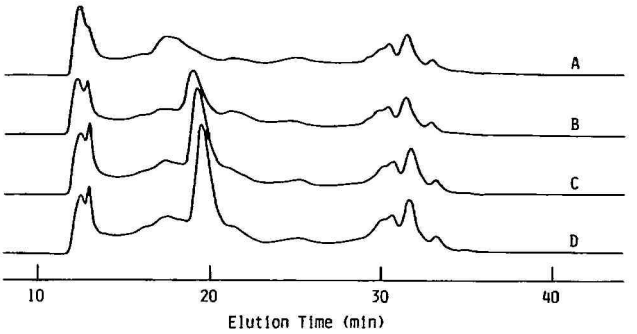

Fig. 1. Chromatograms of rat liver microsomal membrane proteins (0.1 mg) obtained at 280 nm on a TSK G3000SW column in 50 m*M* phosphate buffer (pH 7.0) containing 0.2 *M* sodium chloride, $C_{12}E_8$ and 20% glycerol. $C_{12}E_8$ concentration: 0.005 (A); 0.01 (B); 0.025 (C); 0.05% (D).

RESULTS AND DISCUSSION

Gel filtration

Fig. 1 shows the effect of the detergent concentration in gel filtration. A crude sample of rat liver microsomal membrane proteins was separated with eluents containing various concentrations of $C_{12}E_8$. With decreasing concentration of $C_{12}E_8$, peaks became smaller and also slightly broader. The smaller peaks should be indicative of lower recovery and the broader peaks should result in lower resolution. At concentrations of $\geqslant 0.05\%$, almost the same results were obtained. Therefore, to achieve high recovery and resolution, $C_{12}E_8$ is necessary at a concentration of at least 0.05%. A similar trend was observed for human placenta mitochondrial membrane proteins and with other detergents, although the critical concentrations differed significantly for each detergent. Rather high concentrations of around 1% were

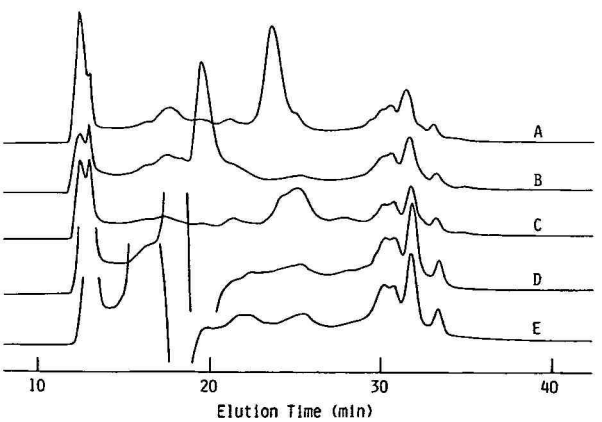

Fig. 2. Chromatograms of rat liver microsomal membrane proteins (0.1 mg) obtained at 280 or 246 nm on a TSK G3000SW column in 50 m*M* phosphate buffer (pH 7.0) containing 0.2 *M* sodium chloride, detergent and 20% glycerol. Detergent: (A) 1.2% octyl glucoside; (B) 0.05% $C_{12}E_8$; (C) 1% CHAPS; (D) 0.1% Emulgen 911; (E) 0.2% Triton X-100.

necessary with octyl glucoside and CHAPS, 0.1 and 0.2%, respectively, with Emulgen 911 and Triton X-100.

Fig. 2 shows the effect of the detergent type. A crude sample of rat liver microsomal membrane proteins was separated with eluents containing various types of detergents. Considerably different patterns were obtained. With Emulgen 911 and Triton X-100, very large positive and negative peaks appeared at elution times between 15 and 20 min. Although the best detergent cannot be specified from these results, it can be said that for some samples or for some components in a sample the separation may be improved by changing the type of detergent.

The sodium chloride concentration was varied from 0.1 to 0.4 *M*, which resulted in almost no change in elution pattern. Changing the glycerol concentration from 0 to 20% also had almost no effect. Therefore, glycerol can be incorporated into the eluent to any concentration in this range, if necessary, for example in order to stabilize membrane proteins. When the flow-rate was reduced from 0.8 to 0.4 ml/min, a slightly higher resolution was attained.

Ion-exchange chromatography

Fig. 3 shows the effect of the detergent concentration in ion-exchange chromatography. A crude sample of rat liver microsomal membrane proteins was separated with eluents containing octyl glucoside at various concentrations. The decrease in the concentration of octyl glucoside to below 0.8% caused a decrease in recovery of protein. However, similar results were obtained at octyl glucoside concentrations above 0.8%. Figs. 4 and 5 also show the effect of the detergent concentration. A crude sample of rat liver microsomal membrane proteins was separated in the presence of various concentrations of $C_{12}E_8$. With decreasing concentration of $C_{12}E_8$, the peak area also decreased, although only slightly. Moreover, it is clear from Fig. 5 that with decreasing detergent concentration the membrane proteins were eluted later as broader peaks. Similar behaviour was observed with all detergents examined here, and also for human placenta mitochondrial membrane proteins. Therefore, it may be said in general that, in ion-exchange chromatography of membrane proteins,

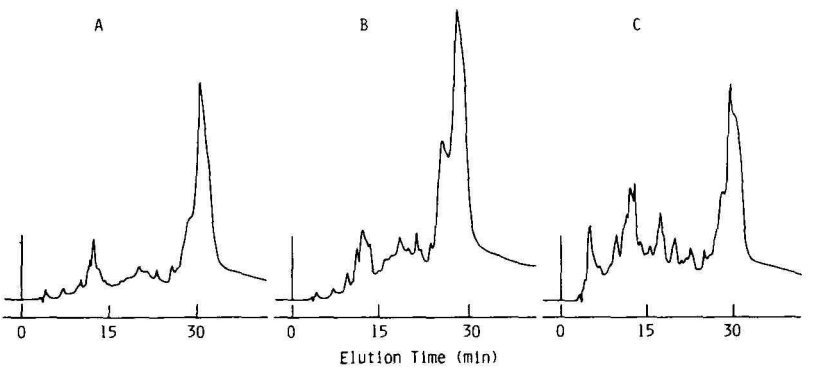

Fig. 3. Chromatograms of rat liver microsomal membrane proteins (2 mg) obtained at 280 nm on TSK DEAE-5PW column with a 60-min linear gradient from 0 to 1 *M* sodium chloride in 20 m*M* Tris–HCl buffer (pH 8.0) containing octyl glucoside and 20% glycerol. Octyl glucoside concentration: 0.2 (A); 0.4 (B); 0.8% (C).

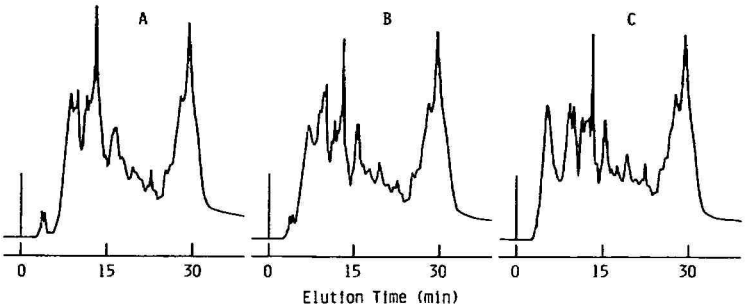

Fig. 4. Chromatograms of rat liver microsomal membrane proteins (2 mg) obtained at 280 nm on a TSK DEAE-5PW column with a 60-min linear gradient from 0 to 1 *M* sodium chloride in 20 m*M* Tris–HCl buffer (pH 8.0) containing $C_{12}E_8$ and 20% glycerol. $C_{12}E_8$ concentration: 0.05 (A); 0.1 (B); 0.2% (C).

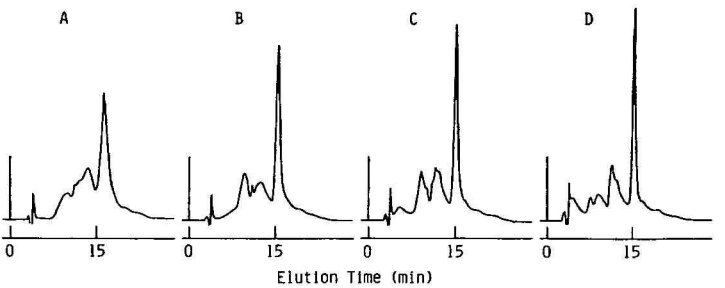

Fig. 5. Chromatograms of rat liver microsomal membrane proteins (2 mg) obtained as in Fig. 4 except the detection was at 417 nm. $C_{12}E_8$ concentration: 0.05 (A); 0.1 (B); 0.2 (C); 0.4% (D).

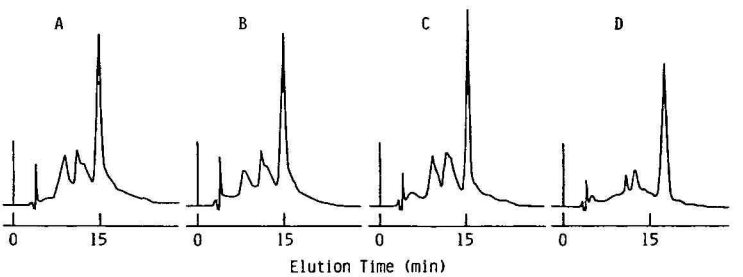

Fig. 6. Chromatograms of rat liver microsomal membrane proteins (2 mg) obtained at 417 nm on a TSK DEAE-5PW column with a 60-min linear gradient from 0 to 1 *M* sodium chloride in 20 m*M* Tris–HCl buffer (pH 8.0) containing detergent and 20% glycerol. Detergent: (A) 0.2% Triton X-100; (B) 0.2% Emulgen 911; (C) 0.2% $C_{12}E_8$; (D) 0.8% octyl glucoside.

the detergent should be incorporated into the eluent at a concentration above a certain level, which varies depending on the detergent. At concentrations below the critical values, membrane proteins are eluted later as broader peaks and in lower yields as the detergent concentration decreases. The critical detergent concentrations were about 0.8% for octyl glucoside and about 0.2% for $C_{12}E_8$, Emulgen 911 and Triton X-100. CHAPS was not examined in ion-exchange chromatography because

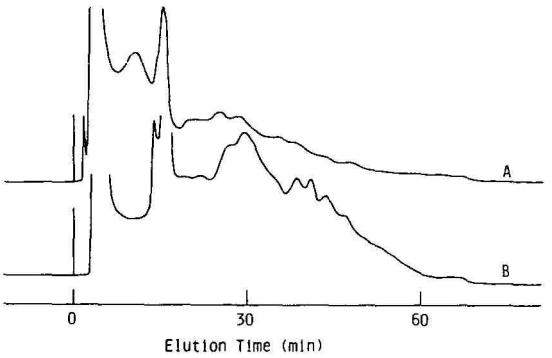

Fig. 7. Chromatograms of rat liver microsomal membrane proteins (2 mg) obtained at 280 nm on a TSK Ether-5PW column with a 60-min linear gradient of ammonium sulphate from 1.5 *M* (A) or 2.25 *M* (B) to 0 in 0.1 *M* Tris–HCl buffer (pH 7.5) containing 0.4% octyl glucoside and 20% glycerol.

it could not be dissolved in the initial eluent to give sufficiently high concentrations.

Fig. 6 shows the effect of the type of detergent. Similar results were obtained with $C_{12}E_8$, Emulgen 911 and Triton X-100. However, $C_{12}E_8$ provided narrower peaks than Emulgen 911 and Triton X-100. With octyl glucoside, the recovery was slightly low. Therefore, $C_{12}E_8$ may be said to be the best of all the detergents examined as regards high resolution and recovery.

The effects of the gradient time, type of buffer and salt, eluent pH, glycerol concentration and flow-rate were similar to those observed for soluble proteins[60,61]. Namely, slightly higher resolution was attained with longer gradient times or higher flow-rates. A change in the type of buffer and salt, eluent pH or glycerol concentration resulted in a change in elution pattern. This suggests that the separation may be improved by manipulating these variables. In addition, membrane proteins were eluted slightly later at higher pH and with a lower glycerol concentration.

Hydrophobic interaction chromatography

Fig. 7 shows the effect of the initial concentration of ammonium sulphate. A crude sample of rat liver microsomal membrane proteins was separated with gradients of ammonium sulphate from 1.0, 1.5 and 2.25 *M* to 0 in the presence of 0.4%

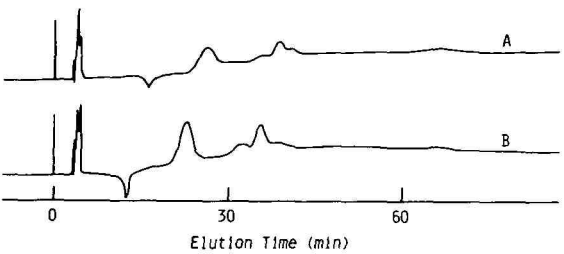

Fig. 8. Chromatograms of rat liver microsomal membrane proteins (2 mg) obtained at 417 nm on a TSK Ether-5PW column with a 60-min linear gradient from 0.1 *M* Tris–HCl buffer (pH 7.5) containing 2.25 *M* ammonium sulphate, 0.4% octyl glucoside and 20% glycerol to 0.1 *M* Tris–HCl buffer (pH 7.5) containing 0.4% (A) or 0.8% (B) octyl glucoside and 20% glycerol.

octyl glucoside. When the initial ammonium sulphate concentration was 2.25 *M*, most of the components in the sample were retained on the column. However, at a concentration of 1.5 *M*, approximately half of the components were eluted with the void volume of the column, and when the initial concentration was 1.0 *M*, most of the components were eluted from the column with no retention. Therefore, in order to retain membrane proteins, it is generally necessary to incorporate around 2 *M* ammonium sulphate into the initial eluent.

Fig. 8 shows an example of the effect of the detergent concentration in hydrophobic interaction chromatography. Membrane proteins were usually eluted earlier as narrower peaks in higher yields as the detergent concentration increased. Therefore, detergents should be incorporated into the eluents at sufficiently high concentrations. However, because the initial eluents contain high concentrations of ammonium sulphate, the dissolution of detergents is ordinarily limited to rather low levels. Accordingly, the detergent should be incorporated into the initial eluent at its maximum concentration. This is also true for the final eluent, as long as the detergent remains solubilized during the whole process of gradient elution. When the initial ammonium sulphate concentration was 2.25 *M*, up to 0.4% octyl glucoside could be dissolved in the initial eluent and 0.8% octyl glucoside could be incorporated into the final eluent without precipitation of the detergent during gradient elution. Fig. 8 shows chromatograms of a crude sample of rat liver microsomal membrane proteins obtained in the presence of 0.4% octyl glucoside in both the initial and final eluents, or in the presence of 0.4% octyl glucoside in the initial eluent and 0.8% octyl glucoside in the final eluent. When the octyl glucoside concentration in the final eluent was increased from 0.4 to 0.8%, membrane proteins were eluted earlier as narrower peaks and in higher yields. Up to 0.01% $C_{12}E_8$ could be dissolved in the initial eluent containing 2 *M* ammonium sulphate, and up to 0.04% could be incorporated in the final eluent without precipitation.

Fig. 9 shows the effect of the detergent type. Different elution patterns were

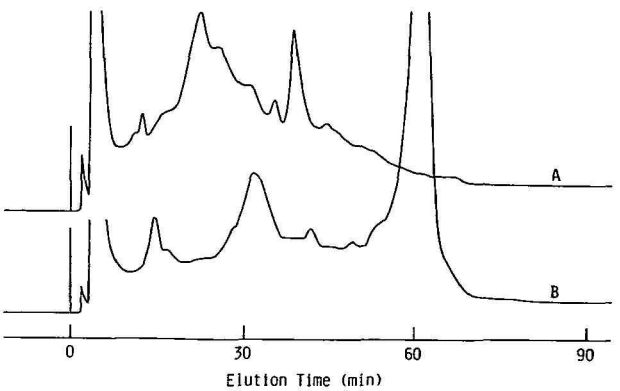

Fig. 9. Chromatograms of rat liver microsomal membrane proteins (2 mg) obtained at 280 nm on a TSK Ether-5PW column with a 60-min linear gradient from 0.1 *M* Tris–HCl buffer (pH 7.5) containing 2.25 *M* ammonium sulphate, 0.4% octyl glucoside and 20% glycerol to 0.1 *M* Tris–HCl buffer (pH 7.5) containing 0.8% octyl glucoside and 20% glycerol (A) or from 0.1 *M* Tris–HCl buffer (pH 7.5) containing 2 *M* ammonium sulphate, 0.01% $C_{12}E_8$ and 20% glycerol to 0.1 *M* Tris–HCl buffer (pH 7.5) containing 0.04% $C_{12}E_8$ and 20% glycerol (B).

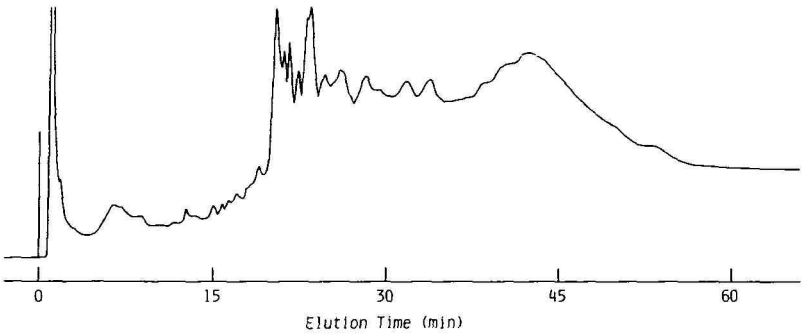

Fig. 10. A chromatogram of rat liver microsomal membrane proteins (0.6 mg) obtained at 220 nm on a TSK Phenyl-5PW RP column with a 2-min linear gradient from 5 to 20% acetonitrile followed by a 48-min linear gradient from 20 to 80% acetonitrile in 0.1% TFA.

obtained with different types of detergents. Therefore, it may be possible to improve some separations by changing the type of detergent. However, only octyl glucoside and $C_{12}E_8$ were successful among the detergents examined here. When Emulgen 911 and Triton X-100 were employed, membrane protein peaks were broad even at the maximum detergent concentrations. With CHAPS, it was difficult to retain membrane proteins on the column.

Reversed-phase chromatography

Fig. 10 shows a chromatogram of a crude sample of rat liver microsomal membrane proteins obtained at a TFA concentration of 0.1%. At 0.05% TFA a slightly different pattern was obtained. These results suggest that reversed-phase chromatography can be employed to separate membrane proteins. However, proteins are usually denatured during such a separation. Therefore, reversed-phase chromatography can be employed only when it is unnecessary to retain the native structures of the proteins.

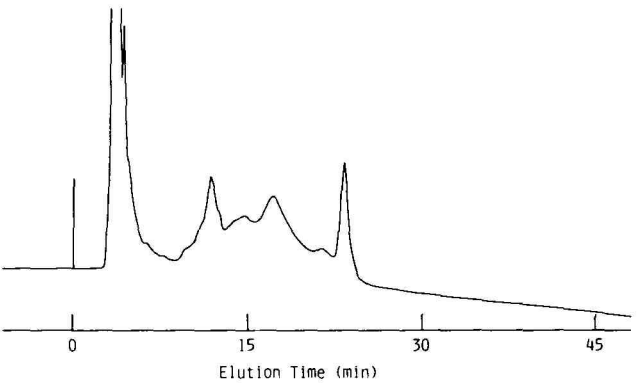

Fig. 11. A chromatogram of human placenta mitochondrial membrane proteins (0.5 mg) obtained at 280 nm on a TSK Chelate-5PW column with a 60-min linear gradient from 0 to 0.2 *M* glycine in 20 m*M* Tris–HCl buffer (pH 8.0) containing 0.5 *M* sodium chloride, 0.4% $C_{12}E_8$ and 20% glycerol.

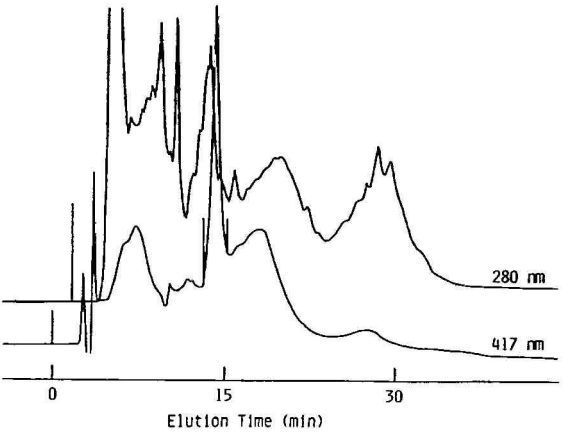

Fig. 12. Separation of crude rat liver microsomal membrane proteins (2 mg) by ion-exchange chromatography on a TSK DEAE-5PW column with a 60-min linear gradient from 0 to 1 *M* sodium chloride in 20 m*M* Tris–HCl buffer (pH 8.0) containing 0.4% $C_{12}E_8$ and 20% glycerol.

Metal chelate affinity chromatography

Fig. 11 shows an example of a separation of human placenta mitochondrial membrane proteins by metal chelate affinity chromatography. This result was obtained with an eluent containing 0.4% $C_{12}E_8$. When the concentration of $C_{12}E_8$ was $\leqslant$0.2%, membrane proteins were eluted later as broader peaks in lower yields, just as in the cases of other modes of HPLC. When octyl glucoside, Emulgen 911 or Triton X-100 was used in place of $C_{12}E_8$, the peaks were small even in the presence of those detergents at high concentrations of around 1%. Therefore $\geqslant$0.4% $C_{12}E_8$ seems to be the first choice for metal chelate affinity chromatography of membrane proteins.

When the eluent pH was varied in the range 7.5–8.5, membrane proteins were eluted earlier and components eluting with the void volume of the column increased at lower pH. The glycerol concentration had little effect on the results in the range 0–20%.

Purification of cytochrome b_5 by HPLC

As shown above, various modes of HPLC have been found to be applicable

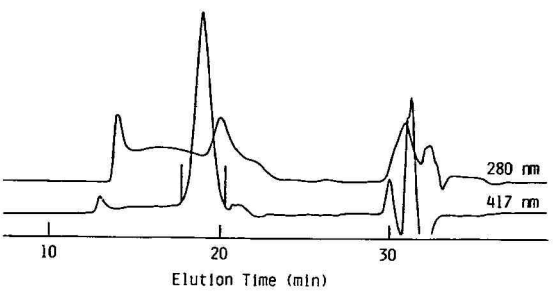

Fig. 13. Separation of the fraction from ion-exchange chromatography (Fig. 12) by gel filtration on a TSK G3000SW column in 50 m*M* phosphate buffer (pH 7.0) containing 0.2 *M* sodium chloride, 0.4% $C_{12}E_8$ and 20% glycerol.

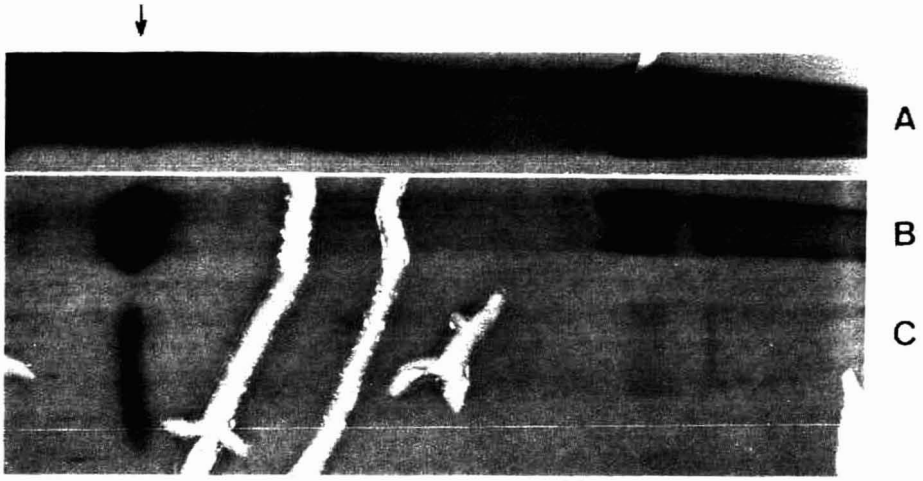

Fig. 14. Purity test of cytochrome b_5 fractions by SDS-PAGE. Samples: A = original crude sample of rat liver microsomal membrane proteins; B = fraction from ion-exchange chromatography; C = fraction from gel filtration. Bands indicated by the arrow correspond to cytochrome b_5.

to the separation of membrane proteins. Therefore, combinations of different modes of HPLC should be very effective in the purification of membrane proteins. Cytochrome b_5 was purified to exemplify the effectiveness of these modes. A crude sample of rat liver microsomal membrane proteins was first separated by ion-exchange chromatography (Fig. 12). The peak appearing at *ca.* 15 min in the chromatogram monitored at 417 nm contained cytochrome b_5, and the column effluent between the two vertical lines was collected. This fraction was subsequently applied to a gel filtration column (Fig. 13). The peak appearing at *ca.* 19 min in the chromatogram monitored at 417 nm was cytochrome b_5, and was collected between the two vertical lines. Fig. 14 shows the result of a purity test of the cytochrome b_5 fractions by sodium dodecyl

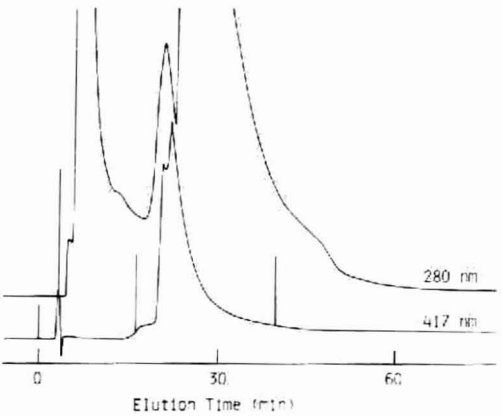

Fig. 15. Separation of crude rat liver microsomal membrane proteins (8.6 mg) by hydrophobic interaction chromatography on a TSK Ether-5PW column with 10-min isocratic elution in 0.1 *M* Tris–HCl buffer (pH 7.5) containing 1.5 *M* ammonium sulphate, 0.01% $C_{12}E_8$ and 20% glycerol followed by 5-min linear gradient to 0.1 *M* Tris–HCl buffer (pH 7.5) containing 0.04% $C_{12}E_8$ and 20% glycerol and then isocratic elution with the final eluent.

