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Contents (continued)

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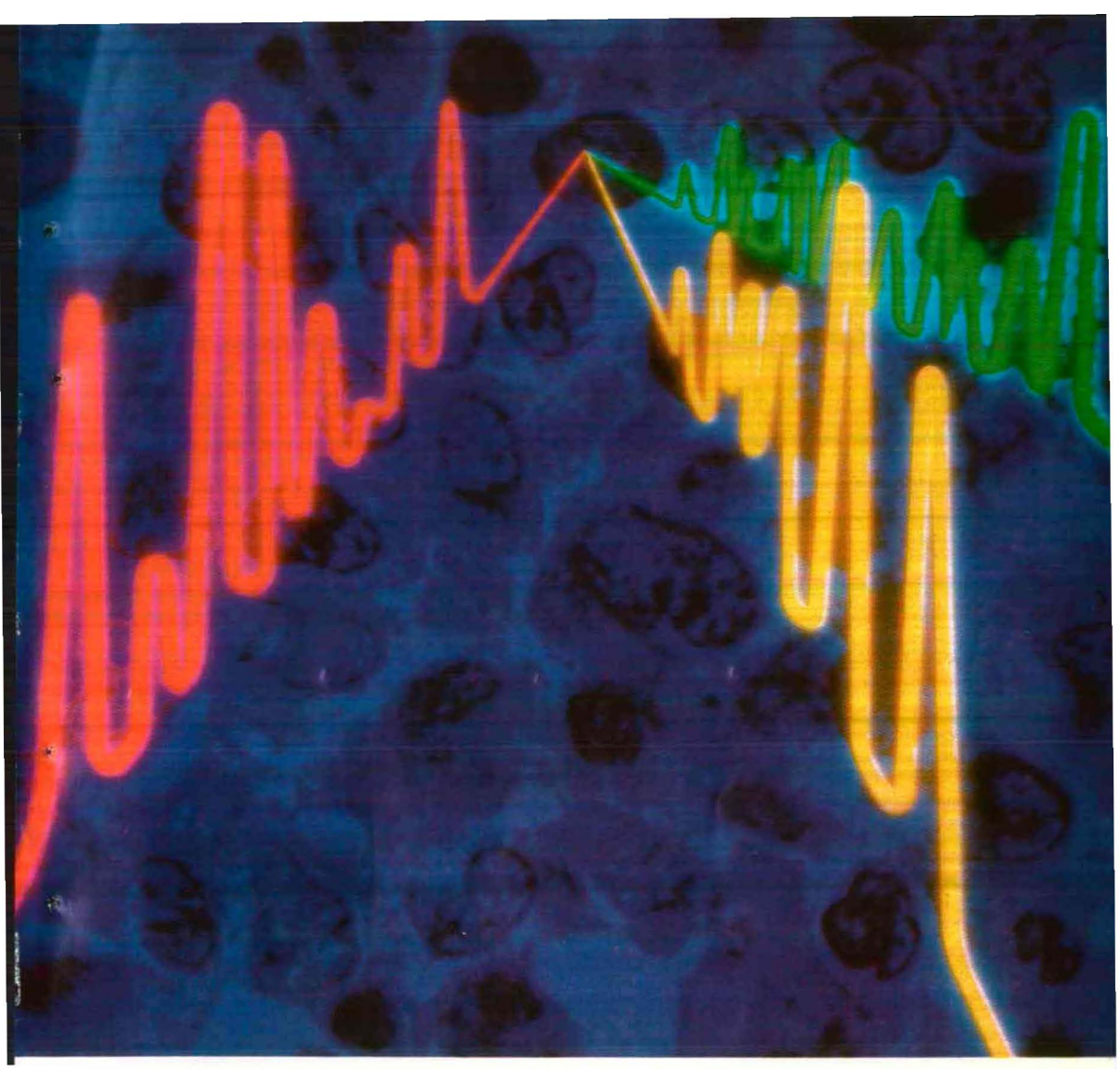

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CHROM. 18 355

INVESTIGATION OF POLAR MODIFIERS IN CARBON DIOXIDE MOBILE PHASES FOR CAPILLARY SUPERCRITICAL FLUID CHROMATOGRAPHY

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(Received November 5th, 1985)

SUMMARY

The effect of low concentrations of polar modifiers on the selectivity of a non-polar carbon dioxide mobile phase in capillary supercritical fluid chromatography (SFC) was determined. The selectivity of mixtures of carbon dioxide–water (approximately 0.3–0.9 mole%) and carbon dioxide–methanol (approximately 0.8 mole%) were compared to the selectivity of pure carbon dioxide using polarity-test mixtures. Under the conditions of this study, water modifiers did not significantly affect selectivity or retention. Slight changes in selectivity and decreased retention, however, were observed for the methanol modifier. These data contrast with those obtained for packed-column SFC in which similar concentrations of modifiers produced significant changes in selectivity and greatly reduced retention, and are consistent with results indicating these previous results were due to modification of the stationary phase rather than changes in fluid phase solubilities.

INTRODUCTION

Capillary supercritical fluid chromatography (SFC) is becoming recognized as a viable analytical methodology. Supercritical fluid mobile phases offer advantages over conventional gas and liquid phases in that their densities approach those of liquids, yet solute diffusivities are up to two orders of magnitude greater and viscosities significantly less than the liquid[1]. The lower viscosities and higher diffusion coefficients result in enhanced chromatographic efficiency and shorter analysis times compared to liquid chromatography. The liquid-like densities give favorable mass transfer properties and allow the separation of less volatile materials than is possible by gas chromatography. In addition, the solvating power of a supercritical fluid is dependent on density and can be controlled as a function of pressure. The advantages of capillary columns in SFC include the low pressure drop which allows pressure programming techniques to be effectively used[2], the ability to produce highly deactivated column surfaces and inert stationary phases[3,4], and the compatibility of flow-rates with gas phase detectors (*e.g.*, flame ionization and mass spectrometric)[5–7].

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Pressure programming in SFC is somewhat analogous to temperature programming in gas chromatography or gradient elution in liquid chromatography. The low pressure drop also allows long column lengths to be used to achieve large numbers of theoretical plates.

In addition to the variable solvating power achieved by controlling the pressure (or density) of a supercritical fluid, selectivity of the mobile phase can also be obtained by altering the chemical properties of the fluid. This is accomplished by using either chemically different fluid systems or by low concentrations of fluid modifiers[8,9]. Usually, low concentrations of a polar or chemically active modifier are added to a less polar and chemically inert fluid system (*e.g.*, methanol to carbon dioxide). The use of a more polar fluid system and/or a polar modified fluid system also increases the maximum solvent power of the mobile phase and provides the potential for elution of more polar and less soluble analytes. The use of modifiers in packed-column SFC has been more extensive than in capillary SFC and has demonstrated that very low concentrations of an appropriate modifier ($<1\%$) can drastically change retention and selectivity[8–11]. Such effects have been accounted for by increased solubility of the solute in the mobile phase and/or modification of the stationary phase or silica surface. However, recent chromatographic evidence[9,11] suggests that deactivation of active column sites by the polar modifiers may be the dominant mechanism.

In this study the influence of low concentrations of water and methanol modifiers on a supercritical carbon dioxide mobile phase in capillary supercritical fluid chromatography was investigated. Polarity-test mixtures containing alkanes and hydroxy-substituted components (to provide both non-specific and specific mobile phase-solute interactions) and a relatively non-selective, 5% phenyl polymethylphenylsiloxane (SE-54) stationary phase were used to monitor mobile phase selectivity effects. Since it is possible essentially to eliminate residual silanol groups on fused-silica capillary column surfaces and significant modification of the polysiloxane stationary phase is unlikely, any changes in selectivity should be accounted for by changes in the mobile phase polarity. The results of this study complement data obtained recently on the solvating power of supercritical fluids and fluid mixtures[12,13] and support the conclusion that modifier effects in previous packed column studies were due to modification of the stationary phase.

EXPERIMENTAL

The primary polarity-test mixture consisted of the following compounds (Chem Service, West Chester, PA, U.S.A.) at approximately 1 mg/ml concentration in methylene chloride: *n*-decane, phenol, 1-octanol, *n*-dodecane, 1-decanol and *n*-tetradecane. A second polarity-test mixture containing the following heavier molecular weight probes was also used: *n*-triacontane (C_{30}), diacetoxyscirpenol (DAS), deoxynivalenol (DON) and *n*-dotriacontane (C_{32}).

The instrumentation used for capillary SFC is similar to that previously described[14,15]. The apparatus utilized a modified Varian 8500 syringe pump to generate a high-pressure and pulse-free flow of mobile phase and a Hewlett-Packard 5700 gas chromatograph to provide constant temperature conditions and flame ionization detection. Constant-pressure operation was controlled and maintained to within 0.2 atm with a microcomputer. Sample introduction was accomplished using a Valco 0.2-

μl C14W HPLC injection valve. The injection valve was connected to the chromatographic column through a splitter device which was adjusted to give approximately a 1:20 flow into the chromatographic column. The chromatographic column was prepared from a length of 12 m × 50 μm I.D. fused-silica tubing (Spectran Corp., Sturbridge, MA, U.S.A.) and coated with a 0.20-μm film-thickness of 5% phenyl polymethylphenylsiloxane stationary phase (SE-54) that was rendered non-extractable by extensive cross-linking with azo-*tert.*-butane[16]. Prior to detection the supercritical mobile phase was decompressed and the linear velocity controlled to approximately 1.5 cm/s by connecting the end of the chromatographic column to a short length (*ca.* 5 cm) of 5 μm I.D. fused-silica restrictor tubing.

Binary fluid mixtures of carbon dioxide–water were produced by equilibrating the carbon dioxide with water in a gas–liquid equilibrium cell placed in-line immediately prior to the injection port. The transfer line and injection port were then maintained at the same temperature as the equilibrium cell to assure a homogeneous fluid mixture. Under a defined set of pressure and temperature conditions the solubility of water in carbon dioxide is accurately known[17–19] (typically ranges between 0.3 and 1.4 mole%) and provides a convenient method of generating a binary mixture. When pure carbon dioxide was used for the mobile phase the fluid was carefully dried prior to loading in the syringe pump by distilling it through activated silica gel. As a further safeguard to ensure that absolutely dry carbon dioxide was delivered to the chromatographic column, an additional drying column, also packed with silica gel, was placed between the syringe pump and injection port. Care was taken to ensure that the pressure drop across this column was negligible. Carbon dioxide–methanol mixtures were prepared by preloading the syringe pump with the proper volume of methanol and then filling the remaining volume with carbon dioxide and allowing the mixture to equilibrate. Isothermal and isobaric chromatographic operating conditions were chosen to provide capacity ratios (k') between 0.5 and 5 and to provide the appropriate equilibrium conditions to generate the carbon dioxide–water mixtures. The void or hold-up times (t_0) used in the k' calculations were obtained from the elution time of the leading edge of the solvent peak which was verified to be a good approximation for the present chromatographic conditions.

RESULTS AND DISCUSSION

The use of fluid modifiers in SFC has tremendous potential for generating highly selective and specific mobile phases. However, it is necessary to understand the modifier interaction mechanism(s) to take full advantage of this potential. By using a capillary chromatographic column with an inert and well-defined surface and stationary phase, the influence of modifiers on the mobile phase can be probed more directly. Two polarity mixtures were used to monitor retention (k') as a function of fluid system. Each mixture contained components that would be expected to exhibit both specific interactions (hydroxy compounds) and non-specific interactions (alkanes) with the polar modifiers. One mixture contained lower-molecular-weight materials that eluted at lower pressures (reduced pressures of 1.1–1.5) and the other mixture contained heavier molecular weight materials that eluted at higher pressures (reduced pressures >2). DAS and DON are trichothecene mycotoxins (sesquiterpenoids) of molecular weights 366 and 296 daltons, respectively, and contain free

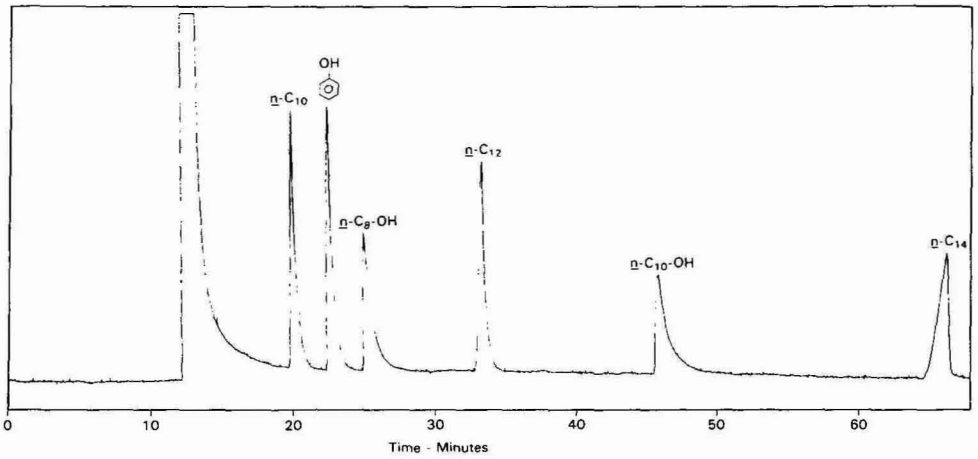

Fig. 1. Typical capillary supercritical fluid chromatogram of a polarity-test mixture. Chromatographic conditions: 12 m × 50 μm fused-silica column coated with 0.20-μm film of cross-linked 5% phenyl polymethylphenylsiloxane (SE-54). Supercritical carbon dioxide mobile phase at 60°C and 80 atm.

hydroxyl and keto groups[20]. A typical supercritical fluid chromatogram of the lower-molecular-weight polarity mixture is shown in Fig. 1. This separation was obtained at 60°C and 80 atm with pure carbon dioxide as the mobile phase. Although the more polar components exhibited slight peak tailing, the column deactivation was acceptable and allowed reproducible elution of these components.

Data comparing the retention (k') for a pure carbon dioxide fluid and a water-modified carbon dioxide fluid system are listed in Table I. Identical chromatographic conditions were employed and at least five replicate runs were made for each fluid system to evaluate reproducibility. To generate the water-modified fluid system the equilibrium cell was heated to 50°C, which at 84 atm produced a fluid mixture of approximately 0.35 mole % water[18]. The chromatographic operating temperature was maintained at 60°C to prevent the possibility of any water condensing from the fluid mixture. Within the limits of the standard deviations of each capacity ratio measurement, the pure carbon dioxide and the water-modified carbon dioxide displayed identical retention for the components in the polarity mixture. This is the expected behavior for the non-polar alkanes since the low modifier concentration

TABLE I

CAPACITY RATIO DATA USING WATER-MODIFIED CARBON DIOXIDE

Chromatographic conditions: 60°C and 84 atm.

Fluid system	*k'*					
	n-C_{10}	*Phenol*	*n-C_8-OH*	*n-C_{12}*	*n-C_{10}-OH*	*n-C_{14}*
CO_2	0.49 ± 0.00	0.70 ± 0.01	0.79 ± 0.01	1.26 ± 0.01	1.95 ± 0.02	3.03 ± 0.02
CO_2–H_2O*	0.49 ± 0.01	0.70 ± 0.01	0.79 ± 0.01	1.26 ± 0.01	1.98 ± 0.04	3.05 ± 0.05

* Equilibrium cell at 50°C; approximately 0.35 mole% water mixture.

TABLE II

CAPACITY RATIO DATA USING METHANOL-MODIFIED CARBON DIOXIDE

Chromatographic conditions: 100°C and 78 atm.

Fluid system	*k'*					
	n-C_{10}	*Phenol*	*n-C_8-OH*	*n-C_{12}*	*n-C_{10}-OH*	*n-C_{14}*
CO_2	0.41 ± 0.01	0.52 ± 0.01	0.66 ± 0.01	1.09 ± 0.01	1.68 ± 0.02	2.72 ± 0.03
CO_2–CH_3OH*	0.42 ± 0.01	0.54 ± 0.01	0.67 ± 0.01	1.08 ± 0.01	1.70 ± 0.02	2.67 ± 0.03

* 0.77 mole% methanol mixture.

would not appreciably change the fluid density and competition for active sites is not important. However, it is significant that the retention of the more polar alcohols was not affected.

This is consistent if the modifier only affects the solvent power of the fluid, since its low concentration would not significantly change the fluid solvating properties. It is also unlikely that the hydrophobic stationary phase was modified by the water. The small change in fluid properties is reasonable if the Hildebrand solubility parameter[21] concept is used to describe the solvent power of the fluid mixture. Recent studies in this laboratory have shown that solubilities change only slightly with the addition of a fluid modifier, which is consistent with the small changes in solvating powers noted from solvatochromic studies of supercritical fluids[12,13].

Data for the same polarity mixture comparing the retention in pure carbon dioxide and in a 0.77 mole% methanol-modified carbon dioxide mixture (Table II) also support these conclusions. Slightly different operating conditions of 78 atm and 100°C were utilized in this comparison. The higher temperature was used to ensure the mixture was above the critical point and in the single-phase equilibrium region[22]. Again, within the limits of the standard deviations of k', no differences in retention existed for the two fluid systems. Although the concentration of the methanol modifier was over twice that of the water modifier, it was insufficient to alter the solvent power of the mobile phase significantly. It is interesting to note that at these conditions of lower pressure and higher temperature, which created a lower density (0.14 *versus* 0.22 g/ml) with a corresponding decrease in mobile phase solvent power, the test probes eluted with lower k' values. This infers that at higher temperatures where solute volatility increases the nature of the fluid becomes less important in determining retention and a gas chromatographic retention mechanism becomes dominant.

Consequently, the second test mixture incorporating larger molecules with lower volatilities was also used to evaluate retention. The k' values of these compounds obtained with pure carbon dioxide, water-modified carbon dioxide and methanol-modified carbon dioxide fluid systems are listed in Table III. In all cases at least five replicate separations were obtained to calculate the capacity ratio data. Operating conditions of 100°C and 168 atm were utilized. At the higher chromatographic operating temperature, two water equilibrium cell temperatures of 50°C and 75°C were utilized which at 168 atm generated water mixtures of approximately 0.55 mole% and 0.90 mole%, respectively[18]. These fluid mixtures, however, did not show any

TABLE III

CAPACITY RATIO DATA FOR TRICHOTHECENE POLARITY MIXTURE

Chromatographic conditions: 100°C and 168 atm.

Fluid system	*k'*			
	n-C$_{30}$	*DAS*	*DON*	*n-C*$_{32}$
CO_2	0.49 ± 0.01	0.66 ± 0.02	1.02 ± 0.03	3.64 ± 0.08
CO_2–H_2O*	0.48 ± 0.00	0.66 ± 0.00	1.04 ± 0.01	3.60 ± 0.03
CO_2–H_2O**	0.47 ± 0.01	0.65 ± 0.01	1.02 ± 0.01	3.58 ± 0.04
CO_2–CH_3OH***	0.45 ± 0.00	0.58 ± 0.01	0.89 ± 0.01	3.25 ± 0.03

* Equilibrium cell at 75°C; approximately 0.90 mole% water mixture.
** Equilibrium cell at 50°C; approximately 0.55 mole% water mixture.
*** 0.77 mole% methanol mixture.

significant retention differences compared to the pure carbon dioxide fluid system. But, with the methanol-modified fluid system significant changes in retention were apparent. Retention decreases of 8–13% for the methanol-modified fluid system occurred, with the more polar DAS and DON showing the greatest decreases. However, these changes were small compared to retention changes (factors of 2–5) observed on packed columns with similar methanol concentrations[9,10]. The small decreases in retention observed with the capillary columns supports the idea that the modifier is changing the solvent power of the mobile phase, with increased selectivity for the more polar compounds. It also supports the conclusion that the drastic changes in retention observed for packed columns are a result of surface and stationary phase modification.

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CHROM. 18 347

HIGH-PERFORMANCE EMITTERS FOR USE IN A SURFACE IONIZATION DETECTOR FOR GAS CHROMATOGRAPHY

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(Received October 28th, 1985)

SUMMARY

An attempt has been made to find good emitter materials for use in a surface ionization detector for gas chromatography. The sensitivity, detectability and stability characteristics were examined for ten refractory metals such as Pt, Ir and Re using tri-*n*-butylamine as a test compound. Of these materials, iridium is the best in terms of detectability. It not only provides six times the detection ability of Pt, but also shows good stability, reproducibility and durability.

INTRODUCTION

The surface ionization detector has recently been developed for gas chromatography[1]. It utilizes a platinum filament emitter which is heated electrically. The emitter connected to the ion collector is positioned 2 mm above the nozzle of the carrier gas exit, which is maintained at a positive potential with respect to the collector. This detector is extremely sensitive to organic compounds such as tri-*n*-butylamine (TBA) which dissociates to species having low ionization potentials. It can be operated in any kind of carrier gas and the addition of air (oxygen) to the detector environment improves the performance.

Studies of the response of this detector have shown that the ionization mechanism involves positive ionization of organic species[2] generated on the hot surface. The molecules being detected decompose on the hot surface into radicals which have lower ionization potentials (IPs) than the molecules and are ionized and partially desorbed. The ion current of the secondary species (s) is dependent on the surface temperature, T (ref. 3)

$$i_s(T) = \frac{n \cdot Y_s(T)}{1 + g_0/g_+ \cdot \exp\left(\frac{\mathrm{IP} - \varphi}{kT}\right)} \quad (1)$$

0021-9673/86/$03.50

where n is the number of molecules impinging on the surface, $Y_s(T)$ is the yield of chemical reactions on the surface, φ is the work function of the surface and g_+/g_0 is the ratio of the statistical weights of the ions and the neutral species. The total ion current is $I(T) = \sum_s i_s(T)$.

Although the principal of the detector has been established, there are many factors which require investigation in order to further the understanding of the detector characteristics as well as improve its operation:

(1) The previous study was made with a platinum emitter which showed good performance. However, it is not known whether platinum is the most suitable material for this purpose. The first priority is therefore to find the emitter material which gives the best response under the desired operating conditions.

(2) The structure and positioning of the emitter.

(3) The effect on the performance of changing the gaseous environment in which the emitter is placed.

(4) The stability, reproducibility and durability.

We have investigated ten types of metals as the emitter material, chosen primarily because of their refractory properties. The response and performance characteristics of these emitters was examined in various gaseous environments. Emphasis is placed on a comparison of the relative ionization abilities of the emitters. In addition, some information on their lifetimes under the optimum operating conditions is presented.

EXPERIMENTAL

Instrument

The laboratory built detector was used with a Shimadzu Model GC-7A Pr F gas chromatograph. The detector components have been described previously[1]. A glass column (3 × 2.6 mm I.D.) containing 10% Apiezon L + 5% potassium hydroxide on Chromosorb W (80–100 mesh) was used. Nitrogen and helium were tested as carrier gases. Samples were prepared using acetone as a solvent, with TBA as the test compound.

Procedure

The emitter was formed from the ten materials in various shapes and then fixed to the ion collector. The materials tested (The Japan Lamp Industrial Co., Tokyo) are listed in Table I. After installation, all the emitters were conditioned by passing sufficient current to reach a temperature of 1200°C while introducing 20 ml/min of air to the surface.

All the emitters were heated to a satisfactorily high temperature well below the respective melting points (at least 300°C) in order to study the effect caused by varying the emitter surface temperature. The emitter temperature was measured by an optical pyrometer Model 760 (Chino Works, Ltd, Tokyo).

None of the column or operating parameters was changed during the study. TBA gave symmetrical peaks. Thus, the peak areas were obtained from the product of the height and width at half-height, and expressed in terms of Coulombs (C) divided by the sample amount (g). Therefore the sensitivity as defined by Patterson[4]

TABLE I

SENSITIVITY AND DETECTION LIMIT FOR THE EMITTER MATERIALS USING TBA AS A TEST SAMPLE

Emitter material	*Work function** *(eV)*	*Melting point** *(°C)*	*Sensitivity*** *(C/g)*	*Detection limit**** *(g/s)*	*Emitter temperature§* *(°C)*
Pt	5.65	1774	1.58	$1.0 \cdot 10^{-14}$	650
Rh	4.98	1966	$2.7 \cdot 10^{-2}$	$1.6 \cdot 10^{-13}$	<650
Mo	4.6	2620	$2.5 \cdot 10^{-1}$	$5.4 \cdot 10^{-14}$	<650
Ir	5.27	2450	6.52	$1.7 \cdot 10^{-15}$	<650
Ni	5.15	1453	$2 \cdot 10^{-2}$	$2.1 \cdot 10^{-13}$	<650
Co	5.0	1495	$3.4 \cdot 10^{-3}$	$1.3 \cdot 10^{-12}$	<650
Re	4.96	3180			
W	4.55	3410			
Ta	4.25	3000			
Pd	5.17	1552			

* These data are taken from ref. 8.
** Measured under the optimum condition for the detection limit.
*** Minimum detectable amount at a signal-to-noise ratio of 2.
§ Temperature for the minimum detectable amount.

was used as one of the criteria for characterization. Measurements of noise were made with a strip-chart recorder and expressed in Amperes. The detectability was calculated as twice the noise divided by the sensitivity.

The stability of the detector with different emitters was investigated by measuring the background current and the noise level. The variation in the background current over 1 h was studied after determining the optimum heating current which gave the best detection limit. Some of the emitters were subjected to a reproducibility test by injecting TBA over a 1-h period.

RESULTS AND DISCUSSION

Sensitivity

Length and position of the emitter. In preliminary studies, we found that the position and length (surface area) of the emitter effect the sensitivity characteristics. The emitter length *vs.* response characteristics were investigated using a platinum filament with a diameter of 0.25 mm. The filament was shaped to a coil of diameter 3 mm, the number of turns of which determined the length of the emitter. The results for TBA are shown in Fig. 1. The signal increases with the length of the emitter and levelled off at about eight turns. The position of the emitter had no significant effect on the sensitivity, particularly for a highly coiled emitter.

Gaseous environment around the emitter. It is well known that, in surface ionization (SI) mass spectrometry, the addition of oxygen raises the work function of the heated SI emitter[5] because of the oxidation on the surface, while organic compounds result in a decrease in the work function due to carbonization of the surface[6], *i.e.*, poisoning of the emitter.

To study the effect of the introduction of air, emitters made from Pt, Ir and

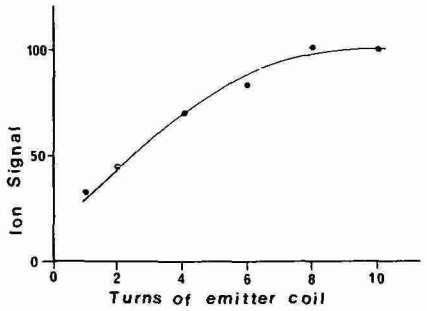

Fig. 1. Dependence of the ion signal on the length of the platinum emitter. Emiter temperature: 650°C. Sample: 0.1 ppm TBA in acetone. GC conditions: column temperature, 150°C; 30 ml/min nitrogen carrier gas mixed with air at 20 ml/min.

Mo were prepared in air (oxygen) using the procedures described in the Experimental section. Presumably the surface of the emitter is oxidized to M_xO_y compounds during this process. When a 1-μl test sample of 1 ppm TBA in acetone was injected repeatedly into these fresh emitters under a 30 ml/min flow of helium and without any other gas, the signal gradually decreased. This trend was found for all the emitters studied, and may be interpreted as the removal of oxygen from the oxidized surface due to reactions between the metallic oxides and the organic compounds on the hot emitter surface. This explanation is consistent with the mentioned phenomenon in SI mass spectrometry.

It can be concluded that an oxidized emitter should be used in order to improve sensitivity and stability and to prevent poisoning. Such an oxidized emitter can be obtained if air (oxygen) is added to the emitter surface from another gas line. Experience has shown that at least 10 ml/min air is sufficient to prevent poisoning of the emitter in the case of helium as carrier gas, as will be discussed later.

A very slight difference was observed in the response when the detector was operated in helium rather than nitrogen as the carrier gas. This result may be attributable to the greater thermal conductivity of helium in comparison to nitrogen which causes a different surface temperature and hence a shift in the response. With the helium carrier at least 10 ml/min air are required to prevent poisoning, which is much more than with nitrogen as the carrier. At present, we do not have an explanation for this observation. In spite of these differences, it can be concluded that the detector is insensitive to the nature of the carrier gas; the sensitivity with helium or nitrogen as carrier is practically identical under optimum conditions.

Emitter material. Fig. 2 illustrates the effect on the response caused by varying the emitter surface temperature, T, for six kinds of emitter materials. All the emitters were formed into single loops from wire of 0.25 mm diameter and then assembled into the detector housing so as to give the same length (area) and position. A 30 ml/min flow of carrier gas (nitrogen) was mixed with the air at 20 ml/min. Since these emitter conditions were carefully controlled, it is certain that the observed large differences in $I(T)$ behaviour are due to the differences in the properties of the emitter materials.

Results could not be obtained for W, Re and Ta which were burnt out after

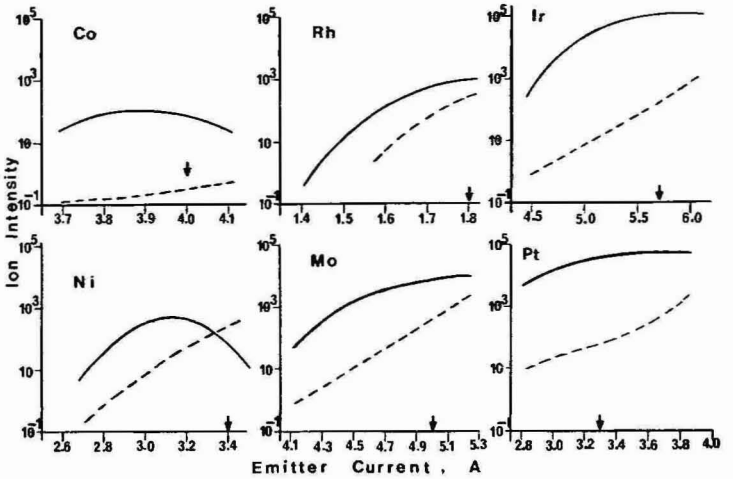

Fig. 2. Dependence of the TBA response (solid line) and background current (dotted line) on the emitter heating current. The operating conditions are the same as in Fig. 1. The arrows indicate the temperature (*ca.* 650°C) above which incandescence can be perceived.

several hours even at emitter temperatures of much less than 650°C. Palladium yielded no response at all, and is thus also omitted from Fig. 2. Fig. 2 shows that both the signal, $I(T)$, and the background current, i_b, increase with the heating current (temperature) and i_b varies widely from material to material. The I *vs.* T curves were bell-shaped for Co and Ni, but with the other emitters the ion signal increased with the heating current and levelled off at saturation values.

The maximum sensitivity for TBA occurs at a different emitter heating current from metal to metal, decreasing in the order Ir, Pt, Mo, Ph, Ni, Co, the value for Ir is times higher than for Pt, which is six times higher than for Mo. The response for cyclohexene followed the same order as that for TBA.

At first we believed that the wide variations in sensitivity could be related to the work function of the emitters, but no relationship has been found (Table I). This suggests that at the high surface temperature the emitter surface converted to an other oxide compound. Presumably the different work functions of the new chemical materials formed on the emitter surface is responsible for the wide variations in sensitivity.

Using Mo, Ir and Pt as emitters, the sensitivity for acetone was measured[4]. Both Mo and Ir are as specific as Pt, as previously reported. The selectivity, defined as the sensitivity ratio, S(TBA)/S(acetone), was $4 \cdot 10^4$ and did not differ significantly among these three emitters.

Detectability

Since the emitters usually showed similar detectabilities and sensitivities (Table I), we picked the three best emitters in terms of sensitivity for the detectability study. Fig. 3 shows the dependence of the signal-to-noise ratio, S/N, on the emitter current, obtained with nitrogen as the carrier gas mixed with air. It should be pointed out that careful adjustment of the heating current is required in order to detect trace

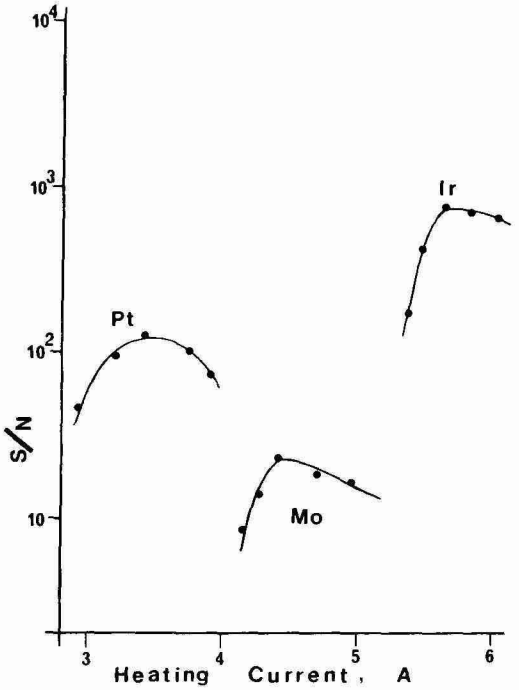

Fig. 3. The signal-to-noise ratio, S/N, *vs.* heating current for various emitters. The operating conditions are as in Fig. 1.

components. Again the iridium emitter provides the best detectability among the three, partly because it gives a lower noise level. There is no obvious explanation for the lower noise characteristics of this emitter.

Stability

Baseline stability. A previous paper[1] reported the stability of the baseline of a surface ionization detector with a hot platinum emitter, noting that about 60 min from switching-on the emitter platinum were required to achieve a stable baseline even at a very low current of $2 \cdot 10^{-11}$ A.f.s. However, the data were obtained for an emitter which was not well defined as regards the initial conditioning and its subsequent usage.

In the present work the thermionic stability with time was investigated in detail for the iridium emitter, which gives the best performance. In the initial conditioning stage with 20 ml/min air after mounting a new iridium emitter in the detector envelope, the background current and the noise level are highest. The large background current is probably attributable to the appearance of Na^+ and K^+ from Na and K as impurity atoms in the iridium material[7]. After heating for 24 h, the background current decreases to *ca.* 1/10 and becomes stable, remaining practically unchanged during the subsequent analytical use. In repeat experiments a stable baseline at a current of $2 \cdot 10^{-11}$ A.f.s. was obtained within half an hour of switching on.

Reproducibility. The effect of the analyte on the stable operation of the detector was investigated. It might be expected that the temperature of the emitter surface

would change with the analyte compounds, caused by the thermal conductivity effect[1]. Based on the experimental *I vs. T* curves shown in Fig. 2, which have a wide flat portion at higher temperatures, no significant effect was apparent.

The second possibility is a change in the surface properties, brought about by alteration of the surface composition due to complex reactions between the surface material and the organic analyte. However, as already mentioned, such a poisoning of the emitter can be prevented if additional air flow is supplied to the detector. For the iridium emitter placed in air, reproducibility tests were performed by replicate injections (ten times) of the 2.6-μl standard sample containing 0.1 ppm TBA. Good reproducibility was achieved: the standard deviation was 1.25 and the coefficient of variation was 1.1% using peak heights (mm).

Durability. If Ir is heated at a current of 6.1 A (*ca.* 1200°C) in the air, it will burn out in 2 days. However, at a heating current of 5.6 A (less than 650°C), which gives the highest detection limit, and at a carrier gas (helium) flow-rate of 40 ml/min mixed with 20 ml/min air, a single emitter can be used continuously for more than 3 months and with no significant drift in sensitivity. The platinum and molybdenum emitters are also capable of withstanding more than one month's analytical use provided they are not heated excessively.

CONCLUSIONS

This study has demonstrated that of the ten materials examined Ir provides the best performance as an emitter for use in a surface ionization gas chromatographic detector, as regards the sensitivity and the detection ability: the sensitivity is 6.52 C/g and the minimum detection limit is $1.7 \cdot 10^{-15}$ g/s for TBA. Also it has very good stability and a low noise level in the presence of air.

There is little difference in performance between the previously studied platinum emitter Ir. Platinum is less expansive and more easily available than Ir. Thus it should also be recommended.

The detector can be built in the laboratory if the gas chromatograph employed is equipped with a conventional flame ionization detector: the iridium (or platinum) emitter can be mounted in the gas flow path through the hole of the envelope which is used for igniting the flame.

Since the further development of this detector seems to be impossible without investigating its applicability, we have already started to explore this field. Preliminary results suggest that either an iridium or platinum emitter may be successfully used for the sensitive gas chromatographic analysis of alkylamines, ethanolamines, nitrosamines, hydrazines, aromatic hydrocarbons, etc.

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NOVEL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ADSORBENTS PREPARED BY IMMOBILIZATION OF MODIFIED CYCLODEXTRINS

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SUMMARY

6-Deoxyamino-β-cyclodextrin has been immobilized through its epoxy glyceride group on hydrophilic gel beads. The retention behaviour of some aromatic and ionic compounds on the resulting gels was studied. The capacity factors of compounds such as mandelic acid and N-benzyloxycarbonylalanine having an aromatic ring and a carboxylic group were increased up to values of 150 by virtue of the hydrophobic and ionic interactions between the aminocyclodextrin moiety and the guest molecules in buffer solution of pH 4–12. Compounds having only an aromatic ring showed moderate capacity factors between 0 and 10, and those having only a carboxylic acid, such as DL-aspartic acid, showed low capacity factors between 0 and 2. There was no difference in the retention behaviour of the D- and L-forms of the substrates. The effects of the pH and the organic solvent content in the mobile phase were examined. The results suggest the occurrence of "host–guest chromatography" with multipoint recognition such as ionic interaction and inclusion complex formation.

INTRODUCTION

The specific interaction between aromatic compounds and the hydrophobic cavity of the cyclodextrin (CD) molecule has been successfully utilized in high-performance liquid chromatography (HPLC)[1–6]. Several polyurethane resins were prepared by cross-linking of epichlorohydrin[7] and of diisocyanates[8]. Another method of CD immobilization was through the use of matrix gels such as silica or polymer gels[9–12], CD has also been used in the mobile phase to separate mandelic acid isomers[13,14].

In our present work, a novel packing material for HPLC was prepared by immobilizing 6-deoxymonoamino-β-CD through its epoxy glyceride group on hy-

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drophilic gel beads. The capacity factors of guest molecules having an aromatic ring together with a carboxylic group were increased by a combination of hydropohobic and ionic effects. The results suggest the occurrence of "host–guest chromatography" with multipoint molecular recognition.

EXPERIMENTAL

Materials

β-CD was a gift from Nihon Shokuhin Kako. The hydrophilic gel beads G3000PW (PW) were supplied by Toyo Soda. Substrates including mandelic acids, methyl mandelates and amino acids were commercial grade used without any purification. N-Benzyloxycarbonylalanine was prepared by the usual methods. All the substrates were in their racemic forms, except for the determination of the association constant in Fig. 8.

Apparatus

The chromatography was accomplished on a Toyo Soda HLC-802 chromatograph equipped with an UV detector (Toyo Soda UV-8). The measurement of the association constant was carried out with an UV spectrometer Jasco Uvidec-1 equipped with a thermostat to maintain the cell compartment at 27.0 ± 0.5°C.

Preparation of bead gels

β-CD was immobilized on a matrix of gel beads as shown in Fig. 1. The bead gel (PW) was treated with epichlorohydrin in an alkaline solution at 45°C for 4 h to modify its active primary hydroxyl groups, as described[15]. The product (epoxy-PW) was then treated with β-cyclodextrin in an alkaline solution, pH 12, at 37°C for 24 h to give β-CD immobilized on the bead gel (CD-PW)[16].

The epoxy content of epoxy-PW was determined by titration methods[17]. After reaction with 1.3 *M* sodium thiosulphate, a titration was carried out using 1/10 *M* hydrochloric acid solution. The result indicated that 620 μmol epoxy group per 1 g dry gel had been introduced. Also, the amount of immobilized β-CD was determined

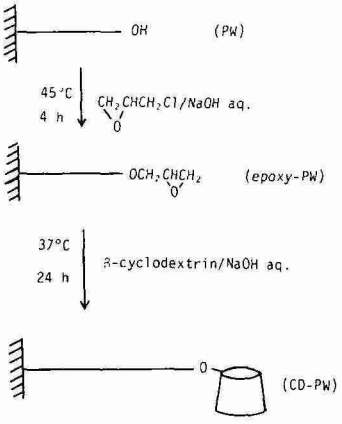


Fig. 1. Preparation of epoxy-PW and CD-PW gels.

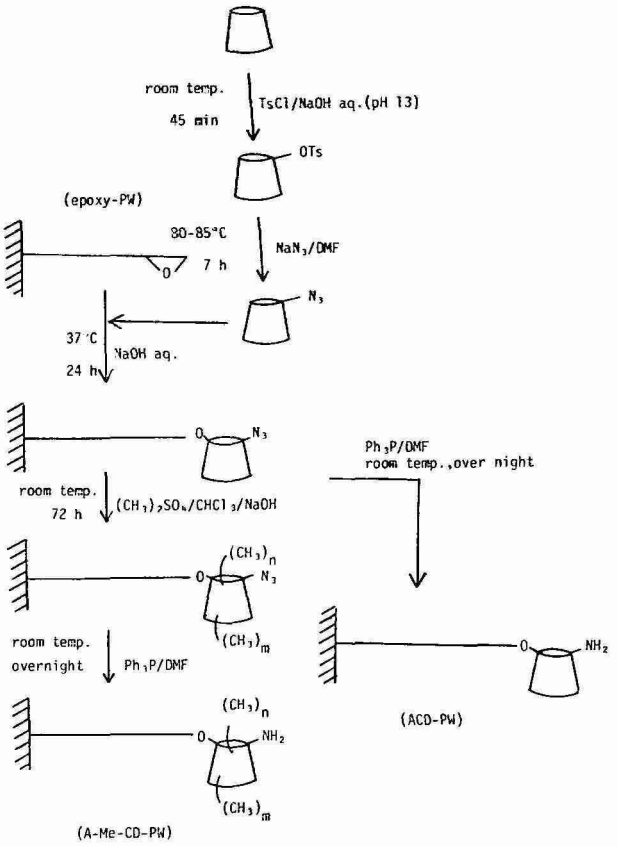


Fig. 2. Preparation of ACD-PW and A-Me-CD-PW gels.

by HPLC analysis of the glucose produced upon hydrolysis with hydrochloric acid. It was shown that 540 μmol of β-CD per 1 g dry gel were immobilized in this preparation.

The immobilization of amino-β-CD and amino-methylated-β-CD on PW gel was carried out as summarized in Fig. 2. The amino-CD gel (ACD-PW) was obtained from epoxy-PW and 6-monoazido-β-CD; the latter was prepared by regiospecific tosylation[18] and substitution by sodium azide[19]. The monoazido-β-CD gel thus obtained was treated with triphenylphosphine in dimethylformamide (DMF), then with ammonium hydroxide to give the desired product. Aminomethyl-β-CD gel (A-Me-CD-PW) was prepared from the 6-monoazido-β-CD gel. This gel was methylated with dimethyl sulphate in chloroform–aqueous sodium hydroxide solution with shaking at room temperature for 72 h. The resulting monoazido-methyl-β-CD gel was treated with triphenylphosphine in DMF, then with ammonium hydroxide to give the desired product. The amino content of the two gels was determined by titration with a pH-stat apparatus as 530 and 230 μmol per 1 g of dried ACD-PW and A-Me-CD-PW, respectively.

Column chromatography

The obtained bead gels were packed by the slurry method in a stainless-steel

column (300 × 4.0 mm I.D.). In the experiments of different pH values, the eluent was usually a mixture of 10% (v/v) acetonitrile and 90% (v/v) 1/40 *M* phosphate buffer between pH 4 and 7 or 90% (v/v) 1/40 *M* carbonate buffer between pH 9 and 11.5. The flow-rate was approximately 0.5–0.7 ml/min. In the experiments on the effect of the organic solvent, the eluent was a mixture of *x*% (v/v) acetonitrile in 1/40 *M* phosphate buffer, pH 7.0, at a flow-rate of 0.6–0.8 ml/min.

Determination of association constant

6-monoamino-β-CD was prepared according to the literature[20] and recrystallized twice. Its elemental analysis, titration of amino group content and ^{13}C NMR spectrum were in accorded with the expected structure.

Benesi–Hildebrand plots were used to determine the binding constant between D- or L-mandelic acids and β-CD or 6-amino-β-CD at 27.0°C. The concentration of mandelic acid was kept constant at $6.00 \cdot 10^{-5}$ *M*. The concentration of β-CD was varied between $1.67 \cdot 10^{-3}$ and $1.00 \cdot 10^{-2}$ *M*. The solutions used were as follows: 1/10 *M* acetate buffer at pH 4.0; 1/15 *M* phosphate buffer at pH 7.0; 1/20 *M* sodium tetraborate–hydrochloric acid buffer in the range pH 8.0–9.2; 1/20 *M* sodium tetraborate–sodium hydroxide buffer in the range pH 9.5–11.2.

RESULTS AND DISCUSSION

Introduction of ACD on the gel beads

As the PW gel polymer has hydroxyl groups in its side chain[21–23], CD units could be introduced after treatment of the gel with epichlorohydrin, as shown in Fig. 1. More than 87% of the resulting epoxy groups reacted with CD to give the CD-PW gel beads. This is the first report of an HPLC adsorbent which contains a CD cavity on a synthetic polymer.

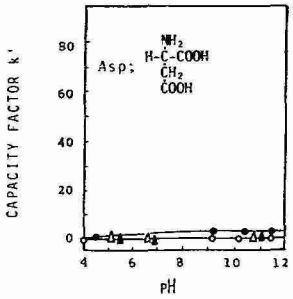
In order to influence the capacity factor, two kinds of modifications of CD molecules were carried out. An amino group was attached to CD by the usual methods of tosylation and azidification as shown in Fig. 2. This "pre-procedure method" gave a sufficient amount of amino groups on the CD-PW gel. Another improvement was achieved by methylation of the hydroxyl group on the CD molecules. By the addition of chloroform, dimethyl sulphate and an aqueous alkaline solution, the methylation, at least on the C-6 hydroxyl group, was easily performed, though there was a 57% decrease in the amino groups.

The resulting ACD-PW and A-Me-CD-PW gel beads are new types of HPLC adsorbents. They can be used at high flow-rates over a wide range of pH for the separation of various aromatic acids such as mandelic acid and N-protected phenylalanine, as will be shown later.

For all of the chiral substrates used in the present experiments, there was no difference in the retention behaviour between the D- and L-forms.

pH dependence of capacity factors

The four types of adsorbents, *i.e.*, PW, CD-PW, A-CD-PW and A-Me-CD-PW, were compared with respect to the capacity factors, k' ($= t_R/t_0 - 1$), of various guest molecules. The pH of the eluent was varied in order to elucidate the interaction between the amino group of CD and the carboxylic group of the substrates.



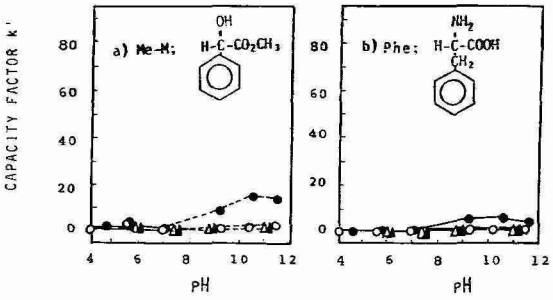


Fig. 3. pH Dependence of the capacity factor, k', of DL-aspartic acid on PW (△), PW, ACD-PW and A-Me-CD-PW with CD-PW (▲), ACD-PW (○) and A-Me-CD-PW (●) gels. Column: 300 × 4.0 mm I.D. Eluent: pH 4–7, acetonitrile–1/40 *M* phosphate buffer (10:90); pH 9–11, acetonitrile–1/40 *M* carbonate buffer (10:90); flow-rate 0.5–0.7 ml/min.

Fig. 4. pH Dependence of the capacity factor, k', of methyl DL-mandelate (a) and DL-phenylalanine (b) on PW (△), CD-PW (▲), ACD-PW (○) and A-Me-CD-PW (●). Other details as in Fig. 3.

The first guest molecule was DL-aspartic acid (Asp). In Fig. 3 the pH dependence of the capacity factor, k', is shown. At neutral pH, this anionic substrate was expected to show an interaction with the positively charged amino group on the CD ring and to exhibit a larger retention volume; however, this was not the case. In the case of PW, CD-PW and ACD-PW, $k' = 0$ at all pH values. Asp was not retained except weakly by A-Me-CD-PW in alkaline solution.

Methyl DL-mandelate (Me-M) and DL-phenylalanine (Phe), which are electrostatically neutral but have a phenyl group, were then examined. The results are shown in Fig. 4. Me-M was not retained on the PW gel. On ACD-PW and A-Me-CD-PW, there was increased retention at alkaline pH, but at strongly alkaline pH the Me-M was probably hydrolyzed to mandelic acid, as will be shown later. Phe was not retained on PW and CD-PW, but on ACD-PW gel showed retention factors of up to 1.0 in alkaline solution. At pH > 9.2, which is the pK_a of the guest molecule, Phe became anionic and was able to interact electrostatically with the amino group in the gels. In addition to the ionic interaction, the A-Me-CD-PW column shows a stronger hydrophobic interaction because of the O-methylation of the CD ring. It exhibited a stronger interaction (k' up to 5.5) than the other gels.

The pH dependence of the capacity ratio of DL-mandelic acid (MA) and N-benzyloxycarbonyl-DL-alanine (Z-Ala), which have both anionic and phenyl groups, was examined (Fig. 5). MA was not retained at all by the PW gel, but was slightly retained by the CD-PW gel. On ACD-PW gel, the capacity factor decreased at pH 3.6 and 10.1, near the pK_a values of MA and ACW-PW. This result indicated an ion-exchange type of retention behaviour and a strong electrostatic interaction between the amino group and the carboxylic group, in contrast to the Asp substrate. The A-Me-CD-PW gel yielded extraordinarily high capacity factors especially at acidic pH. The enhanced hydrophobic interaction was observed by the methylation at the hem of CD ring.

Another substrate, Z-Ala, exhibits similar behaviour to that of MA. The k' values on ACD-PW and A-Me-CD-PW were much higher, up to 150 at acidic pH.

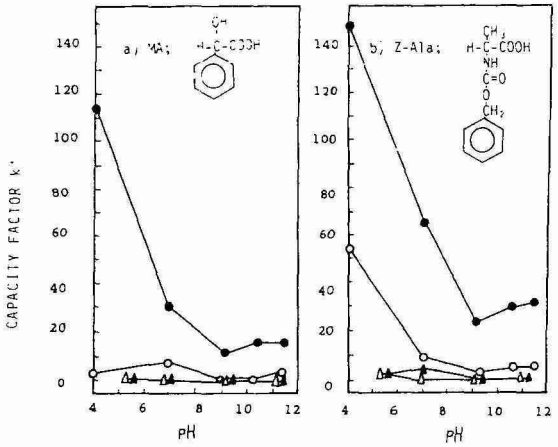


Fig. 5. pH Dependence of the capacity factor, k', of DL-mandelic acid (a) and benzyloxycarbonyl-DL-alanine (b) on PW (△), CD-PW (▲), ACD-PW (○) and A-Me-CD-PW (●). Other details as in Fig. 3.