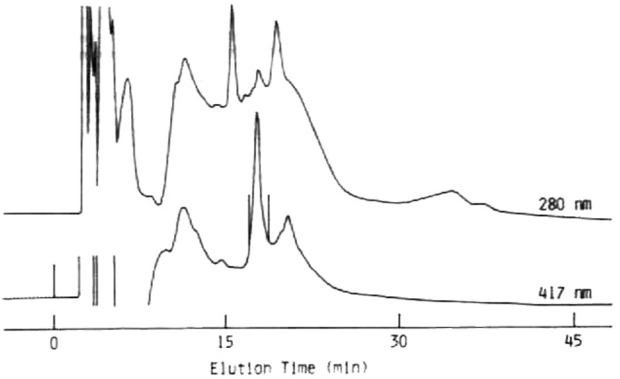

Fig. 16. Separation of the fraction from hydrophobic interaction chromatography (Fig. 15) by ion-exchange chromatography. Conditions as in Fig. 12.

sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). It is clear that the purity of cytochrome b_5 was greatly increased by the ion-exchange chromatography. Furthermore, only a major band corresponding to cytochrome b_5 and a few minor bands are seen in the pattern for the fraction from gel filtration, indicating that rather pure cytochrome b_5 was obtained from a crude sample of solubilized rat liver microsomes by only two steps, namely high-performance ion-exchange chromatography and gel filtration.

Another combination was also investigated. Hydrophobic interaction chromatography was employed in the first step. Fig. 15 shows chromatograms of a crude sample of rat liver microsomal membrane proteins obtained by hydrophobic inter-

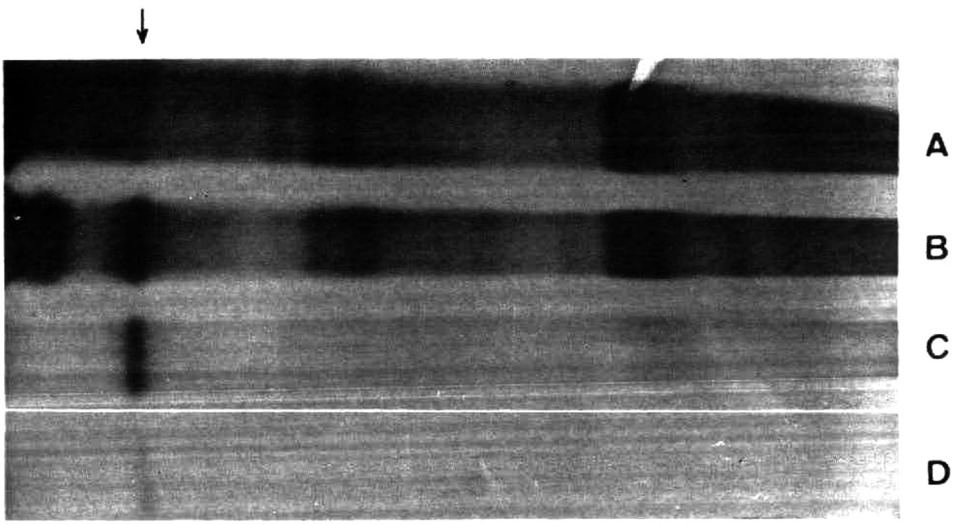

Fig. 17. Purity test of cytochrome b_5 fractions by SDS-PAGE. Samples: A = original crude sample of rat liver microsomal membrane proteins; B = fraction from hydrophobic interaction chromatography; C = fraction from ion-exchange chromatography; D = fraction from gel filtration. Bands indicated by the arrow correspond to cytochrome b_5.

action chromatography. The peak appearing in the chromatogram monitored at 417 nm was fractionated between the two vertical lines. That fraction was then subjected to ion-exchange chromatography after dialysis against the initial eluent of ion-exchange chromatography. The result is shown in Fig. 16. The peak appearing at *ca.* 18 min in the chromatogram monitored at 417 nm was fractionated, further purified by gel filtration and the peak of cytochrome b_5 was collected. The result of a purity test of the cytochrome b_5 fractions is shown in Fig. 17. Although many impurities are seen in the pattern for the fraction from hydrophobic interaction chromatography, cytochrome b_5 was surely enriched. Moreover, contaminants were greatly reduced by ion-exchange chromatography, and finally cytochrome b_5 was purified to near homogeneity by gel filtration.

CONCLUSION

All HPLC modes studied here can be applied to the separation of membrane proteins by selecting appropriate conditions, although the usefulness of each mode differs. Ion-exchange chromatography seems most effective in general. Reversed-phase chromatography also seems very effective when denaturation of proteins is acceptable. The results obtained depend on the chromatographic conditions. In particular, the type and concentration of detergent are especially important. Octaethylene glycol dodecyl ether seems versatile. When the detergent concentration is not sufficiently high, membrane proteins tend to elute late as broad peaks in low yields. The effects of other conditions such as the flow-rate, gradient steepness, type of buffer and salt and eluent pH are similar to those observed for soluble proteins.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TORNALATE® IN SOLUTION DOSAGE FORMS; A SPECIFICITY STUDY

TERRY D. WILSON
Department of Pharmaceutical Sciences, Sterling-Winthrop Research Institute, Rensselaer, NY 12144 (U.S.A.)
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SUMMARY

A reversed-phase high-performance liquid chromatographic method has been applied utilizing ion-pairing to measure Tornalate® in solution dosage forms. Specificity of the method was demonstrated by selectivity for Tornalate analysis, analysis of stressed samples and by peak homogeneity tests. These included the diode-array derived spectral overlay test and fraction collection with rechromatography in an alternate normal phase system. Linearity was also demonstrated in terms of recovery from synthetic samples and detector response.

INTRODUCTION

Tornalate® (bitolterol mesylate) is the di-*p*-toluate ester prodrug of the β-adrenergic N-*tert.*-butylarterenol (colterol). It is used as a bronchodilator for bronchial asthma and reversible bronchospasms. Its *Chemical Abstracts* notation is 4-{2-[(1,1-dimethylethyl)amino]-1-hydroxyethyl}-1,2-phenylene-4-methylbenzoate (ester) methanesulfonate (salt) and it is pictured as number 4 in Fig. 1.

Bitolterol has been assayed in the past by UV spectrophotometry[1] and by phosphorimetry following thin-layer chromatography (TLC)[2]. In addition, an high-performance liquid chromatographic (HPLC) method was used to assay bitolterol in a preformulation stability study which showed the compound to be most stable at pH 3.5 in a water–ethanol (70:30) solvent mixture[3]. This method employed an ODS column with an acetonitrile–5% monosodium citrate in 5% citric acid–water (65:5:30) mobile phase.

A reversed-phase ion-pairing HPLC method has been developed to assay Tornalate in solution forms. This has supplanted a previous HPLC method which used a PAC column with a chloroform–methanol–isopropylamine (85:15:02) mobile phase for stability assay purposes. The reversed-phase method was found to be superior in terms of column stability, batch-to-batch column reproducibility, peak shape and resolution characteristics and system precision.

The present report outlines the results of a specificity study carried out in

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support of this reversed-phase method. It includes linearity of recovery, precision, detector linearity, column selectivity and peak purity data required to show the method is stability-indicating.

EXPERIMENTAL

Reagents

Water was Nanopure from a Sybron/Barnstead Nanopure II system, acetonitrile was HPLC-grade from Fisher Scientific, tetrahydrofuran was HPLC-grade from Burdick and Jackson and acetic and orthophosphoric acid were analytical-reagent grade from Mallinckrodt. Octanesulfonic acid sodium salt was from Eastman-Kodak, chloroform was HPLC-grade from J. T. Baker, methanol was analytical-reagent grade from Mallinckrodt and formic acid was analytical-reagent grade from Aldrich. Ethanol was 200 proof from U.S. Industrial Chemicals and propylene glycol was reagent grade from Fisher.

Compounds studied

The compounds utilized in this study included: bitolterol mesylate, colterol mesylate, colterol monoester mesylate, colterol triester mesylate, ketobitolterol–HCl and acetyloxybitolterol mesylate which were all from Sterling Drug. In addition 4-methylbenzoic acid (*p*-toluic acid) from Eastman-Kodak was used.

Apparatus

Several modular HPLC systems were used in this study consisting of Beckman 110A, Waters 6000A or Varian 5000 pumps; Waters 440 or 441 fixed-wavelength or Kratos SF 770 variable-wavelength UV–VIS detectors; Rheodyne 7125 manual injectors with 20-μl fixed loops and Fisher Recordall 5000 recorders. In addition, a Hewlett Packard 1040A diode array detector was used as was a Hewlett Packard 3357 laboratory automation system. Reversed-phase columns used in this study included Whatman Partisil ODS-3 10-μm, ODS-3 5-μm and IBM and Brownlee RP-18 5-μm columns. The normal phase column used was 5-μm silica gel from Alltech Assoc. All columns were 25 cm $\times$ 4.6 mm I.D. The flow-rate and detection wavelength used in all cases were 1.0 ml/min and 254 nm, respectively.

Mobile phases

A mobile phase consisting of water–acetonitrile–glacial acetic acid–sodium octanesulfonate (380:600:20:0.65, v/v/v/w) was used for the reversed-phase work. Initial variation in the water–acetonitrile ratio from 430:550 to 180:800 was examined keeping acetic acid and octanesulfonate constant with respect to column used and the capacity factor (k') obtained for bitolterol. The alternate normal phase system included tetrahydrofuran–acetonitrile–water–orthophosphoric acid (250:500:250:10) as mobile phase.

Thin-layer chromatography

A TLC system was used to provide confirmation of the stressed sample and placebo results. This consisted of 0.25-mm precoated silica gel 60 F-254 plates from Merck with a mobile phase of chloroform–methanol–formic acid (80:10:10). Follow-

ing 10-μl sample application and drying, the plates were developed to about 10 cm above the point of application, dried in warm air and viewed under both short (254 nm) and long (360 nm) wavelength UV light.

Linearity of recovery from simulated samples

Duplicate sets of simulated samples were prepared for each of the 0.083% (w/v) and 0.033% (w/v) bitolterol mesylate unit dose solutions. These contained 0%, 80%, 100% and 120% of the stated bitolterol levels in addition to the appropriate placebo and were diluted with a water–acetonitrile–acetic acid (380:600:20) dilution solvent to about 0.03 mg/ml for the 100% samples.

Detector linearity

The linearity of detector response–bitolterol mesylate concentration relationship was examined starting with an initial 0.2 mg/ml solution. This was serially diluted 1 to 10 or 1 to 5 with the above dilution solvent.

Selectivity

Solutions of bitolterol mesylate and the analogues listed in compounds studied above, potential impurities and degradation products, were chromatographed separately and together in the ion-pairing reversed-phase system.

Sample assay

Unstressed samples and samples held at 40°C for twelve months as well as a placebo solution stressed for 6 h at 70°C were chromatographed by the reversed-phase system and by the TLC system. In addition, the stressed sample was chromatographed undiluted in this LC system. The peak corresponding to bitolterol mesylate was collected and 20 μl was reinjected into the alternate normal phase HPLC system. The stressed bitolterol mesylate sample was also chromatographed using the HP 1040A detector to obtain spectral data as compared to a standard solution.

RESULTS AND DISCUSSION

The effect of mobile phase composition and reversed-phase column packings on capacity factor as well as separation of Tornalate from its monoester and triester analogues (Fig. 1 numbers 2 and 7 respectively) are shown in Fig. 2. Strongest retention in all cases was provided by the 5-μm spherical reversed-phase packings. In particular the two Brownlee and single IBM columns resulted in a k' value of 10–20 times that found for the irregular particle ODS-3 columns for colterol triester. Similarly bitolterol itself and colterol monoester were retained respectively between 3 and 9 times and between 2 and 4 times as strongly by the spherical as by the irregular bonded phases. While this powerful retention is available in these columns it is not necessarily required. In fact, a method written specifying a less retentive column could be more easily adapted to a more retentive column than the reverse. It can be noted that the differences found between irregular 5-μm and 10-μm ODS-3 columns' retention characteristics were minimal. Conversely the 10-μm ODS-3 intercolumn retention reproducibility was apparent in that the plots for these two columns are nearly coincident. It is evident that small changes in mobile phase composition have

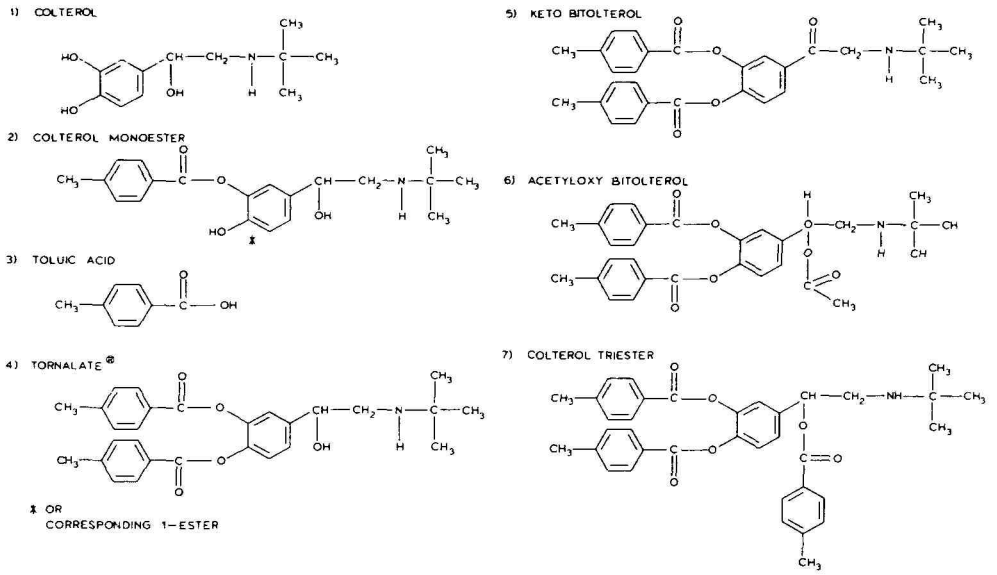

Fig. 1. Structures of compounds investigated in order of elution using reversed-phase system. These include: colterol (1), colterol monoester (2), *p*-toluic acid (3), bitolterol (4), ketobitolterol (5), acetyloxybitolterol (6) and colterol triester (7).

a larger effect on the k' of colterol triester than on either of Tornalate or colterol monoester, there being no hydroxyl groups available in the former for possible interactions with exposed silica hydroxyls.

Tornalate, as a prodrug, has the requirement that a stability-indicating assay method must separate not only potential process inpurities and degradation products

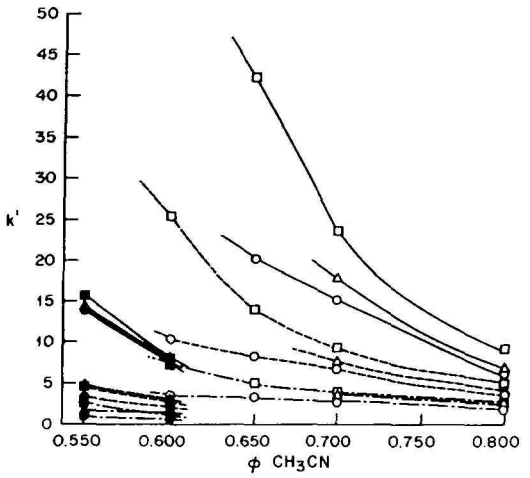

Fig. 2. Relationship between k' for bitolterol (— — —), colterol monoester (— · — · —) and colterol triester (—) and volume fraction of acetonitrile (φ) in the mobile phase showing the effect of reversed-phase column packing. 5-μm Columns, 25 cm × 4.6 mm I.D. [○ = IBM; □ = Brownlee (1); △ = Brownlee (2); ● = Partisil ODS-3]. 10-μm Columns, 25 cm × 4.0 mm I.D. [■ = Partisil ODS-3 (1); ▲ = Partisil ODS-3 (2)].

but also the intended active compound, colterol in this case. This was easily obtained with the present system owing to increased polarity of colterol over bitolterol; the former lacking the di-*p*-methylbenzoates. Overall column selectivity is shown in Fig. 3. The order of elution of bitolterol analogues corresponds to the number given compounds in Fig. 1. Good peak shape was obtained for all components on the 10-μm ODS-3 columns used. Colterol monoester, compound 2, gave two peaks in the chromatogram because of an equilibrium process that occurred in solution in which the 1- and 2-methylbenzoate esters were interconvertible.

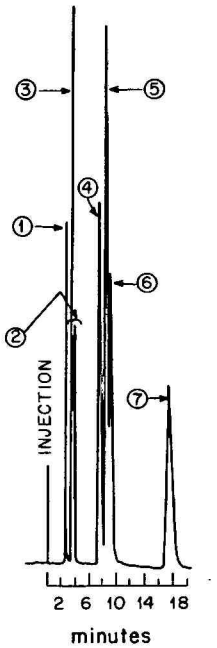

Fig. 3. Chromatogram of a mixed standard containing all compounds listed in Fig. 1 using the mobile phase water–acetonitrile–acetic acid–octanesulfonate (380:600:20:0.65, v/v/v/w). Component 4 is bitolterol.

Linearity of recovery of bitolterol from synthetic samples was obtained with results shown in Table I. Acceptable statistics were found for both the 0.033% and 0.083% (w/v) formulations with average percent recoveries of (100 ± 1)% and percent relative standard deviation (R.S.D.) recoveries of less than 1.5% by peak height or area measurement. Only slightly higher correlation coefficients were found for the 0.083% solution while the 0.033% solution gave slightly better *y*-intercepts.

An indication of system precision is given in Table II. Triplicate injections of fresh standard solutions prepared on day one to three gave the percent R.S.D. values shown, all well below 1%, by peak height or peak area measurement. In addition to a precision requirement of not more than 1.5% for replicate standards, the system suitability test consisted of a resolution factor calculation between bitolterol and colterol triester and an 80% linearity check. The minimum acceptable resolution

TABLE I

BITOLTEROL MESYLATE RECOVERY FROM SIMULATED SAMPLES

Peak height measurement						*Peak area measurement*					
0.33 mg/ml			*0.83 mg/ml*			*0.33 mg/ml*			*0.83 mg/ml*		
Added (mg)	*Found (mg)*	*Recovery (%)*	*Added (mg)*	*Found (mg)*	*Recovery (%)*	*Added (mg)*	*Found (mg)*	*Recovery (%)*	*Added (mg)*	*Found (mg)*	*Recovery (%)*
0.0	0.0	–	0.0	0.0	–	0.0	0.0	–	0.0	0.0	–
0.0	0.0	–	0.0	0.0	–	0.0	0.0	–	0.0	0.0	–
13.2	13.3	100.76	33.2	33.4	100.60	13.2	13.2	100.0	33.2	33.4	100.60
13.2	13.0	98.48	33.2	33.6	101.20	13.2	13.1	99.24	33.2	33.6	101.20
16.5	16.4	99.39	41.6	41.7	100.24	16.5	16.4	99.39	41.6	41.7	100.24
16.5	16.2	98.18	41.6	41.6	100.0	16.5	16.1	97.58	41.6	41.5	99.76
19.8	19.4	97.98	49.9	49.5	99.20	19.8	19.6	98.99	49.9	49.6	99.40
19.8	19.8	100.0	49.9	49.3	98.80	19.8	20.1	101.52	49.9	49.3	98.80
Average recovery (%)		99.13			100.01			99.45			100.00
R.S.D. recovery (%)		1.12			0.890			1.298			0.864
Slope		0.990			0.994			0.997			0.995
Intercept		0.016			0.141			−0.024			0.132
Correlation coefficient		0.99982			0.99989			0.99970			0.99990

TABLE II

SYSTEM PRECISION

	Peak height ($\mu V\ s \cdot 10^{-4}$)			*Peak area ($\mu V\ s \cdot 10^{-5}$)*		
	Day 1	*Day 2*	*Day 3*	*Day 1*	*Day 2*	*Day 3*
	7.252	7.335	7.345	8.433	8.524	8.628
	7.168	7.342	7.363	8.415	8.516	8.630
	7.183	7.332	7.394	8.479	8.486	8.645
Mean	7.201	7.337	7.367	8.442	8.509	8.634
R.S.D. (%)	0.6	0.07	0.3	0.4	0.2	0.1

TABLE III

LINEARITY OF DETECTOR RESPONSE–CONCENTRATION OF BITOLTEROL MESYLATE RELATIONSHIP

Bitolterol mesylate concentration (mg/ml)	*Peak height (· 10^{-3}) (μV s)*	*Peak area (· 10^{-4}) (μV s)*
0.000202	0.7	0.9
0.00202	5.2	6.5
0.00404	10.9	14.8
0.0202	51.8	64.3
0.202	476	640
Correlation coefficient	0.999968	0.999996
R.S.D. of y (%)	1.74	0.61

factor was set at 7 while the 4 in 5 diluted standard had to fall between 78 and 82% of the average standard value.

Results of the linearity of detector response–bitolterol mesylate concentration relationship are shown in Table III. Over a thousand-fold range of dilution, excellent correlation coefficients were obtained with a moderately improved %R.S.D. value found by peak area.

Duplicate HPLC analysis of stressed and unstressed bitolterol mesylate solutions gave results shown in Table IV. No degradation was seen in twelve months at 25°C while a loss of about 7% resulted from the 40°C stress for twelve months. Corroborating evidence was obtained by TLC where 6–8% estimated total inhomogeneity was determined by comparison to diluted reference standard spots. No interference resulted from stressed placebo as shown by the assay values and the blank. Chromatograms in Fig. 4D and E were unstressed and stressed placebo respectively. Fig. 4A is a bitolterol standard with a retention time of about 8 min while Fig. 4B and C represents unstressed and stressed Tornalate solutions. The stressing gave rise to additional components at about 4 and 7 min which show the method to be stability-indicating. Major degradation products have been tentatively identified based

TABLE IV

HPLC ASSAY AND ESTIMATED TOTAL INHOMOGENEITY BY TLC OF STRESSED AND UNSTRESSED BITOLTEROL MESYLATE SOLUTION AND STRESSED PLACEBO

Sample	*Conditions*	*Bitolterol mesylate assay (% claim)*	*Estimated total inhomogeneity (%)*
Solution	12 months, 25°C	100.0, 100.5	1
Solution	12 months, 40°C	92.5, 93.0	6–8
Placebo	6 h, 70°C	0.0, 0.0	–

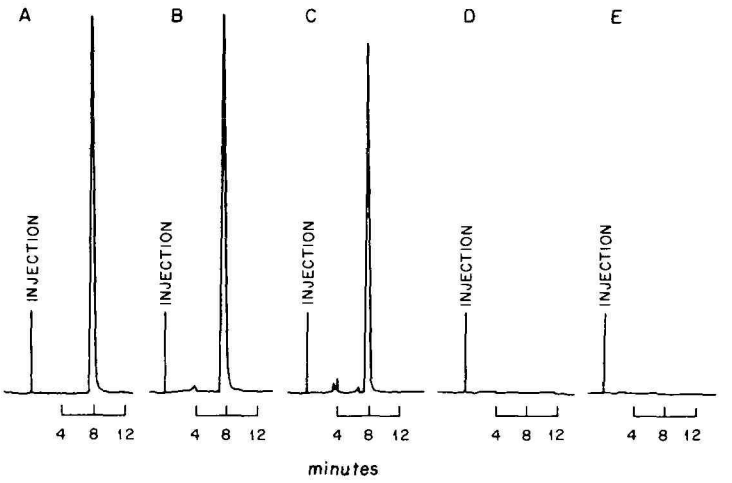

Fig. 4. Chromatograms of bitolterol mesylate reference standard (A), unstressed Tornalate solution (B), Tornalate solution stressed twelve months at 40°C (C), unstresed placebo (D) and placebo stressed 6 h at 70°C (E).

on HPLC retention time data and R_F values from TLC as *p*-toluic acid and colterol monoester. These are seen at R_F 0.62 and 0.41 respectively in Fig. 5, a drawing of the thin-layer chromatogram of standards and stressed and unstressed bitolterol solution samples and placebos.

Homogeneity of the stressed Tornalate solution bitolterol peak was indicated by chromatographing this sample using the HP 1040A diode array detector. When

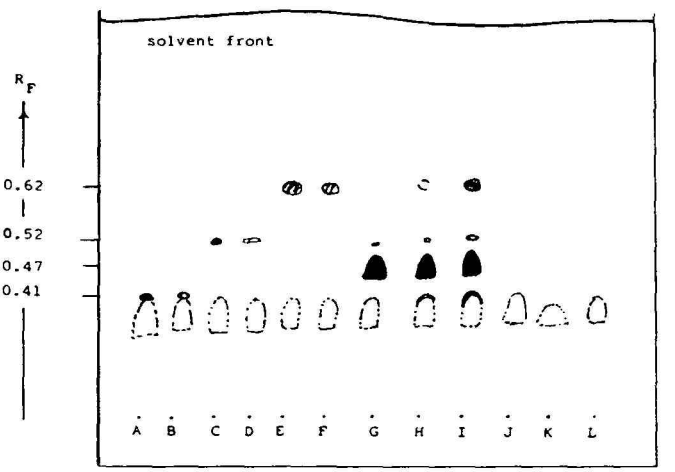

Fig. 5. Thin-layer chromatogram of standards, stressed and unstressed bitolterol samples and placebos. These are identified as: (A) colterol monoester (0.4 μg); (B) colterol monoester (0.2 μg); (C) colterol triester (0.4 μg); (D) colterol triester (0.2 μg); (E) *p*-toluic acid (0.4 μg); (F) *p*-toluic acid (0.2 μg); (G) bitolterol mesylate standard (20 μg); (H) Tornalate solution unstressed; (I) Tornalate solution stressed twelve months at 40°C; (J) unstressed placebo; (K) placebo stressed 6 h at 70°C; (L) and dilution solvent [water–propylene glycol–ethanol (50:25:25)].

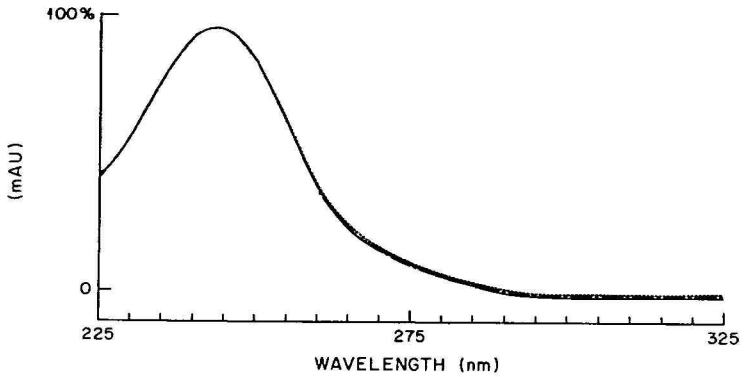

Fig. 6. UV spectral scans of the bitolterol peak in stressed Tornalate solution taken at the upslope (—), apex (----) and downslope (— — —) absorbance normalized and overlaid on a bitolterol reference standard (— · — · —) peak apex scan.

this peak was scanned at the inflection points of the upslope and downslope and at the apex from 225 to 325 nm absorbance normalized and overlaid on a bitolterol standard peak apex scan, Fig. 6 resulted. Any UV absorbing constituent coeluting with the bitolterol peak with sufficiently dissimilar spectrum, would be expected to cause a deviation from the standard spectrum. A spectral shift has been measured in this and other laboratories resulting from as little as 1–3% coeluting impurity[4,5]

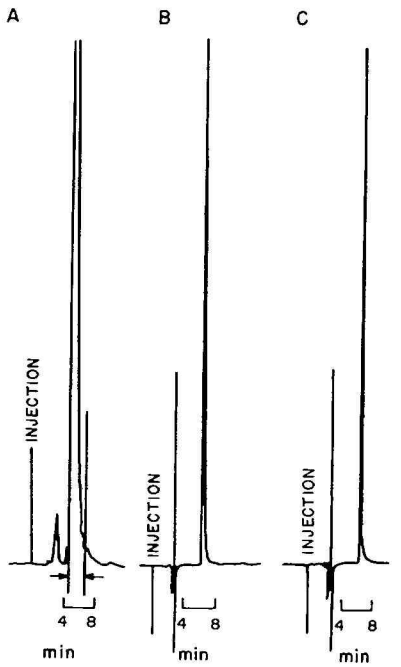

Fig. 7. Chromatogram of undiluted stressed Tornalate solution run in the reversed-phase system (A). Arrows indicate the time of fraction collection. Chromatogram of collected fraction (B) and a bitolterol mesylate standard (C) run in the alternate normal phase system.

whereas caution has been urged in the application of the spectral overlay technique especially where similar spectra are involved[6].

Further evidence for bitolterol peak purity came from the peak trapping experiment in which concentrated, stressed Tornalate solution was chromatographed and the bitolterol fraction was collected. A volume of 20 μl of this fraction shown in Fig. 7A was reinjected into the alternate normal phase system and the chromatogram in Fig. 7B was obtained. This showed only one component at a retention time of 6 min corresponding to that of a standard bitolterol peak in Fig. 7C. The normal phase system which gave capacity factors of 1.3, 1.5 and 1.7 for colterol triester, bitolterol and colterol monoester respectively, revealed by the application of a different separation mechanism that no peaks coeluted with that from bitolterol in the reversed-phase system.

The present results show that the ion-pairing reversed-phase assay method for bitolterol mesylate in Tornalate solution is stability-indicating and specific. In addition the method has the precision and accuracy required for routine analysis of stability samples. Indications of bitolterol peak purity were found using the spectral overlay method and chromatography in an alternate HPLC system. TLC of stressed samples as well, revealed no components other than those seen in the reversed-phase method.

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RAPID AND SENSITIVE DETERMINATION OF DEHYDROASCORBIC ACID IN ADDITION TO ASCORBIC ACID BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING A POST-COLUMN REDUCTION SYSTEM

S. J. ZIEGLER, B. MEIER and O. STICHER*

Pharmazeutisches Institut, Eidgenössische Technische Hochschule Zürich, CH-8092 Zürich (Switzerland)

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SUMMARY

An improved procedure for the direct determination of *l*-dehydroascorbic acid (DHAA), in addition to *l*-ascorbic acid (AA), has been developed. The two biologically active forms of vitamin C were separated using reversed-phase high-performance liquid chromatography. DHAA was reduced to AA with dithiothreitol (DTT) in a post-column reaction system. Complete conversion was achieved at 50°C using a 1-ml reaction coil. Recoveries were in the range of 95–99% and both forms could be detected spectrophotometrically at 267 nm with high sensitivity. Reproducible results [relative standard deviations 2.4% (DHAA) and 1.0% (AA), $n = 5$] were obtained when the method was applied to the analysis of rose hip samples.

INTRODUCTION

Owing to the similar biological activities of *l*-ascorbic acid (AA) and its oxidized form, *l*-dehydroascorbic acid (DHAA), the total vitamin C of a sample is the sum of both forms. It has been reported that methods that assay for only the reduced form of ascorbate in biological samples and foodstuffs may provide misleadingly low vitamin C values[1]. Various methods for the quantitative determination of AA have been proposed and several reviews of AA methodology are available[1–3]. A rapid and selective method for the assay of AA in rose hips has been developed previously in our laboratory[4].

The quantitative determination of DHAA, however, is still problematic. Analyses by currently available chemical methods are time consuming and susceptible to interferences with the matrix containing the vitamin. DHAA can be reduced to AA and subsequent analysis yields the total vitamin C. As most biological samples have much greater content of AA than DHAA, quantitation by difference has the disadvantage of being based on the measurement of a small increase over a large background.

A direct determination using high-performance liquid chromatography (HPLC) is possible[5], but lacks sensitivity owing to the poor UV absorptivity of

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DHAA even at low and, therefore, non-specific wavelengths. Keating and Haddad[6] and Baker *et al.*[7] were successful in enhancing the sensitivity by pre-column derivatization of DHAA with *o*-phenylendiamine (OPD); however, problems with the stability of the derivative in aqueous solutions were experienced. Vanderslice and Higgs[8] separated AA and DHAA using an anion-exchange resin, and fluorescent detection was achieved by a relatively complicated, two-step post-column reaction system involving oxidation of AA to DHAA followed by reaction with OPD. This resulted in significant peak broadening and a long run time of more than 30 min.

As a consequence of these limitations, we have developed a rapid and sensitive method for the direct determination of DHAA and AA. Further, we have applied this procedure to the analysis of rose hips in an effort to determine the total vitamin C content.

EXPERIMENTAL

Fig. 1 shows a schematic diagram of the HPLC system used. Isocratic analyses were performed with a Model 6000A pump, equipped with either a U6K injector or a Wisp 712 autosampler (all from Waters Assoc., Milford, MA, U.S.A.). Three different reversed-phase (RP) column materials were used, LiChrosorb RP-8 (5 μm), Nucleosil C_{18} (7.5 μm) and LiChrosorb RP-18 (5 μm), all with Knauer (Berlin, F.R.G.) column cartridges (250 × 4 mm I.D.). The pumping rate of the mobile phase (0.25% metaphosphoric acid) was 1.0 ml/min. For temperature control of the column and the reaction coil (Model URA 102, 1 ml internal volume) (Kratos, Ramsey, NJ, U.S.A.), a PCRS Model 520 heater system (Kratos) was used. The post-column reagent solution was delivered at a flow-rate of 0.5 ml/min by a URS 051 pumping

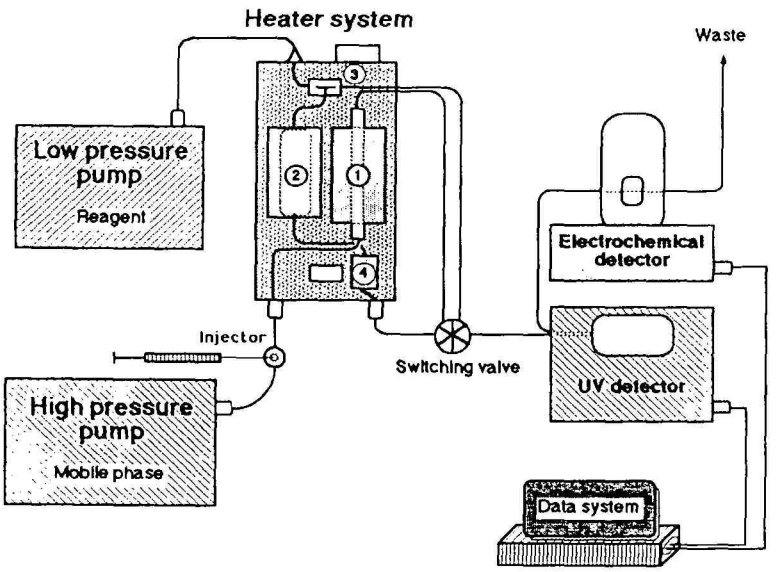

Fig. 1. Schematic diagram of the chromatographic system. 1 = Column; 2 = reaction coil; 3 = vortex mixer; 4 = heat exchanger.

system (Kratos). A Model 7000 switching valve (Rheodyne, Cotati, CA, U.S.A.) allowed the eluent to be routed either to the post-column reaction system or directly to the detectors. The mixed effluent was monitored at 267 nm either with an HP 1040 diode-array detector (Hewlett-Packard, Waldbronn, F.R.G.) or an LC 85 UV detector (Perkin-Elmer, Überlingen, F.R.G.) coupled in series with a Model 4B amperometric detector equipped with a glassy carbon electrode (BAS, West Lafayette, IN, U.S.A.). Owing to high background currents, working potentials above 400 mV *vs.* Ag–AgCl were avoided. For integration, either an SP Model 4000 data system (Spectra Physics, Santa Clara, CA, U.S.A.) or an HP DPU multi-channel integrator with an HP 85 computer was used.

Reagents

l-Ascorbic acid was purchased from Fluka (Buchs, Switzerland). The mobile phase was prepared by dissolving 2.5 g of metaphosphoric acid (Merck, Darmstadt, F.R.G.) in 1 liter of distilled water. The post-column reagent was prepared by dissolving 11.35 g of trisodium phosphate dodecahydrate, 6.65 g of sodium dihydrogen phosphate dihydrate and 150 mg of dithiothreitol (DTT) (all from Fluka) in 500 ml of distilled water, giving a buffered solution of pH 7.6. Mobile phases and reagents were membrane-filtered and deaerated by purging with helium prior to analysis.

Sample preparation

Standards. Stock standard solutions contained between 1 and 80 mg of AA per 100 ml of 1% metaphosphoric acid. AA and DHAA working standard solutions were prepared as follows: 3% bromine solution was added dropwise to 5.00 ml of the stock standard solution in order to oxidize AA to DHAA. Excess of bromine was completely removed by flushing with helium. After addition of a further 5.00 ml of the stock standard solution, the mixture was adjusted to a final volume of 20.00 ml with 1% metaphosphoric acid in a volumetric flask. Volumes of 10 μl of the solution obtained were injected into the chromatographic system or were processed through the clean-up procedure prior to analysis. Identical results were obtained with both methods.

Rose hip samples. Extraction with 1% metaphosphoric acid by means of a Polytron (Kinematica, Kriens, Switzerland) and sample clean-up using Bond-Elut C_{18} (3-ml disposable extraction cartridges) (Analytichem International, Harbor City, CA, U.S.A.) were performed as described previously[4].

Owing to the extreme instability of DHAA in aqueous solutions, the standards and samples cannot be stored for longer than 1 h even when refrigerated and, therefore, must be analyzed immediately after preparation. Calibration runs with freshly prepared working standard solutions were performed daily prior to the analysis of rose hip samples.

RESULTS AND DISCUSSION

Chromatographic separation of AA and DHAA

AA and DHAA could be separated on all the RP column materials used. The best results were obtained with the LiChrosorb RP-18 (5 μm) column owing to a distinctly stronger retardation of AA. The concentration of metaphosphoric acid in

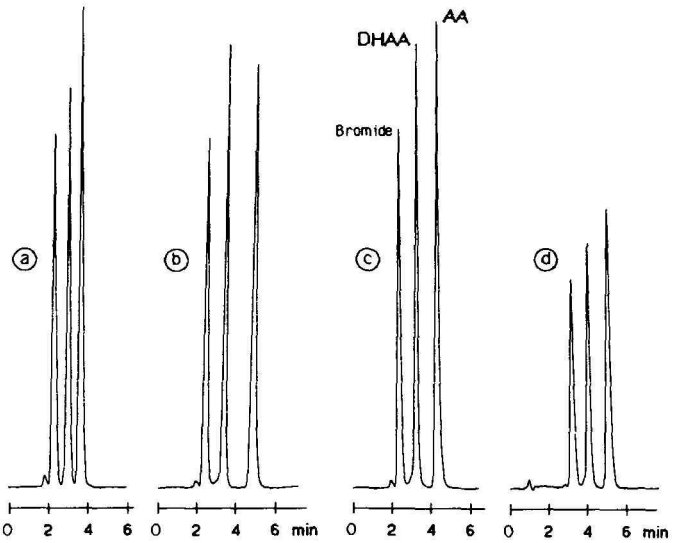

Fig. 2. Effect of the column temperature and of the reaction coil on the separation of DHAA and AA. Column: LiChrosorb RP-18 (5 μm), 250 × 4 mm I.D. Mobile phase: 0.25% metaphosphoric acid (1 ml/min). Injections of 10 μl of a solution containing 4.3 mg/ml of DHAA and 0.1 mg/ml of AA in 1% metaphosphoric acid. Detection at 225 nm. Column temperatures: (a) 20°C; (b) 40°C; (c) and (d) 27°C. Chromatograms a–c were obtained with the post-column reaction system switched off and d after post-column addition of 0.5 ml/min of water and passage through the reaction coil. Bromide (derived from the preparation of DHAA) is only detected owing to the high DHAA concentration needed for the direct spectrophotometric detection and the low wavelength employed.

the mobile phase (0.25%) was reduced from that recommended previously[4] in order to minimize the phosphate buffer concentration needed for the post-column neutralization step.

The influence of column temperature was evaluated over a range 10–50°C. The best separations were achieved at low column temperatures owing to a considerably stronger retardation of AA. Fig. 2a and b shows two examples of chromatograms obtained at 20 and 40°C, respectively. Unfortunately, DHAA elutes rather rapidly from RP columns. While the dead time was measured to be about 87 s (Nucleosil C_{18} column, sodium nitrate peak in water as the mobile phase), the maximum retention time for DHAA was 183 s. Although no difficulties were experienced during the analysis of the rose hip samples, a stronger retardation may be desirable in the analysis of other biological materials, particularly when interfering peaks occur.

Post-column reduction of DHAA

Okamura[9] has described a spectrophotometric method for the determination of AA and DHAA in which DHAA is completely reduced to AA by incubation with DTT. Owing to the rapid conversion, even at low temperatures, a modification of this method for use in a post-column reaction system showed promise. The reaction was found to be dependent on pH, temperature and reaction time. The reduction rate of DHAA by DTT reaches a maximum at pH 6.5–8.0[9]; therefore, the reagent was buffered in order to neutralize the strongly acidic mobile phase.

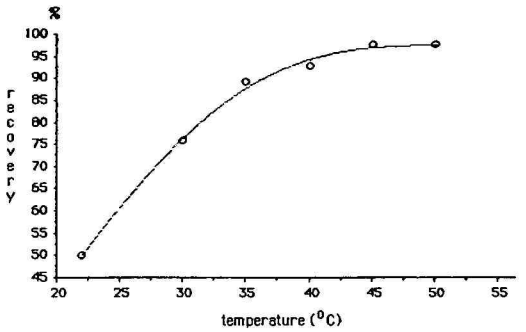

Fig. 3. DHAA reduction rate as a function of the reaction coil temperature.

Fig. 3 shows the relationship between reaction coil temperature and reduction rate. Complete conversion was achieved at temperatures above 45°C with a 1-ml reaction coil, resulting in a reaction time of about 50 s. Peak broadening was minimal and the separation of DHAA and AA was essentially unaffected. Fig. 2c and d show chromatograms obtained with and without the reaction coil.

The mixing of 0.5 ml/min of reagent with 1.0 ml/min of mobile phase caused only a minimal additional pulse, which primarely affected the baseline signal of the electrochemical detector. Almost no interference was observed when using the UV detector at a wavelength of 267 nm.

Detection

The UV absorption characteristics of AA depend strongly on pH. Whereas the absorption maximum at 242 nm in 0.5% metaphosphoric acid was used only for the detection of AA[4], the detection wavelength must be shifted to a higher value when working in a neutral pH range. Fig. 5 shows the UV spectra of both AA and the reduced DHAA taken on-line during a chromatographic run. The absorption maximum is at 267 nm. By using a photodiode-array detector, the identification and purity control of the peaks were greatly facilitated; the conversion of DHAA to AA can be readily validated by superimposing the spectra.

Although the electrochemical detector was superior with respect to sensitivity and selectivity, as described previously[4], our preliminary results now suggest problems such as high background current (originating from the oxidation of DTT) and electrode poisoning, resulting in impaired sensitivity with time and insufficient long-term reproducibility of the detector response. Ways of overcoming these problems have not yet been determined.

Recovery, reproducibility, linearity and sensitivity

The reaction conditions were optimized and the reliability of the method was evaluated by repeated injections of DHAA standards obtained by bromine oxidation as described previously. After post-column reduction, 95–99% (96.7 ± 1.6, $n = 6$ samples) of the original AA was recovered. Results were obtained by direct comparison of the peak area with that of simultaneously injected AA. The reproducibility of the reduction step was 1% [relative standard deviation (R.S.D.), $n = 10$ injections].

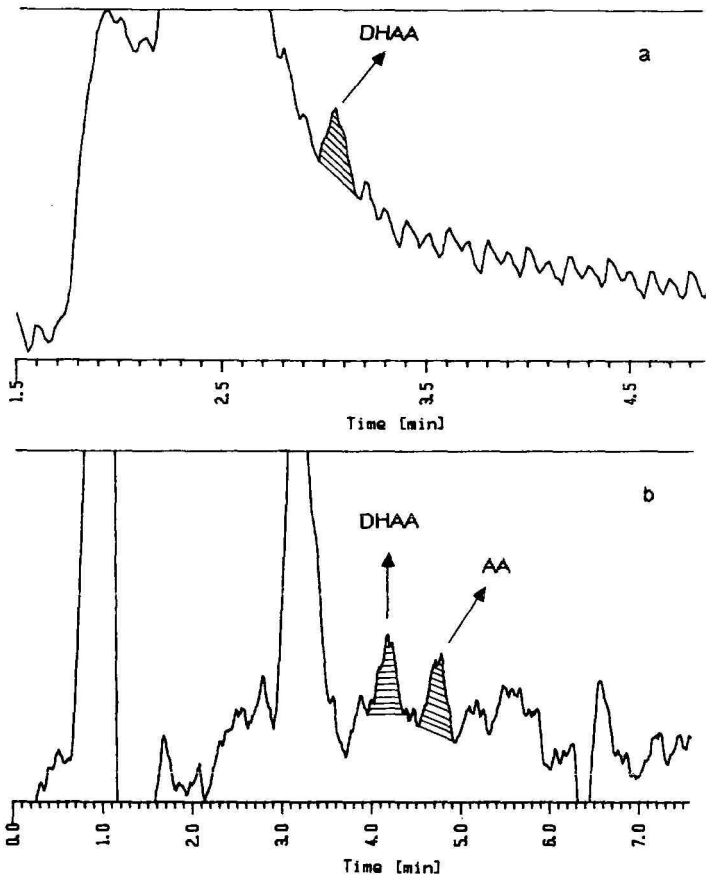

Fig. 4. Evaluation of the detection limits. Column: Nucleosil C_{18} (7.5 μm), 250 × 4 mm I.D.Other conditions as described in the text. (a) Direct detection at 225 nm. Injection volume: 10 μl. Amount injected: $2 \cdot 10^{-7}$ g. (b) Detection at 267 nm after post-column reduction of DHAA. Injection volume: 10 μl. Amount injected: $1.4 \cdot 10^{-9}$ g.

Incomplete conversion or loss of DHAA during the chromatographic run seems unlikely; pre-column reduction of DHAA with subsequent analysis for AA yielded identical recoveries. A small percentage of AA may be converted to diketogulonic acid and further breakdown products during the preparation of DHAA.

The relationship between peak area and concentration was evaluated over the range 0.0064–0.3825 mg/ml and found to be linear (n = 10, correlation coefficient r = 1 of AA and DHAA). The detection limit for DHAA by direct spectrophotometric detection at 225 nm of about $2 \cdot 10^{-7}$ g can be enhanced by a factor of more than 140 by using the proposed post-column system. The detection threshold for DHAA is as sensitive as that for AA and was determined to be $1.4 \cdot 10^{-9}$ g at a signal-to-noise ratio of about 2:1 (Fig. 4).

Assay of rose hip samples

The applicability of the system was demonstrated by the simultaneous deter-

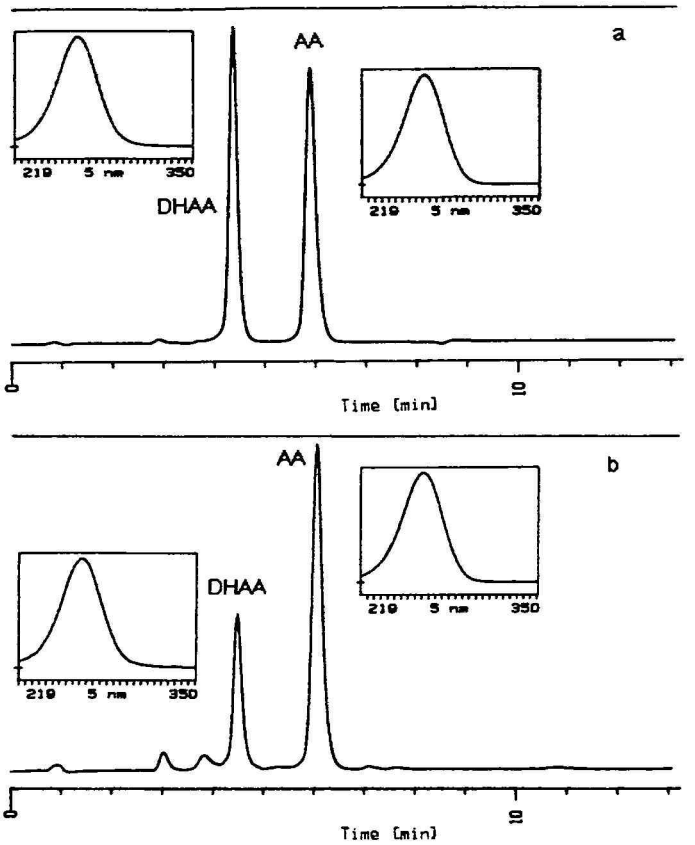

Fig. 5. HPLC elution profiles of (a) a standard solution and (b) a rose hip sample containing 29% DHAA of the total vitamin C content (0.67% dry mass). Column: LiChrosorb RP-18 (5 μm), 250 × 4 mm I.D. Column temperature: 19°C. Other conditions as described in the text. The UV spectra of AA and of the reduced DHAA were taken on-line during the chromatographic run.

mination of AA and DHAA in extracts of rose hips (Fig. 5). The precision of the assay method was checked by multiple analyses of a single sample. The R.S.D. for five measurements was calculated to be 1.0% for AA and 2.4% for DHAA. The sample contained 0.83% of AA and 0.16% of DHAA.

The recoveries of AA and DHAA in different standard solutions added to rose hip samples during sample preparation were found to be 100.1 ± 1.3% and 98.9 ± 1.9%, respectively (n = 5). These results indicate that the assay method has satisfactory accuracy.

As a result of the simultaneous determination of DHAA and AA, the analysis time is nearly the same as that for AA alone[4]. Whereas the chromatographic run time is about 7 min, sample extraction and clean-up, which can be done in triplicate or quadruplicate, require 15–20 min. Complicated and time-consuming pre-column derivatization procedures are not necessary. The use of an autosampler for full automation of the chromatographic system is limited by the labile nature of DHAA in aqueous solution. Although metaphosphoric acid proved to be an excellent stabil-

izing agent for AA, it is of little help with DHAA. A suitably effective alternative has not yet been found.

Results from analyses of various extracts, obtained either from fresh rose hips (frozen and lyophilized) or from commercial products, revealed considerably different vitamin C contents. DHAA values were in the range <1–29% of the total vitamin C content (0.1–1.3% dry mass). Significantly higher DHAA levels were found in lyophilized samples. It has not yet been established whether is a relationship between lyophilization and the DHAA to AA ratio or whether any of the drying procedures using heat are responsible for losses of DHAA.

CONCLUSION

In comparison with other analytical techniques, the method described here is superior with respect to sensitivity, selectivity, accuracy and analysis time. It takes advantage of (1) the high separation performance of RP-HPLC to resolve AA and DHAA, (2) the rapid and complete post-column reduction of DHAA by DTT and (3) the strong UV absorbance of AA. This procedure also proved to be reliable for the routine analysis of the total vitamin C content of rose hips. Moreover, it should also be applicable to the measurement of the DHAA and AA contents of other foodstuffs and biological materials.

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Note

Isotachophoretic determination of the ionic mobilities and ionization constants of weak monoacidic bases by a simple computer-aided slope–intercept method

VLADIMÍR JOKL*, MIROSLAV POLÁŠEK and JAROSLAVA POSPÍCHALOVÁ
Analytical Chemistry Department, Faculty of Pharmacy, Charles University, CS-501 65 Hradec Králové (Czechoslovakia)
(Received December 2nd, 1986)

In recent years isotachophoresis (ITP) has become an attractive analytical tool for the analysis of drugs, especially basic ones. Much attention has been paid to the optimization of the migration process by selecting appropriate electrolyte systems and operating conditions. The ionic mobilities, u, and ionization constants, pK_a, are the fundamental physical-chemical parameters of protolytes that determine (together with the parameters of the leading electrolyte) the effective electrophoretic mobility, $\bar{u}$, of analytes under given conditions and, consequently, the whole course of ITP (the formation and stability of zones, their order and separability). The pK_a values of the analytes are usually known, but this may not be the case if the compounds of interest are, *e.g.*, newly synthesized drugs. The data on ionic mobilities, especially for organic protolytes, are often lacking.