Dependence of capacity factor on organic eluents

In order to clarify the interaction between the hydrophobic CD ring and each guest molecule, the effect of the concentration of the organic solvent on the retention behaviour was examined. In these experiments, an aqueous buffer of pH 7 was mixed with the organic solvents.

The concentration of acetonitrile was varied in the range 0–30% (v/v), and the capacity factors of Me-M, Phe and Asp were examined. The results are summarized in Fig. 6. On the gels CD-PW, ACD-PW and A-Me-CD-PW, Me-M (Fig. 6a) showed a slight change in k' from 0 to 3.5 upon changing the acetonitrile content from 30 to 0%, but on the gel PW there was no change. This change in k' seemed to reflect the change in hydrophobic interaction caused by the CD ring. However, in the case of the Phe substrate (Fig. 6b), there was no change in k' on all the gels. There may be a repulsion between the internal salt of Phe and the hydrophobic cavity of CD. Asp, which has no aromatic moiety and is not expected to interact with the CD cavity, also showed no change in k' on all the gels (Fig. 6c). This suggests that the presence of a phenyl ring is decisive for the interaction with the hydrophobic cavity of CD.

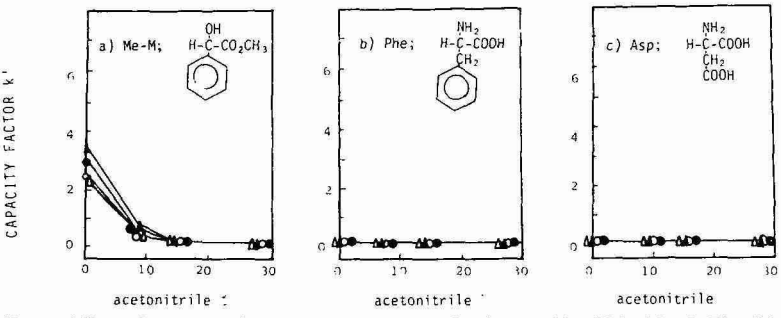


Fig. 6. Effect of the organic solvent on the capacity factor, k', of Me-M (a), Phe (b) and Asp (c) on PW (△), CD-PW (▲), ACD-PW (○), and A-Me-CD-PW (●) gels. Column: 300 × 4.0 mm I.D. Eluent: acetonitrile–1/40 M phosphate buffer pH 7.0 (x: 100 − x); flow rate 0.6–0.8 ml/min.

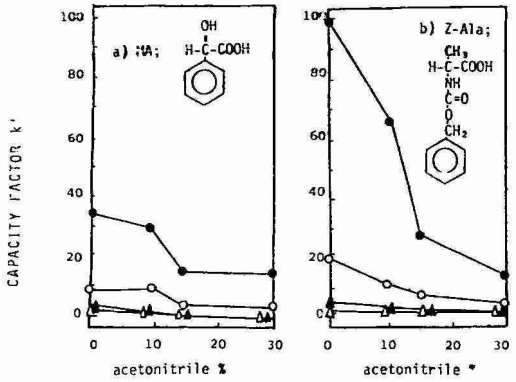


Fig. 7. Effect of the organic solvent on capacity factor, k' of MA (a) and Z-Ala (b) on PW (△), CD-PW (▲), ACD-PW (○) and A-Me-CD-PW (●) gels. Other details as in Fig. 6.

The effects of a mixed organic solvent in the eluent on the capacity factor k' of the guest molecules MA and Z-Ala were examined. The results are summarized in Fig. 7. In the case of MA (Fig. 7a), an effect on the capacity factor was observed on the CD-PW gel but not on the PW gel. This indicated that MA interacts with the hydrophobic cavity of CD. Similar changes in k' were found for Me-M and Phe. On ACD-PW gel, larger changes in capacity factor were observed, again suggesting an interaction between the CD cavity and the guest molecule. An extraordinarily large effect was observed on the A-Me-CD-PW gel, indicating the higher hydrophobicity of the CD cavity upon methylation.

In the case of Z-Ala (Fig. 7b), on PW gel, there was no change in retention. On CD-PW and ACD-PW gels, a small change was observed, being larger with the latter. The A-Me-CD-PW gel yielded the highest capacity factor and was the most sensitive to changes in the content of organic solvent.

From the above results it is presumed that the hydrophobic cavity of CD interacts with the guest molecules.

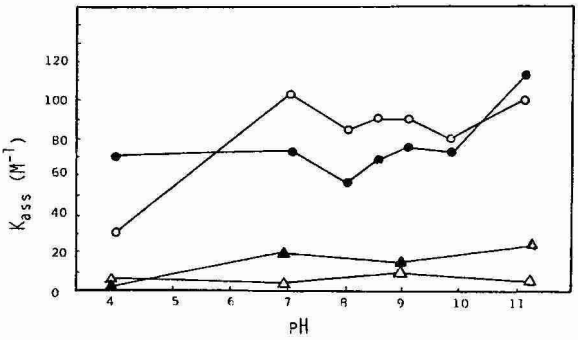
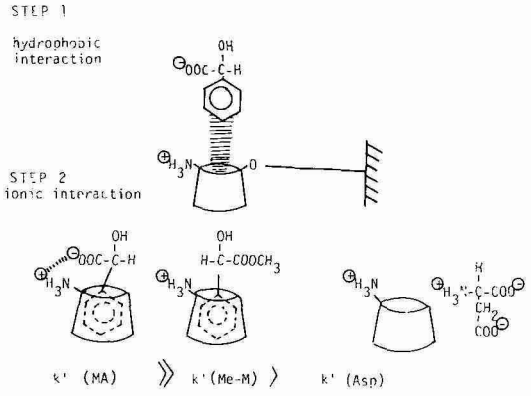


Fig. 8. pH Dependence of the association constannts, K_{ass}, of CD and ACD with D- and L-mandelic acid. [CD] = $1.67 \cdot 10^{-3}$–$1.00 \cdot 10^{-2}$ M, [ACD] = $1.33 \cdot 10^{-3}$ M, [D-, L-mandelic acid] = $6.00 \cdot 10^{-5}$ M; 27 ± 1°C. UV detection: pH 4.0, 225 nm; pH 7.0–11.4, 216 nm. ▲—▲, CD with D-mandelic acid; △—△, CD with L-mandelic acid; ●—●, ACD with D-mandelic acid; ○—○, ACD with L-mandelic acid. Solvents: pH 4.0, 1/10 M acetate buffer; pH 7.0, 1/15 M phosphate buffer; pH 8.0–9.2, 1/20 M sodium tetraborate–hydrochloric acid buffer; pH 9.5–11.2, 1/20 M sodium tetraborate–sodium hydroxide buffer.

In order further to elucidate this interaction, the equilibrium constants, K_{ass}, for binding of β-CD and amino-β-CD to D- and L-mandelic acids were measured in the range pH 4–11. The results (Fig. 8) yielded K_{ass} values at pH 7 of about 16 and 90 M^{-1} for binding of β-CD and of amino-β-CD, respectively, in accord with the capacity factors obtained on the immobilized CD gels.

According to the Pauling–Corey–Koltun steric molecular models, an amino group is embedded inside the CD ring. So it may be presumed that the ionic interaction between a carboxylic group outside the CD ring and this amino group is hindered. Thus, it is necessary that the guest molecule is included into the cavity by hydrophobic interaction, then the included molecule can be held tightly by both ionic and hydrophobic interaction as suggested[24].



Scheme 1. Suggested mechanism.

Scheme 1 illustrates this mechanism for the present packings. In step 1, the hydrophobic moiety of the guest molecule is included in the hydrophobic cavity of CD, forming a host–guest complex. In step 2, the carboxylic moiety participates in a stronger interaction with the amino group. Especially in the case of MA, which has both phenyl and carboxylic groups, a stable complex can be formed by the combined effect of the hydrophobic and ionic interactions, resulting in increased retention.

It may be concluded that the present amino-CD and aminomethyl-CD gels exhibit both hydrophobic and ionic interactions, making them novel adsorbents for HPLC.

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CHROM. 18 348

COMPARATIVE ASSESSMENT OF THE ARTEFACT BACKGROUND ON THERMAL DESORPTION OF TENAX GC AND TENAX TA

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SUMMARY

The artefact background on thermal desorption of pre-conditioned Tenax GC and Tenax TA is compared. Superior blank gas chromatograms were obtained from Tenax TA, and an efficient pre-conditioning procedure to minimise background on heat desorption is proposed. The pooling technique described is suitable for the analysis of trace components of food aromas, especially relatively high-boiling components. The majority of the volatiles identified by combined gas chromatography–mass spectrometry analysis of pooled blank tubes were aliphatic, alicyclic and aromatic hydrocarbons. Many are common food aroma components, and their existence in blank experiments must be monitored carefully.

INTRODUCTION

Over recent years, the adsorption of volatile components onto synthetic porous polymers has been used widely as a means of investigating food aromas, and Tenax GC has been reported frequently as the adsorbent[1–6]. The aroma compounds are retrieved for subsequent analysis by thermal desorption or by solvent elution. Heat desorption has the advantage of rapidity and also that the aroma isolated has no solvent odour to interfere with any sensory analysis[7,8].

Tenax GC is a polymer based on 2,6-diphenyl-*p*-phenylene oxide[9]. It has a high affinity for organic compounds[10] and is regarded as the best porous polymer adsorbent when relatively high-boiling aroma components are of interest[11,12]. Unlike other polymers, *e.g.* the Chromosorbs and Porapaks, it has a very high temperature stability of 380–400°C[13,14] and is, therefore, particularly suited to heat desorption. It is fairly hydrophobic, and high recoveries of volatiles are obtained quickly on thermal elution[10,15]. Its thermal stability accounts for relatively low background levels, which can be decreased further by pre-conditioning at an elevated temperature, *e.g.* 200–360°C for 0.5–48 h under a stream of purified inert gas[10,16,17]. Nevertheless, the presence of certain artefacts on blank gas chromatograms has been established[18]. In order to characterise these potential contaminants, Lewis and Williams heated unconditioned Tenax GC at 250°C for 6 h under a purified nitrogen flow of 30 ml

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min^{-1}, and identified several aliphatic and aromatic hydrocarbons in the effluent together with 27 unknown components[19]. Even after careful clean-up of the polymer, some artefacts remain[20] and in an attempt to overcome this problem, Tenax TA has become available recently[20].

In this paper we compare the background produced on heat-desorbing pre-conditioned Tenax GC and Tenax TA adsorption tubes treated identically, and describe a method for preparing Tenax TA adsorption tubes showing a minimal background on thermal elution.

EXPERIMENTAL

Preparation and conditioning of Tenax tubes

Tenax GC. Single tubes were prepared as follows. Acid (conc. hydrochloric acid)-washed glass tubes (20 cm × 4 mm I.D., 6 mm O.D.) were silanised by injecting 1 μl Silyl 8 (Pierce) under a nitrogen flow of 50 ml min^{-1} at 200°C for 30 min. The deactivated tubes were then packed with 200 mg Tenax GC (60–80 mesh, Chrompack) pre-conditioned under a nitrogen flow of 50 ml min^{-1} at 275°C for 24 h. The packed tubes were plugged with silanised glass wool (Phase Separations) and capped. Immediately before use, the tubes were conditioned under a nitrogen flow of 50 ml min^{-1} at 250°C for 2 min, cooled to room temperature, and this procedure repeated.

Pooled tubes were obtained by heat-desorbing thirteen single tubes, prepared as described above, onto another (collector) tube equilibrated at −76°C. Heat desorption occurred at 250°C for 1 min under a nitrogen flow of 30 ml min^{-1}, maintained for 2 min after heating.

All nitrogen supplies (BOC, CP grade) were dried and purified using 200 g molecular sieves 5A and 13X (BDH).

Tenax TA (tubes I). Single and pooled Tenax TA (60–80 mesh, Chrompack) tubes were prepared as described above for Tenax GC.

Tenax TA (tubes II). Single and pooled Tenax TA tubes were prepared as described above except that the Tenax TA was pre-conditioned at 340°C for 2 h under nitrogen. The packed tubes were then re-conditioned at 340°C for 2 h under nitrogen. Immediately before use, the tubes were further conditioned under nitrogen at 300°C for 15 min, cooled to room temperature, and this procedure repeated.

Gas chromatography

Single and pooled tubes were analysed by capillary gas chromatography (GC) using a Perkin Elmer Sigma 2B instrument. The normal capillary injector system was not used. Instead, the Tenax tube was attached to the helium carrier gas line and flushed with helium at 250 ml min^{-1} (1 p.s.i.) for 5 s to remove air. The remote end of the tube was then immediately attached to a fused-silica bonded phase DB5 open tubular GC column (60 m × 0.32 mm I.D. × 1 μm film; J & W Scientific), the first 0.3 m of which formed a U-trap cooled in liquid nitrogen. The helium pressure was increased to 20 p.s.i. (3 ml min^{-1} flow through the column). All connections were made using appropriate zero dead volume unions with graphite ferrules or Viton O-rings.

The Tenax was heated to 250°C over a period of 1 min and maintained at 250°C for an additional minute. During this time, helium carrier gas transferred any heat-desorbed volatiles into the cold trap, and the helium flow was maintained for

10 min after heating. The cold trap was then removed from the liquid nitrogen and fed into the GC oven, the temperature program being simultaneously started.

Other relevant GC conditions of analysis were: injector setting, 30°C; flame ionisation detector, 250°C; the oven temperature was increased from 30 to 55°C at 1°C min^{-1}, and from 55 to 200°C at 2°C min^{-1}, and held at 200°C until the end of the run; nitrogen make-up gas, hydrogen and air flows of 30, 30 and 500 ml min^{-1}, respectively; attenuation, $\times 64$ ($32 \cdot 10^{-11}$ A f.s.). This attenuation setting is typical of that used in this laboratory for the analysis of trace volatile components of food aromas.

Gas chromatography–mass spectrometry

Components were identified as far as possible by gas chromatography–mass spectrometry (GC–MS) analysis of pooled tubes using a Perkin-Elmer Sigma 3 gas chromatograph interfaced via a single-stage all glass jet separator at 250°C to a Kratos MS 25 mass spectrometer linked on-line to a Kratos DS 50S data processing system and equipped with a computer-controlled multipeak monitoring (MPM) unit. The GC conditions described above were used. Significant operating parameters of the mass spectrometer were: ionisation voltage, 70 eV; ionisation current, 100 μA; source temperature, 200°C; accelerating voltage, 4 kV; resolution, 600; scan speed, 1 s per decade, repetitive throughout the run.

RESULTS AND DISCUSSION

After evaluating a range of conditioning parameters, including different temperatures and different flow-rates for varying time periods, best results were obtained

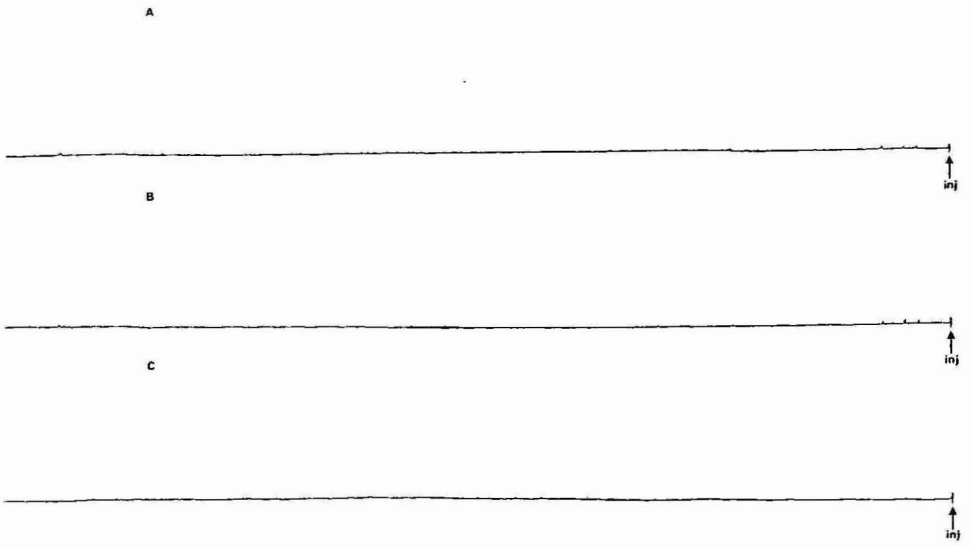

Fig. 1. Gas chromatograms obtained on heat-desorption of single Tenax tubes. (A) Tenax GC pre-conditioned under nitrogen at 275°C for 24 h and 250°C for 2 min (twice); (B) Tenax TA (tubes I) pre-conditioned under nitrogen at 275°C for 24 h and 250°C for 2 min (twice); (C) Tenax TA (tubes II) pre-conditioned under nitrogen at 340°C for 4 h and 300°C for 15 min (twice).

as described in the Experimental section. Experience also showed that the additional brief conditioning treatment immediately before use was particularly beneficial for minimising background on blank gas chromatograms. This technique was therefore implemented in all the comparisons made in this study.

Gas chromatograms obtained from single Tenax GC and Tenax TA (tubes I) tubes conditioned identically are shown in Fig. 1A and B. The baselines obtained are good and there is no significant difference between the two. This small amount of background was reduced further to negligible proportions (as shown in Fig. 1C) by subjecting Tenax TA to a more rigorous heat-conditioning treatment (tubes II). For some food aromas, where the main sensory contributors are present in fairly large concentrations, single tube analyses are adequate, and the excellent baseline from Tenax TA shown in Fig. 1C is certainly acceptable.

However, in many instances nowadays, flavour research has advanced to the point where the main requirement is to analyse trace components, and the pooling technique described in the Experimental section is useful. Clearly, the number of tubes pooled depends on the composition of the aroma being analysed, and in this laboratory, up to fourteen pooled tubes have been used on different occasions.

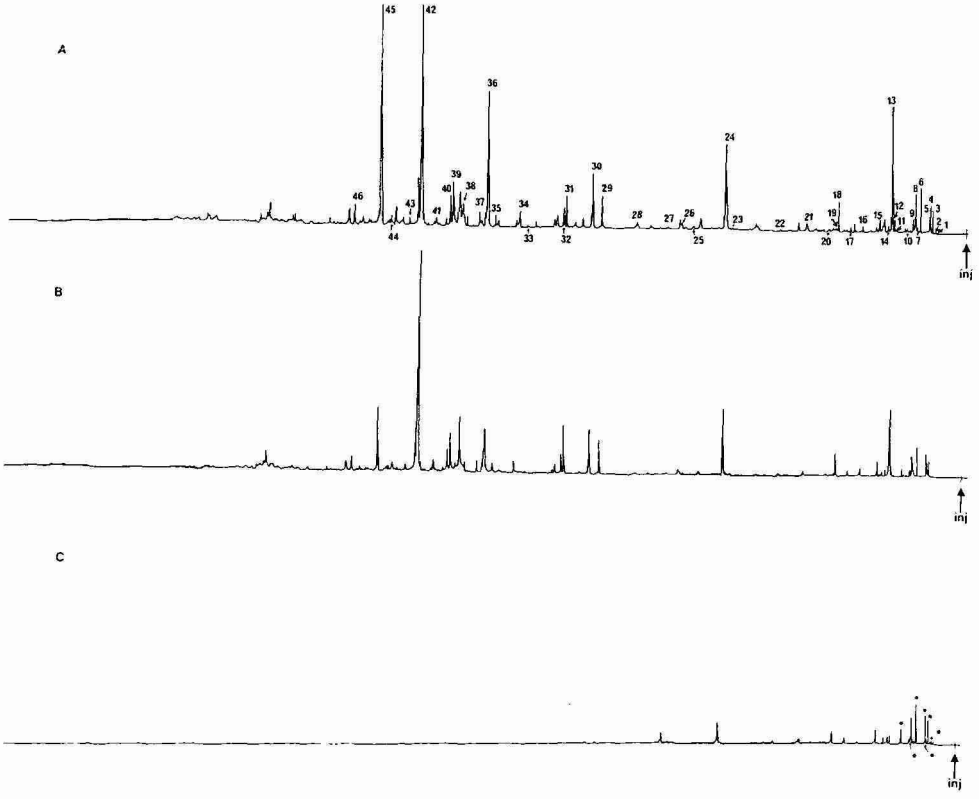

Fig. 2. Gas chromatograms obtained on heat-desorption of pooled Tenax tubes. (A) Tenax GC preconditioned under nitrogen at 275°C for 24 h and 250°C for 2 min (twice); (B) Tenax TA (tubes I) preconditioned under nitrogen at 275°C for 24 h and 250°C for 2 min (twice); (C) Tenax TA (tubes II) preconditioned under nitrogen at 340°C for 4 h and 300°C for 15 min (twice). (For peak identification see Table I.)

TABLE I

COMPONENTS IDENTIFIED BY GC–MS ANALYSIS OF POOLED TUBES

Peak numbers correlate with Fig. 2.

Component and class	*Peak number*	*Component and class*	*Peak number*
Aliphatic hydrocarbons		*Benzenoids*	
Butane*	5	Benzene	18
Pentane	9	Toluene	24
Hexane	15	Ethylbenzene	29
Heptane	21	Propylbenzene	35
Octane	26	Butylbenzene	44
Nonane	32	Xylenes	30, 31
Decane	39	A trimethylbenzene	40
Undecane	46	C_3 alkylbenzenes	33, 37, 38, 42
Methylpropane*	3	A methylstyrene or indan	43
2-Methylbutane*	6	Benzaldehyde	36
2-Methylpentane	13	Acetophenone	45
3-Methylpentane	14	*Halogenated compounds*	
3-Methylhexane	20	Dichloromethane*	11
2-Methylheptane	23	Trichlorofluoromethane*	7
A branched-chain hydrocarbon	19	1,1,1-Trichloroethane	17
Methylpropene*	4	1,1,2-Trichloro-1,2,2-trifluoroethane	10
? 2,2-Dimethylhex-3-ene	25	Tetrachloroethene	27
Alicyclic hydrocarbons		A dichlorobenzene	41
Cyclohexane	18	*Miscellaneous compounds*	
Methylcyclopentane	16	Carbon dioxide**	1
Methylcyclohexane	22	Carbonyl sulphide*	2
Ethylcyclohexane	28	Acetone	8
Propyl or isopropylcyclohexane	34	Trimethylsilanol***	12

* Component contributed by the pooling gas.
** Component contributed by the pooling coolant (carbon dioxide).
*** Component contributed by silanisation of glass tubes.

Gas chromatograms obtained from fourteen pooled Tenax GC and Tenax TA (tubes I) tubes conditioned identically are shown in Fig. 2A and B. Neither is acceptable as a blank chromatogram. The more rigorous heat-conditioning of Tenax TA (tubes II) before pooling produced the chromatogram shown in Fig. 2C, which represents the best baseline obtained from fourteen pooled tubes. Experiments involving the use of different temperatures for different times for conditioning the Tenax TA, and also the use of purified helium instead of nitrogen as purge gas, whether purified as described in the Experimental section or using a rare gas purifier (British Oxygen, RGP MK 3), resulted in inferior blank chromatograms. Pellizzari *et al.*[21] have reported that extracting Tenax GC with acetone for 18 h reduced background levels, but this procedure did not improve blank chromatograms when applied to Tenax TA in this study. The peaks asterisked in Fig. 2C are, in fact, contributed by the pooling gas and could not be minimised further. For the analysis of relatively high-boiling components, Fig. 2C is an acceptable blank, but generally speaking, the technique is being pushed to its limits under the conditions described, as far as very low boiling components are concerned.

Components identified by combined GC–MS analysis of pooled tubes are listed in Table I, in which the peak numbers correlate with Fig. 2. In all cases, mass spectra agreed with those in the *Eight-Peak Index of Mass Spectra*[22]. The majority of the components identified were aliphatic, alicyclic or aromatic hydrocarbons. Many are commonly-occurring food aroma components[23], and therefore their existence in blank experiments needs to be monitored with care.

CONCLUSION

Superior blank chromatograms were obtained from Tenax TA than from Tenax GC. The most efficient pre-conditioning procedure for Tenax TA, in order to minimise artefact background on thermal desorption, consisted of heating the adsorbent under purified nitrogen for 4 h at 340°C and then at 300°C for two separate periods of 15 min immediately before use [see Experimental section, *Tenax TA (tubes II)*]. The pooling technique described is suitable for the analysis of trace components of food aromas, and especially so for relatively high-boiling components.

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CHROM. 18 324

STABILITY-INDICATING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR LACTIC ACID IN LOTIONS

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SUMMARY

A stability-indicating high-performance liquid chromatographic method using an amine column and a UV detector at 214 nm is developed for lactic acid in dermatological products. The detector response for lactic acid is linear in the range 8.8–39.9 μg. Repeatability of the chromatographic peak area is 0.9% relative standard deviation (R.S.D.) (n = 6). Precision of the method is 1.3% R.S.D. (n = 9). Recovery of lactic acid from lactate lotion is 100.4% with 0.4% R.S.D. (n = 9). The method is particularly suitable for products containing parabens.

INTRODUCTION

Lactic acid has been widely used in the food industry as an additive, acidulant and preservative[1]. The hygroscopic properties of sodium lactate and lactic acid–sodium lactate mixtures were found to amplify the emollient properties of hand cream[2], and have also been utilized in cosmetic formulations and in the treatment of skin diseases. In 1977, Van Scott *et al.*[3] found applications of lactic acid in the treatment of dry skin and acne. They also found that keratoses of the skin may be successfully prevented or treated with lactic acid[4].