ITP data obtained under strictly defined conditions allow the estimation of the u and pK_a values of protolytes. In contrast to other instrumental methods, ITP has certain advantages: minimum consumption of the sample and no special demands on its purity. The u and pK_a obtained by ITP should be fully sufficient for the above optimization purposes, even though such values do not possess thermodynamic or absolute character.

For fully ionized compounds with known pK_a, u can easily be evaluated by ITP, *e.g.*, by measuring the potential gradient in the sample zone relative to a reference compound[1]. Several computer-aided ITP methods for simultaneous determination of u and pK_a have been devised, involving measurement of a series of effective mobilities (or relative step heights) for a sample in a number of leading electrolytes of various pH_L values. Some methods minimized the difference between experimental and simulated ITP data by refining the input u and pK_a values[2,3], or a set of experimental ITP data is processed by non-linear regression involving iterative calculations of the steady state[4]. Another original approach lies in the measurement of the zone conductivity of the compound in two different leading electrolytes and construction of the isolines of these conductivities (the so-called isoconductors) in an u–pK_a system of coordinates; the intersection of such lines yields the required values[5]. All these methods[2–5] involve relatively complicated calculations and they have been applied only in connection with anionic ITP.

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Direct determination of the ionic mobilities of weak bases including drugs ($pK_a < 6$) by cationic ITP under the conditions in which they are completely protonated is beset by obstacles[6] caused by the relatively high effective mobility of the hydrogen ion which cannot serve as a terminator for low-mobility base cations (it penetrates through the zones and disturbs ITP migration). In this paper a simple slope–intercept method is presented for determining u and pK_a for such bases by using a linear transformation of the $\bar{u}_{X,X} = f(pH_X)$ function (which is computed from only two experimental points). The applicability of this method is exemplified by evaluation of the parameters of weak organic bases (including drugs) in acetate leading electrolytes.

EXPERIMENTAL

Apparatus

A commercial ITP analyzer (URJVT, Spišská Nová Ves, Czechoslovakia) equipped with a single PTFE capillary column (200 mm × 0.3 mm), a conductivity detector and a 30-μl sampling valve was employed in all experiments.

Materials

All chemicals and drugs were of analytical or pharmacopoeial purity and were used without additional purification. Formic acid and acetic acid were purified by isothermal diffusion; twice-distilled water was used as a solvent.

Operating electrolyte systems

The leading electrolytes were prepared from standardized stock solutions of potassium hydroxide and acetic acid without any additives (Table I). The terminating electrolyte was always 0.01 *M* in formic acid; hence H^+ was the terminating ion[6].

Procedure

Aqueous solutions containing tetraethylammonium iodide and the base studied (or its hydrochloride for medazepam), each 0.2 m*M*, were injected. Chlordiazepoxide was initially dissolved in 0.1 *M* hydrochloric acid; its injected solution was 10 m*M* in hydrochloric acid. The analysis was performed at a driving current of 50 μA which was switched to 20 μA after 800 s; under such conditions, the time elapsed

TABLE I

ELECTROLYTE SYSTEMS

	Leading electrolyte		*Terminating electrolyte*
	System 1	*System 2*	
Cation	K^+	K^+	H^+
c_L (m*M*)	10	10	
Counter ion	Acetate	Acetate	Formate
$c_{R,L}$ (m*M*)	40	13	10
pH_L (calculated)	4.28	5.28	

between the start of analysis and the passage of the first zone boundary through the detector was approximately 15–17 min. All analyses were carried out in duplicate or triplicate.

Calculations

All mobilities are expressed in $10^9 \cdot m^2\ V^{-1}\ s^{-1}$. The effective mobilities, $\bar{u}_{X,X}$, of the bases were determined with the use of tetraethylammonium ($u = 33.8$) as the reference ion, according to

$$\bar{u}_{X,X} = 60.9/(h_{X,\,rel} + 0.80) \quad (1)$$

where $h_{X,\,rel}$ is the relative step height of a base X[7].

The evaluation of u_X and $pK_{a,X}$ for bases was performed by means of the IQ-151 32-kbyte microcomputer (ZPA Nový Bor, Czechoslovakia) and our own program written in BASIC 6. The program involves an iteration procedure for minimizing the RFQ function[8] and occupies 1.6 kbytes of the computer random access memory (RAM); a single computation takes about 90 s. Alternatively, the calculation can be accomplished by using a programmable pocket calculator, *e.g.*, Texas Instruments TI-59; a single calculation takes about 15 min.

RESULTS AND DISCUSSION

The effective mobility, $\bar{u}_{X,X}$, of a base X in its own zone is defined by

$$\bar{u}_{X,X} = [H]_X u_X/([H]_X + K_{a,X}) \quad (2)$$

which can be transformed to the equation of a straight line

$$\bar{u}_{X,X} = -K_{a,X}\bar{u}_{X,X}/[H]_X + u_X \quad (3)$$

with a slope of $-K_{a,X}$ and an intercept u_X. To evaluate the parameters of this straight line it is sufficient to determine the effective mobilities, $\bar{u}_{X,X}$, in two leading electrolytes having the same fixed concentration of the leading ion, c_L, but two different concentrations of the same counter ion, $c_{R,L}$ (and, consequently, differing in pH_L). The starting input data are: the ionic mobilities of the leading ion K^+ (76.1), of acetate as the counter ion (42.4) and of H^+ (362.4); $pK_{a,R}$ (4.76) and c_L as constants, $c_{R,L1}$, $c_{R,L2}$ and corresponding experimental values of $\bar{u}_{X,X1}$ and $\bar{u}_{X,X2}$ as variables. Initially the starting values of u_X and $pK_{a,X}$, *i.e.*, $(u_X)_0$ and $(pK_{a,X})_0$ are calculated by using the original $[H]_L$ values. Thereafter, $(u_X)_0$ and $(pK_{a,X})_0$ are used to calculate $[H]_X$ by the RFQ method (a subroutine) and the whole cycle is repeated with the refined $(u_X)_i$ and $(pK_{a,X})_i$ values until the difference between $(pK_{a,X})_{i+1}$ and $(pK_{a,X})_i$ is less than an arbitrary constant, here 0.01 (*cf.*, Fig. 1). As shown in Fig. 2, the simulated function $\bar{u}_{X,X} = f(\bar{u}_{X,X}/[H]_X)$ is not rectilinear after the first approximation, when $[H]_L$ is substituted for $[H]_X$, since the real $[H]_X$ in the zones of the bases is different (lower in this instance). Therefore a straight line connecting the two experimental points would give erroneous values of u_X and $pK_{a,X}$. Iterative approximations of $[H]_X$ make

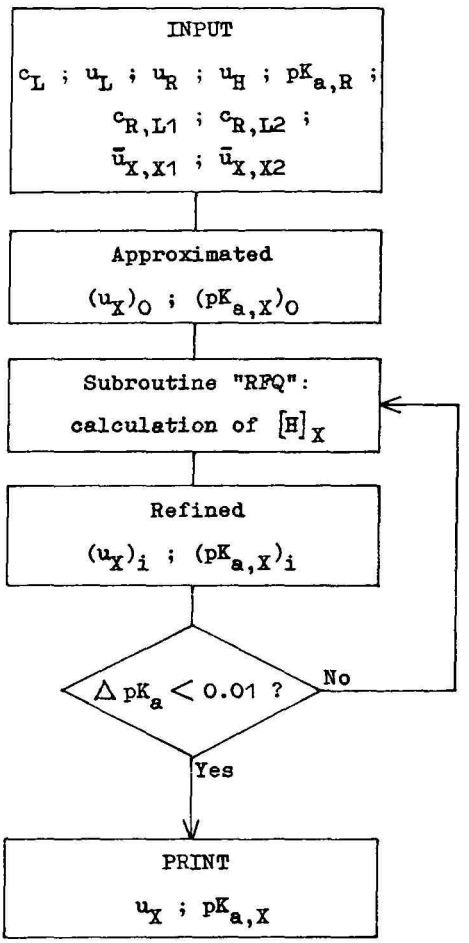

Fig. 1. Schematic flow chart of the program for computing u and pK_a for weak bases from the pairs of experimental values of the effective mobilities, $\bar{u}_{X,X1}$ and $\bar{u}_{X,X2}$.

the function 3 straighter and the calculated u_X and $pK_{a,X}$ converge to the true end values.

The parameters computed for some bases are presented in Table II. The effect of temperature and ionic strength are neglected; it may be presumed that they are compensated to a certain extent by the relative measurement of the mobilities (*cf.*, ref. 5). Hence it seems that the errors in the measurement of the effective mobilities will be the main source of uncertainty in the results. As seen in Table II, the computed $pK_{a,X}$ values are in acceptable agreement with earlier published data for most of the bases. The rather large difference between the computed and literature $pK_{a,X}$ values for 2-methylpyridine (and also the poor reproducibility of the computed result) is connected most probably with difficulties in the determination of the effective mobilities of this base since the values are very close to that of the reference ion. The cause of the large difference in the case of medazepam is not clear; the ITP behaviour

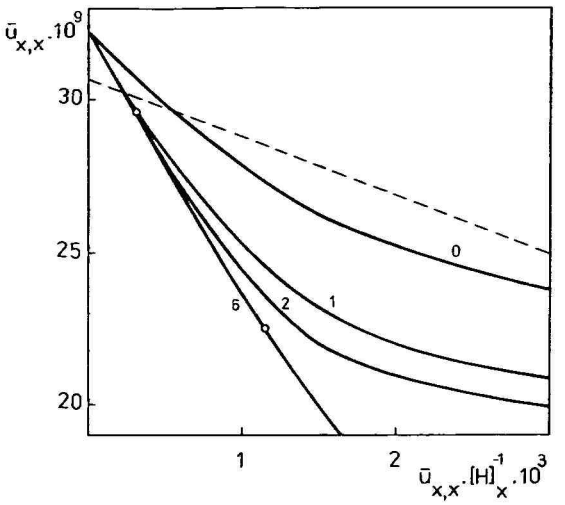

Fig. 2. Convergence pathway of the computation: simulated function $\bar{u}_{x,x} = f(\bar{u}_{x,x}/[H]_x)$ for 1,10-phenanthroline ($u_x = 32.3$, $pK_{a,x} = 5.07$); the two experimental points are shown. Solid curves: 0, first approximation ($pH_x = pH_L$); 1, 2, 6, number of the iteration cycle. The dashed line connects the experimental points (not seen in the diagram) in the first approximation.

of medazepam indicates that it is a substantially stronger base than, *e.g.*, chlordiazepoxide (medazepam exhibits a considerably smaller difference in the effective mobilities determined in the two electrolyte systems employed).

The limitations of the method are first the need for sufficient buffering capacity

TABLE II

PARAMETERS FOR BASES EVALUATED FROM ISOTACHOPHORETIC DATA

$pK_{a,x}$ = ionization constant; u_x = ionic mobility; A, values computed in this study as means of two independent ITP determinations with standard deviations in parentheses; B, literature values (from ref. 11 except where stated otherwise).

Base	$pK_{a,x}$		$u_x \cdot 10^9$ ($m^2\ V^{-1}\ s^{-1}$)	
	A	*B*	*A*	*B*
Aniline	4.67 (0.01)	4.60	38.7 (0.3)	
4-Chloroaniline	4.17 (0.01)	3.98	31.7 (0.4)	
Pyridine	5.13 (0.01)	5.20	51.1 (0.2)	
2-Methylpyridine	6.42 (0.20)	5.97	41.5 (0.4)	
1,10-Phenanthroline	5.07 (0.01)	4.96	32.3 (0.3)	
Hexamethylenetetramine	4.90 (0.01)	5.18	36.8 (0.1)	
6-Aminohexanoic acid	4.44 (0.01)	4.37*	30.0 (0.4)	28.8*
Histidine	6.03 (0.02)	6.04*	29.2 (0.1)	29.6*
4-Aminoantipyrine	4.41 (0.04)	4.18	25.0 (0.5)	
Aminophenazone	5.18 (0.01)	4.94	24.2 (0.1)	
Chlordiazepoxide	4.89 (0.02)	4.6**	20.8 (0.1)	
Medazepam	5.61 (0.01)	4.4**	23.1 (0.2)	

* Ref. 9.
** Ref. 10.

of the counter-ion system in the zones of the bases, and secondly a reliably ascertainable difference between the two experimental values of the effective mobilities of the compound studied. The impossibility of termination at very low effective mobilities is another methodological limitation. Apparently the acetate system can be used to examine bases with a practical range of basicities given approximately by $pK_{a,X} = pK_{a,R} {}^{+1.3}_{-0.4}$. The calculation is fairly sensitive, especially under the extreme limiting conditions, to the reliability of the experimental $\bar{u}_{X,X}$ data. For example, satisfactory convergence is not attained for β-alanine ($pK_a = 3.55$) when calculating its pK_a and u from the actual values of the effective mobilities ($\bar{u}_{X,X1} = 20.0$, $\bar{u}_{X,X2} = 11.8$); in this instance, $pH_X < 4$ and the zone exhibits distinct inversion of mobility with respect to terminating H^+. Analogous $\bar{u}_{X,X}$ values of 17.7 and 10.5 (simulated by using the RFQ function) do not differ greatly from the original ones, but here the calculation converges to the expected result.

In spite of the discussed limitations, the method proposed would be of use, because of its simplicity, to laboratories possessing only modest computation facilities. The results are sufficient for the purposes of prediction and optimization of ITP analysis. The study of compounds exhibiting basicities outside the above recommended range would be possible by using another counter-ion system provided that the effective mobilities are not affected by the base–counter ion interactions and that the free base does not precipitate under the given conditions.

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Note

Response correlation among d.c. and low-frequency a.c. regimes in electron-capture detection*

K. W. MICHAEL SIU* and SHIER S. BERMAN
Division of Chemistry, National Research Council of Canada, Montreal Road, Ottawa, Ontario (Canada)
and
WALTER A. AUE
Department of Chemistry, Dalhousie University, Halifax, Nova Scotia (Canada)
(Received January 9th, 1987)

A recent study of ours[1] speculatively compared a.c. response in the low-frequency range of an electron-capture detector with d.c. response in the same detector. In particular, response at very low frequencies, *e.g.* around 10 Hz, was thought to arise from the regular-field phase of a.c. ("regular" meaning that the radioactive foil serves as the cathode). Response at higher frequencies, from about 100 to 1000 Hz, was considered to originate from both phases, with the "reversed"-field phase (the foil as the anode) adding to the the roughly constant regular-field contribution.

Based on this model we predicted that, in the very low frequency range, a.c. response should correspond to *one half* of d.c. response. This should be the case at any voltage as long as the d.c. potential and the +/− a.c. rectangular wave amplitude were to be the same. Only the regular-field contribution would be observed, despite

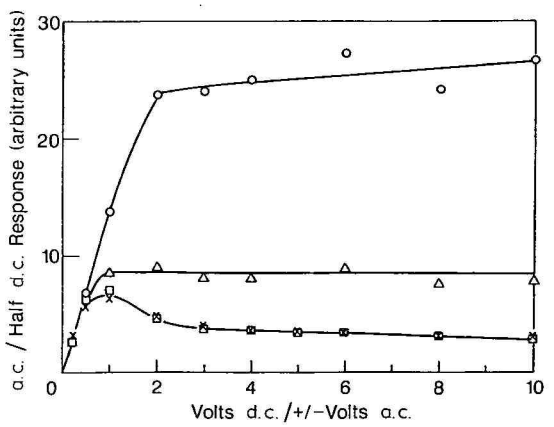

Fig. 1. Response profiles under d.c. and a.c. regimes. Conditions: Varian electron-capture detector, a.c. rectangular wave. ×, d.c. (plotted at half value); □, 4 Hz; △, 100 Hz; ○, 1000 Hz.

* NRCC 27335.

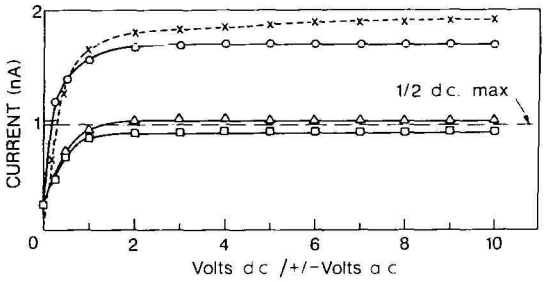

Fig. 2. Current profiles under d.c. and a.c. regimes. Same conditions and symbols as in Fig. 1.

the fact that the electron capture *reaction* is much stronger in the reversed-field than in the regular-field phase.

Owing to the importance that this prediction has for our attempt to interpret the observed behaviour of an electron capture detector driven by an a.c. field, we decided to confirm or deny its validity by direct experiment.

The latter was carried out on a Varian ^{63}Ni electron-capture detector (the detector "A" of our earlier report) under conditions similar in d.c. and a.c., and again with 10 pg lindane as analyte[1].

In Fig. 1, the response at three a.c. frequencies is plotted against *half* the d.c. response. Shown are measurements at 4 Hz (the lowest possible frequency before significant oscillation was registered), 100 Hz and 1000 Hz. Response was also measured at 10 Hz and turned out to be practically identical with the 4 Hz profile of Fig. 1. Fig. 2, for the sake of completeness, provides the corresponding current (baseline) profiles.

These data demonstrate clearly that, in the very low frequency range, a.c. response amounts to half the corresponding d.c. response and, by implication, arises almost completely from the regular-field phase.

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Note

Analysis of coke oven gas by gas chromatography

PERTTI KOKKONEN*
Department of Chemistry, University of Oulu, 90570 Oulu (Finland)
ERKKI OJANIEMI
Research Centre, Rautaruukki Oy, 92170 Raahensalo (Finland)
and
RISTO PÖYKIÖ
Department of Chemistry, University of Oulu, 90570 Oulu (Finland)
(Received November 10th, 1986)

The use of gas chromatography (GC) in the analysis of coke oven gas has been reported by several workers[1–5]. The method has been used to control the purification and chemical recovery process of coke oven gas in iron and steel works. Compared with classical procedures of gas analysis, *e.g.*, IR and UV–fluorescence spectroscopy, chemiluminescence, gamma absorption and titrimetry, GC increases the speed and precision of determinations[6,7]. Doran and Cross[8] studied a sample containing O_2, N_2, CH_4, CO, CO_2, C_2H_6, C_3H_8 and n-C_4H_{10} using a molecular sieve and Chromosorb P columns. Terry and Futrell[9] analysed gases containing C_2–C_6 hydrocarbons in the presence of O_2 and N_2 using a three-column system. For the analysis of mixtures of permanent gases and C_1–C_2 hydrocarbons, Marchio[10] used Porapak and molecular sieve columns.

Although much work has been carried out in this field, no papers on the dependence of the different operational parameters on the separation of these molecules have been published. We have therefore carried out experiments with various packing materials, column lengths, temperatures and flow-rates of the carrier gas in order to establish the optimal conditions for coke oven gas analysis.

EXPERIMENTAL

Column packings

As the stationary phase we used molecular sieves 5A (60–80 and 80–100 mesh) (Ohio Valley Specialty Chemical), Porapak QS (80–100 mesh) (Waters) and Chromosorb 102 (60–80 mesh) (Manville Products). The columns were filled in the usual manner with pure stationary phase material. Each column was pre-conditioned at a temperature at least 30 K higher than its working temperature. All the connections between the columns (1/8 in. O.D.) and the valves and also the column tubes were made of stainless steel. Columns of Porapak QS (500 × 0.2 cm I.D., 80–100 mesh), Chromosorb 102 (360 × 0.2 cm I.D., 60–80 mesh) and Carbosieve S-II (300 × 0.2 cm I.D., 100–120 mesh) were supplied by Perkin-Elmer.

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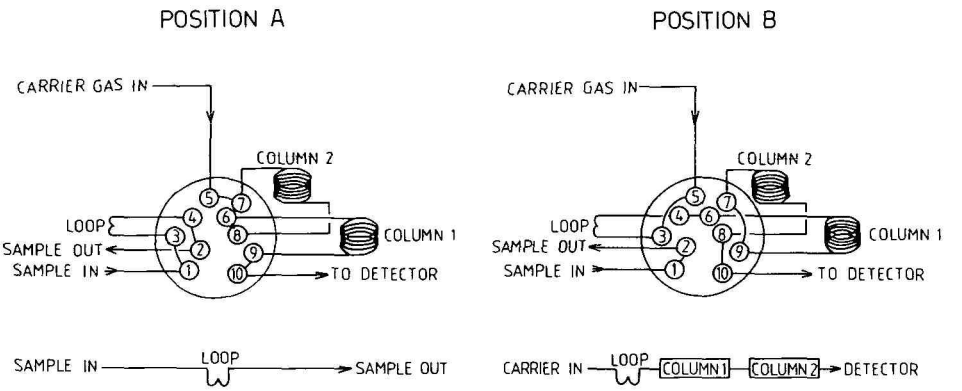


Fig. 1. Schematic diagram of gas sampling and column switching during the analytical run.

Apparatus

The analyses are accomplished by using a Perkin-Elmer Model Sigma 300 dual-column temperature-programmed gas chromatograph equipped with an integrator (Perkin-Elmer LCI-100), instruments for thermal conductivity (TCD) and flame ionization detection (FID), a ten-port valve and a 2.5-ml sample loop. The flow circuit of the instrument is shown in Fig. 1.

Column 1 refers to a Porapak QS or Chromosorb 102 column and column 2 contains molecular sieves. The sample is introduced by means of a gas-sampling valve from a sample loop to the Porapak QS (or Chromosorb 102) column. With the valve in position B, injection of the sample into column 1 permits a portion of the sample to flow into column 2. The separation of CO, CO_2, C_2H_6 and H_2S molecules takes place in this first column, but H_2, O_2, N_2 and CH_4 pass quickly into the molecular sieve column, where they are separated. The valve is thrown from position B to position A just before the CO_2 would elute from the porous polymer, and the components that were in the porous polymer column enter the detector directly. Instead of pure helium, a gas mixture containing hydrogen (8.6 ± 0.3%) mixed with helium manufactured by AGA (Oulu, Finland) was used as the carrier gas. This gas mixture, together with the programmed polarity changing of the recorder at the beginning of each run and back again after the H_2 peak, make it possible to transform an M-shaped negative peak of hydrogen into a normal positive peak for the quantitative determination. The composition of the calibration gas supplied by AGA was as follows: O_2, 0.989 ± 0.020; CO_2, 1.99 ± 0.04; C_2H_6, 2.10 ± 0.04; N_2, 3.05 ± 0.06; CO, 6.89 ± 0.14; CH_4, 26.17 ± 0.26%; and H_2S, 4920 ± 246 ppm; remainder H_2 (*ca.* 58.319%).

The temperature programmes were as follows: time (1), 3–10 min at the initial temperature (15–35°C), after which the column oven was heated to 60°C at a rate (1) of 20–25°C/min, held for time (2) = 0 min in all instances; this was followed by an immediate rise from 60°C to 225°C at a rate (2) of 4–32°C/min, then held for time (3) = 25–35 min. The injector and detector temperatures were 150°C. The flow-rate of the carrier gas varied between 30 and 50 ml/min.

TABLE I

INFLUENCE OF THE OVEN TEMPERATURE, THE FLOW-RATE OF THE CARRIER GAS AND THE PARTICLE SIZE OF THE PACKING MATERIAL ON THE RETENTION TIMES, t_R, OF H_2, O_2, N_2 AND CO GASES USING A MOLECULAR SIEVE 5A COLUMN (200 × 0.2 cm I.D.)

Injector and detector (TCD) temperatures, 150°C.

Component	*Flow-rate (ml/min)*	*Particle size (mesh)*	*t_R (min)*			*Flow-rate (ml/min)*	*t_R (min)*		
			40°C	*50°C*	*60°C*		*40°C*	*50°C*	*60°C*
H_2	30	60–80	0.518	0.504	0.498	50	0.406	0.398	0.401
O_2	30		1.314	1.148	1.074	50	1.021	0.954	0.818
N_2	30		3.05	2.433	2.01	50	2.38	1.829	1.578
CO	30		16.40	11.24	8.62	50	12.66	8.76	6.62
H_2	30	80–100	0.668	0.657	0.650	50	0.520	0.502	0.500
O_2	30		1.489	1.404	1.281	50	1.169	1.090	0.997
N_2	30		3.609	3.116	2.60	50	2.82	2.44	2.03
CO	30		17.17	13.46	9.97	50	13.59	10.10	7.67

RESULTS AND DISCUSSION

The first approach was to test various packing materials and their ability to separate the gas components. Several runs were carried out to find the optimal working conditions. Figs. 2–4 show the chromatograms for different types of packing material. Apart from H_2, O_2 and N_2, the order of separation of the other gas components in the mixture is different for all the columns studied. The peak were verified for all the columns studied. The peaks were verified for all the column types by the standard addition method.

The separation of the O_2 and N_2 using a single Carbosieve S-II column involves long retention times, and the whole run therefore takes a long time although

TABLE II

INFLUENCE OF THE OVEN TEMPERATURE, THE FLOW-RATE OF THE CARRIER GAS AND THE PARTICLE SIZE OF THE PACKING MATERIAL ON THE RETENTION TIMES, t_R, OF H_2, O_2, N_2 AND CO GASES USING A MOLECULAR SIEVE 5A COLUMN (100 × 0.2 cm I.D.)

Injector and detector (TCD) temperatures, 150°C.

Component	*Flow-rate (ml/min)*	*Particle size (mesh)*	*t_R (min)*		*Flow-rate (ml/min)*	*t_R (min)*	
			20°C	*40°C*		*20°C*	*40°C*
H_2	30	60–80	0.262	0.253	50	0.210	0.184
O_2	30		0.666	0.541	50	0.449	0.417
N_2	30		1.98	1.261	50	1.481	0.998
CO	30		12.51	6.15	50	9.20	4.64
H_2	30	80–100	0.276	0.262	50	0.213	0.210
O_2	30		0.696	0.545	50	0.552	0.434
N_2	30		1.85	1.193	50	1.474	0.950
CO	30		15.35	6.94	50	11.59	5.51

a heating rate of 32°C/min is used to raise the oven temperature from 60 to 225°C. In addition this column does not separate H_2S. We therefore considered the influence of the different operational parameters on the separation of the gas components using only Porapak QS and Chromosorb 102 columns together with the molecular sieve 5A column.

Next we tested the influence of the flow-rate of the carrier gas, the oven temperature, the particle size of the packing material and the length of the column on the separation of H_2, O_2, N_2 and CO using the single colecular sieve column. The gas mixture was prepared by mixing nearly equal volumes of the pure components. The results are given in Tables I and II. The retention times and the chromatograms (not presented) show that the separation of H_2 and O_2 sets the limit for the operational parameters. When a flow-rate of 50 ml/min and a column 1 m long filled with 60–80 mesh molecular sieves are used, the highest temperature for a quantitative analysis is 40°C. Tables I and II also show the normal behaviour of the operational parameters: the retention times of the gas components increase with increasing length of the column and decreasing particle size of the packing material, and decrease with increasing oven temperature and flow-rate of the carrier gas. The most influential of these parameters seems to be the length of the column; the particle size of the packing material has only a minor effect on the retention times under otherwise constant conditions.

The third approach was to compare the polymer columns, Porapak QS and Chromosorb 102, with each other using the double column system. Because the 1-m molecular sieve column is full enough to separate the first three components (H_2, O_2 and N_2), we used it together with the two above-mentioned polymer columns.

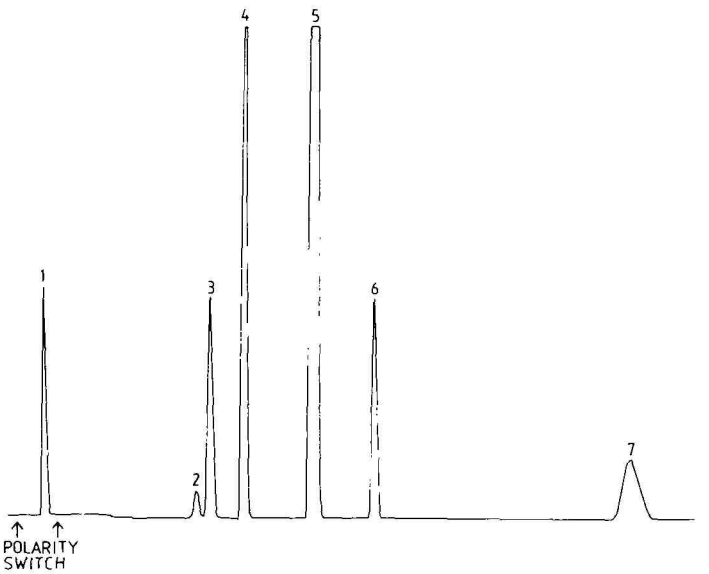


Fig. 2. Chromatogram of the separation of a gas mixture. Column 1 = stainless steel, 300 cm × 2 mm I.D., Carbosieve S-II, 100–120 mesh. Carrier gas, helium–hydrogen mixture, flow-rate 30 ml/min. Oven temperature programme: 20°C for 3 min, then 10°C/min to 60°C, held for 0 min, 32°C/min to 225°C, held for 25 min. Peaks: 1 = H_2; 2 = O_2; 3 = N_2; 4 = CO; 5 = CH_4; 6 = CO_2; 7 = C_2H_6.

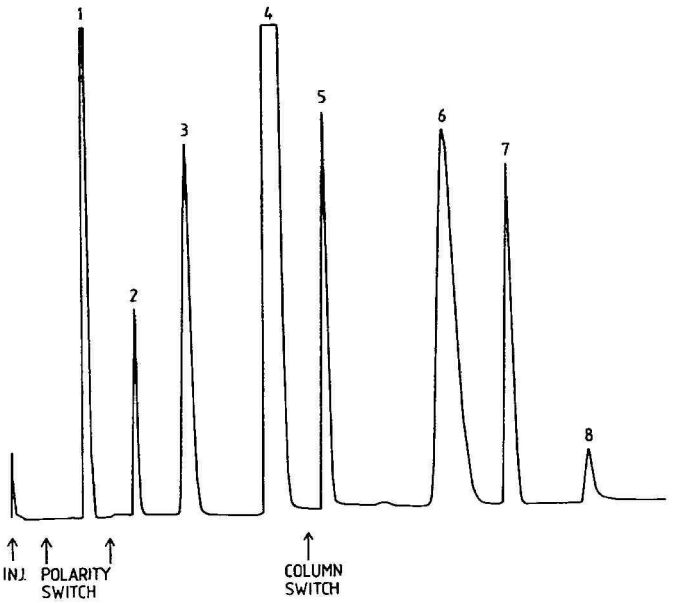
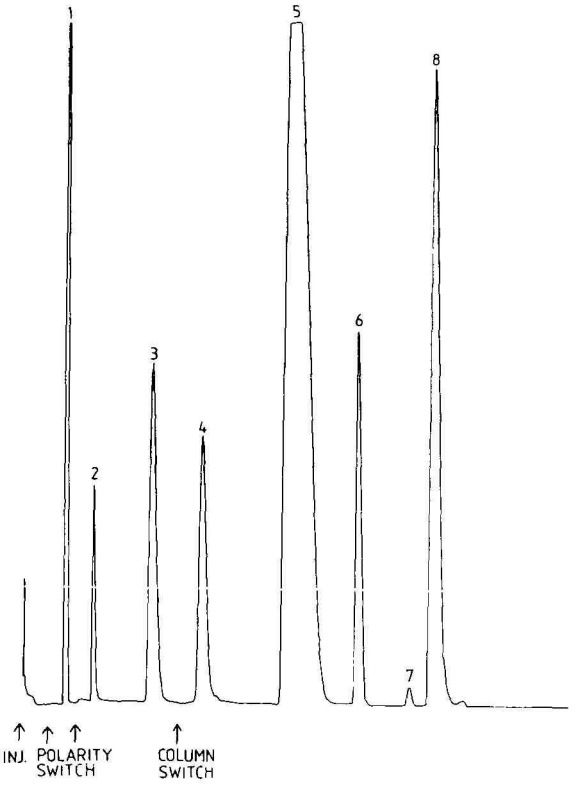


Fig. 3. Typical chromatogram of the separation of permanent and hydrocarbon gases. Column 1 = stainless steel, 500 cm × 2 mm I.D., Porapak QS, 80–100 mesh. Column 2 = stainless steel, 100 cm × 2 mm, I.D., molecular sieve 5A, 60–80 mesh. Carrier gas, helium–hydrogen mixture, flow-rate 10 ml/min. Oven temperature programme: 25°C for 8.5 min, then 25°C/min to 60°C, held for 0 min, 4°C/min to 225°C, held for 25 min. Peaks: 1 = H_2; 2 = O_2; 3 = N_2; 4 = CH_4; 5 = CO_2; 6 = CO, 7 = C_2H_6; 8 = H_2S.

It was found that with the Porapak QS column the critical step in the gas mixture analyses is the separation of CO and C_2H_6. We tested the dependence of Δt_R, the difference between the retention times of C_2H_6 and CO gases, on the flow-rate of the carrier gas and the oven heating rate (2) using the following operational parameters: time (1) = 8.5 min, time (2) = 0 min, time (3) = 25 min, initial oven temperature = 25°C and rate (1) = 25°C/min. The results are given in Fig. 5. Sufficient separation of CO and C_2H_6 for quantitative determination using the molecular sieve column 5A (1 m × 1/8 in. O.D., 60–80 mesh) and the Porapak QS column (5 m × 1/8 in O.D., 80–100 mesh) is achieved when the flow-rate of the carrier gas is 20 ml/min and the oven heating rate (2) is 4°C/min. The total time for the separation of all the components from which H_2S (19.61 min) is the last one. A significantly better separation is achieved with a lower flow-rate (10 ml/min), as shown in Fig. 2. The retention time of H_2S is then 22.65 min.

When the Chromosorb 102 and molecular sieve column system is used, the critical step in the analyses is the separation of H_2S and CO. The operational parameters are given in Fig. 4. It seems that the separation of the components is sufficient at a flow-rate of 50 ml/min when the heating rate (2) is 15°C/min or higher (Fig. 5). When a flow-rate of 50 ml/min and a heating rate (2) of 15°C/min are used, the retention time of the last component is 10.31 min. Fig 5 shows that Δt_R for the separation of CO and H_2S at a constant heating rate (2) increases with decreasing flow-rate of the carrier gas.



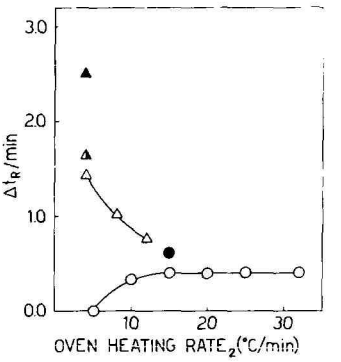


Fig. 4. Chromatogram of the separation of a gas mixture. Column 1 = stainless steel, 360 cm × 2 mm, I.D., Chromosorb 102, 60–80 mesh. Column 2 = stainless steel, 100 cm × 2 mm, I.D., molecular sieve 5A, 60–80 mesh. Carrier gas, helium–hydrogen mixture, flow-rate 30 ml/min. Oven temperature programme: 15°C for 5.5 min, then 20°C/min to 60°C, held for 0 min, 15°C/min to 250°C, held for 35 min. Peaks: 1 = H_2; 2 = O_2; 3 = N_2; 4 = CO_2; 5 = CH_4; 6 = C_2H_6; 7 = H_2S; 8 = CO.

Fig. 5. Δt_R versus heating rate (2) for the separation of successive peaks. The triangles refer to the Porapak QS column and the separation of the CO and C_2H_6 peaks. The symbols △, ▲ and ▲ refer to flow-rates of 30, 20 and 10 ml/min, respectively. The circles relate to the separation of H_2S and CO with the Chromosorb 102 column. The symbols ○ and ● refer to flow-rates of 50 and 30 ml/min, respectively. Oven temperature programmes as in Figs. 3 and 4, except that rate (2) varies.

It can be concluded that a single column system is not sufficient for coke oven gas analysis, although the Carbosieve S-II column alone is able to separate all components except H_2S from the gas mixture studied. When a double column system (molecular sieve column + polymer column) is used, the results show that the shorter Chromosorb 102 column yields a good separation of all the components in nearly half of the time required by the Porapak QS column. The Chromosorb 102 packing material is hence recommended for the GC analysis of coke oven gas.

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Note

Application of a gas chromatographic capillary-to-capillary column-switching system to the analysis of complex illicit drug samples

HELMUT NEUMANN* and HANS-PETER MEYER
Fachgruppe Chemie, Kriminaltechnisches Institut, Bundeskriminalamt, D-6200 Wiesbaden (F.R.G.)
(First received October 8th, 1986; revised manuscript received December 5th, 1986)

Progress in the analysis of complex drug samples using capillary columns in gas chromatography (GC) is well documented[1–3]. In this area we have studied in detail the trace impurity profiles of illicit heroin[4,5] and the determination of alkaloids in opium and crude morphine[6]. However even when using highly efficient and perfectly deactivated dimethylsiloxane capillary columns, some problems such as insufficient separation of certain parts of the impurity profiles, decomposition during the GC process and total masking (overlap) of important components by interfering matrix compounds, remained unsolved.

To address these problems thoroughly, we made use of two capillary columns of different selectivity coupled by a switching device, which should permit separations and determinations that are impossible with a single column[7–10]. The heart-cutting mode, *i.e.*, the transfer of timed cuts from the first to the second column, with enhanced resolution on the second column was applied to improve the separation of certain parts of a heroin impurity profile, to study the GC decomposition of thebaine and to the unmasking of important impurity components. Preliminary results are reported in this paper.

EXPERIMENTAL

All chromatography was performed on a Sichromat 2 GC system (Siemens, Karlsruhe, F.R.G.). The dual-oven gas chromatograph was equipped with a Siemens double T-piece for column-switching purposes (Deans' principle of pneumatic switching). With regard to the flow resistance, we prepared the double T-piece with a 0.23 mm O.D. plantinum–iridium coupling capillary. A 20 m × 0.25 mm I.D. fused-silica capillary column coated with OV-1 or SE-54 (see the legends of the figures) with a 0.15-μm film thickness was installed in the first oven. In order to optimize the switching conditions, the end of this column was coupled with a 5 cm × 0.32 mm I.D. uncoated fused-silica tube by a column-connecting unit (Gerstel, Mülheim, F.R.G.). In the second oven, OV-17 cross-linked fused-silica columns with a film thickness of 0.25 μm and different dimensions (5 m × 0.32 mm I.D. and 20 m × 0.32 mm I.D.) were installed. Helium was used as the carrier gas.

Trapping on the second column was considered essential for peak focusing and to avoid mixed polarity in the retention behaviour within the second column.

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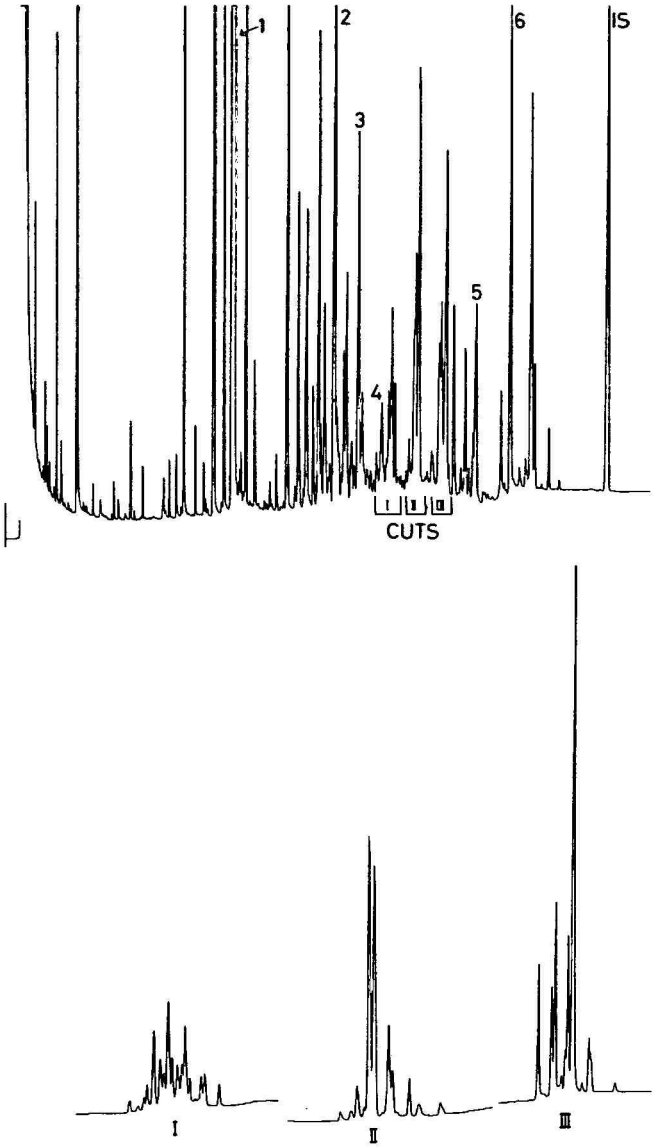

Fig. 1. Chromatograms generated by the trace impurities of an illicit heroin sample. Top: complete chromatogram generated on the first column (SE-54). Oven temperature programme: initial temperature 160°C, raised at 12°C/min to 240°C, 5°C/min to 280°C, then 20°C/min to 330°C. Bottom: separations of selected cuts I–III on the second column (5 m OV-17). Oven temperature programmes: (I) initial temperature 150°C, raised at 8°C/min to 290°C; (II) initial temperature 150°C, raised at 20°C/min to 240°C, then 5°C/min to 290°C; (III) initial temperature 150°C, raised at 20°C/min to 240°C, then 8°C/min to 290°C. Peaks: 1 = acetylthebaol; 2 = 4-acetoxy-3,6-dimethoxy-5-[2-(N-methylacetamido)]ethylphenanthrene; 3 = N-acetylnorlaudanosine; 4 = narcotine; 5 = N-acetylnornarcotine; 6 = (*E*)-N-acetylanhydronornarceine; IS (internal standard) = dotetracontane.

Therefore, a liquid carbon dioxide cold trap (Siemens) was used. Trapping started 2 min before column switching and stopped with the start of the temperature pro-

gramme of the second oven. Switching times and the trapping period were automatically controlled by the controlling device of the Sichromat 2.

Sampling was carried out with a split injector (temperature 300°C; splitter flow-rate 60 ml/min), and for monitoring the effluent from both the first and second columns two flame-ionization detectors (335°C) were used. Further experimental details are given in the legends of the figures.

Samples were prepared as described previously[4–6]. Standard thebaine was obtained from Boehringer-Ingelheim (Ingelheim, F.R.G.), Merck (Darmstadt, F.R.G.) and Knoll (Ludwigshafen, F.R.G.).

RESULTS AND DISCUSSION

Enhanced resolution of heroin trace impurity profiles

The identification of compounds in certain parts of the trace impurity profiles of illicit heroin is still difficult, mainly owing to incomplete chromatographic resolution of these parts of the chromatogram, as indicated by overlapping of peaks in regions I–III in Fig. 1 (top).

Column-switching capillary GC permitted this problem to be addressed by cutting these parts of the profile to a second column with a more polar stationary phase, OV-17 (Fig. 1). Peak group I, which contained the one previously identified peak in this area of the chromatogram (peak 4 = narcotine on the first column coated with SE-54) was separated from 6 peaks into 17, peak group II from 5 into 13, and peak group III from 6 into 12. A comparison of the results of the statistical theory of multi-component chromatograms, which showed that a random chromatogram will never contain more than 37% of its potential peaks[11], supports the considerable progress made here. Fig. 1 also demonstrates that some major peaks with good shapes and without signs of overlapping separate into more peaks on the second column. The major peak in peak group II is an example.

The combination of a non-polar SE-54 first column for an overall good separation of the complex mixture with a medium-polarity OV-17 column for further separation of the problematic parts of the chromatogram therefore offers new approaches to this problem. As indicated by similar work on tobacco smoke samples[12], this technique will improve the ability to identify the impurity compounds in question by mass spectrometry, which is being studied separately.

Study of a decomposition problem in the GC analysis of opium

Capillary GC proved to be a good method for the direct determination of major and minor constituents of opium after silylation without the need for prior extraction or column separation[6]. We used two capillary columns to study further the problem of thebaine decomposition during normal GC analysis, studied previously by a combination of GC and thin-layer chromatographic (TLC) experiments[13].

Fig. 2 (top) shows that on non-polar phases such as OV-1, thebaine chromatographs at lower concentrations as a single peak without obvious signs of decomposition. However, when the alkaloid group is "cut" to a second column, a different picture is obtained. Whereas morphine (peak 3) and codeine (peak 1) again result in single peaks, the "pure" thebaine peak obtained on the first column (peak

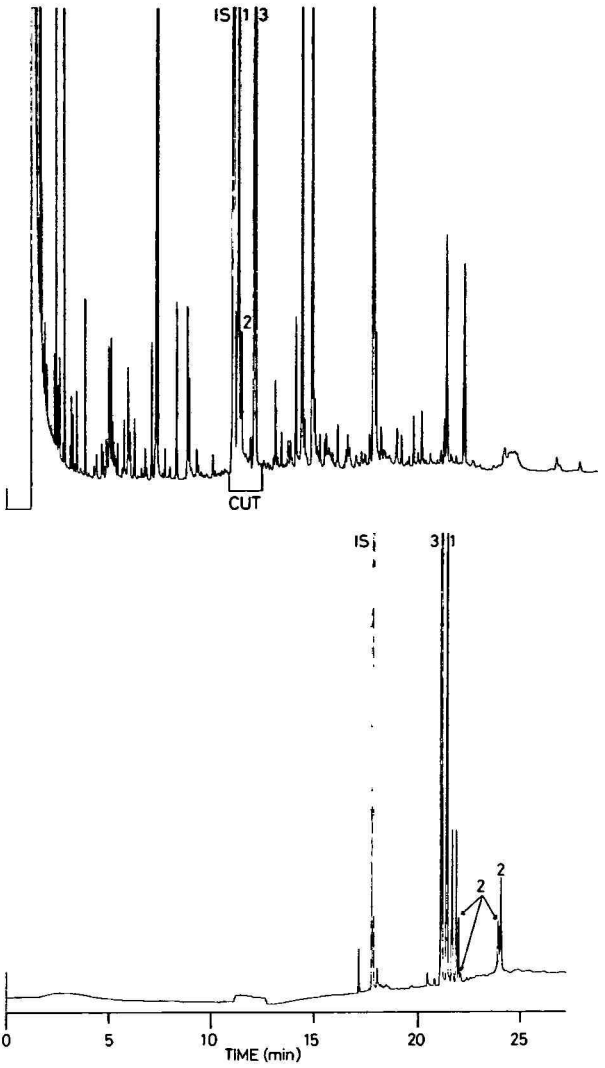

Fig. 2. Top: gas chromatogram of an opium sample on the first column (OV-1). Oven temperature programme: initial temperature 150°C, raised at 9°C/min to 320°C. Bottom: separation of the marked cut fraction on the second column (20 m OV-17). Oven temperature programme: initial temperature 150°C, raised at 25°C/min to 240°C, 4°C/min to 270°C, 25°C/min to 290°C. Peaks: 1 = codeine-TMS; 2 = thebaine; 3 = morphine-TMS; IS = tetracosane (TMS = trimethylsilyl derivative).

2) is separated to four major peaks [Fig. 2 (bottom), peak group 2]. This result was also obtained with different samples of standard thebaine. It is in substantial agreement with previous work[13] in which TLC identified four major decomposition products generated under normal GC conditions. Fig. 2 demonstrates that the instrumental set-up used, with an independent second oven, is capable of complete separation of the cut fraction on the second column within the normal running time of the sample on the first column. It should also be noted that only a combination of

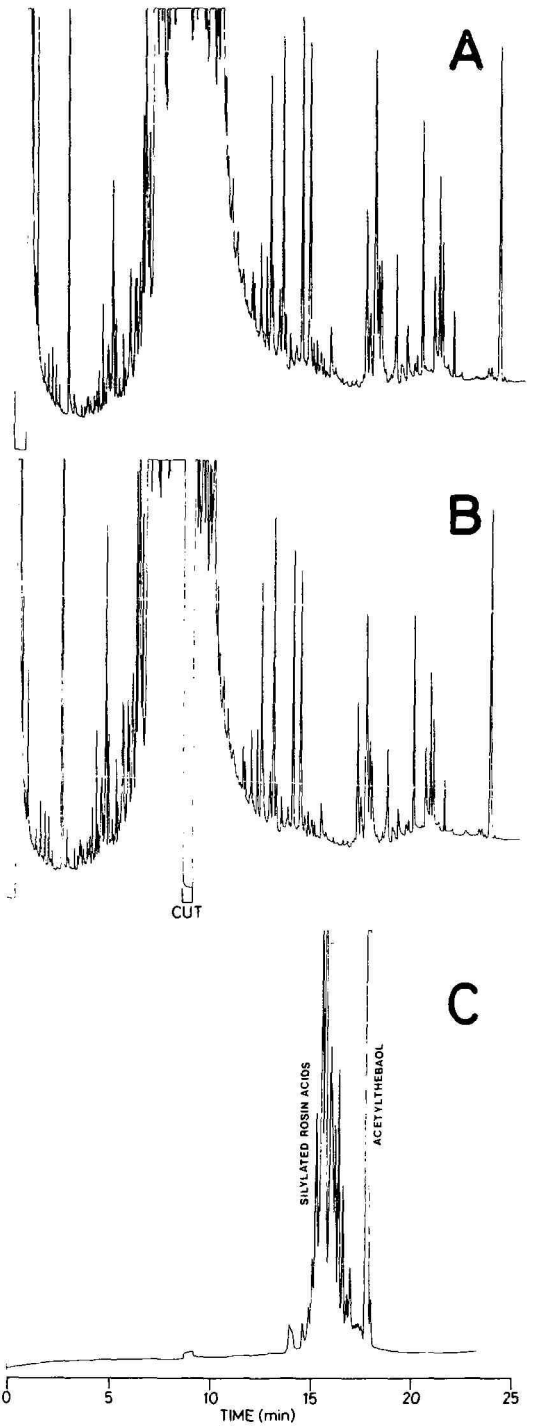

Fig. 3. Chromatograms of trace impurities of an illicit heroin sample diluted with rosin. (A) Complete chromatogram generated on the first column (SE-54). Oven temperature programme as in Fig. 1 (top). (B) Chromatogram of the cut run on the first column (conditions as in A). (C) Separation of the selected cut on the second column (5 m OV-17). Oven temperature programme: initial temperature 150°C, raised at 8°C/min to 290°C.

two columns with different selectivities can solve the problem, as the OV-17 capillary column alone is not suitable for the analysis of high-boiling components such as narcotine[14].

As shown here, the technique used is capable of indicating efficiently if a "pure" peak represents an intact compound or some degradation occurs during the chromatographic process.

Separation and determination of totally masked heroin impurity components

Recently, rosin or colophony was identified in illicit heroin samples from Southwest Asia[15]. Because this diluent consists mainly of rosin acids, it is enriched in the trace impurity extracts obtained by extraction of a slightly acidic aqueous solution of the heroin sample[4,5]. The result, as demonstrated in Fig. 3A, is that an important part of the impurity chromatogram is masked by these compounds. In this region, for instance, the impurity component acetylthebaol[16] is eluted. Fig. 3B shows that this part of the chromatogram can be transferred to a second column, and here (Fig. 3C) acetylthebaol could be separated nearly quantitively from the accompanying rosin matrix. For sample comparisons it is therefore possible to determine the interesting components, even if they are masked by large amounts of an interfering substance or group of substances.

CONCLUSION

A commercially available capillary-to-capillary column-switching instrument has been applied successfully to achieve a better resolution of certain parts of very complex mixtures of opium and heroin impurities and diluents. The superior characteristics of this technique in comparison with capillary GC on a single column have been demonstrated by three different examples. At present, however, the application of column-switching capillary GC on a routine basis is difficult owing to the extreme complexity of the technology involved.