Several methods have been described for the determination of lactic acid. The colorimetric method[5] using complexation with ferric ion is not very rugged and accurate results could be obtained only with strict control of experimental conditions. Gas chromatographic methods[1,6,7] require cumbersome derivatization of the acid before analysis. The liquid chromatographic methods are relatively simple, and they were developed mainly for lactic acid in either food products or plasma[8–12]. However, these high-performance liquid chromatographic (HPLC) methods are not applicable to dermatological products due to excipient interference or deterioration of column performance by excipients. Also, some of these methods require expensive columns and operations at elevated temperatures. This paper describes the development of a specific, accurate and precise HPLC method for lactic acid in lotions.

EXPERIMENTAL

Apparatus

Chromatography was performed using a Waters Assoc. (Milford, MA, U.S.A.) Model 204 chromatograph with a M-6000A pump, a U6K injector and a UV detector (Model 441) set at 214 nm. A 30 cm × 3.9 mm I.D. μBondapak amine column (Waters Assoc.) was connected to the HPLC system. Output was monitored on an OmniScribe recorder (Houston Instruments, Houston, TX, U.S.A.) and a Model 3352B laboratory data system (Hewlett-Packard, Avondale, PA, U.S.A.). A Hewlett-Packard Model 1040A diode array spectrophotometric detector was used in HPLC peak purity evaluation.

Reagents

Lactic acid (88% solution) was purchased from McKesson Chemical Co. (Buffalo, NY, U.S.A.). HPLC-grade acetonitrile and ACS-grade methanol and sodium hydroxide were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). HPLC-grade phosphoric acid (85%) was purchased from Fisher Scientiific (Fairlawn, NJ, U.S.A.). A lactate lotion, Lac-Hydrin® from Westwood Pharmaceuticals (Buffalo, NY, U.S.A.), and its placebo were used in validating the method. The excipients in Lac-Hydrin include light mineral oil, glyceryl stearate, PEG-100 stearate, propylene glycol, polyoxy 40 stearate, glycerin, magnesium aluminum silicate, laureth-4, cetyl alcohol, methyl cellulose, fragrance, quaternium-15, and methyl and propyl parabens.

HPLC operating conditions

The eluent was water–acetonitrile (1:1) containing 3.2 m*M* ammonium dihydrogen phosphate. The flow-rate was 1.0 ml/min. Injection volume was 20 μl. Peak areas were used to quantitate lactic acid.

Sample and standard preparation

Lactic acid reagent, which contains *ca.* 88% lactic acid, was standardized by titration as described in ref. 13. The sample and standard solutions for HPLC analysis were prepared as follows.

A sample equivalent to 120 mg of lactic acid was weighed into a 150-ml beaker, 20 ml of 0.1 *N* sodium hydroxide was added, and the beaker was covered with a watch glass and boiled gently on a hot plate for 20 min. After cooling to room temperature, it was diluted to *ca.* 40 ml with water, adjusted to pH 6.0 with dilute phosphoric acid and then quantitatively transferred and diluted to 100 ml with water. The solution was then clarified by filtration.

RESULTS AND DISCUSSION

Choice of detector

Lactic acid is a poor UV absorber, and it can only be detected at wavelengths below 220 nm. Derivatization as a means of improving detection appears possible but would unnecessarily lengthen the procedure. Detection by refractive index detector is less sensitive than UV detection at 214 nm. Therefore, the method was developed using the latter.

Chromatography

Polystyrene-based ion-exchange columns have found wide application to the analysis of organic acids such as lactic acid but they require column operation at elevated temperatures[8–10]. The reported separations on other types of columns (amine and octadecylsilane) for lactic acid[11,12] are not adequate for analyzing lotion-type products due to excipient interferences. Using an ion-pairing approach we were able to improve this separation.

Excellent separation of lactic acid from excipients was obtained using a Waters μBondapak C_{18} column and 0.005 *M* tetrabutylammonium hydroxide (pH adjusted to 7 with phosphoric acid) as eluent. However, this system is not suitable for analyzing products containing parabens, a common preservative in topical products. Heating the sample with 0.1 *N* sodium hydroxide during sample preparation, to hydrolyze intermolecular esters of lactic acid, also hydrolyzes parabens to *p*-hydroxybenzoic acid. The latter elutes very late, thus extending the chromatographic run time to over 40 min (Fig. 1).

Efforts to reduce the analysis time by changing the ion-pairing agent or by adding methanol to the eluent were not successful due to interference from other positive or negative peaks. Also, it was noted that some product excipients were held up on th column unless a stronger eluent with 20% or more methanol is used. Circumventing the problem of late-eluting components by the use of two columns and the column switching technique led to a temporary digression of the baseline in

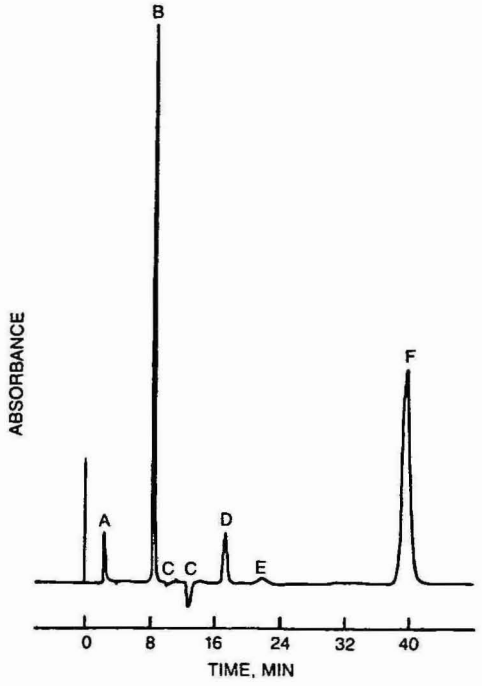
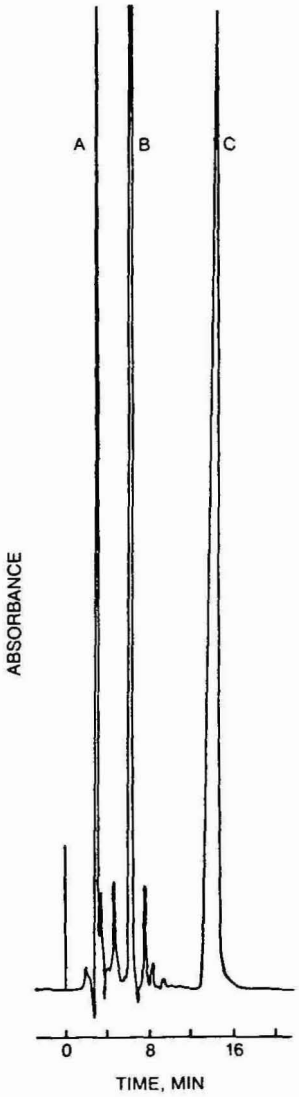


Fig. 1. Chromatogram of lactate lotion. Column: μBondapak C_{18}; eluent: 0.005 *M* tetrabutylammonium hydroxide in water (pH adjusted to 7.0 with phosphoric acid); flow-rate: 1.0 ml/min. Peaks: A = solvent front; B = lactic acid; C = negative peak; D = impurity of lactic acid; E = excipient; F = late-eluting excipient peak.

chromatograms, which interfered with the quantitation of the lactic acid peak. The digression is apparently due to pressure transients during column switching by the HPSC Model 410 system (Autochrom, Milford, MA, U.S.A.), which became more prominent at 214 nm, the wavelength of detection.

The problem of late-eluting peaks and non-eluting components was successfully resolved using a μBondapak amine column and 3.2 mM phosphate buffer in



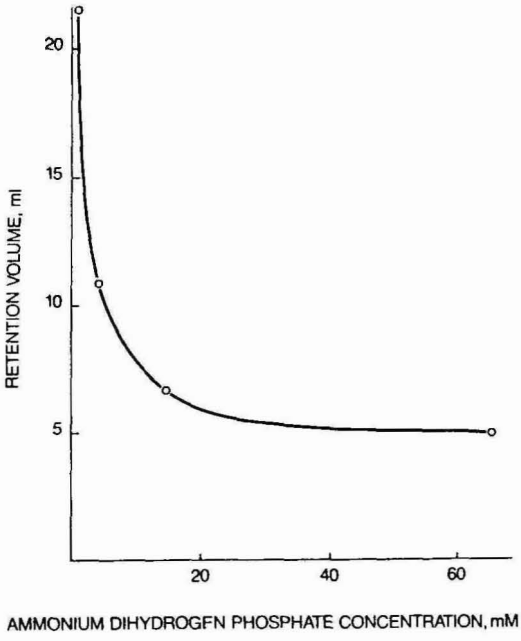


Fig. 2. Typical chromatogram of lactate lotion sample. Eluent: water–acetonitrile (1:1) containing 3.2 mM ammonium dihydrogen phosphate; flow-rate: 1.0 ml/min. Peaks: A = solvent front; B = excipient peak; C = lactic acid.

Fig. 3. Effect of ionic strength on retention of lactic acid. Mobile phase: 50% acetonitrile–water (pH = 5.3) containing various amount of ammonium dihydrogen phosphate.

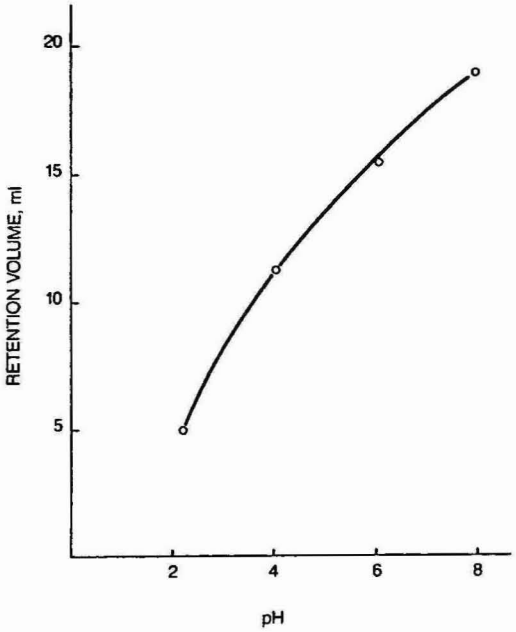


Fig. 4. Effect of pH on retention of lactic acid.

acetonitrile–water (1:1) as eluent (Fig. 2). The lactic acid peak is well resolved from the excipient peaks. As shown in the method validation section, it is well suited for quantitation. The amine column, although normally operating as a reversed-phase column, acts as an ion-exchange column in this system. Change in the cation by replacing the ammonium ion in the eluent with potassium ion or tetramethylammonium ion has no effect on the retention time. An increase in ionic strength with ammonium dihydrogen phosphate, while keeping the pH constant, decreased the retention volume (Fig. 3). An increase in the pH of the eluent led to an increase in retention volume (Fig. 4). These observations support the ion-exchange separation mechanism of an acid. A log plot of the capacity factor k' *vs.* concentration of acetonitrile in the eluent (Fig. 5) showed a linear relationship, typical of reversed-phase chromatography and well-known in ion-exchange chromatography[14].

Internal standard

No suitable internal standard was found, although we tested several compounds: 2-bromoacetic acid, 2-hydroxybutyric acid, ammonium acetate, maleic acid, hexanoic acid, *p*-hydroxybenzoic acid, ethyl glycolate, glycolic acid, ammonium oxalate and glyceric acid. Only glycolic acid resolved well from lactic acid, but the peak showed tailing. Because of the lack of a good internal standard, we developed the method using external standard only.

Chromatographic precision

Chromatographic precision of the method was determined by six replicate injections of a lactic acid standard (1.1 mg/ml). The lactic acid peak area was reproduced with a relative standard deviation (R.S.D.) of 0.9%.

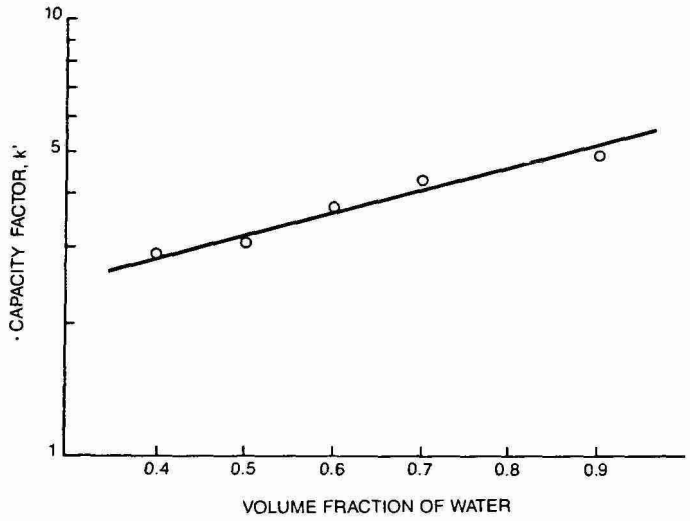


Fig. 5. Effect of solvent strength on retention of lactic acid at constant pH and ionic strength. Eluent: acetonitrile–water with 3.2 m*M* ammonium dihydrogen phosphate (pH 5.3).

Linearity of detector response

Seven lactic acid standard solutions in the concentration range 0.44–2.0 mg/ml were prepared and analyzed. Linear regression analysis of the peak area data gave a correlation coefficient (r) of 0.9996 and a y-intercept equivalent to −0.2% of the normal response (12% lactic acid lotion).

Method validation

Placebo of lactic acid lotion was analyzed by this method. No interference

TABLE I

ACCURACY DATA

Spiked sample		*Lactic acid*	
Placebo (g)	*Lactic acid (mg)**	*Assay (mg)*	*% Recovery*
0.5	119.9	120.6	100.6
0.5	119.9	119.6	99.8
0.5	119.9	121.1	101.0
1.0	119.9	120.7	100.7
1.0	119.9	119.6	99.8
1.0	119.9	119.8	99.9
1.5	119.9	120.6	100.6
1.5	119.9	120.5	100.5
1.5	119.9	120.6	100.5
		Mean	100.4
		R.S.D.	0.4%

* Spiked as aqueous lactic acid solution.

TABLE II

PRECISION DATA

Lactate lotion (mg)	*Lactic acid assay (%)*
494.6	13.06
493.3	12.69
505.4	12.64
1002.6	12.83
1016.3	12.63
992.2	13.08
1503.4	12.71
1517.2	12.81
1500.0	12.83
Mean	12.81
R.S.D.	1.3%

peaks at the retention time of lactic acid were observed. Nine synthetic samples were made by spiking the placebo with solutions containing known amounts of standard lactic acid at levels corresponding to 67–200% of the normal concentration. As shown in Table I, the average recovery obtained by the method is 100.4% (0.4% R.S.D.). The lack of any trend in recovery values with increase in amount of placebo suggests that the lactic acid/placebo ratio has no effect on the assay. Nine samples of lactate lotion with a label content of 12% lactic acid were analyzed by the HPLC

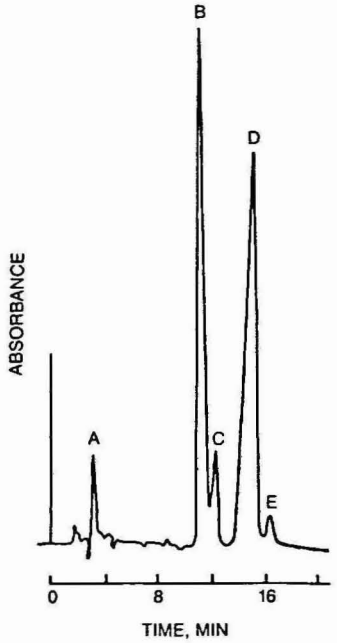


Fig. 6. Chromatogram of lactic acid and its possible degradation products. Peaks: A = solvent front; B = pyruvic acid; C = acetic acid; D = lactic acid; E = impurity from pyruvic acid.

procedure. The average of nine assays is 12.81%, with a relative standard deviation of 1.3% (Table II). The lack of any trend in assay results with change in sample size used for analysis indicates that larger or smaller samples can be used for analysis. The specificity of the method was checked by chromatographing a mixture of lactic acid with its possible degradation products. Fig. 6 shows that pyruvic acid and acetic acid peaks are resolved from the lactic acid peak. Oxalic acid and acetaldehyde were retained on the column. The purity of the separated lactic acid was further checked by recording the UV spectra (200–400 nm) at different points on the lactic acid peak using the diode array spectrophotometric detector. When normalized, all the spectra were found to be superimposable. Similar spectra were observed for the lactic acid peak of the lactate lotion sample stored at 30°C for 55 months. Hence, any degradation of placebo or lactic acid does not lead to interferences in the assay. Therefore, the method is stability-indicating for lactic acid.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Suresh Marathe for providing the compounds screened as internal standards, and Mr. Terry Murray for providing the lactate lotion and placebo. Thanks are also due to Dr. Samir Hanna for helpful discussions during the course of this work.

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CHROM. 18 385

Note

Effect of surfactants on electrophoretic zone mobility and its application to the separation of adenosine nucleotides

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The migration distance in electrophoresis using a support is affected by the physicochemical nature of the support. One of the main factors is the capillary action from both the electrode cells to the centre of the support[1–3]. This results in different migration distances depending on the spotting position on the support.

In the course of a study on the component of the supporting solution in zone electrophoresis, we found that the migration distances from different positions on a support showed the same values by the use of a supporting solution containing a surfactant. This means that it is not necessary to take care over the spotting position and that it becomes easier to evaluate the zone mobility theoretically. We now describe the effect of the surfactant on the zone mobility of picric acid on various supports and its application to the separation of adenosine nucleotides.

MATERIAL AND METHODS[3]

Sodium dodecyl benzenesulphonate (SDBS), laurylpyridinium chloride (LPC) and sodium dodecyl sulphate (SDS) of guaranteed grade were purchased from Wako Chemicals (Osaka, Japan), adenosine nucleotides from Sigma (St. Louis, MO, U.S.A.). Four different kinds of papers were used as supports: Toyoroshi No. 51A (51A, pure cellulose paper), Sartorius Membranfilter (AC, acetyl-cellulose paper), Whatman DEAE-cellulose paper (DE81, anion-exchange cellulose paper) and Whatman phosphate paper (P81, cation-exchange cellulose paper). All the papers were cut to a size of 40 × 1 cm before use.

A known weight of surfactant was dissolved in 0.1 *M* sodium chloride solution or in a phosphate buffer solution (pH = 7.0, ionic strength = 0.1), prepared by mixing equimolar amounts of disodium hydrogen phosphate (0.2 *M*) and sodium dihydrogen phosphate (0.2 *M*). Solutions with the surfactant or without were used as the supporting solutions. A strip (40 × 1 cm) of a filter-paper was dipped in a supporting solution and the excess of solution on the paper was removed by another paper. A 5-μl volume of a sample solution was spotted and its position marked by a lead pencil. The strips were then dipped in hexane in a migration chamber. A

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constant voltage (1000 V per 30 cm) was applied for 30 min at *ca.* 20°C. The electric current varied with the chemical nature of the support and supporting solution. The position of adenosine nucleotides on the strip was detected by irradiation with UV light (253 nm).

RESULTS AND DISCUSSION

The electrophoretic zone mobility is affected by many factors[4]. In order to evaluate the observed migration distances, it is necessary to know the chemical nature of a supporting solution and the physicochemical nature of the support. We spotted a sample solution at three different positions on various supports and then carried out the electrophoresis.

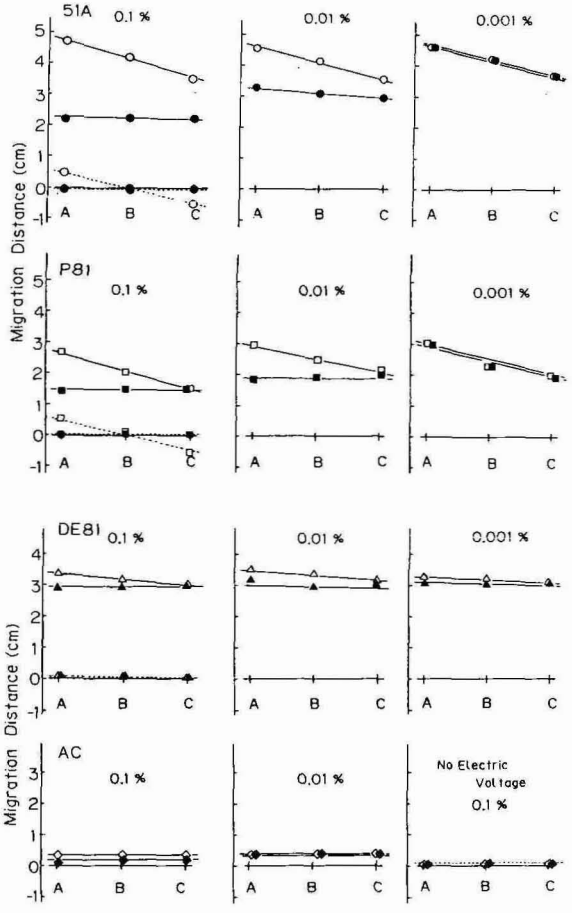

Fig. 1. Effect of SDBS on the migration distances of picric acid from different spotting positions on various supports. Sample: 10^{-3} *M* picric acid. Supporting solutions: 0.1 *M* sodium chloride solution (○, □, △, ◇) and 0.1 *M* sodium chloride containing SDBS (●, ■, ▲, ◆); the percentages of SDBS is shown. Supports: 51A, P81, DE81 and AC. Spotting positions: A, 5 cm to the cathodic side from the centre of the support; B, the centre of the support; C, 5 cm to the anodic side from the centre. Electrophoresis conditions: 1000 V per 30 cm, 30 min, 20°C. The dotted lines show the movements due to capillary action.

The observed migration distances of picric acid from three positions on various supports with or without the surfactant (SDBS) are shown in Fig. 1. The dotted lines show the movements observed when the sample was allowed to stand under conditions similar to those of the electrophoresis but in the absence of an applied voltage. When 0.1% (w/w) of SDBS was added to the supporting solution, the movements due to capillary action were almost zero on all supports. The effects of SDBS depended on its concentration and varied with the nature of the support.

Taking 51A paper as an example, when electrophoresis was carried out in neat 0.1 *M* sodium chloride solution as a supporting solution, different migration distances were obtained from different positions where the capillary action is different. The slope of the plots of migration distance *vs.* spotting position is proportional to the strength of the capillary action. Generally, the direction of the capillary action is towards the centre of the support from both sides and usually disappears in 1 h, depending on the physicochemical natures of the supporting solution and the support[5]. Upon addition of 0.1% (w/w) surfactant to the supporting solution, we were surprised to see that the migration distances from three different positions showed the same values irrespective of the spotting position, as shown in Fig. 1. The effect of the surfactant decreased with decreasing concentration. When 0.1% (w/w) surfactant was added to the supporting solution and the support allowed to stand in the migration chamber for 30 min without an electric voltage, no migration of picric acid was observed. It was assumed that there was little capillary action because of the lowering of the surface tension upon adding the surfactant[6].

In a separate experiment[7], when electrophoresis was carried out without dipping the support into hexane, the supporting solution containing 0.1% (w/w) SDBS evaporated more rapidly than usual, again because of the lowering of the surface tension.

In addition to the effect on the capillary action, the fact that all the migration distances of picric acid in 0.1% (w/w) SDBS were less than in the absence of SDBS

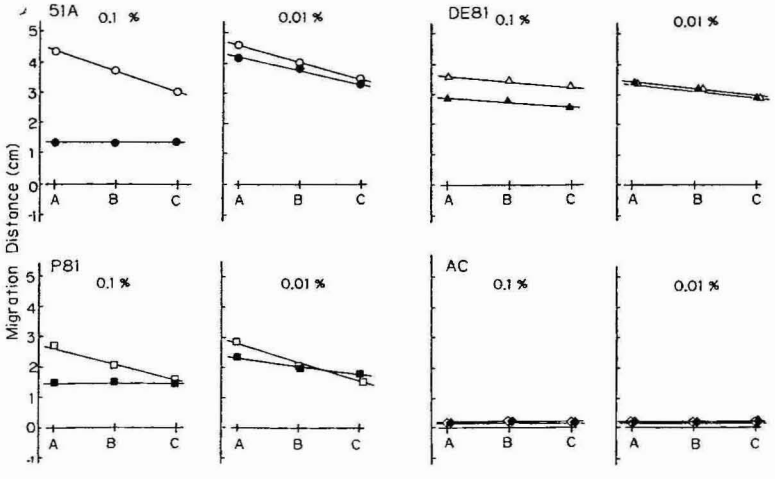

Fig. 2. Effect of SDS on the migration distances of picric acid from different spotting positions on various supports. Electrophoresis conditions and symbols as in Fig. 1. SDS was substituted for SDBS.

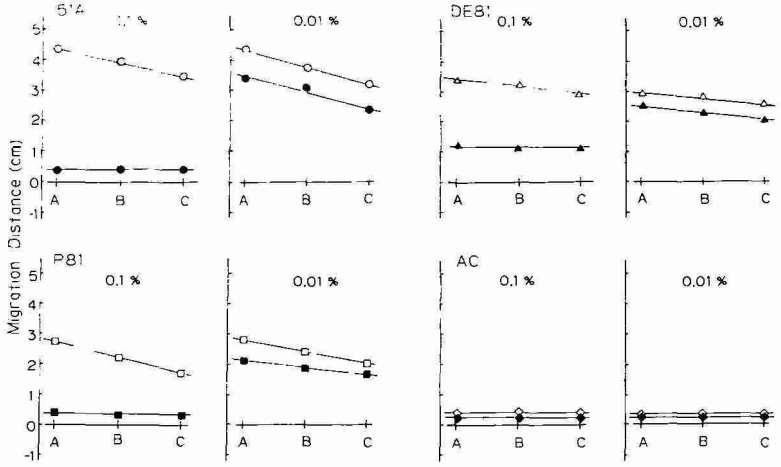

Fig. 3. Effect of LPC on the migration distances of picric acid from different spotting positions on various supports. Electrophoresis conditions and symbols as in Fig. 1. LPC was substituted for SDBS.

means that there is an interaction between picric acid and SDBS. Thus, adding an excess of SDBS to the supporting solution is not profitable.

Previously, we found[8] that the capillary action increases in the order AC $\approx$ DE81 < 51A < P81. This order is also true of the effect of SDBS; the effect of SDBS was large on P81 and 51A papers, but slight on AC and DE papers.

The effects of SDS and LPC were similar, as shown in Figs. 2 and 3, respectively; at 0.1% (w/w) concentration, LPC is the most effective and SDBS and SDS

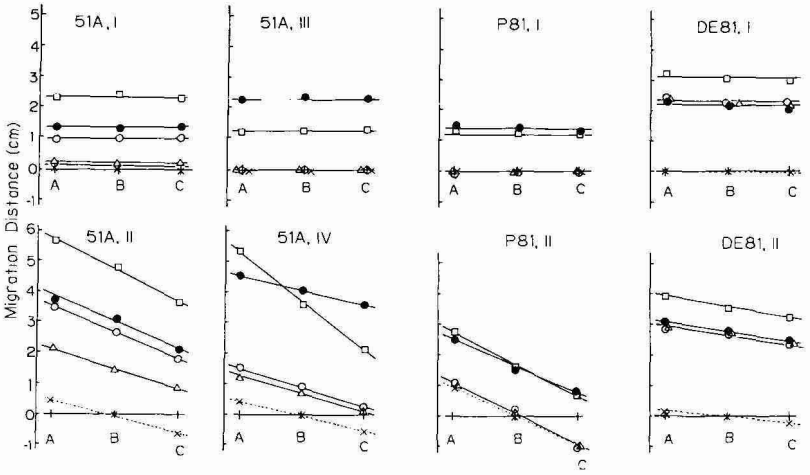

Fig. 4. Separation of adenosine nucleotides in a supporting solution containing SDBS on various supports. Sample: 5 μl of $5 \cdot 10^{-3}$ *M* solution; ○—○, AMP; △—△, cAMP; □—□, ATP; ●—●, picric acid; × · · · ×, the movement of picric acid due to capillary action. Supports: 51A, P81, DE81, AC. Supporting solutions: phosphate buffer (pH = 7.0, ionic strength = 0.1) + 0.1% SDBS (I); phosphate buffer (II); 0.1 *M* sodium chloride + 0.1% SDBS (III); 0.1 *M* sodium chloride (IV). Electrophoresis conditions: 1000 V per 30 cm, 30 min, 20°C.

are equivalent. The effects of the chemical structure or charge of the surfactants remain unknown. From these results, we recommend the addition of *ca.* 0.1% (w/w) surfactant to the supporting solution.

A supporting solution containing a surfactant was then applied to the separation of adenosine nucleotides. Fig. 4 shows the observed migration distances of adenosine nucleotides and picric acid in 0.1% (w/w) SDBS–phosphate buffer together with those in phosphate buffers. In the experiments described in the previous section, we used 0.1 *M* sodium chloride solution as the supporting solution. However, for greater reproducibility of experimental results, it is desirable to use a solution of high buffering capacity as a supporting solution. In the supporting solution containing SDBS, the migration distances of the nucleotides showed the same values irrespective of the spotting positions, as expected. A good separation of adenosine 5′-monophosphate (AMP) from adenosine cyclic-3′,5′-monophosphate (cAMP) was achieved only on 51A paper in phosphate buffer, but not in 0.1 *M* sodium chloride solution. In the case of P81 or DE81, AMP and cAMP were not separated. Probably, the ionic moieties of the support affected the movement of the anions of the nucleotides. The fact that all the migration distances of the nucleotides in SDBS solutions were shorter than those solutions without surfactant suggests the occurrence of an interaction between the nucleotides and SDBS.

It is concluded that the presence of a surfactant in a supporting solution markedly affects the capillary action of a support and interacts with the sample to an extent dependent on the chemical nature of the latter.

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CHROM. 18 369

Note

Microscopic investigations of the surface structure of carbon–silica adsorbents

III. Heterogeneity of the carbon layer

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The influence of the heterogeneity of adsorbents on their chromatographic performance has been described[1,2]. Thus studies of the crystalline structure and crystal defects of adsorbents are very important. Bethge and Heydenreich[3] stated that the "microstructure" is related to the crystallographic structure and micromorphology of solids as well as to local inhomogeneities in the chemical composition. Based on the results of electron microscope studies, Kranaukhov[4] classified porous materials according to whether they had a corpuscular or spongy structure. Corpuscular systems are formed by particles of various shapes connected to one another. In this case, the pores represent the interstices between the particles. For spongy structures a system of channels and cavities is developed wherein individual particles cannot be distinguished. Sometimes both these two types are combined: particles of the corpuscular system may have internal spongy porosity, and large pores of the spongy system may involve small particles.

Dubinin[5] reported that fossil coals carbonized at temperatures of 500–900°C consist of an ordered part (carbon crystallites) and an amorphous part (high-carbon-number radicals). The structure of the crystallites resembles that of graphite, and they are irregularly spaced throughout the coal. The radicals are bound with carbon atoms. Carbonized organic materials generally contain crystallites 0.6–3 μm in diameter. The number of layers of carbon atoms in the crystallites increases from two to five with increasing diameter.

Boehm[6] distinguished three forms of carbon: graphite, diamond and black microcrystalline carbon. The last one, formally called amorphous, can be obtained by pyrolysis of organic materials. It consists of graphite-like layers of limited size stacked parallel to one another without ordering. Sometimes there is a considerable content of disorganized tetrahydrally bonded carbon, often cross-linking different layers. These defects cause a regular array of carbon bonds to be disturbed by forming free valences, which become sites of heterogeneity at the carbon surface.

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TABLE I

CONDITIONS FOR MODIFICATION OF SILICA GEL AND THE SURFACE PROPERTIES OF THE ADSORBENTS INVESTIGATED

S = Specific surface area; L = average layer thickness of deposited carbon; C = amount of carbon = the loss of mass of carbosils during heat treatment in the air.

Adsorbent	*Modifier*	*Time of pyrolysis (h)*	*S* (m^2/g)	*C* (%, w/w)	*L* (Å)
Initial silica gel	Unmodified	–	150.7	–	–
Carbosil A1	Dichloromethane	1	112.2	0.8	0.29
Carbosil A2	Pentanol	3	151.2	1.2	0.44
Carbosil A3	1. Dichloromethane	1			
	2. Pentanol	3	138.2	1.76	0.65
Carbosil A4	1. Pentanol	3			
	2. Dichloromethane	1	109.5	2.21	0.81

The aim of this study was to investigate the microstructure of the carbon layer formed by pyrolysis of pentanol or dichloromethane and a mixture of these two compounds.

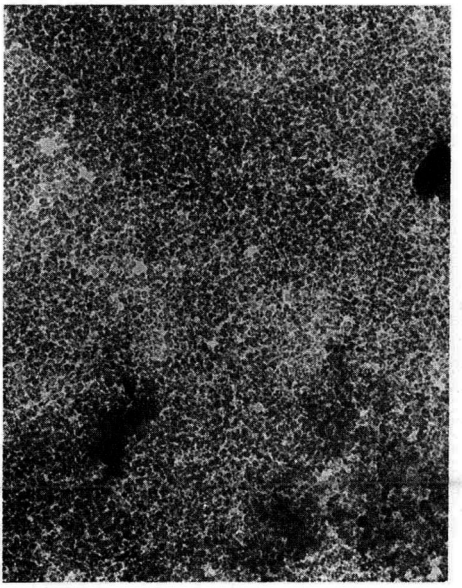
EXPERIMENTAL

The silica adsorbents were prepared as described previously[7]. Different amounts of carbon were deposited on their surfaces by pyrolysis of dichloromethane and pentanol (Table I).

Samples for transmission electron microscopy were prepared by dissolving the silica in hydrofluoric acid according to the method described previously[8]. Because all the silica may not dissolve in the acid it possible that a small amount of it remains on the carbon layer. Finally, the thin carbon layer with a small amount of silica was examined by using a JEM 200 A transmission electron microscope at an acceleration voltage of 200 kV. The sample was cooled with nitrogen. The bright field (BF), dark field (DF) and selected area diffraction (SADP) techniques were used. In each experiment the sample orientation was perpendicular to that of the electron beam (goniometer position $x = 0$, $y = 0$). The results for four adsorbents (A1, A2, A3, A4) were recorded on 600 photoplates. Representative micrographs are illustrated.

RESULTS AND DISCUSSION

Figs. 1 and 2 show the carbon layer deposited on adsorbent A1. Figs. 3 and 4 that deposited on adsorbent A2. The former layer was prepared by pyrolysis of dichloromethane, the latter by pyrolysis of pentanol. A magnification of 20 000 × (Figs. 1 and 3) revealed differences between these two samples. When a magnification of 100 000 × was used (Figs. 2 and 4), it was found that the carbon layer on adsorbents A1 and A2 consisted of small agglomerates, the microstructure of sample A2 being finer. Differences in the microstructure were also found for adsorbents A3 and A4. Fig. 5 shows the electron diffraction pattern obtained from the same area of the carbon layer as presented in Figs. 1 and 3. This kind of pattern is typical for non-



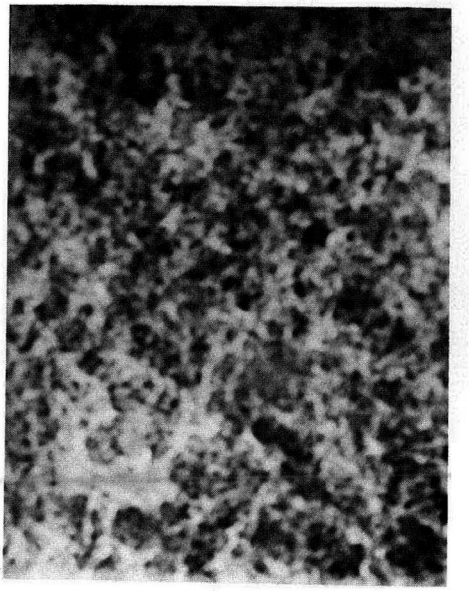


Fig. 1. Carbon layer deposited on adsorbent A1 (magnification 20 000 ×).

Fig. 2. Carbon layer deposited on adsorbent A1 (magnification 100 000 ×).

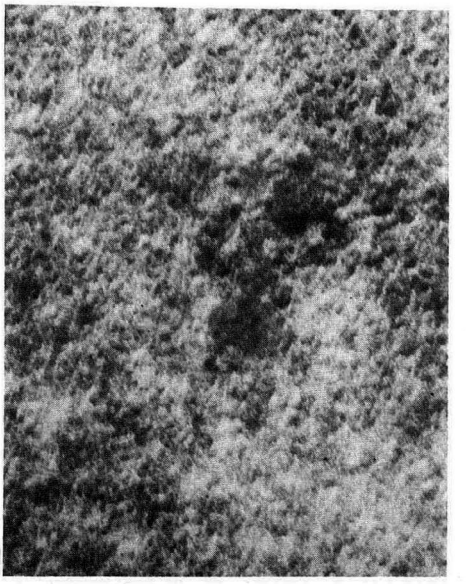


Fig. 3. Carbon layer deposited on adsorbent A2 (magnification 20 000 ×).

Fig. 4. Carbon layer deposited on adsorbent A2 (magnification 100 000 ×).

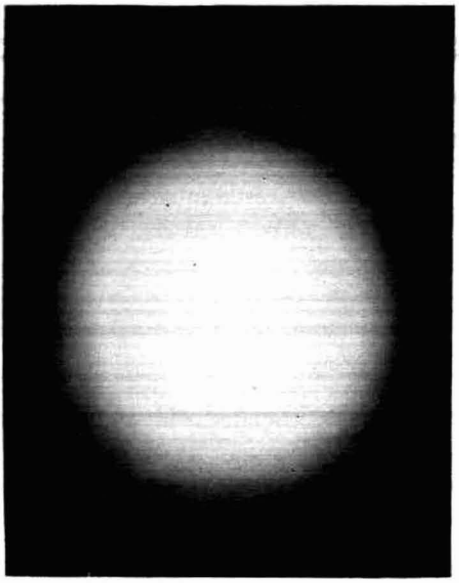
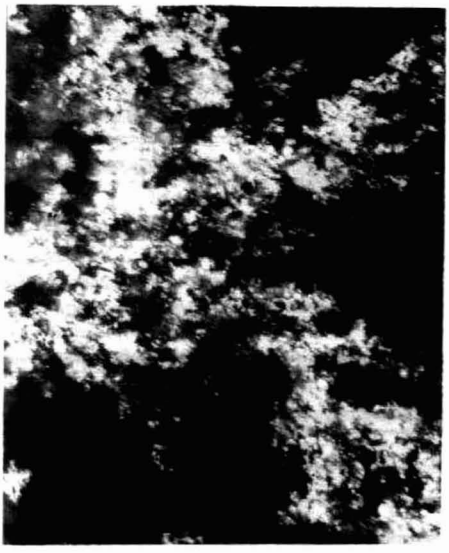


Fig. 5. Electron diffraction pattern showing the amorphous character of the carbon layer on adsorbents A1 and A2.

Fig. 6. BF image of the carbon layer on adsorbent A1 (magnification 20 000 ×).



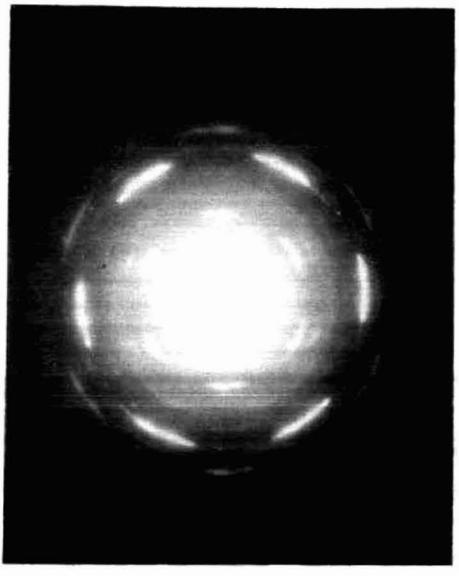


Fig. 7. DF image showing the distribution of crystallites in the area presented by the BF image (Fig. 6).

Fig. 8. Diffraction pattern obtained from the area shown in Fig. 6.

crystalline (amorphous) structures[9] and was observed in all the four samples.

According to the classification of the porosity types given by Kranaukov[4], the carbon layer on the adsorbents A1–A4 had corpuscular character. Dubinin[5] stated that such a system, also called polydisperse, can be regarded as a sign of inhomogeneity. This means that in the amorphous part of the carbon layer the homogeneity is disturbed by microporosity (microporous structure).

Figs. 6–18 show the regions of the carbon layer where crystallites were found. For observations of the crystalline phase, dark field (DF) and selected area diffraction (SADP) were used.

Figs. 6–8 show the crystalline phase found in the carbon layer of adsorbent A1. Fig. 6 shows that the carbon layer is amorphous, similar to that in Fig. 1, without features and crystallographic details. The diffraction pattern obtained from this area (Fig. 8) shows that small amounts of crystallites are present. Fig. 7 shows the distribution of crystallites in the area equal to that of the photoplates. The screen magnification was 20 000 ×, *i.e.*, 1 cm on the photoplates corresponds to about 0.5 μm; the size of each photoplate is 9 × 6 cm. Each photoplate provides information on an area of carbon layer equal to 13.5 μm^2. In the area mentioned above the concentration of crystallites is high (Fig. 7). Their sizes are $\leqslant$ 500 Å.

The carbon layer on sample A1 was obtained by pyrolysis of dichloromethane in a very simple reaction:

$$CH_2Cl_2 \xrightarrow{500°C} C + 2\,HCl \qquad (1)$$

According to Leboda[10], the IR spectra of many carbon-silica adsorbents obtained by pyrolysis of dichloromethane show that homogeneous carbon is obtained in this

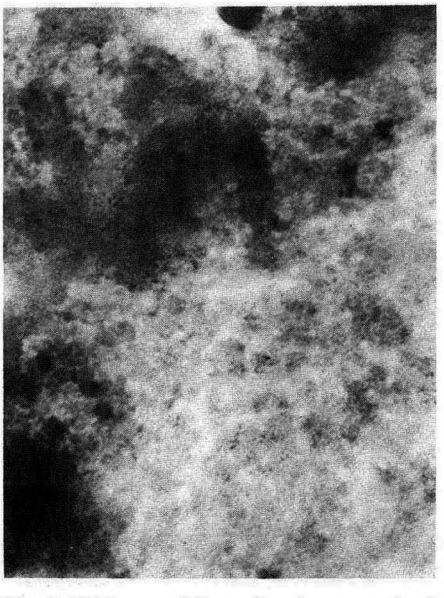
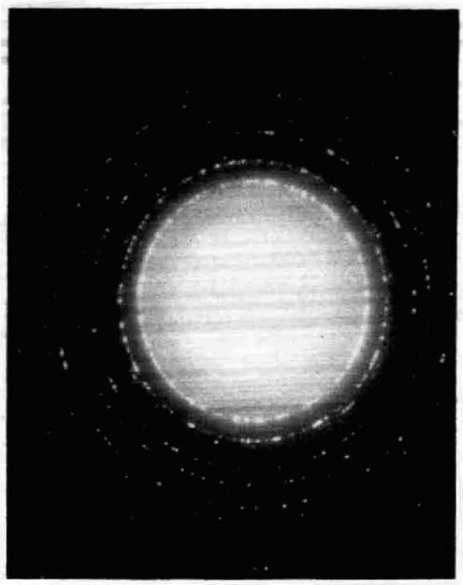


Fig. 9. BF image of the carbon layer on adsorbent A2 (magnification 20 000 ×).

Fig. 10. DF image showing the distribution of crystallites in the area presented by the BF image (Fig. 9).



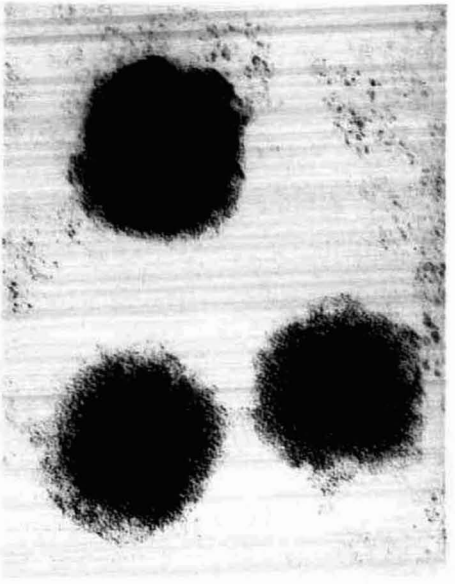


Fig. 11. Diffraction pattern obtained from the area shown in Fig. 9.

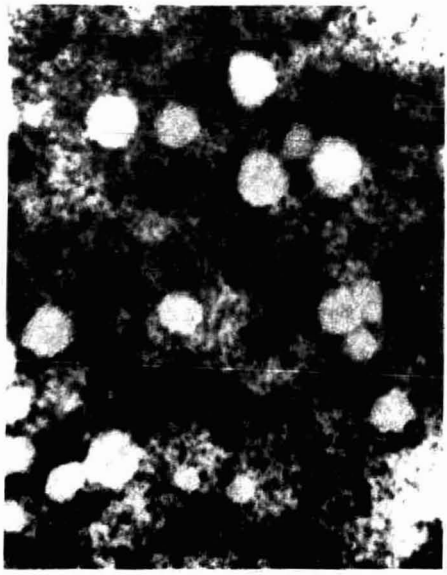
Fig. 12. BF image showing the carbon layer on adsorbent A4.

reaction. However, the adsorption energy, E, for n-hexane on these carbosils showed three clear maxima[10]. Therefore, it has been suggested that the carbon layer obtained in reaction 1 consists of energetically non-equivalent carbon particles (agglomerates), etc. The results presented here show that the carbon layer obtained in reaction 1 is mostly amorphous and consists of agglomerates of about 200–300 Å (Fig. 6) and of a small amount of crystallites of < 500 Å (Figs. 7, 8). Furthermore the considerably complicated shape of the adsorption energy curves obtained by a chromatographic method[10] was caused by the fact that microcrystalline areas are present in amorphous geometrically non-equivalent carbon layers.

Figs. 9–11 show the carbon layer on adsorbent A2. It should be noted that the mechanism of formation of the carbon deposit on the silica gel surface during dichloromethane decomposition is totally different from that during alcohol pyrolysis. In reaction 1 carbon is formed due to thermal decomposition of dichloromethane, while alcohol pyrolysis is preceded by adsorption and chemisorption of alcohol molecules on the surface of the carrier. The IR spectrum shows that the adsorbents obtained by pyrolysis of alcohols have a very complicted chemical structure, but chromatographic experiments have shown that they are energetically homogenous[10].

The bright field image (Fig. 9) shows that the carbon layer on adsorbent A2 consists of agglomerates, but, in comparison to adsorbent A1 (Fig. 6), the microstructure of carbon is finer. The dark field image (Fig. 10) and SADP (Fig. 11) show crystallites to be present in sample A2. The size of the crystallites in sample A2 is similar to that in sample A1, but their distribution in the former over the same area of the photoplate is rarer (smaller concentration of crystals).

Figs. 12–15 show the carbon layer on adsorbent A4 which was prepared by



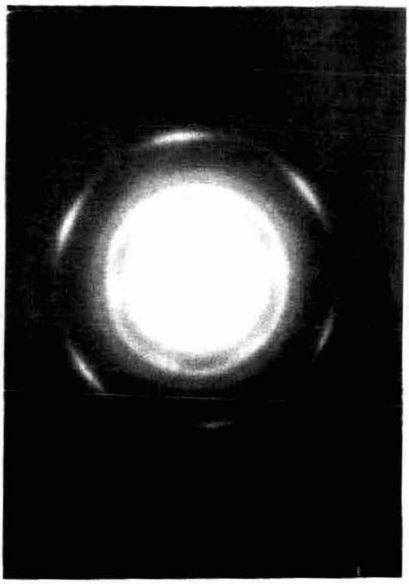
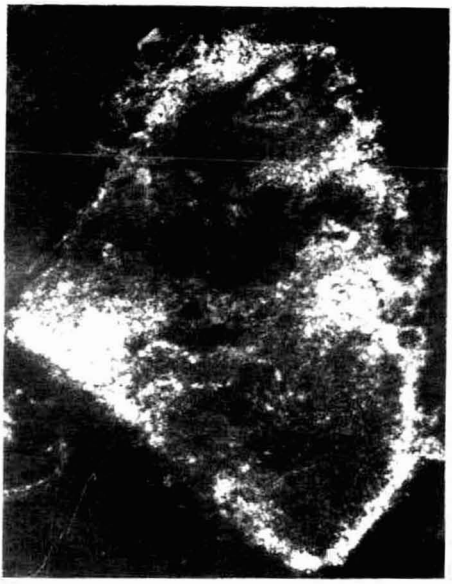


Fig. 13. DF image showing the crystalline character of the agglomerates.

Fig. 14. Diffraction pattern corresponding to Fig. 12.



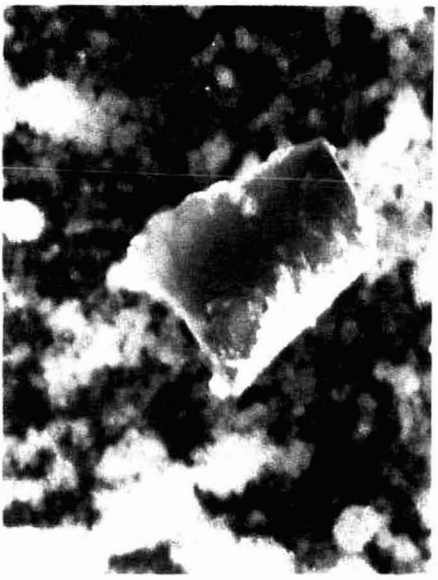


Fig. 15. Morphology of a single crystal which very often occurs in the carbon layer on adsorbent A2 (magnification 20 000 ×).

Fig. 16. Morphology of a single crystal which very often occurs in the carbon layer on adsorbent A4 (magnification 20 000 ×).

pyrolysis first of pentanol and then of dichloromethane. The BF image (Fig. 12) shows that the carbon layer consists of agglomerates of a few μm in size. These agglomerates have their own microstructure. DF (Fig. 13) and SADP (Fig. 14) show the crystalline character of the agglomerates. In contrast to adsorbent A4, adsorbent A3 was prepared by pyrolysis first of dichloromethane and then of pentanol. The fact that for adsorbent A3 no similar pictures were obtained is surprising. It can be supposed that crystallites formed upon pyrolysis of the alcohol act as nuclei for the growth of carbon crystalites formed upon thermal decomposition of dichloromethane. These results confirm our previous study, where the replica method was used[7].

Figs. 15 and 16 show the characteristic objects found in each of the four samples. The diffraction patterns formed from the area of these objects (Fig. 17) shows their crystalline structure with hexagonal symmetry. Andrews *et al.*[11] reported that a single crystal in the electron beam will give rise to a more or less regular and ideally symmetrical arrangement of spots, each of which has a different value of (hkl)*. Spots which are equidistant from the centre and diametrically opposite are associated with the same set of crystal planes and are indexed as (hkl) and $(\bar{h}\bar{k}\bar{l})$. They are single crystal type spot patterns. It is quite possible that these objects represent one of the crystalline forms of silica, since a similar diffraction pattern was obtained for silica gel heated at 600°C. The method of sample preparation involves the dissolution of silica gel in hydrofluoric acid. However, during pyrolysis in the autoclave, amorphous silica may change to a crystalline form. As amorphous silica is easier to dissolve in hydrofluoric acid than its crystalline forms, some of the latter may remain attracted to the carbon layer.

Fig. 17. Diffraction pattern corresponding to Fig. 15.