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Note

Quantitative determination of the ligand in Phenyl-Sepharose FF with proton nuclear magnetic resonance and derivative ultraviolet spectroscopy

BO-LENNART JOHANSSON* and INGRID DREVIN
Pharmacia AB, Biotechnology, Department of Quality Control, S-751 82 Uppsala 1 (Sweden)
(Received December 8th, 1986)

Phenyl-Sepharose FF, a derivative of the cross-linked agarose gel Sepharose FF, is a gel for large-scale hydrophobic interaction chromatography (HIC). The phenyl groups are coupled to the agarose gel via the reaction of phenyl glycidyl ether with Sepharose FF.

Quality control of the ligand content of different HIC gels demands simple and accurate methods with high precision[1]. UV-absorbing ligands covalently coupled to a gel matrix can easily be quantified spectrophotometrically if a solubilizing medium is chosen[2]. Recently, spectrophotometric methods for the determination of phenyl ligands in Phenyl-Sepharose CL-4B[3] and Phenyl-Superose[4] have been reported. These methods differ in the method of solubilizing the gel matrix. Phenyl-Sepharose CL-4B is hydrolysed by hydrochloric acid whereas Phenyl-Superose, which is cross-linked to a higher degree[5], requires boron tribromide[4]. The chemical stability of Phenyl-Sepharose FF is intermediate between those of these two gels, but hydrochloric acid can be used to solubilize the gel.

This paper describes the modifications of the UV method used for Phenyl-Sepharose CL-4B to suit the new support (Sepharose FF). It also describes the elimination of disturbing absorption bands from Sepharose FF by second-derivative UV absorption spectroscopy. For elucidation of systematic errors a ^{1}H NMR method was also applied.

EXPERIMENTAL

Chemicals and apparatus

Hydrochloric acid, acetone, methanol and phenoxyethanol were of analytical-reagent grade. Phenyl-Sepharose FF and Sepharose FF were obtained from Pharmacia (Uppsala, Sweden). Deuterium chloride and [$^2H_6$]dimethyl sulphoxide (isotopic purity greater than 99.5%) were purchased from Ciba-Geigy (Basle, Switzerland).

A Shimadzu UV-240 spectrophotometer, equipped with a PR-1 graphic printer, OPI-2 option program/interface and matched 1-cm quartz cells, was used for the spectrophotometric measurements. The scanning speed was 50 nm/min. The second-derivative spectra were derivatives of stored data with slit width 1 nm and derivative wavelength difference 1 nm. The wavelength range was 250–300 nm.

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The ^1H NMR spectra were recorded with a Jeol FX 200 199.5-Hz instrument. In the pulsed NMR experiments the number of pulses was 100, the pulse time 7 μs, the pulse delay 15 s, the acquisition time 2 s and the delay between pulse and acquisition 50 μs. The spectral range explored was 2000 Hz.

Sample pre-treatment

About 2 ml of homogenized Sepharose 6 FF or Phenyl-Sepharose FF were transferred into a glass filter funnel (G-4), washed with water, shrunk with acetone and finally dried at 70°C for 15 h and stored in a desiccator.

Determination of the ligand content by second-derivative UV spectroscopy

A 20-mg amount of the dried gel was hydrolysed at 20°C with 2.0 ml of concentrated hydrochloric acid for 15 min and the hydrolysed gel was diluted to 10.0 ml with methanol. An aliquot of this solution was further diluted 10-fold before the second-derivative spectrum between 250 and 300 nm was registered. The absorbance was evaluated according to Fig. 2.

A calibration graph for phenoxyethanol in solutions of hydrolysed unsubstituted gel matrix was constructed for the concentration range 0.05–0.2 m*M*.

Determination of the ligand content by ^1H NMR spectroscopy

The dried gel (20 mg) was hydrolysed with 200 μl of concentrated deuterium chloride at 70°C for 45 s and then cooled in an ice-bath. The sample was diluted with 1.00 ml of [$^2H_6$]dimethyl sulphoxide and a ^1H NMR spectrum was registered for the solution. The peaks from the isotopic impurities in [$^2H_6$]dimethyl sulphoxide served as internal standard. Standard solutions of phenoxyethanol in hydrolysed unsubstituted Sepharose 6 FF in the concentration range 5–12 m*M* were registered in the same way.

RESULTS AND DISCUSSION

Zero-order and second-derivative UV spectroscopy

Fig. 1 shows the absorption spectra of hydrolysed Phenyl-Sepharose FF and unsubstituted agarose matrix (Sepharose FF). It also shows the interferences from Sepharose FF on the spectrum of the phenyl groups. The lesser cross-linked gel Sepharose CL-4B does not give this type of interference (see Fig. 3 in ref. 3). Moreover, the UV response of hydrolysed Sepharose FF varies from one gel sample to another. Finally, the calibration graph for phenoxyethanol based on the measurement of the absorbance at 273 nm minus a blank value at that wavelength gives an intercept above the origin.

The observed interferences are probably caused by residues of large polysaccharide units of the gel matrix which result in light scattering. It can be seen that longer hydrolysis times do not decrease the absorbance contribution from Sepharose FF. As the background UV signal varies slowly with wavelength (Fig. 1), it should be possible to eliminate this interference by registering the second-derivative spectrum instead of the zero-order spectrum[6,7].

The second-derivative spectra of hydrolysed Phenyl-Sepharose FF and Sepharose FF are depicted in Fig. 2 which shows that the interference from the agarose

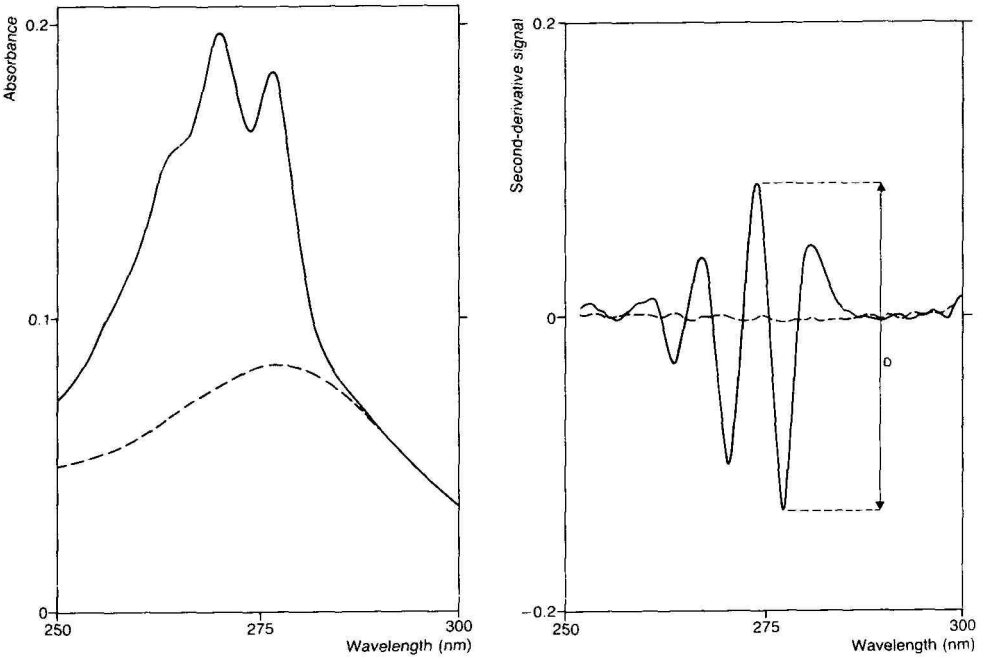

Fig. 1. UV spectra of hydrolysed Phenyl-Sepharose FF (solid line) and Sepharose FF (broken line).

Fig. 2. Second-derivative UV spectra from Fig. 1. Solid line, hydrolysed Phenyl-Sepharose FF; broken line, hydrolysed Sepharose FF.

matrix is removed. Phenoxyethanol has been shown to have a spectrum very similar to that of the bonded phenyl groups coupled to the gel[3] and was used as calibration standard also in this study. The equation of the calibration graph is

$$y = 3.71x + 2.90 \cdot 10^{-4} \quad (1)$$

where y is the absorbance D in Fig. 2 and x the concentration of phenoxyethanol in mM. Eqn. 1 indicates that the systematic error observed for the calibration graph in the zero-order UV method is eliminated. The correlation coefficient of the calibration graph calculated by linear regression was 0.999 over the investigated concentration range.

A series of six different development batches were analysed by second-derivative UV absorbance spectroscopy and the results are presented in Table I. The pooled standard deviation (s) was estimated to be 0.01 μmol/mg dry gel where s had 15 degrees of freedom.

^1H NMR spectroscopy

In order to study systematic errors, a ^{1}H NMR method was also applied to the determination of the ligand content. The NMR method used in this study was the same as that developed for Phenyl-Sepharose CL-4B[3] except that Phenyl-Sepharose FF was hydroysed in concentrated instead of 6.3 M deuterium chloride and

TABLE I

DEGREE OF SUBSTITUTION ON DIFFERENT DEVELOPMENT BATCHES OF PHENYL-SEPHAROSE FF BY TWO INDEPENDENT METHODS

Degree of substitution (μmol/mg dry gel)*	
Second-derivative UV spectroscopy	*^1H NMR spectroscopy*
0.45 ± 0.02	0.43 ± 0.03
0.32 ± 0.01	0.29 ± 0.03
0.32 ± 0.01	0.31 ± 0.02
0.25 ± 0.02	–**
0.16 ± 0.02	–**
0.64 ± 0.02	0.62 ± 0.03

* Values reported with a confidence interval of t = 95%; see text for details.
** Not analysed.

that phenol was substituted for phenoxyethanol as a calibration standard. The calibration graph for phenoxyethanol was linear and had an intercept at the origin. The pulse delay (15 s) was chosen so that the aromatic protons had time to relax towards their equilibrium value. The importance of this has been discussed earlier[1,3].

The results from four different development batches are presented in Table I. A pooled standard deviation (s) of 0.02 μmol/mg dry gel was achieved where s had 5 degrees of freedom.

CONCLUSION

This study has demonstrated the use of second-derivative UV absorption spectroscopy for the determination of the ligand content in hydrolysed Phenyl-Sepharose FF. Interferences due to broad-band matrix absorption are eliminated. Comparison of the phenyl content of Phenyl-Sepharose FF determined by the two methods described here shows that the methods consistently yield the same results at a confidence level of 95%. Therefore, it is concluded that no significant systematic errors are present.

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Note

Interaction of mannose-binding proteins with different types of immobilized affinity ligands

HANS-JOACHIM GABIUS* and REINHILD ENGELHARDT
Max-Planck-Institut für Experimentelle Medizin, Abteilung Chemie, Hermann-Rein-Strasse 3, D-3400 Göttingen (F.R.G.)
(Received December 16th, 1986)

Immobilized glycoproteins or carbohydrates have been widely employed for the purification of a variety of plant lectins which are versatile tools for the structural investigation and localization of glycoconjugates. Besides their well known presence in plants, endogenous carbohydrate-binding proteins without enzymatic activity (lectins) are now being detected in a steadily increasing number of organisms and cell types and have also been detected in vertebrates[1,2]. Here they appear to be involved in a variety of physiologically significant recognitive interactions[3,4]. Since they are also present in tumours, forming a new class of tumour markers and targets for therapy[5] and participating in the pathogenesis and spread of cancer at different stages[6], their chromatographic purification from sources of limited quantity like solid tumours requires thorough standardization for the best possible yields.

By using a series of immobilized ligands for the Ca^{2+}-dependent mannose-binding protein, consisting of natural glycoproteins, synthetic neoglycoproteins and immobilized D-mannose, we here demonstrate significant quantitative differences in the yield of this type of lectin from three different sources.

EXPERIMENTAL

Materials

Sepharose 4B and concanavalin A-Sepharose 4B were obtained from Pharmacia Fine Chemicals (Freiburg, F.R.G.), *p*-nitrophenyl-α-D-mannopyranoside, RNase B, mannan, invertase and D-mannose were from Sigma (Munich, F.R.G.) and cyanogen bromide and divinyl sulphone were from Merck (Darmstadt, F.R.G.). Human liver and a fibroadenoma from rat mammary gland were kindly provided by the department of pathology in Göttingen and the Zentralinstitut für Versuchstierzucht (Hannover, F.R.G.), respectively. Livers from roe-deers were donated by R. Engelhardt. The commercially available glycoproteins were purified by affinity chromatography on concanavalin A-Sepharose 4B prior to the coupling procedure.

Preparation of neoglycoproteins

Neoglycoproteins containing α-mannopyranoside residues were synthesized by diazo coupling of *p*-aminophenyl glycosides, prepared from *p*-nitrophenyl glycosides

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by catalytic reduction in the presence of palladium on charcoal, or coupling of *p*-isothiocyanate glycosides, prepared from *p*-aminophenyl glycosides by reaction with thiophosgene, to bovine serum albumin[7]. After purification of the conjugates by gel filtration and extensive dialysis against distilled water, the mannose content was determined by the resorcinol–sulphuric acid method[8].

Preparation of affinity column supports

Glycoproteins were coupled to Sepharose 4B which had been activated by cyanogen bromide[9]. To exclude any non-specific binding during preparation of the lectin to the immobilized protein, chemically deglycosylated glycoproteins were also coupled to Sepharose 4B. D-Mannose was coupled to the resin after activation of the resin by divinyl sulphone[10], using 12 ml of divinyl sulphone per 100 ml of packed gel. To demonstrate coupling, the plant lectin concanavalin A was passed through a small column and was specifically eluted by D-mannose. Protein coupled to the gel was estimated in the commonly employed manner by determining the amount of protein in the supernatant and washes after the coupling reaction, but before blocking with ethanolamine and further washing, additionally ascertained by the use of iodinated mannan.

Preparation of mannose-binding proteins by affinity chromatography

Frozen and thawed tissues (30 g), trimmed of connective tissue, necrotic parts and fat, were blended in a Waring blender with 250 ml of cold acetone for 1 min and processed as described[11,12]. Briefly, the resulting acetone powder was subsequently extracted with salt medium (20 m*M* Tris–HCl, pH 7.8, containing 0.2 *M* sodium chloride, 1 m*M* dithiothreitol and 0.1 m*M* phenylmethanesulphonyl fluoride) and detergent medium (20 m*M* Tris–HCl, pH 7.8, containing 2% peroxide-free Triton X-100, 0.4 *M* potassium chloride, 1 m*M* dithiothreitol and 0.1 m*M* phenylmethanesulphonyl fluoride). After centrifugation the supernatants from both extractions were pooled and brought to a final concentration of 1.25 *M* and 25 m*M* calcium chloride. This solution was passed over a precolumn of Sepharose 4B to avoid further binding to the unsubstituted column material and then aliquots were recirculated over 18-ml capacity columns of each affinity medium for 10 h. The Ca^{2+}-dependent mannose-binding proteins were eluted by EDTA. The analytical procedures and ascertainment of carbohydrate specificity with fluoresceinylated (*i.e.* fluorescein isothiocyanate labelled) neoglycoproteins have been described in detail[11–13]. Protein was determined by the dye-binding assay adapted for microtitre plates[14] and the yield is given as the mean of two independent preparations.

RESULTS AND DISCUSSION

The influence of the type of affinity ligand, immobilized on Sepharose, on the qualitative and quantitative aspects of purification of mammalian Ca^{2+}-dependent mannose-binding proteins was investigated under identical conditions. To allow a more general answer to the problem of optimal affinity-ligand selection, we have used different types of mammalian species and tissue as starting material. A series of affinity columns was prepared by coupling mannosylated bovine serum albumin (BSA), containing 4 and 18 mannose residues per BSA molecule, respectively, RNase

B, which bears one high-mannose type oligosaccharide[15], the high-mannose type glycoproteins invertase[16] and mannan[17] with high degrees of glycosylation and differences in branching and the sugar D-mannose itself to the column support. For comparative purposes, the protein concentration on the gels was adjusted to 2 ± 0.2 mg/ml. This amount of coupling also reduced the occurrence of leakage, notable at higher protein densities especially for mannosylated BSA with high mannose content, coupled to the amino groups of the protein, and assured constant conditions during the analysis. Interference from binding to the column support of proteins non-specifically or even specifically, known for serum amyloid P component, a lectin with specificity for the cyclic 4,6-pyruvate acetal of galactose in Sepharose[18], was excluded by the use of a precolumn. This precolumn was also indispensable to prevent galactose-dependent binding to invertase.

The material, purified by affinity chromatography, gave an identical electrophoretic pattern for all types of affinity ligands (Fig. 1). Human liver contained a protein having an apparent molecular weight of 30 000, rat fibroadenoma gave a protein band at a similar molecular weight, whereas two protein bands having apparent molecular weights of 58 000 and 22 000 were present in preparations from roe-deer liver. The yields of these proteins for each type of affinity ligand, summarized

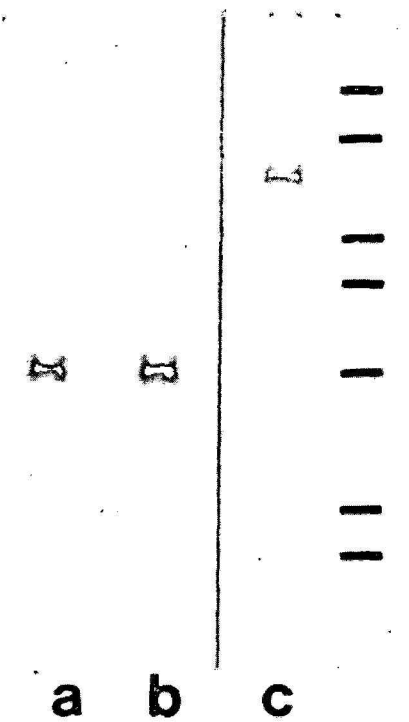

Fig. 1. Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate of mannose-binding proteins from human liver (a), rat fibroadenoma (b) and roe-deer liver (c). Standards for molecular weight designations, indicated by bars, are: phosphorylase b (97 000); bovine serum albumin (66 000); egg albumin (44 000); glyceraldehyde-3-phosphate dehydrogenase (36 000); carbonic anhydrase (29 000); β-lactoglobin (18 400) and lysozyme (14 300).

TABLE I

EFFECT OF THE TYPE OF IMMOBILIZED LIGAND ON THE YIELD OF Ca^{2+}-DEPENDENT MANNOSE-BINDING PROTEINS

Yields are given in μg per 5 g liver or tumour.

Tissue	*Ligand*					
	*BSA_4**	*BSA_{18}***	*RNase B*	*Mannan*	*Invertase*	D-*Mannose*
Human liver	30	46	63	86	68	89
Rat fibroadenoma	12	17	19	28	31	202
Roe-deer liver	34	82	450	680	585	1250

* Mannosylated bovine serum albumin with 4 sugar residues.
** Mannosylated bovine serum albumin with 18 sugar residues.

in Table I, demonstrated clear differences, revealing that immobilized D-mannose was most efficient. A comparison between the various immobilized glycoproteins showed that the results for mannan were very favourable, with decreased yields from immobilized invertase, RNase B and the two neoglycoproteins.

Similar mannose-binding proteins have been reported from rat liver with yields of 3.2[19] and 4.6 μg/g liver[20], using mannan-Sepharose, of 140 and 10 μg/g liver[21] using RNase B or mannose and of 1.5 and 2 μg/g liver[22] using invertase or mannose respectively. Since the protein densities on the column and the conditions differed, no clear answer as to the selection of the affinity ligand could be drawn.

Whereas a similar protein has also recently been described for rat mammary adenocarcinoma[23], the different molecular weights in the preparations from roe-deer liver underscore that variations in the molecular weights of related proteins can occur in different species, as noted for fucose-binding lectins from rat, mouse and human liver[24]. Differences in the subunit-composition, isolated by use of different affinity ligands, as reported for the rat liver mannose-specific lectin[21], could not be detected. In conclusions, these studies reveal that under controlled conditions the yield of a mannose-specific lectin is consistently dependent on the type of immobilized affinity ligand employed. Careful selection among possible choices can therefore lead to significant quantitative increases in the protein, enabling structural studies with less starting material. This result may also be of relevance to other types of lectins with different specificities.

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Note

Rapid analysis of coffee flavour by gas chromatography using a pyrolyzer

K. HARADA, O. NISHIMURA and S. MIHARA*
Ogawa & Co., Ltd., 6-32-9 Akabanenishi, Kita-ku, Tokyo 115 (Japan)
(Received December 16th, 1986)

Gianturco[1] discussed the formation and rate of production of volatile compounds during the roasting coffee beans. As the roasting time was increased, both the number and size of the chromatographic peaks increased. This phenomenon indicates that most components of coffee aroma arise from the roasting process[1,2]. Baltes[3] has recently described phenol formation during coffee roasting.

The Curie-point principle has been widely used as a method for analyzing non-volatile materials[4,5]. First a sample is rapidly heated to a temperature that is sufficient to decompose it to volatile substances. These are then analyzed by gas chromatography (GC). Colenutt and Thorburn[6] adopted a conventional pyrolysis GC system for the analysis of volatile compounds. In their system, desorption is achieved in the pyrolysis unit, but no pyrolysis occurs.

This paper presents a simple and rapid method for analyzing the flavour of coffee grounds using a gas chromatograph equipped with a pyrolyzer. The results obtained with this method were compared with those from conventional methods, *i.e.*, headspace analysis and simultaneous distillation–extraction (SDE).

EXPERIMENTAL

Gas chromatography

A 50-m fused-silica capillary column (0.22 mm I.D.) coated with Carbowax 20M was used to separate volatiles transferred to the column through the splitter injection port. The splitting ratio was adjusted to 1:50 with carrier gas (nitrogen) at 22 p.s.i. The linear flow-rate was 42 cm/s and the column flow-rate was 2.0 ml/min. The injection port temperature was 200°C. The column temperature was increased from 80 to 200°C at 2°C/min. The gas chromatograph used was an Hitachi Model 163 equipped with a flame ionization detector heated to 250°C.

Gas chromatography–mass spectrometry (GC–MS)

An Hitachi Model M-80B mass spectrometer–gas chromatograph (Hitachi Model 663) equipped with an Hitachi Model 0101 data system was used under the following conditions: ionization voltage, 70 eV; emission current, 80 μA; ion acceleration voltage, 3100 V; ion-source temperature, 200°C.

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Curie-point pyrolyzer

Volatiles were removed from a *ca.* 10-mg sample of Colombian Arabica coffee grounds by heating at 235°C in a ferromagnetic sample support in the pyrolyzer. The total heating time was 3 s. The pyrolyzer can easily be connected to the original injection port of the gas chromatograph or the gas chromatograph–mass spectrometer unit.

Headspace sampling

A 150-g amount of coffee grounds was placed in a 500-ml headspace vessel equipped with an Allihn condenser. The trapping column (170 mm × 6.4 mm I.D.) containing *ca.* 2 g of Porapak Q (60–80 mesh) was fixed to the top end of the Allihn condenser. A 400-ml volume of water at 70°C was poured into the vessel. The extraction of the coffee grounds and the trapping of headspace volatiles were carried out in a water-bath at 70°C stirred at a constant rate. The headspace vessel was purged for 2.5 h by the passage of nitrogen at a flow-rate of 240 ml/min. The entrapment column was reversed[7] and heated at 180°C for 0.5 h. The volatiles collected in the trapping were then backflushed to a glass trap chilled with solid carbon dioxide[7]. The trap was rinsed with 30 μl of diethyl ether. This permitted efficient transfer of the trapped volatiles into a vial for storage until analysis.

Simultaneous distillation–extraction (SDE)

A Nickerson-Likens extractor as modified by Schultz *et al.*[8] was used. A 400-g amount of coffee grounds in 1 l of water was added to a 2-l flask, and 150 ml of dichloromethane were added to a 300-ml flask. The distillation head was attached and then both flasks were heated to boiling. The distillation was carried out for 6 h at atmospheric pressure. The extract was dried over anhydrous sodium sulphate for 12 h and then evaporated to *ca.* 0.5 ml for instrumental analysis.

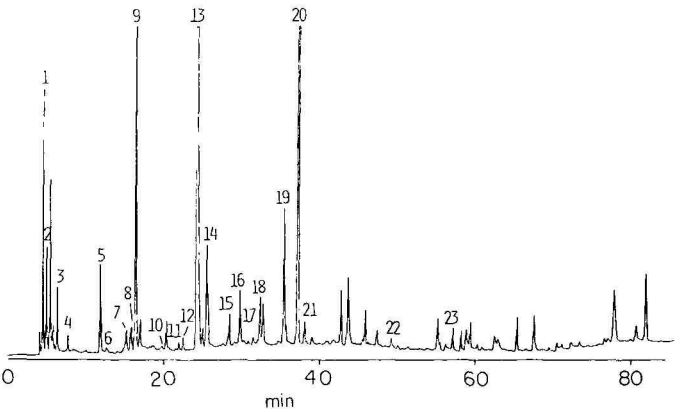

Fig. 1. Gas chromatogram of the volatiles from coffee grounds desorbed in the Curie-point pyrolyzer at 235°C. Peaks: 1 = acetaldehyde; 2 = acetone; 3 = 2,3-butanedione; 4 = 2,3-pentanedione; 5 = pyridine; 6 = pyrazine; 7 = 2-methylpyrazine; 8 = acetoin (3-hydroxy-2-butanone); 9 = acetol (2-ketopropyl alcohol); 10 = 2,3-dimethylpyrazine; 11 = 2-ethyl-6-methylpyrazine; 12 = 2-ethyl-5-methylpyrazine; 13 = acetic acid; 14 = furfural; 15 = 2-acetylfuran; 16 = propionic acid; 17 = furfuryl acetate; 18 = 5-methylfurfural; 19 = γ-butyrolactone; 20 = furfuryl alcohol; 21 = isovaleric acid; 22 = guaiacol; 23 = phenol.