* The set of Miller indices which are reciprocal intercepts in units of cell of crystal. If the plane cuts one of the axes on the negative side of the origin the corresponding Miller indices are negative $(\bar{h}\bar{k}\bar{l})$.

At present it is not possible to solve the problem of the crystal structure of the objects examined. Examination of the adsorbents by means of X-ray diffraction does not reveal the diffraction lines corresponding to the Bragg reflections, because of the low sensitivity of this method. Moreover, as the effect of radiation damage was very strong, the measurements had to be made very quickly and could be made only for one position of the goniometer ($x = 0$, $y = 0$). In this way, diffraction patterns were obtained for accidentally crystallographic directions.

CONCLUSIONS

(1) The carbon layer deposited on the adsorbents A1–A4 consists mainly of amorphous carbon.

(2) In each of the four samples, crystallites were found to occupy only a small part of the carbon layer.

(3) In general, two types of crystallites were distinguished; (a) large ones, about 10–50 μm in size, gave a diffraction pattern with three-fold symmetry (Fig. 17) and are considered to be a crystalline form of silica; (b) small ones, about 500 Å in size, considered to be a graphite-like form of carbon.

The heterogeneous character of the carbon layer revealed by the electron microscope studies confirmed the earlier chromatographic results obtained for carbon–silica adsorbents[7,10]. Moreover, the electron microscope experiments allow us to conclude that both the chemical and crystallographic structure of the carrier (pure or partially carbonized silica) affect the character of the deposited carbon layer.

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CHROM. 18 374

Note

Preparation of support grains for transmission electron microscopic studies

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Diatomaceous supports are formed from diatomaceous earth or diatoms. Diatoms possess a very complicated crystalline structure composed mainly of aluminosilicates. Transmission electron microscopic (TEM) studies on the morphology and crystalline structure of supports are possible, but a sample of the particles of the support thinner than 800 Å is required.

Barna[1] proposed an ion milling unit for preparing samples for bulk materials and a layer system for TEM studies. The aim of this work was to employ an ion milling unit for thinning support grains and to obtain suitable samples for TEM studies. The support grains (each kind of support for chromatography can be used) were embedded in the resin and placed in a small holder of diameter 3 mm. After polymerization of the resin the sample was polished on both sides. When the thickness of the sample was 50–70 μm the sample holder was placed in the ion milling equipment. Experiments were carried out several times. The average time of the thinning process was 1–1.5 h. The operating voltage was 8 kV and the ion beam current was 200 μA. The advantage of the direct thinning method is that studies can be carried out on the morphology of the support inside the grain, while the replica method used previously allowed only the topography of the grain surface to be determined[2].

The small particles of diatoms shown in Fig. 1 are agglomerated into larger particles, so the support grain consists of a mass of diatomite fragments fused at the contact point, giving rise to macropores as shown in Fig. 2. Fig. 3 shows micropores originating from the natural undestroyed structure of diatoms, and Fig. 4 a "flat" surface of SiO_2 monocrystals, which often occur in the particles of the support[2]. Other types of monocrystals that were also found in thinned grains are shown in Fig. 5.

Figs. 6, 7 and 8 show the bright field (BF), the dark field (DF) and the diffraction pattern, respectively, at the same location on the support particles. The BF depicts a fine structure of the grain with black intrusions, which, as shown in the diffraction pattern (Fig. 8), possess a crystalline character. The DF shows the distri-

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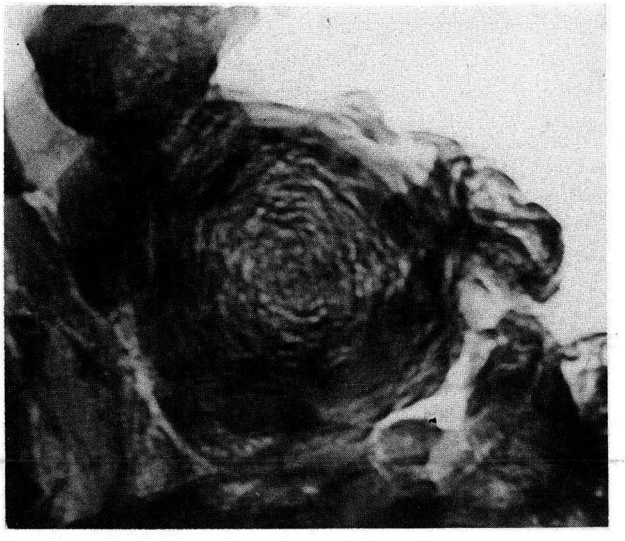

Fig. 1. Interior of support grain, showing that it consists of small particles of diatoms. (TEM, magnification 50 000 ×).

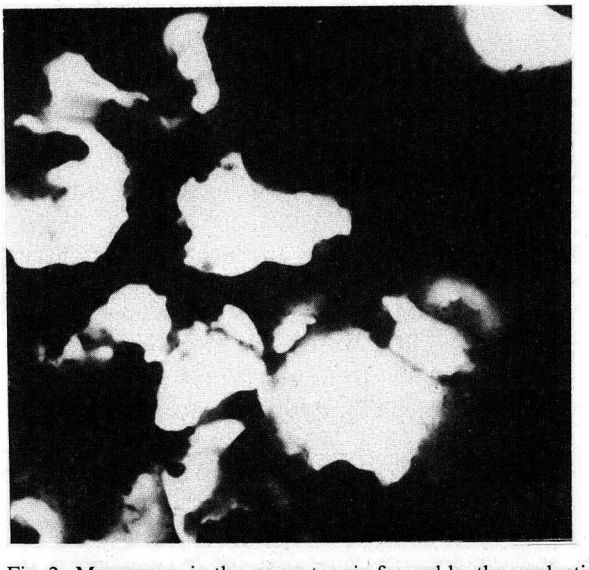

Fig. 2. Macropores in the support grain formed by the production process (TEM, magnification 500 ×).

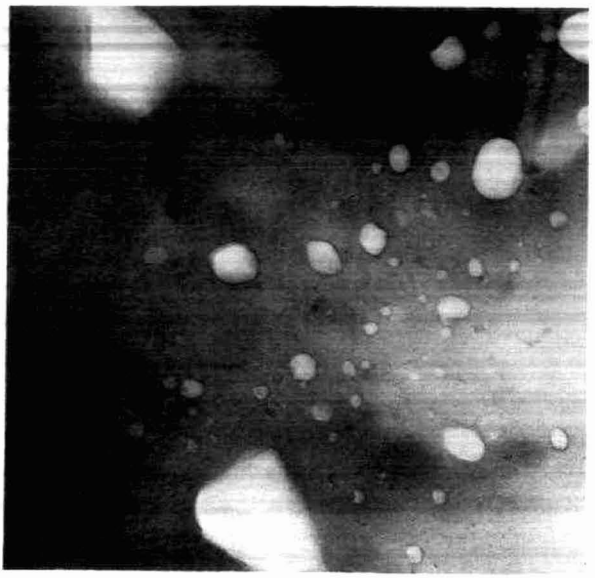

Fig. 3. Micropores originating from natural undestroyed structure of diatom (TEM, magnification 50 000 ×).

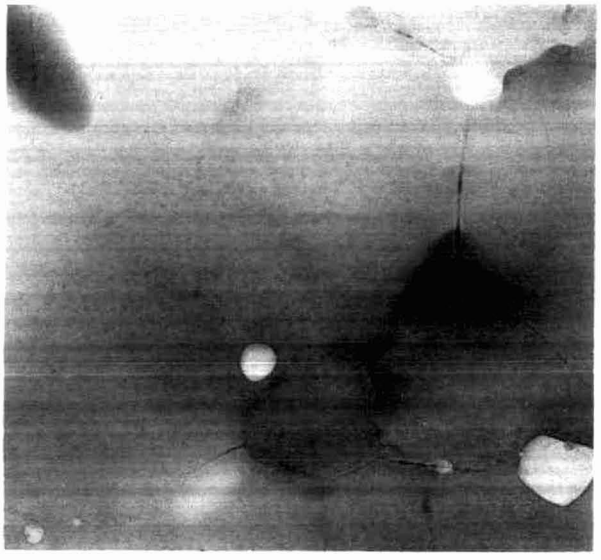

Fig. 4. "Flat" surface of monocrystal of SiO_2 as one of the main components of the support grain (TEM, magnification 50 000 ×).

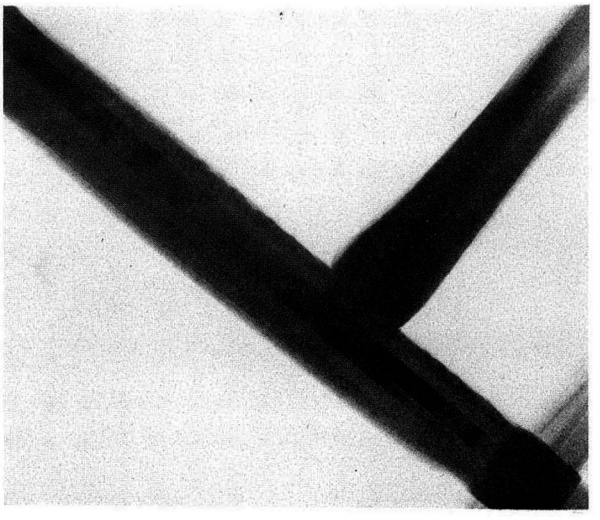

Fig. 5. Small crystallities that often occur in the grain.

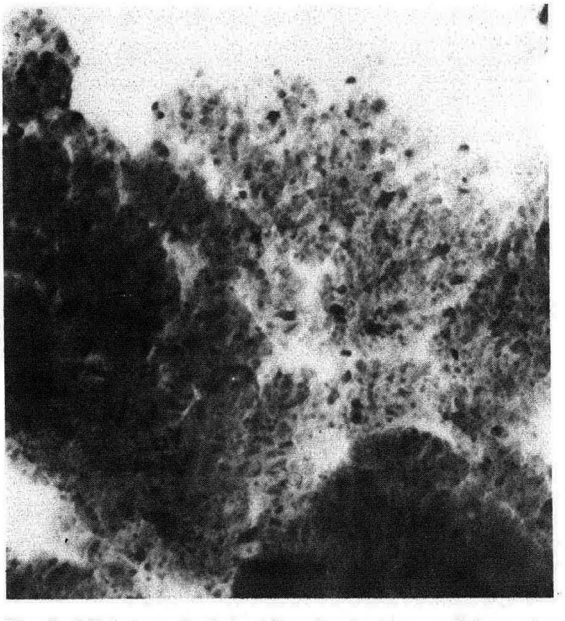

Fig. 6. BF image, depicting the microstructure of the grain with black intrusions (TEM, magnification 50 000 ×).

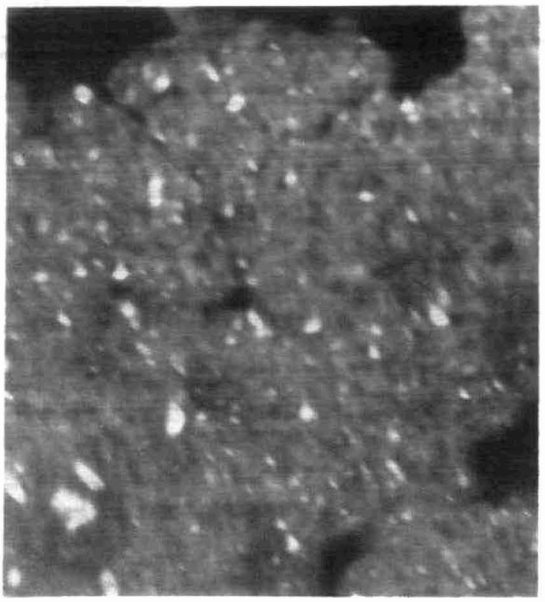

Fig. 7. DF image showing the distribution of crystals in the area represented by the BF.

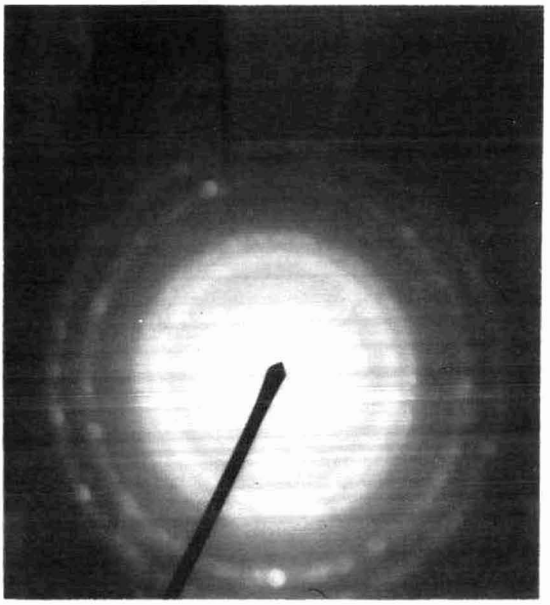

Fig. 8. Diffraction pattern illustrating the crystalline character of the intrusions.

bution of crystals in the area obtained from the reflex marked in the diffraction pattern.

CONCLUSIONS

Employing the ion-etching method for the preparation of support grain samples provides new possibilities for studying these materials by conventional TEM and energy loss spectroscopy, high-resolution electron microscopy and other techniques. By using the method described above, the crystal structure of the support, which undoubtedly influences its chromatographic performance, can be better distinguished.

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CHROM. 18 383

Note

Separation of 7α- and 7β-methoxycarbonylmethyl steroids by preparative high-performance liquid chromatography: comparison with thin-layer chromatography

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The 7α- and 7β-carboxymethyl steroid derivatives have been used either as haptens in radioimmunoassays[1,2] or as ligands in affinity chromatography[3]. We have described[4,5] a general method for introducing a carboxymethyl chain at the C-7 site in the 3-hydroxy-5-ene and 4-en-3-oxo-steroid series (Fig. 1). After protecting the functional groups, bromination by N-bromosuccinimide and condensation with sodium ethyl malonate is followed by saponification and decarboxylation. The subsequent removal of the protective functional groups yields equal proportions of the 7-carboxymethyl derivatives in the two possible orientations. Separation of the epimers before hydrolysis of the 3-ethylene ketal is preferable in order to obtain higher ΔR_F values and to allow easier identification of the epimers through NMR analysis

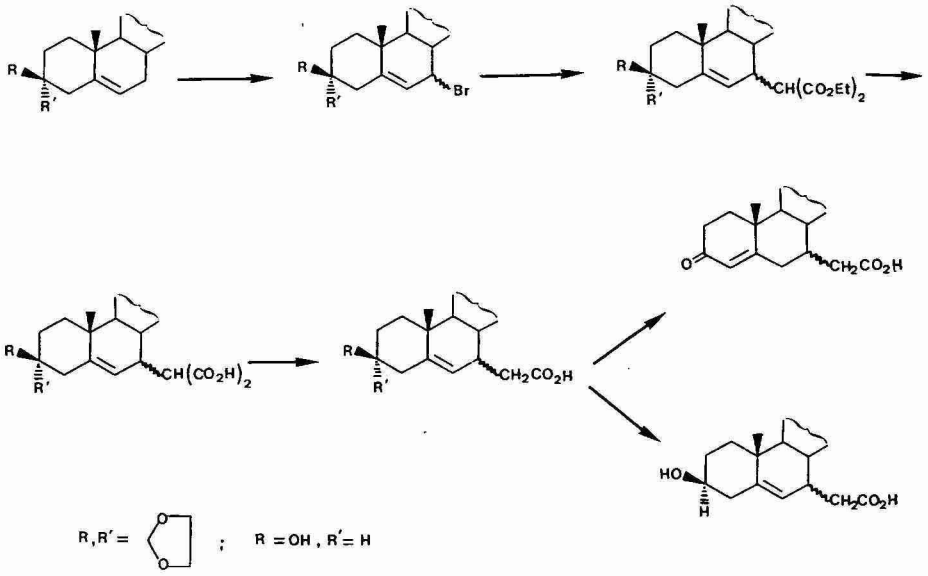

Fig. 1. Flow chart for the preparation of 7-carboxymethyl steroids.

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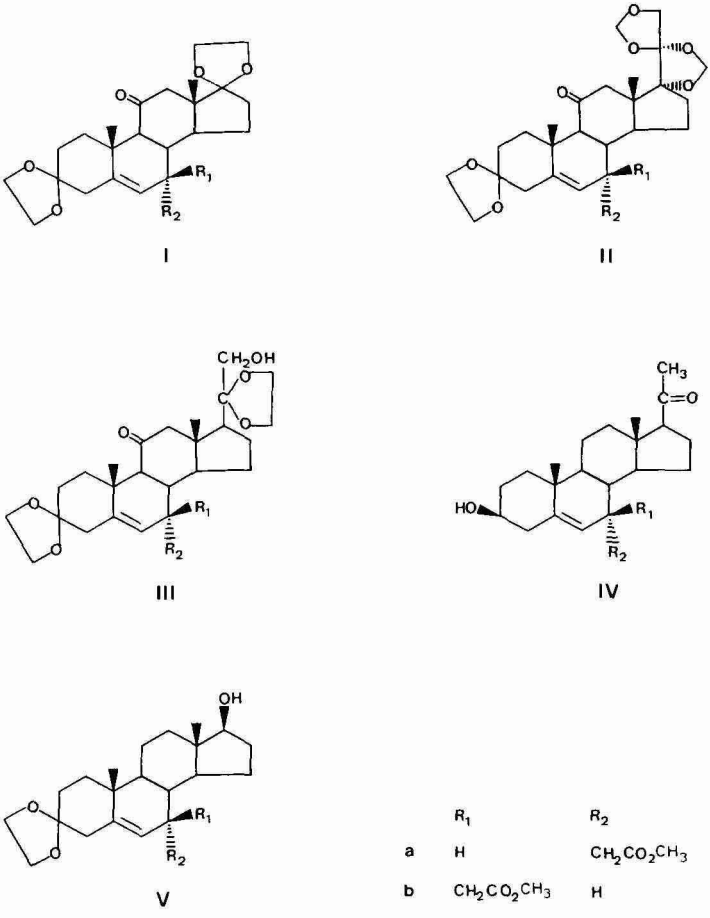
of the proton signal at C-6[4,5]. After preliminary experiments, the separation of the 7α and 7β epimers was found to be more convenient as their methyl ester derivatives on a normal phase rather than as their acid forms on a C_{18} bonded reversed-phase. Furthermore, the methyl derivatives, which are conveniently separated using mixtures of volatile solvents, require a purification step in the course of their formation.

MATERIALS AND METHODS

Thin-layer chromatography (TLC) was carried out on silica gel plates (60 F_{254}; Merck, Darmstadt, F.R.G.) after activation at 110°C for 30 min, but without presaturation in the solvent tank. A 6000 A liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a U6-K injector, a Waters UV detector and a Waters Model 401 difference refractometer was used for analytical and semi-preparative high-performance liquid chromatography (HPLC). The columns (Waters) were μPorasil (30 cm × 4 mm I.D.), average particle size 10 μm, or reversed-phase μBon-



	R_1	R_2
a	H	$CH_2CO_2CH_3$
b	$CH_2CO_2CH_3$	H

Fig. 2. The pairs of epimers studied.

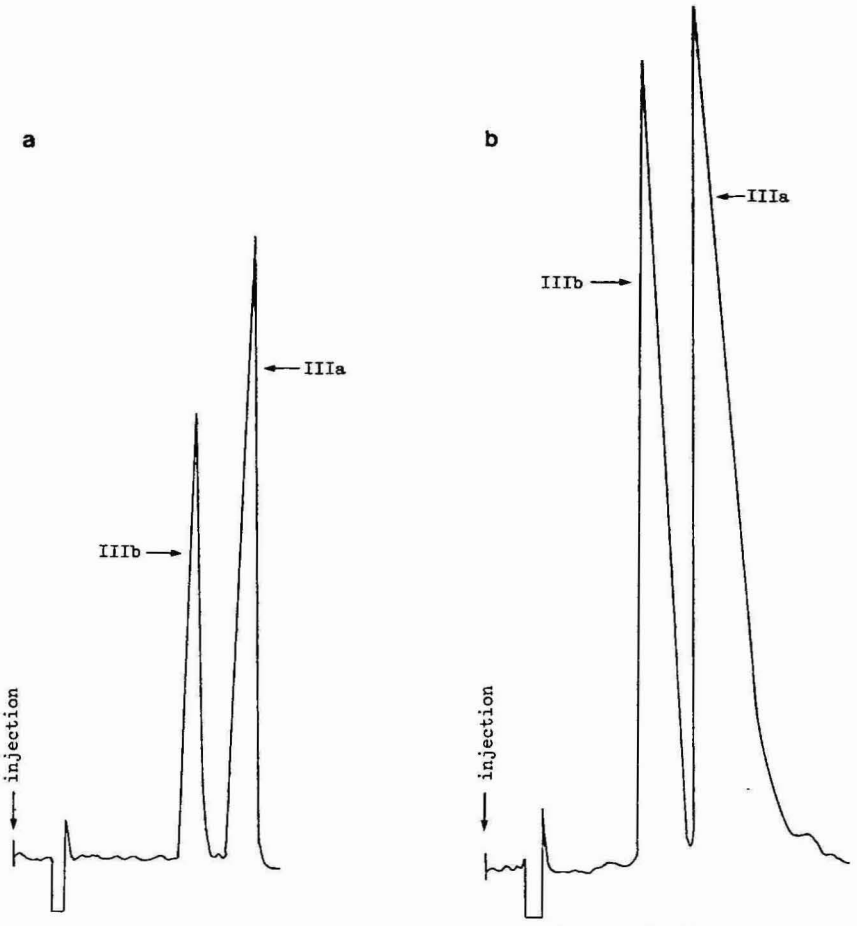


Fig. 3. Separation of IIIa and IIIb. (a) Analytical HPLC: flow-rate 2 ml/min, t_0 = 1.6 min. (b) Semi-preparative HPLC: flow-rate 8 ml/min, t_0 = 1.6 min.

TABLE I

TLC R_F VALUES AND SELECTIVITY FACTORS, k'_α/k'_β, OF 7α- AND 7β-EPIMERS IN VARIOUS SOLVENT SYSTEMS

Capacity factor, $k' = (1 - R_F)/R_F$.

Sample	*Hexane–ethyl acetate (30:70)*			*Ethyl acetate*			*Benzene–diethyl ether (60:40)*			*Benzene–diethyl ether (30:70)*		
	$R_{F\beta}$	$R_{F\alpha}$	k'_α/k'_β	$R_{F\beta}$	$R_{F\alpha}$	k'_α/k'_β	$R_{F\beta}$	$R_{F\alpha}$	k'_α/k'_β	$R_{F\beta}$	$R_{F\alpha}$	k'_α/k'_β
I	0.44	0.40	1.18	0.54	0.50	1.18	0.35	0.30	1.26	0.39	0.34	1.24
II	0.46	0.43	1.13	0.58	0.56	1.09	0.42	0.37	1.23	0.44	0.40	1.18
III	0.14	0.13	1.09	0.34	0.33	1.05	0.07	0.06	1.18	0.07	0.06	1.18
IV	0.37	0.36	1.04	0.50	0.48	1.08	0.27	0.26	1.06	0.27	0.26	1.05
V	0.39	0.36	1.14	0.49	0.45	1.17	0.28	0.24	1.23	0.29	0.26	1.16

dapak C_{18} (30 cm × 4 mm I.D.), average particle size 10 μm, in the case of analytical separation, μPorasil (30 cm × 7.8 mm I.D.), average particle size 10 μm, in the case of semi-preparative separations and PrePak (30 cm × 5.7 cm I.D.), average particle size 35–75 μm, in the case of preparative separations. Solvents for analytical and semi-preparative separations (Carlo Erba, RS HPLC) were filtered through Millipore filters (0.45 μm) and degassed by ultrasonication for 10 min, while those for preparative separations were pure grade, distilled and filtered through Millipore filters (0.45 μm) before use.

The amounts injected were usually up to 25 μg in 25 μl of solvent in analytical HPLC, at a flow-rate of 2 ml/min, up to 50 mg in 500 μl in semi-preparative HPLC, at a flow-rate of 8 ml/min and up to 2 g in 10 ml for preparative HPLC, at a flow-rate of 200 ml/min.

The following epimer pairs were studied (Fig. 2): 7α-methoxycarbonyl-5-pregnene-3,11,17-trione 3,17-bisethylene ketal (Ia) and the 7β epimer (Ib) derived from adrenosterone; 7α-methoxycarbonylmethyl-17α,20,20,21-bismethylenedioxy-5-pregnene-3,11,20-trione 3-ethylene ketal (IIa) and the 7β epimer (IIb) derived from cortisone; 7α-methoxycarbonylmethyl-21-hydroxy-5-pregnene-3,11,20-trione 3,20-bisethylene ketal (IIIa) and the 7β epimer (IIIb) derived from 11-dehydrocorticosterone; 3β-hydroxy-7α-methoxycarbonylmethyl-5-pregnen-20-one (IVa) and the 7β epimer (IVb) derived from pregnenolone; 17β-hydroxy-7α-methoxycarbonylmethyl-5-androsten-3-one 3-ethylene ketal (Va) and the 7β epimer (Vb) derived from testosterone.

RESULTS AND DISCUSSION

The TLC separation of the epimers was undertaken in various solvents (Table I) and it was found that hexane–ethyl acetate would be the most useful solvent for the analytical HPLC separation. However, the cortisone epimers (IIa and IIb) were eluted too rapidly. The separation was improved by decreasing the solvent strength and using 75:25 proportions. This mixture could not be applied to preparative or semi-preparative HPLC due to the low solubility of the compounds, the concentration of the injected sample being 100 times higher. Thus part of the ethyl acetate and

TABLE II

TLC R_F VALUES AND SELECTIVITY FACTORS, k'_α/k'_β, OF 7α- AND 7β-EPIMERS IN HEXANE–ETHYL ACETATE–DICHLOROMETHANE

Capacity factor, $k' = (1 - R_F)/R_F$.

Sample	*Hexane–ethyl acetate–dichloromethane (25:60:15)*			*Hexane–ethyl acetate–dichloromethane (30:50:30)*		
	$R_{F\beta}$	$R_{F\alpha}$	k'_α/k'_β	$R_{F\beta}$	$R_{F\alpha}$	k'_α/k'_β
I	0.39	0.33	1.30	0.39	0.34	1.24
II	0.41	0.37	1.19	0.45	0.41	1.18
III	0.12	0.11	1.10	0.10	0.09	1.12
IV	0.32	0.31	1.05	0.32	0.31	1.05
V	0.32	0.29	1.15	0.33	0.30	1.15

TABLE III

INFLUENCE OF THE ETHYL ACETATE AND DICHLOROMETHANE PROPORTIONS ON THE SELECTIVITY FACTORS, k'_α/k'_β, IN ANALYTICAL HPLC t_0 = 1.6 min

Sample: IIa and IIb. Capacity factor, $k' = (t_R - t_0)/t_0$.

Hexane–ethyl acetate–dichloromethane	$t_{R\beta}$ *(min)*	$t_{R\alpha}$ *(min)*	k'_α/k'_β
50:45:00	3.4	4.0	1.33
50:30:30	3.2	3.8	1.37
75:25:00	7.2	8.2	1.18
75:20:10	7.2	8.4	1.21

hexane was replaced with dichloromethane. The same solvent strength was maintained, but the compounds exhibited a remarkably higher solubility. TLC of the epimers with this ternary mixture (Table II) showed that the addition of a third solvent has little influence on the selectivity factors, k'_α/k'_β. Similarly, Table III shows that, at the same solvent strength, a comparable variation of the ethyl acetate and dichloromethane proportions did not significantly alter the selectivity factors in analytical HPLC. Table IV shows the conditions used for separation of the various pairs of epimers with ternary mixtures in analytical HPLC.

An attempt to separate cortisone derivatives (IIa and IIb) by preparative HPLC using the solvent system described for analytical HPLC revealed that the capacity factors, $k' = (t_R - t_0)/t_0$, were greater than 15, resulting in a poor resolution. The direct transposition of the conditions for analytical HPLC to those suitable for preparative HPLC by means of a simple relationship was shown not to be feasible, thus necessitating several trials to define the most suitable solvent mixtures. However, this study permitted the identification of solvent systems which can be used directly to perform separations in semi-preparative HPLC (Fig. 3) at sample concentrations similar those ones used in preparative HPLC. Conditions for the separation of each pair of epimers in preparative HPLC are given in Table V. All compounds were separated in 90% yield. Analytical HPLC and NMR assays revealed that the impurities were less than 2%. Table V shows also a comparison of the results of the TLC separations either with the solvent mixture used for preparative HPLC or that already found to be optimal for TLC. Use of TLC to predict suitable HPLC conditions has already been suggested[6,7]. Decreasing the solvent strength of the optimum ternary mixture for TLC allows the extrapolation of the results to preparative HPLC. The TLC/HPLC solvent-strength ratio should be of the same order for each epimer pair with a R_F range between 0.1 and 0.25.

This study of the separation of 7-methoxycarbonylmethyl steroid epimers using TLC and analytical, semi-preparative and preparative HPLC allowed the establishment of a ternary mixture of solvents suitable for the resolution of the epimer pairs within a wide range of sample concentrations injected. Among these different liquid chromatography methods, the closest analogy was found between preparative HPLC and TLC, permitting the extrapolation of the conditions used for the latter to preparative HPLC.

TABLE IV

RETENTION TIMES, t_R, AND SELECTIVITY FACTORS, k'_α/k'_β, OF 7α- AND 7β-EPIMERS IN ANALYTICAL HPLC t_0 = 1.6 min

Capacity factor, $k' = (t_R - t_0)/t_0$.

Sample	*Hexane–ethyl acetate–dichloromethane*	$t_{R\beta}$ *(min)*	k'_β	$t_{R\alpha}$ *(min)*	k'_α	k'_α/k'_β
I	70:20:10	9.8	5.12	14.8	8.25	1.60
II	75:25:00	7.2	3.50	8.2	4.12	1.18
III	25:50:25	7.0	3.37	8.8	4.50	1.33
IV	60:20:20	10.2	5.37	11.4	6.12	1.13
V	60:20:20	10.4	5.50	13.4	7.37	1.34

TABLE V

COMPARISON BETWEEN RETENTION TIMES, t_R (min), AND SELECTIVITY FACTORS, k'_α/k'_β, OF 7α- AND 7β-EPIMERS IN PREPARATIVE HPLC (t_0 = 2.55 min) AND R_F VALUES AND SELECTIVITY FACTORS IN TLC

Capacity factors, k': in TLC, $(1 - R_F)/R_F$; in HPLC, $(t_R - t_0)/t_0$.

Sample	*Preparative HPLC*				*TLC*			*TLC: optimum separation*			
	Hexane–ethyl acetate–dichloromethane	$t_R\beta$*	$t_R\alpha$*	k'_α/k'_β	$R_F\beta$	$R_F\alpha$	k'_α/k'_β	*Hexane–ethyl acetate–dichloromethane*	$R_F\beta$	$R_F\alpha$	k'_α/k'_β
I	50:30:20	19	25	1.37	0.24	0.20	1.26	25:60:15	0.39	0.33	1.3
II	50:30:20	17.2	21	1.25	0.26	0.23	1.18	25:60:15	0.41	0.37	1.19
III	20:50:30	21.8	24.7	1.15	0.10	0.09	1.12	0:100:0	0.34	0.32	1.10
IV	40:40:20	17.2	18.7	1.10	0.20	0.18	1.14	10:60:30	0.37	0.34	1.10
V	40:40:20	18	21	1.20	0.20	0.18	1.14	10:60:30	0.37	0.34	1.14

* Measured after recycling once.

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CHROM. 18 392

Note

Preparation and gel permeation chromatographic properties of pullulan spheres

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Gel permeation chromatography (GPC) in aqueous media has been achieved with the use of porous gels prepared from water-soluble natural polysaccharides, *e.g.*, dextran and agar, and synthetic polymers, *e.g.*, poly(vinyl alcohol) and poly(acrylamide)[1]. However, at high molecular weights, M_{lim}, the pressure resistance becomes a problem. For the purpose of resolving this problem, we have synthesized new packings from cellulose[2,3] and poly(γ-methyl L-glutamate)[4,5]. These gels allow remarkably high flow-rates in GPC.

Here we report the application of a new material, pullulan (Fig. 1) to GPC packings. Pullulan is a water-soluble polysaccharide first used in industry in Japan in 1978. It has been shown to be a linear polymer of maltotriose containing maltotetraose and shows no abnormality such as crystallization and gelation [6–11]. The preparation and the excellent GPC properties of pullulan spheres are also described.

EXPERIMENTAL AND RESULTS

Pullulan spheres were prepared as follows: 60 g of pullulan were suspended in 600 ml of formamide and dissolved by vigorous stirring at 540°C. A 180-ml volume of pyridine and 400 ml of acetic anhydride were added and the mixture was stirred at 50°C for 48 h. Pullulan acetate was obtained by reprecipitation from 7 l of water. It was then dissolved in dichloromethane to give a 1.1–3.7% (w/w) solution. This solution was poured into 2.0% (w/w) poly(vinyl alcohol) aqueous solution at 30°C

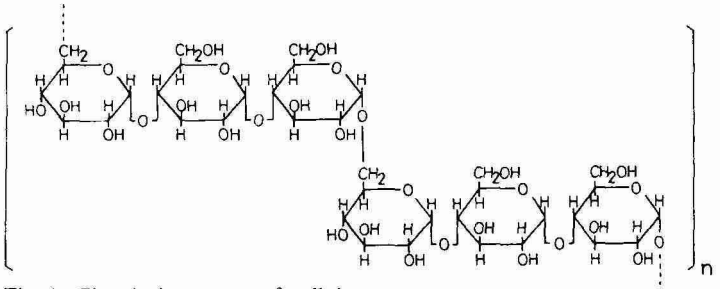

Fig. 1. Chemical structure of pullulan.

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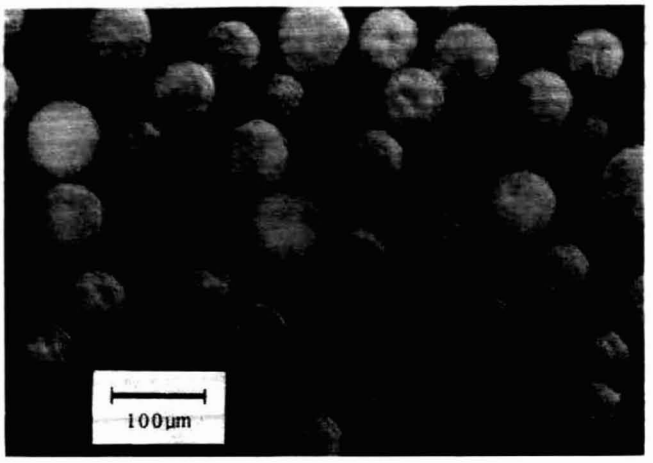

Fig. 2. Optical micrograph of pullulan spheres.

and stirred vigorously. Spheres of pullulan acetate were produced upon gradual evaporation of dichloromethane (sphering process). These beads were filtered off and then suspended in 300 ml of 10 *M* sodium hydroxide–methanol solution and stirred at room temperature for 24 h (saponification process). The saponified beads were cross-linked by epichlorohydrin in 165 ml of acetone–dimethyl sulphoxide at 60°C (cross-linking process). The total yield of the cross-linked pullulan spheres was 70–90%. In this study, the GPC properties of spheres of diameter from 44 to 105 μm were investigated. Fig. 2 shows a typical optical micrograph of the pullulan spheres prepared.

The pullulan spheres were examined in aqueous systems, packed in a glass

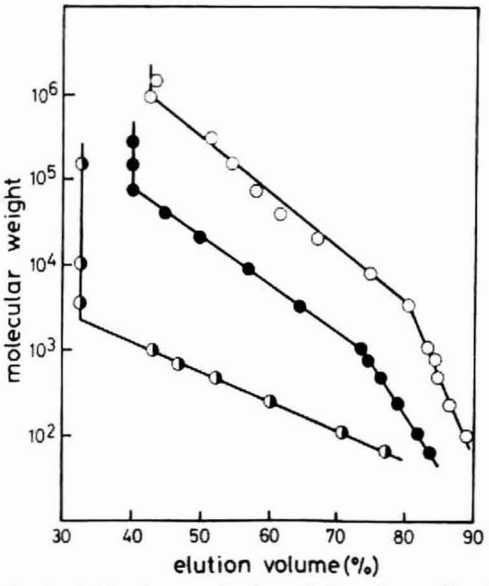

Fig. 3. Calibration graphs for pullulan spheres: ○—○, P-5; ●—●, P-3; ◑—◑, P-1.

TABLE I

PREPARATION CONDITIONS AND M_{lim} VALUES FOR PULLULAN SPHERES

Gel No.	*Polymer* concentration*	*Acetone/DMSO ratio***	M_{lim}***
P-1	3.7	1.0	2000
P-2	2.2	1.0	20 000
P-3	1.5	1.0	70 000
P-4	1.1	0.67	1 000 000
P-5	1.1	1.0	850 000
P-6	1.1	2.3	170 000
P-7	1.1	4.0	50 000
P-8	1.1	9.0	35 000

* The concentration of pullulan acetate (%, w/w) in the sphering process.
** The position of the suspension medium in the cross-linking process.
*** Excluded molecular weight.

column (30 cm × 5 mm I.D.). Poly(ethylene glycol) was used as a standard sample. As shown by the calibration curves in Fig. 3, pullulans show typical GPC behaviours. The exclusion limits, M_{lim}, are summarized in Table I. The value of M_{lim} varied according to the preparation conditions, for example, the concentration of pullulan acetate and the composition of suspension media in the sphering and the cross-linking processes, respectively; it is also related to the pore size of the network of pullulan spheres. Consequently, it is believed that the pullulan content in the media has direct effects upon the formation of the pullulan network.

By adjusting the preparation conditions, pullulan spheres with M_{lim} from 10^3

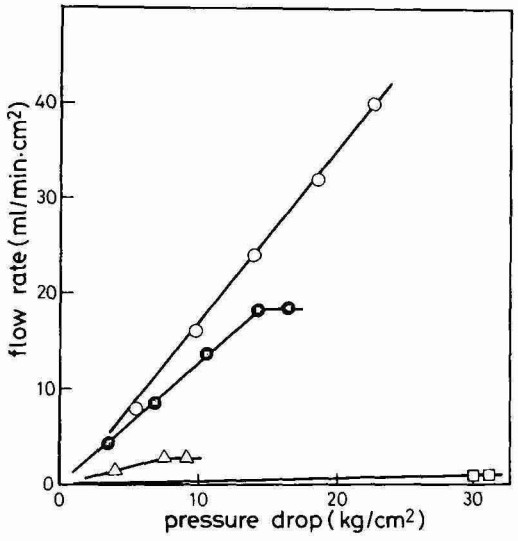

Fig. 4. Relationship between the flow-rate and the pressure drop. ○—○, Pullulan spheres P-7 (M_{lim}, $5 \cdot 10^4$); ●—●, Sephadex G-50m (M_{lim}, $1 \cdot 10^4$); △—△, Sephadex G-150m (M_{lim}, $15 \cdot 10^4$); □—□, Bio-Gel P-30 (M_{lim}, $5 \cdot 10^4$).

to 10^6 can be produced. Usually, macroporous gels with large M_{lim} such as 10^6 are manufactured by using a diluent in the sphering process[2–5]. However, for pullulan spheres, values of M_{lim} up to 10^6 can be obtained even in the absence of a diluent. This is a noteworthy characteristic of pullulan material.

High-speed GPC is desired in aqueous systems. In this context, the pressure resistance is an important property. Fig. 4 shows the relationship between the flow-rate and the pressure drop, compared with the behaviour observed for Sephadex gels and Bio-Gel. All measurements were made by use of a 15 cm × 4 mm I.D. metal column packed with spheres ranging from 44 to 105 μm in diameter. The straight line in Fig. 4 indicates that these pullulan spheres form rigid and stable packing materials for high-pressure chromatography.

It was confirmed that there are no problems in using pullulan spheres for GPC. For example, the packing exhibits almost no interactions (hydrophobic interactions) with sample substrates and can be used over a wide range of pH because it is not ionic.

In conclusion, this study is the first example of the sphering of pullulan and its application to GPC as a packing material. Pullulan spheres give a wide range of M_{lim} and have good properties, such as high flow-rates and no interaction with substrates, for chromatography.

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CHROM. 18 391

Note

Gel chromatography of pyridoxalated and glutaraldehyde-treated human haemoglobin on Superose™ 12

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The characterization and batch monitoring of chemically modified haemoglobins (Hb), under investigation as possible oxygen-carrying and offloading infusable fluids, needs sensitive methods of analytical fractionation. Rapid micromethods are usually preferred[1–3].

In the present work we used a recently developed variant of gel chromatography on monodisperse microbeads of Superose™ 12 (Pharmacia) with a fast protein liquid chromatography (FPLC) apparatus (Pharmacia)[4]. The results are compared with those achieved by gel chromatography on Sepharose 6B. Irregularities and discrepancies observed during gel chromatography of the labile haemoglobin preparations are discussed as well as certain implications concerning the retention times of haemoglobins in the bloodstream.

MATERIALS AND METHODS

Human stroma-free haemoglobin (SFH) and its derivatives (PHIR-PG) modified with pyridoxal-5'-phosphate, borohydride and glutaraldehyde were prepared as before[2]. The samples, usually containing 60 g Hb/l, were filtered through a 0.22-μm sterile Millipore filter prior to application to an HR 10/30 chromatographic column prepacked with Superose™ 12 (ref. 4). A 0.05 *M* phosphate buffer pH 7.0 containing 0.15 *M* sodium chloride was degassed, filtered through a sterile 0.22-μm filter and used for equilibration of the column and elution of the SFH samples. The chromatographic column was connected to the Pharmacia FPLC system and separations were achieved according to the instruction manual[4]. A gel-filtration calibration kit of standard proteins for molecular weight determination (Pharmacia) was used. Standard gel chromatography was performed on columns of Sepharose 6B (55 × 1.7 cm) and Sephacryl S-200 (70.5 × 1.7 cm) at a flow-rate of 0.13 ml/min.

RESULTS AND DISCUSSION

Fig. 1 shows a typical elution pattern of a mixture of standard native proteins

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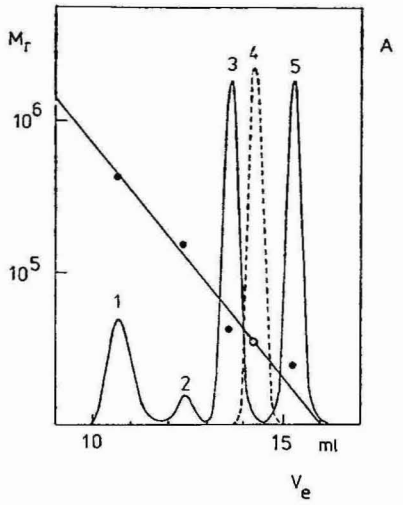

Fig. 1. Elution profiles of proteins and calibration curve for M_r estimation on Superose™ 12. Curves: 1, ferritin; 2, aldolase; 3, ovalbumin; 4, native human haemoglobin (separate experiment); 5, chymotrypsinogen. Buffer: 0.15 *M* sodium chloride, 0.05 *M* sodium phosphate pH 7.0. Flow-rate : 0.4 ml/min. *A* = Absorbance at 280 nm.

on Superose™ 12. The haemoglobin samples were chromatographed separately under the same conditions. Repeated experiments led to very sharp and reproducible separations. The plots of V_e (elution volume) *versus* log M_r (relative molecular mass) at the two flow-rates used, 0.4 and 0.9 ml/min, were identical. The separation on Superose™ 12 usually required about 25 min in contrast to 13–15 h on Sepharose 6B. Moreover, the peaks were markedly more distinct on Superose™ 12. The V_e/log M_r relationship was not exactly linear with the given calibration kit, evidently due to the effects of factors other than M_r, *e.g.*, molecular size, shape and various interactions with the gel matrix which influenced the V_e values. The haemoglobin tetramer Hb_4 (M_r 64 500) is known to dissociate in aqueous solution into subunits of lower M_r. The statistical ratio of dissociated to undissociated haemoglobin molecules at equilibrium in a given buffer solution depends on the Hb concentration[5]. Therefore, gel chromatography of haemoglobin on either Sephadex G-100 (ref. 5), Sepharose 6B (ref. 2) or Superose™ 12 led always to higher V_e values than expected[5]. However, when a standard amount of native haemoglobin (either freshly prepared or stored dry with sucrose[2]), *e.g.*, 1 mg in 0.1 ml, was repeatedly applied to a given column and chromatographed under standard conditions, reproducible V_e values were achieved for the haemoglobin moiety, corresponding to a mean M_r of 37 000. In the above sense, haemoglobin can be used as a coloured substance for M_r calibration.

An irregularity was observed repeatedly during gel chromatography of native haemoglobin on columns of Sephacryl S-200. In parallel experiments, where haemoglobin was chromatographed on Sepharose 6B and Superose™ 12 columns without any problems, on Sephacryl S-200 the haemoglobin was denatured spontaneously after about 1 h, flocculated in the column and the whole chromatographic procedure deteriorated. In contrast to native haemoglobin, the modified samples (PHIR-PG)

were chromatographed without problems under the same conditions on the same Sephacryl S-200 column. The reason might be either the stabilization of Hb molecules of PHIR-PG by glutaraldehyde, *cf.*, Fig. 3, or hydrophobic interactions of the native haemoglobin with the cell matrix of Sephacryl S-200, leading to denaturation.

The elution curve 1 in Fig. 2 indicates the presence of three main molecular populations in modified haemoglobin of the type PHIR-PG. The peaks correspond to M_r 240 000 (22%), 120 000 (23%) and 43 000 (55%) which indicates roughly the prevalence of polycondensates Hb_{16}, Hb_8 and of subunits Hb_2 + Hb_3 (in ratio *ca.* 2:1). Intramolecularly stabilized tetramers Hb_4 (Fig. 3) which are also present in modified haemoglobins, according to other analytical methods[1,6–8], are believed to be hidden between the two higher peaks.

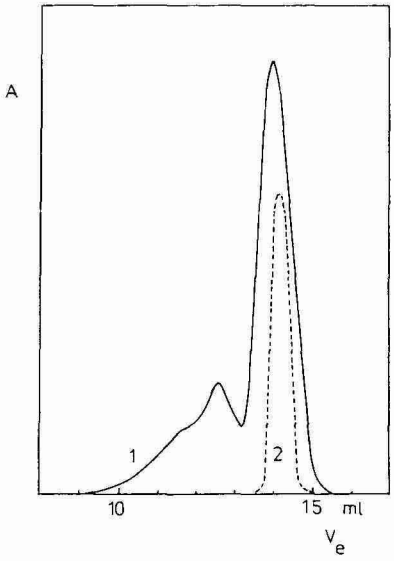

Fig. 2. Gel chromatography of haemoglobin samples on Superose™ 12. Curves: 1, human haemoglobin treated with pyridoxal-5'-phosphate, glutaraldehyde and borohydride (PHIR-PG); 2, native human stroma-free haemoglobin (SFH). Other conditions as in Fig. 1. *A* = Absorbance at 280 nm.

The fact that the biggest peak (55%) of PHIR-PG and the peak of native haemoglobin had very similar elution volumes was felt to be a discrepancy since in biological experiments[7] (*cf.*, ref. 6) PHIR-PG and native haemoglobin differed markedly in their half retention times ($T/2$) in the bloodstream of rats. After infusion of haemoglobin solutions, PHIR-PG showed a $T/2$ of 11–18 h while native haemoglobin showed $T/2$ = 1.5–2 h due to dissociation of Hb_4 into subunits and their rapid renal filtration. The prolonged $T/2$ of modified polycondensed haemoglobin (PHIR-PG) was not surprising. More puzzling was the high amount of Hb_2 and Hb_3 in PHIR-PG during gel chromatography as well as of Hb_1 in other experiments, where sodium dodecyl sulphate (SDS)-treated PHIR-PG was analyzed by gel permeation techniques[1,6,8].

An explanation could be found in terms of the very simple scheme shown in

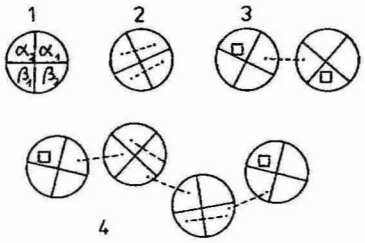

Fig. 3. A simplified scheme of modified haemoglobin molecules assumed to be present in PHIR-PG samples. Circles: haemoglobin tetramers Hb_4. Squares: bound pyridoxalphosphate. Dashed lines: glutaraldehyde cross-links. 1, Native tetramer Hb_4 with subunits; 2, intramolecularly cross-linked Hb_4 (after full dissociation, two "true" dimers would appear); 3, pyridoxalated Hb_8 with one intermolecular cross-link (after dissociation, two pyridoxalated and four intact monomers as well as one cross-linked "untrue" dimer would appear); 4, pyridoxalated cross-linked Hb_{16}, assumed to form after full dissociation of two pyridoxalated and five intact monomers, one "untrue" trimer, two "untrue" and one "true" dimers.

Fig. 3. Hypothetically, some of the Hb molecules and subunits, which have been altered by the reaction with pyridoxal-5'-phosphate and covalently bound by glutaraldehyde, do not dissociate, while those remaining intact are able to dissociate (partly in water and fully in SDS-containing media) and can enter the gel cavities or pass through the gel network during gel permeation. Moreover, about 10–20% of haemoglobin molecules in modified Hb are known to be unaffected by the chemicals added. A different treatment of chemically modified haemoglobin molecules and subunits, even if they are of the same size as the native ones, in the kidney must be assumed, too.

In conclusion, gel chromatography of proteins on Superose™ 12 proved most useful in shortening significantly the analysis time and in sharpening the elution peaks in comparison with gel chromatography on, *e.g.*, Sepharose 6B. Gel chromatography on Superose™ 12 together with isoelectric focusing remain the most suitable methods for a direct characterization of the molecular heterogeneity of intact haemoglobin products, unchanged by a supplementary addition of denaturing agents, *e.g.*, SDS[2,6–8] or 4-hydroxymercuribenzoate[3].

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Note

Three methods for quantitative determination of the ligand in Phenyl-Sepharose CL-4B

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Phenyl-Sepharose CL-4B is a derivative of the cross-linked agarose gel Sepharose CL-4B. The phenyl groups are coupled to the gel matrix via the reaction of phenyl glycidyl ether with Sepharose CL-4B. The partial structure is:

$$\text{Sepharose CL-4B–O–CH}_2\text{–CH(OH)–CH}_2\text{–O–R}$$

where R is the phenyl group. Phenyl-Sepharose CL-4B is a gel intended for use in hydrophobic interaction chromatography (HIC), mainly for the separation and purification of proteins and peptides[1–9].

Quality control of the ligand content of different HIC gels demands simple methods with high precision and accuracy[10]. Furthermore, a knowledge of the ligand density is important for retention model studies[11]. In this paper we describe three independent methods for the quantitative determination of the ligand content in Phenyl-Sepharose CL-4B. The methods are based on UV spectrophotometry and ^{1}H NMR spectrometry and analysis of the carbon content of unsubstituted and substituted Sepharose CL-4B. Recently, Genieser *et al.*[12] described a gas chromatographic method.