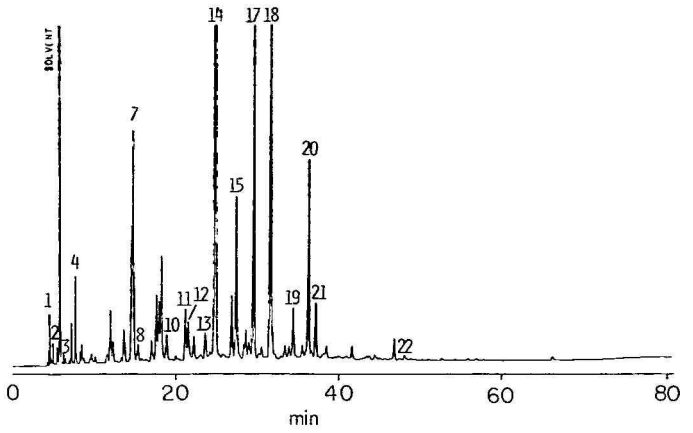

Fig. 2. Gas chromatogram of the headspace volatiles from a coffee brew. See Fig. 1 for peak identification.

RESULTS AND DISCUSSION

Fig. 1 shows a gas chromatogram of the volatiles from coffee grounds obtained in the Curie-point pyrolyzer at 235°C. When the pyrolyzer was used at different heating temperatures ranging from 170 to 235°C (total heating time per sample: 3 s), the qualitative composition of the volatiles from the coffee grounds did not change. The effluent gas from the outlet of the pyrolyzer had a coffee-like odour. Since the above heating conditions are much milder than the roasting conditions used for coffee beans (180–215°C for 15–17 min or 260°C for 5 min)[2], the influence of the pyrolysis of the coffee grounds may be neglected in the analysis of the volatiles. The mixture of compounds in Fig. 1 was analyzed by GC–MS and identified by comparing MS and retention time data with those of individual compounds.

The headspace analysis (adsorbent trap) (Fig. 2) and SDE methods (Fig. 3)

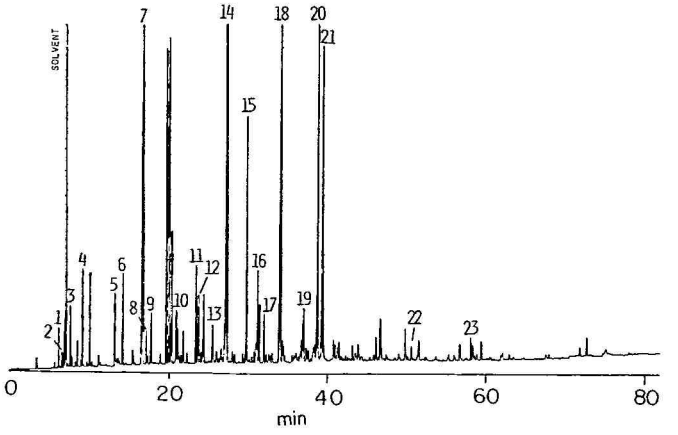

Fig. 3. Gas chromatogram of the volatiles obtained from coffee grounds by SDE. See Fig. 1 for peak identification.

were chosen in order to compare the results for the qualitative analysis of volatile compounds from coffee grounds. The dependence of the headspace method on compound volatility is obvious. The peak area % drops off quite quickly as the vapour pressure of the compound decreases.

The SDE method gave very good recoveries[9]. Its disadvantages include the problems of the solvent front overlapping with low boiling compounds and artifact formation due to thermally induced change. Changing to SDE had a very negative effect on the recovery of polar substances, *e.g.*, acetic acid (peak 13).

The pyrolyzer method offers a very simple and efficient means of isolating flavours from a very small amount of coffee grounds. It is possible to analyze both low boiling compounds, *e.g.*, acetaldehyde (peak 1) and acetone (2) and high boiling compounds, *e.g.*, guaiacol (22) and phenol (23) using the pyrolyzer method, as shown in Fig. 1. Consequently, this method is very simple and useful for the analysis of flavour compounds in food.

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Note

High-performance liquid chromatographic system for the separation of tricyclic antidepressant and related drugs using ODS-Hypersil

R. GILL* and S. O. WANOGHO
Central Research Establishment, Home Office Forensic Science Service, Aldermaston, Reading, Berkshire, RG7 4PN (U.K.)
(Received January 13th, 1987)

Experience in high-performance liquid chromatography (HPLC) has shown that different commercial packing materials of nominally the same type (*e.g.* ODS-silica) can have very different chromatographic properties. The consequent problems arising from the need to transfer methods between different locations have led Forensic Science Laboratories in the U.K. to standardise on the materials used for routine work[1] and bulk purchases of a silica (Spherisorb S5W) and an ODS-silica (ODS-Hypersil) have been made. These materials have proved to be effective for the vast majority of HPLC separations required for toxicology casework and the concept of using only two types of column has proved to be convenient and economical. Eluents have been developed to use with the standard materials for various drug classes and, in each case, retention data for groups of compounds of forensic interest have been published. This information serves as a guide to the identification of unknown compounds and the quantification of specific drugs using HPLC. At the present time data have been published for barbiturates[2,3], local anaesthetics[4], amphetamines[5], narcotic analgesics[5], ergot alkaloids[6], benzodiazepines[7], analgesic and anti-inflammatory drugs[8] and thiazide diuretics[9]. A further system for basic drug screening on silica has also been developed[10].

In the present paper we describe an eluent for the separation of tricyclic antidepressant and related drugs on ODS-Hypersil, an important group of compounds often encountered in forensic toxicology casework. Numerous HPLC methods for the analysis of specific antidepressants and metabolites have been published and some of the literature has been reviewed[11–13]. The HPLC system finally selected is a modification of a procedure by Kabra *et al.*[14] recommended for the determination of a few antidepressants in plasma or serum. In the present work retention data are presented for 27 drugs.

EXPERIMENTAL

Materials

Acetonitrile (HPLC grade) was obtained from Rathburn Chemicals (Walkerburn, U.K.) and phosphoric acid (AristaR grade) from BDH (Poole, U.K.). Water was distilled in glass in the laboratory. All other chemicals were analytical grade

from BDH except for *n*-nonylamine which came from Aldrich (Gillingham, U.K.).

The HPLC packing material used was 5-μm ODS-Hypersil from Shandon Southern Products (Runcorn, U.K.).

All drugs came from the drug collection of the Central Research Establishment, Home Office Forensic Science Service.

Chromatography

The HPLC equipment consisted of a Waters M6000 pump, a Rheodyne injection valve (Model 7120) fitted with a 20-μl sample loop and a Cecil CE272 variable-wavelength UV detector operated at 230 nm. The stainless-steel column (16 cm × 5 mm I.D., Shandon Southern Products) was packed with ODS-silica using a conventional slurry procedure in which the material was dispersed in isopropanol and pumped with hexane.

The eluent was prepared by mixing acetonitrile (300 ml) with a pH 3 phosphate buffer (700 ml). The aqueous buffer was prepared by adding *n*-nonylamine (0.6 ml) to aqueous sodium dihydrogen phosphate (0.01 *M*, 1000 ml) and then adjusting the pH to 3.0 by the dropwise addition of phosphoric acid[14]. A flow-rate of 2 ml/min was used.

Drug samples were dissolved in acetonitrile–water (30:70, v/v) for injection onto the column. Retention data are expressed as capacity ratios, k', which are defined by $k' = (t_R - t_0)/t_0$, where t_R and t_0 are the retention times of the drug and a non-retained compound, respectively. Injections of acetonitrile were used to determine t_0.

RESULTS AND DISCUSSION

The HPLC system chosen for the separation of the tricyclic antidepressant and related drugs was a modification of a published procedure[14]. The original method

TABLE I

HPLC RETENTION DATA FOR TRICYCLIC ANTIDEPRESSANT AND RELATED DRUGS (ARRANGED IN ORDER OF ELUTION)

Compound	*k'*	*Compound*	*k'*
Azatadine*	0	Protriptyline	3.60
Viloxazine	0.17	Cyproheptadine*	4.17
Nomifensine	0.42	Imipramine	4.17
Dibenzepin	0.50	Nortriptyline	4.58
Zimeldine	0.67	Maprotiline	4.92
Triprolidine*	1.17	Amitriptyline	5.42
Oxypertine	1.33	Trimipramine	6.17
Noxiptyline	1.63	Butriptyline	7.33
Opipramol	1.63	Clomipramine	9.92
Mianserin	1.92	Deptropine*	10.40
Doxepin	2.27	Iprindole	10.83
Mebhydrolin*	2.48	Lofepramine	> 30
Desipramine	3.60	Trazodone	> 30
Dothiepin	3.60		

* Antihistamine drugs.

TABLE II

HPLC RETENTION DATA FOR TRICYCLIC ANTIDEPRESSANT AND RELATED DRUGS (ARRANGED IN ALPHABETICAL ORDER)

Compound	*k'*	*Compound*	*k'*
Amitriptyline	5.42	Mebhydrolin*	2.48
Azatadine*	0	Mianserin	1.92
Butriptyline	7.33	Nomifensine	0.42
Clomipramine	9.92	Nortriptyline	4.58
Cyproheptadine*	4.17	Noxiptyline	1.63
Deptropine*	10.40	Opipramol	1.63
Desipramine	3.60	Oxypertine	1.33
Dibenzepin	0.50	Protriptyline	3.60
Dothiepin	3.60	Trazodone	>30
Doxepin	2.27	Trimipramine	6.17
Imipramine	4.17	Triprolidine*	1.17
Iprindole	10.83	Viloxazine	0.17
Lofepramine	>30	Zimeldine	0.67
Maprotiline	4.92		

* Antihistamine drugs.

used an ODS-Ultrasphere column and an eluent containing 21% acetonitrile with a pH 3 phosphate buffer containing *n*-nonylamine. An identical aqueous buffer was used in the present work but an increase in the concentration of acetonitrile, to 30%, was found to be necessary with the ODS-Hypersil column to achieve an appropriate retention range for the compounds studied. Basic drugs often give poor peak shapes on ODS-silica columns and require the addition of amine modifiers to the eluent to control peak shapes[15]. The *n*-nonylamine used in the aqueous buffer was found to

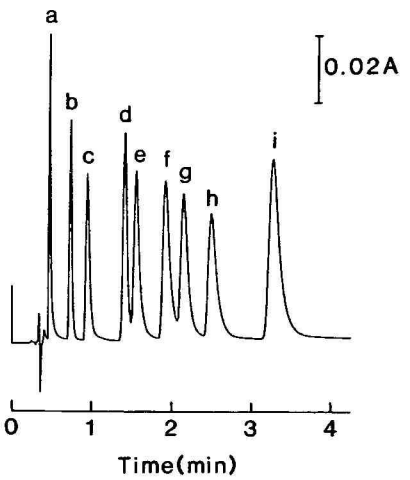

Fig. 1. Chromatography of tricyclic antidepressant drugs on ODS-silica. Column: ODS-Hypersil, 5 μm (16 cm × 5 mm I.D.); eluent: 30% acetonitrile containing a phosphate buffer (pH 3) and nonylamine; flow-rate: 2 ml/min; detection: UV absorbance (230 nm). Peaks: a = zimeldine; b = noxiptyline; c = doxepin; d = protriptyline; e = imipramine; f = amitriptyline; g = trimipramine; h = butriptyline; i = clomipramine.

be a very satisfactory additive, maintaining symmetrical peak shapes for the tricylic antidepressants on ODS-Hypersil.

Table I gives the retention data (k' values) for 27 drugs arranged in their order of elution. The list includes 22 tricyclic antidepressant drugs with the remaining five compounds being antihistamine drugs, selected as having chemical structures similar to those of the antidepressants. The antidepressant drugs include all 17 such compounds available for prescription in the U.K. in June 1986 along with five others (dibenzepin, nomifensine, noxiptyline, opipramol and zimeldine).

Table I indicates that most drugs show some retention within the range $k' = 0$ to 11 with the exceptions of azatadine which is not retained at all, and lofepramine and trazodone which show very long retention times with the eluent used. Table II presents the same data arranged by alphabetical order of the drug names to facilitate the rapid retrieval of information for a specific compound. The good peak shapes obtained for the present system are demonstrated in Fig. 1, which shows the separation of nine tricyclic antidepressant drugs on the 16-cm column.

No attempt has been made in the present note to demonstrate the use of the HPLC system for the determination of tricyclic antidepressants in biological fluids although such applications are feasible. The original paper by Kabra *et al.*[14] includes a description of an extraction procedure for serum and plasma which may be directly applicable. Further work is required to explore the range of drugs which may be analysed using this HPLC system.

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Letter to the Editor

Comments on enhanced peak response due to solvent interaction

Sir,

There has been a series of claims[1,2] and counter claims[3] recently concerning the change in liquid chromatographic (LC) peak response due to solvent interactions. This effect, if it in fact exists, has far ranging implications to quantitation in LC in general. We present here measurements in our laboratory on the same compounds. We thus show that the "anomalous" peak responses can be readily explained based on well-known behaviors in LC columns and LC detectors.

All eluents used are reagent grade material without further purification. A conventional chromatographic system was used. It consisted of a reciprocating pump (Milton Roy, Riviera Beach, FL, U.S.A., Model 196-0066), a 25 cm × 4.6 mm, 5-μm C_{18} column (Alltech, Deerfield, IL, U.S.A.), a 20-μl sample loop at a conventional injection value (Rheodyne, Berkeley, CA, U.S.A., Model 7010) and a commercial UV variable-wavelength detector (ISCO, Lincoln, NE, U.S.A., Model V[4]) without a reference cell. A flow-rate of 1.0 ml/min is used throughout. The output of the UV detector is connected to an IBM PC/AT computer with an analog interface (Data Translation, Marlboro, MA, U.S.A., Model DT2827 with ATLAB software). The computer takes readings every 0.1 s. Typically at least 100 of these data points define an analyte peak. The area is determined by locating and determining the peak start/-stop points manually, interpolating a baseline, and summing all intermediate points after baseline correction. The test compounds were captopril (Squibb, Rolling Meadows, IL, U.S.A.), nadolol and bendroflumethiazide (U.S. PC, Rockville, MD, U.S.A.). The eluents used were methanol–water–phosphoric acid (40:60:0.04) for captopril, methanol–0.1 *M* acetate buffer (pH 5) (35:65) for nadolol, and methanol–0.1 *M* acetate buffer (pH 5) (50:50) for bendroflumethiazide. The wavelengths used were 214 nm, 270 nm and 270 nm respectively. Care was taken to use freshly prepared solutions. The amounts injected were in the 1 μg range, which minimize column saturation effects. The detector was operated within its linear range, typically using the 0.05 a.u. full scale.

The results of our investigation are shown in Table I. Since the determinations of peak areas and peak heights are greatly influenced by the interpolated chromatographic baseline, the trials included in Table I are only those for which a stable baseline is achieved throughout the entire chromatogram, *i.e.* detector drift or baseline shifts are not present. The nadolol peak exhibits substantial tailing, and this resulted in poorer statistics in determining the peak areas. This is particularly true when ethanol is used as the solvent, when the signal does not return to the original baseline until after 2–3 peak widths. All of the peaks are well resolved from the refractive index disturbance at the void volume, and the injected quantities are small, so that artifacts of the detector are not expected to be important. Several conclusions can be drawn:

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(1) There is no statistically significant change in the areas of the peaks when the solvent is changed. This is in agreement with Berridge[3] but in variance with Perlman and Kirschbaum[1,2]. In fact it will be truly surprising if the areas did change. For retained peaks, one can infer that the *environment* of the species at the detector is constant regardless of its original solvent. Assuming there are no instrumental artifacts, Beer's law should hold and the integrated response (area) should match the total amount of material injected in the limit of low concentrations. In conventional spectrophotometry, we try to reproduce the final solvent (environment) when measuring unknowns and standards. The *history* of the sample, however, is never known to be a factor provided chemical reaction did not take place. The types of interactions suggested in ref. 2, *i.e.* intramolecular hydrogen bonding, are not permanent, and should be invariant once the species is put back into the environment of the chromatographic eluent. It is possible that for certain systems[4], the column itself promotes chemical changes, but then the effect cannot be classified as an enhanced peak response. In fact, if chemical changes lead to spectroscopic changes, it is highly likely that one can observe a second peak in the chromatogram, such as Fig. 7 in ref. 5.

(2) We have measured the molar absorptivities of captopril at 214 nm in 100% water, methanol–water (90:10), and ethanol–water (90:10), and found that the ratios are 1.0, 0.81, and 0.63 respectively. This indicates a substantial solvent effect is present, which can be explained by a hypothesis such as intramolecular hydrogen bonding. We believe that the results in ref. 2 (Fig. 1) can be explained in this way. The aztreonam peak is *not* retained, so that the environments of the measurements reflect those of the original solvents, and not the constant environment of the eluent. However, these variations in the areas are not related to the relative areas in the chromatographic runs in Table I, since the solvent there is of constant composition. Measurements of peak responses at the void volume are also subject to known interferences from the changing refractive index created by the solvent injected.

(3) There is no significant change in retention times of the solutes on changing solvents. We note that because of the asymmetric peak shapes, the peak maximum cannot be used directly to determine the retention time. For these compounds, errors of up to 5% can be introduced if the peak maximum rather than the peak centroid is used. The slight variations in retention times reported in ref. 3 are thus not conclusive.

TABLE I

DETECTOR RESPONSES FOR CAPTOPRIL, NADOLOL AND BENDROFLUMETHIAZIDE

Solute	*Solvent*	*No. of trials*	*Area*	*Height*
Captopril	Water	3	6410 ± 30	101 ± 0.5
	Methanol	8	6480 ± 50	62 ± 1.0
	Ethanol	5	6390 ± 20	43 ± 0.3
Nadolol	Water	5	6774 ± 117	108 ± 2.3
	Methanol	4	6696 ± 135	90 ± 2.8
	Ethanol	2	6398 ± 220	79 ± 2.0
Bendroflumethiazide	Methanol	5	3400 ± 30	51 ± 0.8
	Acetonitrile	5	3400 ± 30	54 ± 0.7
	Ethanol	4	3410 ± 40	40 ± 1.0

(4) Table I shows that there is a clear trend in the observed peak heights as a function of the initial solvents. As the eluting strength of the solvent increases, the peak height decreases because the peak becomes broader. This can be readily explained as being the result of dynamic gradient-elution caused by the injected solvent[6,7]. In going from water to methanol to ethanol in the injected plug, the elution strength increases and the solute is spread out more and more in the beginning section of the column. This is analogous to using larger and larger injection loops for the same injected amount, and band broadening eventually becomes important. The eluent quickly equilibrates once again and the whole (broadened) band moves down the column as before. This accounts for the invariance in the retention times for the solutes in different solvents. This is also why the peak heights are almost constant in ref. 3, where a much smaller sample loop is used. The effect of the solvent plug is then minimized.

In summary, we can conclude that there is no anomalous behavior in response for this group of compounds in LC, unless the detector is used in its non-linear range (Fig. 1 of ref. 3), chemical reaction or decomposition is present[5], or the eluent environment is not maintained when the solute elutes[2]. All of the unusual responses reported so far can be explained based on well-known behaviors in LC.

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Ames Laboratory-USDOE and Department of Chemistry K. C. CHAN
Iowa State University, Ames, IA 50011 (U.S.A.) EDWARD S. YEUNG*

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OBITUARY

PROFESSOR Dr. ERICH VON HAYEK

Erich von Hayek died on December 12th, 1986, aged 83, in Innsbruck, Austria. He was past-president of the association of Austrian Chemists and had been Professor of Inorganic and Analytical Chemistry at the University of Innsbruck and Rector of the University in 1966/1967.

One of his outstanding characteristics was that he did not limit himself to research in the fields of inorganic and analytical chemistry but was also active in physical and technical chemistry.

He was well known for his seminal work on the synthesis of crystalline basic salts and also on high-valence states of transition metals. On the medical side he did important work on bone minerals.

He published in 1959 one of the first reviews in this journal: "Adsorptions- und Fällungschromatographie Anorganischer Verbindungen" and was an authority in the field of inorganic chromatography.

During the whole of his life he took full advantage of the ski and mountain climbing opportunities offered by the Tyrolian mountains, and kept up his interest in science, attending lectures up to the last days of his life.

He will be sadly missed.

AWARD

1987 DAL NOGARE AWARD

The 1987 Dal Nogare Award has been presented by the Chromatography Forum of the Delaware Valley to Dr. Fred Regnier of Purdue University. Dr. Regnier has received the award for his achievements in the field of chromatography, particularly for his contributions in the study of fundamental principles of macromolecular retention and separation. The Dal Nogare Award has been given by the Chromatography Forum of the Delaware Valley annually since 1972 in recognition of scientists who have contributed significantly to the understanding and practice of chromatography. The presentation of the award was held on Tuesday, March 10, 1987, at the Pittsburgh Conference held in Atlantic City, NJ, U.S.A.

Fred E. Regnier is Professor of Biochemistry, Department of Biochemistry at Purdue University. During the period of 1969 to 1976 he held the position of Assistant and Associate Professor of Biochemistry at Purdue. He served as Associate Director of the Agriculture Experimental Station at

Purdue from 1976 to 1977. During the period 1965 to 1968 he was a Postdoctorate Research Associate at Oklahoma State University (1965–1966), University of Chicago (1966–1967), and Harvard University (1968–1969). Dr. Regnier received his Ph.D. degree in Chemistry from Oklahoma State University at Stillwater in 1965. Previously he had received his B.S. degree at Nebraska State College. He is the author of 70 publications on various aspects of chemistry, biochemistry, and particularly chromatographic techniques and applications. He received the David B. Hime Award for Achievements in Chromatography, presented by the Chicago Chromatography Discussion Group in 1982. Dr. Regnier serves on Editorial Boards for *Analytical Biochemistry, Journal of Liquid Chromatography,* and the *Liquid Chromatography Magazine.* He has five patents concerning chromatography and biochemistry. He is a member of Phi Lambda Upsilon and Sigma Xi honorary societies, the American Chemical Society and the American Society of Biological Chemists.

ANNOUNCEMENTS OF MEETINGS

4th TRIANGLE CHROMATOGRAPHY SYMPOSIUM AND INSTRUMENT EXHIBIT, RALEIGH, NC, U.S.A., MAY 14, 1987

The symposium will be held at the Jane S. McKimmon Center, North Carolina State University, Raleigh, NC, U.S.A. The symposium, sponsored by the Triangle Chromatography Discussion Group and the North Carolina Section of the American Chemical Society, will feature invited lectures covering all aspects of chromatography and will include an instrument exhibit.

For further information, please contact: Joseph R. Hudson, Jr., Rhone-Poulenc, Inc., Agrochemical Division, P.O. Box 12014, Research Triangle Park, NC 27709, U.S.A. Tel.: (919) 549-2501.

1st INTERNATIONAL SYMPOSIUM ON THE INTERFACE BETWEEN ANALYTICAL CHEMISTRY AND MICROBIOLOGY, APPLICATIONS OF CHROMATOGRAPHY AND MASS SPECTROMETRY, COLUMBIA, SC, U.S.A., JUNE 3–5, 1987

This new international symposium, sponsored by the College of Science and Mathematics and by the School of Medicine of the University of South Carolina, will serve to forge connections between analytical chemistry and microbiology at a level that emphasizes both the fundamental development and practical application of chromatographic and mass spectrometric methods. The depth of growing interdisciplinary efforts by both analytical chemists and microbiologists necessitates a symposium where both groups of researchers can meet on a common ground. The symposium will be planned with ample opportunity for discussion in large groups as well as in informal settings.

Plenary lectures will be presented by speakers invited by the international organizing committee. Contributions in the form of presented papers and posters are also solicited from participants. A book covering the plenary talks presented at the meeting will be published by Plenum after the meeting. A copy of this book will be distributed free to all registered participants. Papers or posters presented at the meeting may also be submitted to the *Journal of Microbiological Methods.*

The symposium will have an interdisciplinary flavor, emphasizing analytical chemical applications of chromatography and mass spectrometry in microbiology. Topics relevant to the meeting include: chemotaxonomy; direct analysis of chemical markers for microorganisms; GC, LC and MS in environmental microbiology; applications of GC, LC and MS in the food industry; analysis of tissues and fluids for bacteria and fungi; rapid detection and identification of biological materials; clinical microbiology applications; computer assisted pattern recognition/data handling; sample handling/preparation; pyrolysis GC, pyrolysis MS, pyrolysis GC–MS of microorganisms; derivatization methods coupled to GC, GC–MS, LC, and LC–MS; headspace analysis of volatile fermentation products; high-resolution MS methods.

The meeting will be held at the conference facilities of the University of South Carolina in Columbia, SC, U.S.A. Lodging will be provided in single or double rooms. There are excellent airline connections to Columbia through Atlanta and Charlotte from New York, Washington, Houston, Dallas, and the west coast, as well as several direct connections from New York and Newark. Registration

and lodging fees should be paid in advance; checks should be made out in U.S. dollars.

Mailing address for symposia correspondence and registration fees: 1st International Symposium on the Interface between Analytical Chemistry and Microbiology, P.O. Box 7126, Columbia, SC 29202, U.S.A.

7th ANNUAL AMERICAN–EASTERN EUROPEAN COLLOQUIUM AND CONFERENCE ON LIQUID CHROMATOGRAPHY, BUDAPEST, HUNGARY, JULY 6–8, 1987

The Budapest Chromatography Symposium will be organized by: the Hungarian Chemical Society, the Interchrom Association and the Hungarian Pharmacological Society. The main topics of the conference will be: theoretical aspects of chromatography; high-performance liquid chromatography; thin-layer chromatography; electrophoretic methods; supercritical fluid chromatography; gas chromatography; calculation methods, computers and quantitative evaluation; chiral recognition in chromatography; stationary phases, instrumentation and chromatographic systems; preparative application of chromatography; chromatographic separation of amino acids, peptides and proteins, amines, nucleic acids, biologically active natural products, drugs and metabolites; free communications.

Oral lectures and poster presentations will be accepted. Anyone wishing to contribute an original paper should submit an abstract of 350–500 words in English not later than March 15, 1987. The official language of the conference is English.

An international exhibition of chromatographic equipment, stationary phases, materials, solvents and books will be held during the conference. The site of the symposium will be the building of research and teaching of the Semmelweis University of Medicine, VIII. Nagyvárad tér 4, 1089 Budapest, Hungary.

A volume will be published containing selected papers presented in the conference provided they are submitted at the meeting and found suitable for publication.

For further information, please contact: Dr. Huba Kalász, Department of Pharmacology, Semmelweis University of Medicine, Nagyvárad tér 4, P.O. Box 370, Budapest, Hungary. Tel.: (361) 137070 ext. 258, telex: 225070.

7th INTERNATIONAL SYMPOSIUM ON AFFINITY CHROMATOGRAPHY AND INTERFACIAL MACROMOLECULAR INTERACTIONS, OBERAMMERGAU, F.R.G., AUGUST 17–21, 1987

The Organizers and the Advisory Committees invite you to participate in the 7th International Symposium on Affinity Chromatography and Interfacial Macromolecular Interactions taking place in Oberammergau, F.R.G., August 17–21, 1987.

These symposia go back to a symposium held 1976 in Birmingham, U.K. Last year the International Interest Group in Biorecognition Technology (IIGBT) was constituted to provide a continuing 'umbrella' organization guaranteeing the future of these leading symposia.

The aims of this year's symposium are to bring together prominent experts from basic research institutions and industry to focus on the molecular mechanisms involved in biorecognition and macromolecular surface interactions with the concept of transposing these findings into new technologies. A special aim of the symposium is to give particularly the younger research workers a chance to present their latest scientific results to a congenial but critical audience.

The main topics of the symposium are: fundamentals of molecular recognition; new developments in affinity separations; analytical and clinical applications; and affinity therapy/biocompatability. Poster sessions are organized to complement the sections with free contributions and to cover additional topics. They should contain new and unpublished information. There will also be a chance for strategy discussions on the role of biorecognition and surface technology in the coming years to the 21st century. The papers presented by the speakers at the symposium will be published in a special symposium volume. The deadline for receiving the final "camera ready" manuscripts will be August 17, 1987. The proceedings will be available to the participants of the symposium at a special reduced price.

The symposium takes place only 50 miles south of Munich, the capital city of Bavaria. Munich which houses three universities and ten Max-Planck-Institutes has produced decisive contributions to the natural sciences in the past decades. The small (*ca.* 5000 inhabitants) picturesque town of Oberammergau (altitude *ca.* 800 m) lies in a valley surrounded by mountains up to 1700 m. It is famous for its woodcarvings and religious plays. This locality provides an ideal setting for an interdisciplinary exchange of ideas and information among the participants during and after the sessions.

For further details, please contact: Professor Dr. H.P. Jennissen, Scientific Secretariat, Institut für Physiologische Chemie und Ernährungsphysiologie, Universität München, Veterinärstrasse 13, D-8000 München, F.R.G.

PROCESS ANALYSIS AND CONTROL SYMPOSIUM SCHEDULED FOR FACSS '87

A symposium on process analysis and control will be part of the 1987 FACSS (Federation of Analytical Chemistry and Spectroscopic Societies) meeting, to be held in Detroit, MI, U.S.A. on October 4–9, 1987. The symposium is a forum for the presentation of research in the following areas: chemometrics in service of process analysis and control; the role of analyzer data in process modeling, optimization, and control; spectroscopic instrumentation for process analysis; new developments in process chromatography; process analyzers, sensors, and transducers.

For further information about the symposium, or submitting papers, contact: FACSS Assistant Program Chair, Dr. Deborah Illman, Center for Process Analytical Chemistry, BG-10, University of Washington, Seattle, WA 98195, U.S.A.

Dr. Illman is Associate Director at the Center for Process Analytical Chemistry (CPAC). The Center is an industry/university cooperative that began operation 3 years ago with a 5-year grant from the National Science Foundation. CPAC, currently sponsored by 30 organizations, is dedicated to the interdisciplinary advancement of on-line, real-time process analysis and control.

4th SYMPOSIUM ON HANDLING OF ENVIRONMENTAL SAMPLES IN CHROMATOGRAPHY, BASLE, SWITZERLAND, APRIL 27–29, 1988

The fourth meeting in this series (previous symposia took place in Lausanne, Freiburg and Palma de Mallorca) will be held in Basle, Switzerland, on April 27–29, 1988. The organisation of the meeting is in the hands of the International Association of Environmental Analytical Chemistry. A strong industrial participation is planned. The symposium will feature invited and contributed lectures and posters. It is the intention of the organisers to bring together specialists in this field who can give a good account of the state of the art in their respective specialty and to present first-hand experience in sample handling.

For further information and for submission of contributions please contact: Professor R.W. Frei, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. Tel.: (020) 5485379.

CALENDAR OF FORTHCOMING MEETINGS

April 14, 1987 Rockville, MD, U.S.A.	**CHROMEXPO-1987** Contact: Mrs. Janet Cunningham, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772. (Further details published in Vol. 387.)
April 27–May 1, 1987 Sydney, Australia	**9th Australian Symposium on Analytical Chemistry** Contact: The Secretary 9AC, Mr. John ames, P.O. Box 137, North Ryde, N.S.W. 2133, Australia. Tel.: (020) 887-8688. (Further details published in Vol. 350, No. 2.)

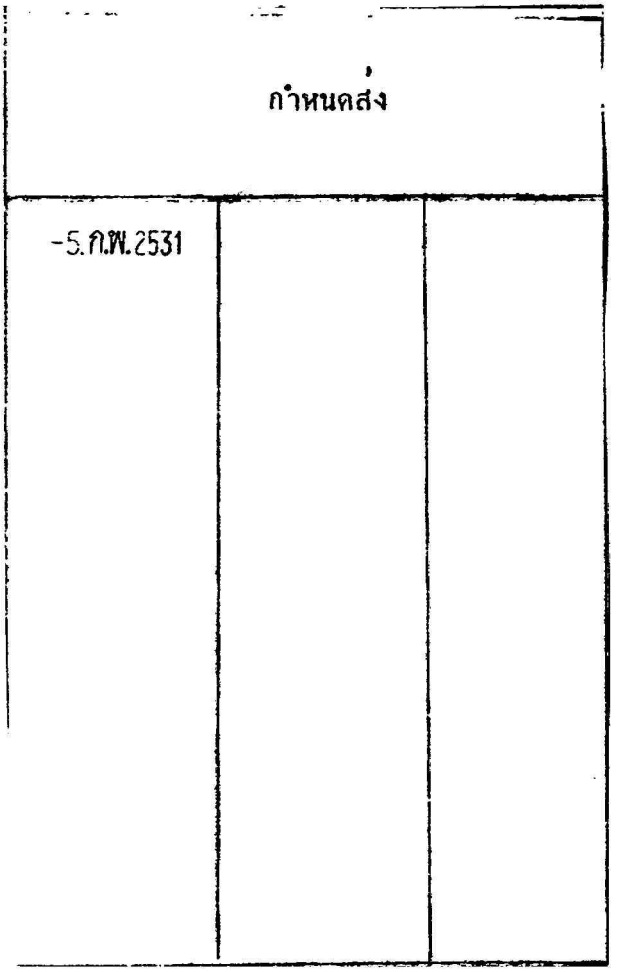
April 30, 1987 London, U.K.	**Spring Symposium and Annual General Meeting "Advances in HPLC"** Contact: The Executive Secretary, The Chromatographic Society, Trent Polytechnic, Burton Street, Nottingham NG1 4BU, U.K.
May 4–5, 1987 Washington, DC, U.S.A.	**3rd Preparative-Scale Liquid Chromatography Symposium** Contact: Symposium Manager, 3rd WSPLC, Barr Enterprices, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772. (Further details published in Vol. 369, No. 2.)
May 5–8, 1987 Boulder, CO, U.S.A.	**9th Symposium on Biotechnology for Fuels and Chemicals** Contact: Charles D. Scott, 9th Symposium on Biotechnology, Oak Ridge National Laboratory, P.O. Box X, Oak Ridge, TN 37831, U.S.A.
May 11–14, 1987 Ghent, Belgium	**2nd International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences** Contact: Dr. W. Bayens, State University of Ghent, Laboratory of Pharmaceutical Chemistry and Drug Quality Control, Harelbekestraat 72, B-9000 Ghent, Belgium. (Further details published in Vol. 354.)
May 13–15, 1987 Amsterdam, The Netherlands	**Scientific Computing and Automation (Europe)** Contact: K. Foley, Scientific Computing and Automation, P.O. Box 330, 1000 AH Amsterdam, The Netherlands. Tel.: (020) 5862 828. (Further details published in Vol. 362, No. 3.)
May 14, 1987 Raleigh, NC, U.S.A.	**4th Triangle Chromatography Symposium and Instrument Exhibit** Contact: Joseph R. Hudson, Jr., Rhone-Poulenc, Inc., Agrochemical Division, P.O. Box 12014, Research Triangle Park, NC 27709, U.S.A.
May 18–20, 1987 Wayzata, MN, U.S.A.	**Minnesota Chromatography Forum Spring Symposium "A Focus on Capillary Chromatographic Techniques"** Contact: Meeting Management, Inc., 1421 East Wayzata Boulevard, Suite 50, Wayzata, MN 55391, U.S.A. Tel.: (612) 473-0318.
May 19–21, 1987 Riva Del Garda, Italy	**8th International Symposium on Capillary Chromatography** Contact: Dr. P. Sandra, Research Institute for Chromatography, P.O. Box 91, B-8610 Wevelgem, Belgium. (Further details published in Vol. 369, No. 2.)
May 21–23, 1987 Aspen, CO, U.S.A.	**Applications of Chromatography and Spectroscopy to Clinical Medicine** Contact: Dr. Paul V. Fennessey, Department of Pediatrics, University of Colorado Health Sciences Center, Denver, CO 80262, U.S.A. Tel.: (303) 394-7286.
May 24–27, 1987 Columbia, SC, U.S.A.	**1st International Symposium on the Interface between Analytical Chemistry and Microbiology** Contact: 1st International Symposium on the Interface between Analytical Chemistry and Microbiology, P.O. Box 7126, Columbia, SC 29202, U.S.A. (Further details published in Vol. 360. No. 2.)

May 27–29, 1987 Budapest, Hungary	**ARCH '87, Automated Reasoning in Chemistry** Contact: ARCH '87 Conference, Mrs. O. Enyedy, Secretary, Institute of Isotopes of the Hungarian Academy of Sciences, P.O. Box 77, H-1525 Budapest, Hungary.
June 1–4, 1987 Loen, Norway	**Euro Food Chem IV, 4th European Conference on Food Chemistry** Contact: Euro Food Chem IV, Conference Secretariat, Norwegian Food Research Institute, P.O. Box 50, N-1432 Ås-NLH, Norway. (Further details published in Vol. 363, No. 2.)
June 3–5, 1987 Columbia, SC, U.S.A.	**1st International Symposium on the Interface Between Analytical Chemistry and Microbiology** Contact: 1st International Symposium on the Interface Between Analytical Chemistry and Microbiology, P.O. Box 7126, Columbia, SC 29202, U.S.A.
June 7–12, 1987 St. Louis, MO, U.S.A.	**8th International Symposium on Organosilicon Chemistry** Contact: Washington University, Campus Box 1150, St. Louis, MO 63130, U.S.A.
June 12–15, 1987 Lund, Sweden	**International Symposium on Titration Techniques** Contact: "Symposium on Titration Techniques", c/o The Swedish Chemical Society, Wallingatan 26 A, S-111 24 Stockholm, Sweden.
June 15–19, 1987 Sils-Maria, Switzerland	**Capillary Gas Chromatography** Contact: Dr. M. Lederer, Postfach 101, CH-7514 Sils-Maria, Switzerland. (Further details published in Vol. 389, No. 2.)
June 21–26, 1987 Toronto, Canada	**XXV *Colloquium* Spectroscopium Internationale** Contact: Mr. L. Forget, Executive Secretary CSI XXV, National Research Council of Canada, Ottawa, K1A 0R6 Canada. Tel.: (613) 993-9009, telex: 053-3145. (Further details published in Vol. 330, No. 2.)
June 28–July 4, 1987 Amsterdam, The Netherlands	**HPLC '87, 11th International Symposium on Column Liquid Chromatography** Contact: Organisatie Bureau Amsterdam bv, Europaplein, 1078 GZ Amsterdam, The Netherlands. Tel.: (31) 20-440807, telex: 13499 raico nl. (Further details published in Vol. 331, No. 2 and Vol. 366.)
July 6–8, 1987 Budapest, Hungary	**7th Annual American–Eastern European Colloquium and Conference on Liquid Chromatography** Contact: Dr. H. Kalász, Department of Pharmacology, Semmelweis University of Medicine, Nagyvárad tér 4, P.O. Box 370, 1445 Budapest, Hungary. Tel.: (361) 137070, ext. 258, telex 225070.
Aug. 2–6, 1987 Denver, CO, U.S.A.	**29th Rocky Mountain Conference** Contact: Michael Reddy, U.S. Geological Survey, 5293 Ward Road, Arvada, CO 80002, U.S.A. Tel.: (303) 236-3617.
Aug. 12–19, 1987 Budapest, Hungary	**World Congress of Theoretical Organic Chemists.** Contact: E.A. Lang, WATOC CONGRESS, Hungarian Chemical Society, Anker köz 1, H-1061 Budapest, Hungary.

Aug. 17–21, 1987 Oberammergau, F.R.G.	**7th International Symposium on Affinity Chromatography and Interfacial Macromolecular Interactions** Contact: Prof. Dr. H.P. Jennissen, Institut für Physiologie, Physiologische Chemie und Ernährungsphysiologie, Universität München, Veterinärstr. 13, D-8000 München 22, F.R.G.
Aug. 25–30, 1987 Beijing, China	**8th International Conference on Computers in Chemical Research and Education** Contact: Cheng Qian, 345 Lingling Road, 200032 Shanghai, China. Telex: 33354 SIOC CN.
Sept. 5–6, 1987 Leiden, The Netherlands	**International FIP Satellite Symposium on Disposition and Delivery of Peptide Drugs** Contact: Secretariat of the Symposium on Disposition and Delivery of Peptide Drugs, Mrs. W.M. Gerritsen-Brusche, Center for Bio-Pharmaceutical Sciences, P.O. Box 9502, 2300 RA Leiden, The Netherlands.
Sept. 7–11, 1987 Paris, France	**Euroanalysis VI, European Conference on all Aspects of Analytical Sciences** Contact: G.A.M.S., 88 Boulevard Malsherbes, 75008 Paris, France. (Further details published in Vol. 357, No. 3.)
Sept. 8–10, 1987 Berlin, F.R.G.	**24th International Symposium on Advances in Chromatography** Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. (Further details published in Vol. 387.)
Sept. 8–11, 1987 Guildford, U.K.	**7th International Bioanalytical Forum on Bioanalysis of Drugs and Metabolites, especially Anti-inflammatory and Cardiovascular** Contact: Dr. E. Reid, Guildford Academic Associates, 72 The Chase, Guildford, Surrey GU2 5UL, U.K. Tel.: 0483-65324.
Sept. 13–18, 1987 New York, NY, U.S.A.	**26th Eastern Analytical Symposium** Contact: Joseph P. Luongo, AT&T Bell Labs. 1A-352, 600 Mountain Ave, Murray Hill, NJ 07974, U.S.A.
Sept. 14–17, 1987 San Francisco, VA, U.S.A.	**101st AOAC Annual International Meeting and Exposition on Analytical Methodology** Contact: AOAC Office, 1111 N. 19th Street, Suite 210, Arlington, VA 22209, U.S.A. Tel.: (703) 522-3032. (Further details published in Vol. 387.)
Sept. 22–25, 1987 Selvino, Italy	**4th International Symposium on Instrumental Thin-Layer Chromatography (Planar Chromatography)** Contact: Dr. Helmut Traitler, NESTEC Ltd., Nestlé Research Department, CH-1800 Vevey, Switzerland. Tel.: (021) 51 01 11. (Further details published in Vol. 363, No. 2.)
Sept. 23–25, 1987 Barcelona, Spain	**International Symposium on Pharmaceutical and Biomedical Analysis** Contact: Dr. Emilio Gelpi, Symposium Secretariat, International Symposium on Pharmaceutical and Biomedical Analysis, Palau de Congressos, Avgda. Reina Mª. Cristina s/n, 08004 Barcelona, Spain. Tel.: (325) 30 00-223 99 40, telex: 53.117 foimb-e.

Sept. 28–30, 1987 Barcelona, Spain	**International Symposium on Applied Mass Spectrometry in the Health Sciences** Contact: Dr. Emilio Gelpi, Symposium Secretariat, International Symposium on Applied Mass Spectrometry in the Health Sciences, Palau de Congressos. Dept. de Convencions, Avgda. Reina M.ª Cristina s/n, 08004 Barcelona, Spain. Tel.: (325) 30 00-223 99 40, telex: 53.117 foimb-e.
Sept. 28–Oct. 1, 1987 Gaithersburg, MD, U.S.A.	**Accuracy in Trace Analysis – Accomplishments, Goals, Challenges** Contact: Harry Hertz, A309 Chemistry Building, National Bureau of Standards, Gaithersburgh, MD 20899, U.S.A. Tel.: (301) 921 2851.
Sept. 28–Oct. 2, 1987 Amsterdam, The Netherlands	**2nd Amsterdam HPLC Summercourse** Contact: Dr. J.C. Kraak, Laboratory for Analytical Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands.
Sept. 29–30, 1987 Würzburg, F.R.G.	**Bioflavour '87** Contact: Prof. Dr. P. Schreier, University of Würzburg, Food Chemistry, Am Hubland, D-8700 Würzburg, F.R.G.
Oct. 4–9, 1987 Detroit, MI, U.S.A.	**FACSS XIV, Federation of Analytical Chemistry and Spectroscopy Societies** Contact: Dr. Steve Swarin, Publicity Chairman, Analytical Chemistry Department, General Motors Research Laboratories, Warren, MI 48090-9055, U.S.A. Tel.: (313) 986-0806. (Further details published in Vol. 387.)
Oct. 12–16, 1987 Sils-Maria, Switzerland	**5th Symposium and Workshop on Ion Chromatography** Contact: Workshop Office IAEAC, M. Frei-Hausler, Postfach 46, CH-4123 Allschwil 2, Switzerland. (Further details published in Vol. 366.)
Oct. 12–17, 1987 Varna, Bulgaria	**6th Danube Symposium on Chromatography** Contact: 6th Danube Symposium on Chromatography, Scientific Council on Chromatography of the Bulgarian Academy of Sciences, Centre of Chemistry, BU-1040 Sofia, Bulgaria. Tel.: 7131 (3591), telex: 22729 ech ban bg. (Further details published in Vol. 387.)
Oct. 19–25, 1987 Beijing, China	**2nd Beijing Conference and Exhibition on Instrumental Analysis** Contact: Secretariat of the Beijing Conference and Exhibition on Instrumental Analysis, Room 4311, Beijing Exhibition Centre Hotel, Beijing, China. Tel.: 890541 ext. 481 or 415.
Oct. 20–22, 1987 Sopron, Hungary	**3rd Symposium on the Analysis of Steroids** Contact: Prof. S. Görög, c/o Hungarian Chemical Society, Anker köz 1, H-1061 Budapest, Hungary.
Nov. 2–4, 1987 Washington, DC, U.S.A.	**7th International Symposium on High-performance Liquid Chromatography of Proteins, Peptides and Polynucleotides** Contact: Shirley E. Schlessinger, Symposium Manager, 7th International Symposium on HPLC of Proteins, Peptides and Polynucleotides, 400 East Randolph, Chicago, IL 60601, U.S.A. Tel.: (312) 527-2011. (Further details published in Vol. 387.)

Feb. 1–3, 1988 Baden-Baden, F.R.G.	**2nd International Symposium on Preparative and Up Scale Liquid Chromatography** Contact: Prof. Dr. K. Unger, Universität Mainz, Joachim-Becher-Weg 24, D-6500 Mainz, F.R.G. Tel.: 06131-395745. (Further details published in Vol. 387.)
April 18–21, 1988 Las Vegas, NV, U.S.A.	**Flow Analysis IV, An International Conference on Flow Analysis** Contact: Dr. Gilbert E. Pacey, Department of Chemistry, Miami University, Oxford, OH 45056, U.S.A. (Further details published in Vol. 357, No. 3.)
April 27–29, 1988 Basle, Switzerland	**4th Symposium on Handling of Environmental Samples in Chromatography** Contact: Professor R.W. Frei, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. Tel.: (020) 5485379.
May 18–20, 1988 Amsterdam, The Netherlands	**CAC–88, 4th International Conference on Chemometrics in Analytical Chemistry** Contact: CAC–88, Laboratory for Analytical Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands. Tel.: (020)-5223541 (Dr. Smit). (Further details published in Vol. 369, No. 2.)
June 5–11, 1988 Frankfurt am Main, F.R.G.	**ACHEMA 88. International Meeting on Chemical Engineering and Biotechnology, 22nd Exhibition-Congress** Contact: DECHEMA, Organisation ACHEMA, Postfach 97 01 46, D-6000 Frankfurt am Main 97, F.R.G.
June 19–24, 1988 Washington, DC, U.S.A.	**HPLC '88, 12th International Symposium on Column Liquid Chromatography** Contact: Symposium Manager, Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898 3772. (Further details published in Vol. 363, No. 2.)
Sept. 5–8, 1988 Jena, G.D.R.	**COMPANA '88, 4th Conference on Computer Application in Analytical Chemistry** Contact: Professor Dr. K. Danzer, c/o Friedrich Schiller University Jena, Department of Chemistry, Steiger 3, 6900 Jena, G.D.R. Tel.: Jena 82 25028, telex: 05886134 uni dd.
July 30–August 5, 1989 Cambridge, U.K.	**SAC 89, International Conference on Analytical Chemistry** Contact: SAC 89, Royal Society of Chemistry, Analytical Division, Burlington House, London W1V 0BN, U.K. Tel.: (01) 437-8656.
Aug. 28–Sept. 1, 1989 Wiesbaden, F.R.G.	**11th International Symposium on Microchemical Techniques** Contact: Gesellschaft Deutscher Chemiker, Abt. Tagungen, P.O. Box 900440, D-6000 Frankfurt/Main 90, F.R.G. Tel.: (069) 79 17-366/360, telex: 4170497 gdch d.
Aug. 27–31, 1990 Vienna, Austria	**7th European Conference on Analytical Chemistry, "Euroanalysis 7"** Contact: Prof. Robert Kellner, Austrian Society for Microchemistry and Analytical Chemistry, Institute for Analytical Chemistry, Technical University of Vienna, Getreidemarkt 1, A-1060 Vienna, Austria.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PUBLICATION SCHEDULE FOR 1987

Journal of Chromatography and Journal of Chromatography, Biomedical Applications

MONTH	J	F	M	A	M	J	J	
Journal of Chromatography	384 385 386 387	388/1 388/2 389/1	389/2 390/1 390/2 391/1	391/2 392 393/1 393/2	393/3 394/1 394/2 394/3			The publication schedule for further issues will be published later.
Bibliography Section		412/1		412/2				
Cumulative Indexes, Vols. 351-400								
Biomedical Applications	413	414/1	414/2 415/1	415/2 416/1	416/2	417/1	417/2 418	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 362, No. 3, pp. 461–464. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

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

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

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

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

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

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Chiral HPLC columns for optical resolution

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Examples of New chiral HPLC columns

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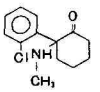
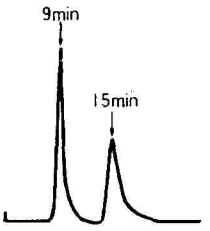
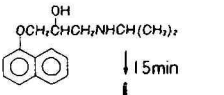
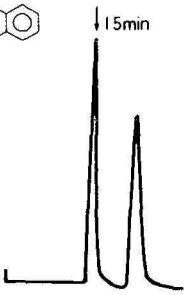




Column: 4.6mm ID × 250mm
Eluent: ethanol-H_2O (95:5)
Flow rate: 0.5ml/min
Detection: UV254nm

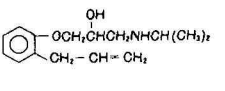
CHIRALCEL OD



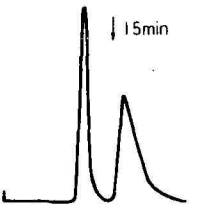


Column: 4.6mm ID × 250mm
Eluent: hexane-2-propanol-diethylamine (80:20:0.1)
Flow rate: 0.5ml/min
Detection: UV254nm

CHIRALCEL OD



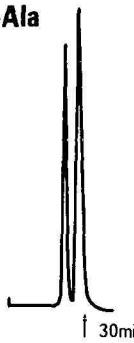
Sample: 150mg



Column: 20mm ID × 500mm
Eluent: hexane-2-propanol-diethylamine (80:20:0.1)
Flow rate: 12ml/min
Detection: UV254nm

CHIRALPAK WE

DL-Ala



Column: 4.6mm ID × 250mm
Eluent: aq 0.25mM $CuSO_4$
Flow rate: 1.0ml/min
Detection: UV230nm Temp. 35°C

Name	Separation
CHIRALPAK OT(+) CHIRALPAK OP(+)	Compounds possesing aromatic group (see: Technical Brochure No.1)
CHIRALCEL OA CHIRALCEL OB CHIRALCEL OC CHIRALCEL OD CHIRALCEL OK CHIRALCEL CA-1	(see: Technical Brochure No.1, No.3)
CHIRALPAK WH CHIRALPAK WM CHIRALPAK WE	DL-Amino acid or its derivative (see: Technical Brochure No.2, No.4)

Size of column

Analytical Column	4.6mm (I.D.) × 250mm (L)
Precolumn	4.6mm (I.D.) × 50mm (L)
Semipreparative column	10mm (I.D.) × 250mm or (500mm)
	20mm (I.D.) × 250mm or (500mm)

Preparation of pure enantiomer (~kg) with Chiral column are acceptable.

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DAICEL CHEMICAL INDUSTRIES, LTD.

Tokyo
8-1, Kasumigaseki 3-chome,
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Phone: 03(507)3151, 3189
Telex: 222-4632 DAICEL J
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New York
Pan-Am Bldg., 200 Park Avenue,
New York, N.Y. 10166-0130, U.S.A.
Phone: (212)878-6765, 6766
Telex. (23)236154 DCC UR
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DAICEL (U.S.A.), INC.
611 west 6th Street, Room 2152
Los Angeles California 90017, U.S.A.
Phone: (213)629-3656
Telex: 215515 DCIL UR
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DAICEL (EUROPA) GmbH
Königsallee 92a.
4000 Düsseldorf 1, F.R. Germany
Phone: (0211)134158
Telex: (41)8588042 DCEL D
FAX: (0211)879-8329