EXPERIMENTAL

Chemicals and apparatus

Phenyl-Sepharose CL-4B and Sepharose CL-4B were obtained from Pharmacia, Uppsala, Sweden. Deuterium chloride and [^{2}H]dimethyl sulphoxide (DMSO-d_6; isotopic purity greater than 99.5%) were from Ciba-Geigy, Basel. Hydrochloric acid, acetone, methanol and phenol were of *pro analysi* quality, whereas 2-phenoxyethanol was of purum quality.

A Shimadzu spectrophotometer UV-240 with a graphic printer PR-1 and a 1-cm cell was used for spectrophotometric measurements. The ^{1}H NMR spectra were recorded with a Jeol Fx 200 instrument at 199.5 MHz. In the pulsed NMR experiments the number of pulses was 100, the pulse time 7 μs, the pulse delay 20 s, the acquisition time 2 s, and the delay between pulse and acquisition 50 μs. The spectral range explored was 2000 Hz.

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Sample pretreatment

About 5 ml of homogenized Sepharose CL-4B or Phenyl-Sepharose CL-4B were transferred to a glass filter funnel (G3). The gel was washed first with 5 × 5 ml of distilled water and then with 2 × 5 ml of water–acetone (1:1). The gel was shrunk with 5 × 5 ml of acetone: after each addition it was homogenized and sucked dry with a water pump. Finally, the gel was dried at 105°C for 15 h.

The degree of substitution by UV spectrophotometry

Hydrolysis and preparation of the sample solution. The dried gel (20 mg) was hydrolysed at 20°C with 1.00 ml of concentrated hydrochloric acid for 10 min. The hydrolysed gel was diluted with methanol to 10.0 ml. This solution was further diluted 20 times with methanol before the absorbance was registered at λ_{max} (270.5 nm).

Determination of the molar absorptivity of phenoxyethanol. Four standard solutions of phenoxyethanol in the concentration range 0.09–0.31 m*M* were used in the determination of the molar absorptivity (ε). In accordance with the gel sample solution phenoxyethanol was dissolved in methanol acidified with 0.06 *M* hydrochloric acid.

The degree of substitution by ^1H NMR spectrometry

The dried gel (20 mg) was hydrolysed with 200 μl of 6.3 *M* deuterium chloride at 70°C for 45 s and then cooled in an ice-bath. The sample was diluted with 2.00 ml of DMSO-d_6, and from this mixture two aliquots of 1.00 ml were taken. To one of the aliquots 100 μl of DMSO-d_6 were added, and to the other 100 μl of DMSO-d_6 containing 14.9 μmol of phenol were added. NMR spectra of these solutions were registered. The peaks from the aromatic protons were integrated, and the peak from the isotopic impurities of DMSO-d_6 served as internal standard (Fig. 1). Standard solutions of phenol, in the concentration range 4–17 m*M*, were registered in the same way. The relaxation times were measured by the inversal recovery method[13].

The degree of substitution by carbon analysis

Phenyl-Sepharose CL-4B and its corresponding unsubstituted Sepharose CL-4B were pretreated as above. The carbon content was determined in both gels.

Calculation. The amount of phenyl glycidyl ether (*X*) in milligrams coupled to 100 mg Sepharose CL-4B has been calculated from the following equation:

$$Z = \frac{100\left(Y + \frac{108.10}{150.18}X\right)}{100 + X} \tag{1}$$

Conversion of this equation gives

$$X = \frac{100(Z - Y)}{71.98 - Z} \tag{2}$$

where *Y* and *Z* are the degrees of carbon content in weight percent of Sepharose CL-4B and Phenyl-Sepharose CL-4B, respectively, 150.18 g/mol is the molecular weight of the phenyl glycidyl ether and 108.10 g/mol is the weight of the carbon

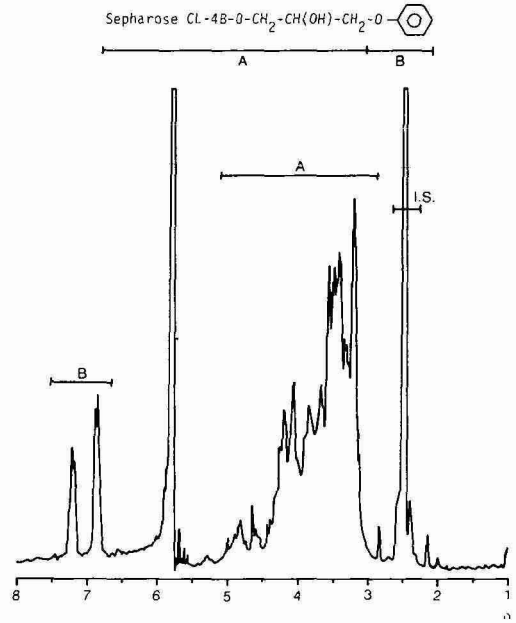

Fig. 1. ^1H NMR spectrum of partially hydrolysed Phenyl-Sepharose CL-4B in DMSO-d_6. The small amount of DMSO containing ^1H was used as internal standard (I.S.).

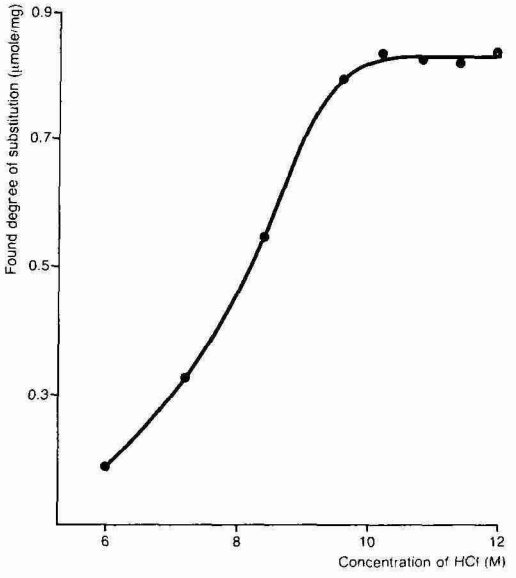

Fig. 2. Dependence of the spectrophotometrically found ligand content in Phenyl-Sepharose CL-4B on the concentration of hydrochloric acid used in the hydrolysis step.

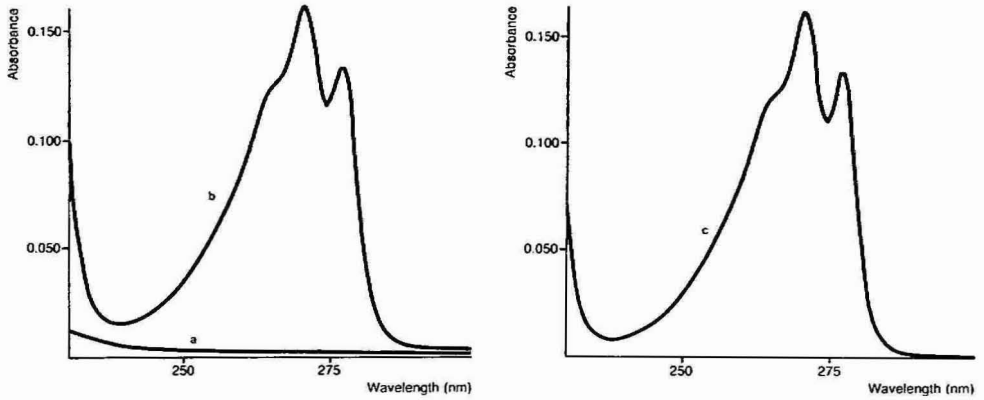

Fig. 3. UV spectra of hydrolysed Sepharose CL-4B (a) and Phenyl-Sepharose CL-4B (b) and of 0.10 m*M* 2-phenoxyethanol (c). For details see Experimental section.

atoms in the same ether. From this it follows that the degree of substitution (*S*) can be written as:

$$S = \frac{X \cdot 10^3}{150.18\ (100 + X)}\ \mu\text{mol/mg} \qquad (3)$$

RESULTS AND DISCUSSION

Ultraviolet spectrophotometry

UV-absorbing ligands covalently coupled to a gel matrix can be quantified spectrophotometrically if a solubilizing medium is chosen [14]. In this method we have used hydrochloric acid to cleave the glycosidic linkages in Sepharose CL-4B. More specifically, 10 *M* hydrochloric acid is required for the total dissolution of the dried gel in methanol (Fig. 2). A spectrum of hydrolysed Phenyl-Sepharose CL-4B is depicted in Fig. 3, which also shows that the interference from the gel matrix is low.

TABLE I

DEGREE OF SUBSTITUTION ON DIFFERENT BATCHES OF PHENYL-SEPHAROSE CL-4B DETERMINED BY THREE METHODS

Year of production	*Degree of substitution (μmol/mg dry gel)**		
	*Spectrophotometry***	*^1H NMR (spectroscopy****	*Carbon analysis§*
1981	0.80 ± 0.05	0.89 ± 0.04	0.83 ± 0.11
1983	0.89 ± 0.03	0.93 ± 0.04	0.89 ± 0.22
1983	0.86 ± 0.05	0.88 ± 0.04	0.83 ± 0.22

* Values reported with a confidence interval of 95%.
** Pooled S.D. = 0.033 (degrees of freedom = 42).
*** Pooled S.D. = 0.017 (degrees of freedom = 3).
§ Pooled S.D. = 0.14 (degrees of freedom = 9).

TABLE II

SPECTROPHOTOMETRIC DETERMINATION OF THE LIGAND ON DIFFERENT BATCHES OF PHENYL-SEPHAROSE CL-4B

Year of production	*Batch No.*	*Degree of substitution (μmol/mg dry gel)**	*Year of production*	*Batch No.*	*Degree of substitution (μmol/mg dry gel)**
1977	9705	0.93 ± 0.05	1983	28 567	0.83 ± 0.05
1979	8565	0.90 ± 0.05	1983	32 351	0.89 ± 0.03
1979	12 514	0.81 ± 0.05	1983	33 555	0.86 ± 0.03
1980	16 110	0.88 ± 0.03	1983	33 777	0.86 ± 0.05
1980	18 581	0.80 ± 0.05	1984	35 016	0.80 ± 0.02
1981	19 418	0.80 ± 0.05	1985	38 578	1.01 ± 0.05
1982	19 419	0.93 ± 0.03	1985	00 323	0.97 ± 0.02
1982	28 566	0.80 ± 0.05			

* Values reported with a confidence interval of 95% calculated with a pooled S.D. = 0.033 (degrees of freedom = 42).

Furthermore, the spectrum of bonded phenyl groups coupled to the gel with the corresponding glycidyl ether is very similar to that of free 2-phenoxyethanol (Fig. 3). The molar absorptivity (ε) of 2-phenoxyethanol was determined to $1.76 \cdot 10^3\ M^{-1}\ cm^{-1}$. This value was used in the calculation of the degree of substitution. The results from fifteen different batches, produced between 1977 and 1985, are presented in Tables I and II.

^1H NMR spectroscopy

The hydrolysis procedure of Phenyl-Sepharose CL-4B, described above, solubilizes the gel in DMSO-d_6. The hydrolysed gel can be characterized as short polygalactanes having ether-linked substituents. A typical NMR spectrum of such a solution is shown in Fig. 1. For the determination of the amount of phenyl groups the NMR signal from the aromatic protons are integrated and summarized. The reliability of the standard addition method has been verified by showing that the calibration graph of phenol was linear and had an intercept at the origin. Furthermore, the pulse delay has been chosen so that the aromatic protons had time to relax towards their equilibrium value. The importance of the operating parameter pulse delay is illustrated by the fact that the relaxation times of the phenyl protons are shorter when the phenyl group is coupled to the gel (*ca.* 1.0 s) compare with free uncoupled phenol molecules (*ca.* 6.7 s). The reliability of the integrated absorption signals has further been confirmed by showing that unsubstituted Sepharose CL-4B does not interfere with the evaluation of the peak areas.

The degree of substitution was determined on three different batches (Table I).

Carbon analysis

The degree of substitution was determined by carbon analysis on three batches of Phenyl-Sepharose CL-4B (Table I). The greater uncertainty of this method com-

pared with the two others can be deduced from the fact that the carbon contents in unsubstituted and substituted Sepharose CL-4B are of the same magnitude[10].

CONCLUSION

An important consideration in the selection of an analytical method is the accuracy and the precision of the result. Comparison of the phenyl content in Phenyl-Sepharose CL-4B determined by the three methods described shows that they consistently yield results showing only random differences at a 95% confidence level (Table I). Therefore it is concluded that no significant systematic errors are present. However, the higher precision of the NMR method and the UV method (Table I) makes these methods more reliable for quality control of the ligand content. The UV method is now used in the authors' laboratory, mainly because of the simpler instrumentation involved.

In this study the ligand density is determined on a dried gel. Compared with sampling of a certain settled gel volume this procedure is simpler and more reliable[10,15]. However, if a rough estimate of the density is wanted per millilitre of settled gel the dry weight of Phenyl-Sepharose CL-4B can be used (*ca.* 32 mg/ml). Moreover, from the molecular weight of phenyl glycidyl ether (150 g/mol) and anhydrodisaccharide unit of agarose (306 g/mol), it can easily be calculated that a ligand density of 0.90 μmol/mg dry gel means that one phenyl group is linked to a hexasaccharide unit. However, this calculation does not take account of the contribution from the cross-linker.

ACKNOWLEDGEMENT

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CHROM. 18 433

Note

Isolation of artemisinin (qinghaosu) and its separation from artemisitene using the Ito multilayer coil separator–extractor and isolation of arteannuin B

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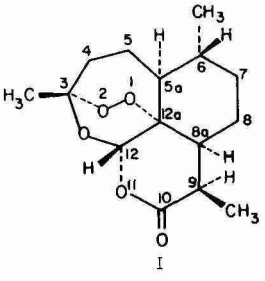
The isolation of the known endoperoxide artemisinin (qinghaosu, I), a promising antimalarial agent[1], from *Artemisia annua* collected in the United States has been reported[2]. Recently, we isolated from *A. annua* a new sesquiterpene endoperoxide, artemisitene (II), which was separated from artemisinin by repeated normal-phase high-performance liquid chromatography (HPLC)[3]. Another sesquiterpene found in *A. annua*, arteannuin B (III), may be of biosynthetic importance in the production of artemisinin. This compound has been identified[4], but the procedure for obtaining it from the plant has not been published.



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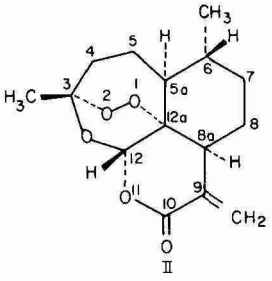

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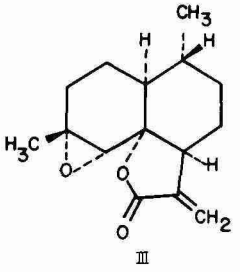

III

Isolation of artemisinin involves chromatography of the crude plant extract on silica gel with chloroform–ethyl acetate as eluent[2]. Because artemisinin and artemisitene co-chromatograph on silica gel and co-crystallize from widely different solvent systems, crystalline samples of artemisinin frequently contain as much as 10% of artemisitene. The difficulty and expense of this purification procedure for large-scale production of artemisinin, as well as the contamination of the material so obtained, led us to search for an alternative isolation procedure.

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We describe here: the use of the Ito multilayer coil separator–extractor to achieve the isolation of artemisinin and arteannuin B from *A. annua* extracts, the use of this apparatus to separate artemisinin from artemisitene and the isolation of arteannuin B from *A. annua* by chromatography on silica gel.

EXPERIMENTAL AND RESULTS

Apparatus

An Ito multilayer coil separator–extractor (P.C. Inc., Potomac, MD, U.S.A.) was equipped with a No. 10 gauge (2.6 mm I.D.) coil, volume 400 ml. The stationary phase was isooctane–ethyl acetate (7:3), and the mobile phase consisted of methanol–water (6:4). Stationary and mobile phases were equilibrated before use.

Isolation of artemisinin and arteannuin B from A. annua

The top half of *A. annua* plants, grown in Silver Spring, MD, U.S.A., were harvested in August 1985. The center stalk was removed, and the plant material, which was air dried and coarsely ground (122.7 g), was extracted with light petroleum as previously described[2]. HPLC assay with electrochemical detection (ED)[5] of the extract showed the presence of 150 mg (0.12% yield) of artemisinin. The crude extract was taken up in *ca.* 15 ml of a 1:1 mixture of mobile and stationary phases for injection.

The coil was filled with stationary phase, then the entire samples was introduced at once. The coil was rotated at 800 rpm, and mobile phase was pumped through at *ca.* 2 ml/min. Those fractions containing artemisinin (elution volume *ca.* 400 ml), as determined by HPLC–ED, were combined, and the methanol was removed under reduced pressure at 35°C. The remaining aqueous suspension was extracted with diethyl ether (4 ×), the ether solution was dried ($MgSO_4$) and the diethyl ether evaporated. Crystallization of the residue from 4 ml of cyclohexane afforded 123 mg (82% recovery) of artemisinin which, after a second recrystallization, melted at 153–154°C (*cf.* ref. 6: m.p. 156–157°C); the IR spectrum was identical with that of pure material.

Earlier fractions (elution volume 200 ml) contained arteannuin B as determined by HPLC (UV detection at 220 nm), as well as artemisitene (ED). They were combined and worked up as for artemisinin (see above). Triturating the oily residue with ether afforded 83 mg of crystalline arteannuin B, m.p. 148.5–150°C (*cf.* ref. 4: m.p. 152°C) with IR and NMR spectra identical with those reported[4]. Artemisitene remained in the mother liquor, from which it could be isolated in trace amounts (< 1 mg) by silica gel chromatography, eluting with 5% ethyl acetate in chloroform.

Separation of artemisinin from artemisitene

The apparatus and conditions were identical to those described above. The crystalline sample of artemisinin (590 mg) containing *ca.* 10% of artemisitene was introduced in 8 ml of ethyl acetate–methanol–isooctane (3:3:2). When the mobile phase began to be eluted (after 50 ml), fractions of 7 ml each were collected. Artemisitene was eluted in fractions 14–22. These were combined, the organic solvent was removed at 35°C, and the aqueous residue was extracted with methylene chloride (3 ×). After drying ($MgSO_4$), the solvent was evaporated, and the residue recrystallized

from diethyl ether–light petroleum to yield 54 mg (91% recovery) of artemisitene, m.p. 163.5–164.5°C (*cf.* ref. 3: m.p. 161–162°C). Artemisinin was eluted in fractions 35–55. After workup as for artemisitene (see above) recrystallization from diethyl ether–light petroleum afforded 438 mg (82% recovery) of artemisinin, m.p. 150 152°C.

Isolation of arteannuin B by column chromatography

Dried *A. annua* (West Virginia, October 1984, 40.2 kg) was extracted by covering the plant material with Freon TF (1,1,2-trichloro-1,2,2-trifluoroethane) in two 55-gallon drums for 165 h. After evaporation of the solvent, 1.22 kg of residue remained. This was taken up in methylene chloride (450 ml), and waxes (654 g), precipitated by addition of 4 l of acetonitrile, were removed by filtration. Evaporation of solvent left 350 g of residue, which was adsorbed on 400 g of silica gel (E. Merck, silica gel 60). This was placed on top of a column containing 5.25 kg of silica gel, and the column was eluted with methylene chloride–ethyl acetate (25:1). Fractions containing arteannuin B (elution volume 19–23 l) were combined. After removal of solvents, arteannuin B (8.4 g) was obtained by crystallization of the residue (39 g) from cyclohexane. A second extraction (46 h) followed by chromatography gave an additional 2.7 g of arteannuin B, for a total yield of 0.027% of material identical with that described above (*Isolation of artemisinin and arteannuin B from A. annua*), m.p. 152–154°C.

DISCUSSION

Contamination of artemisinin with varying amounts of artemisitene is a complication and a potential hindrance in its development as an antimalarial drug. Our ability to effect a wide separation of artemisinin and artemisitene on the Ito apparatus means that artemisinin can be obtained free of artemisitene on a reasonably large scale and in a short time. Moreover, artemisitene can be isolated in sufficient quantity for further chemical transformations. For this separation of artemisinin from artemisitene, the Ito apparatus is superior to HPLC because considerably larger amounts can be injected and because the recovery is better than that obtained by normal-phase HPLC.

The stationary phase mixture used in the Ito multilayer coil separator–extractor is readily recovered by flash distillation on a rotary evaporator. The mobile phase, consisting of methanol and water, is the only material consumed in this isolation of artemisinin and arteannuin B from extracts of *A. annua*. Both aspects make this a very economical procedure. We are now trying to develop methods that would allow the isolation of multi-gram quantities of artemisinin from plant extract using the Ito apparatus.

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CHROM. 18 386

Note

Chromatographic separation and determination of tryptophan in foodstuffs after barytic hydrolysis using Fractogel TSK HW 40 S

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(Received December 2nd, 1985)

A knowledge of the total tryptophan content of food products is of major importance because tryptophan is an essential amino acid for man and monogastric animals. The degradation of the tryptophan molecule as a result of hydrolysis by hydrochloric acid requires the use of alkaline media[1], such as sodium hydroxide[2,3] or barium hydroxide[4], which implies a separation of tryptophan by a method other than ion-exchange chromatography. The destruction of tryptophan in the sodium hydroxide hydrolysates after acidification[3] led us to choose barytic hydrolysis and to investigate new supports for the adsorption chromatography of tryptophan.

The use of carbohydrate gels such as starch gel[2] or Sephadex G-25F[4] described in the earliest publications requires a considerable time to obtain a fair chromatographic separation. Sephadex G-10 has been used to study the complete composition of wheat grains as a function of nitrogen content[5]. However, such supports have a wide particle size distribution. Nowadays, soft supports are available, based on different chemical natures. We have deliberately excluded the study of expensive high-performance liquid chromatographic supports (*e.g.*, C_{18} silica gels) and focused on gels for low-pressure chromatography. In addition to dextran gels, modern supports such as Bio-Gel P-2 (ref. 6) and Trisacryl GF 05 (ref. 7) are known to adsorb tryptophan. Fractogel TSK HW 40 S has also been reported by the manufacturer[8] to be a potential support for the adsorption chromatography of tryptophan in pure water.

In this paper, we describe a comparison of the chromatographic parameters of tryptophan obtained using several gels: Fractogel TSK HW 40 S, Fractogel PGM 2000, Sephadex G-25 Superfine, Sephadex G-10, Sephacryl S-200 Superfine, Sephasorb HP Ultrafine, Trisacryl GF 05, Ultrogel AcA-202 and Bio-Gel P-2. Of these gels, the two Fractogels clearly exhibited the best resolution factor and the smallest peak width, giving a higher sensitivity and better accuracy in the determination of tryptophan. An *o*-phthaldialdehyde fluorimetric detection method adapted from Roth[9] was used. Under these conditions the recovery of protein tryptophan reaches 94 ± 1.7% in foodstuffs and 98 ± 1% in purified proteins.

EXPERIMENTAL

The samples used were either hen egg white lysozyme (Merck, 17 000 units

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mg^{-1}), with a purity of 90.4% determined by spectrophotometry[10], Kjeldahl nitrogen determination and amino acid analysis, or plant flours and ground foodstuffs, with lysozyme as an internal standard.

Barytic hydrolyses were performed according to Slump and Schreuder[4] using 100–300 mg of flour with 4.2 g of barium hydroxide octahydrate and 8 ml of Milli-Q water in 30-ml polymethylpentene plastic tubes. The samples were heated in an autoclave, in the absence of oxygen, for 16–18 h at 125°C (1.4 bar). The hydrolysates, chilled in an ice-bath, were acidified with 3 *N* hydrochloric acid to pH 3.5 using a Metrohm pHstat, diluted to 50 ml with water, then centrifuged at 14 000 *g*.

External calibrations were conducted with Calbiochem A-grade L-tryptophan, the purity of which was measured by spectrophotometry using a molar asbsorptivity of 5670 mol l^{-1} cm^{-1} at 279 nm and pH 3[11]. About 0.1 ml was injected on to the column using a Cheminert 20 PTFE sample loop.

A 300 × 9 mm I.D. Altex glass column was utilized, filled to 200 mm with one of the following gels: Fractogel TSK HW 40 S, Fractogel PGM 2000, 32–63 μm (Merck); Sephadex G-25 Superfine, Sephadex G-10, Sephacryl S-200 Superfine and Sephasorb HP Ultrafine (Pharmacia); Trisacryl GF 05 and Ultrogel AcA-202 (IBF); and Bio-Gel P-2, 200–4000 mesh (Bio-Rad Labs.).

Elutions were performed at a flow-rate of 30 ml h^{-1} (Milton-Roy Minipump) with pH 3.25 citrate buffer[12], in which phenol was replaced with caprylic acid, at room temperature. The eluate was mixed with the *o*-phthaldialdehyde (OPA) reagent (0.5 g of OPA dissolved in 5 ml of 95% ethanol, to which were added 2 ml of 2-mercaptoethanol and 2 ml of 30% Brij 35 solution, followed by dilution to 1 l with 1 *M* borate buffer, pH 10.6) delivered at the same flow-rate. After reaction for 90 s the effluent was monitored through a 2-mm cell with an Aminco fluoro-monitor, using a Corning 7-51 excitation filter and a Wratten 2A emission filter. The signal was recorded and integrated using a Spectra-Physics Autolab AA.

RESULTS AND DISCUSSION

In Fig. 1 are compared the chromatographic elution profiles obtained with Fractogel HW 40 S, Sephadex G-25, Sephadex G-10 and Bio-Gel P-2. These profiles exhibit two peaks: the first eluted corresponds to amino acids and primary amines reacting with OPA and the second to tryptophan. The profiles obtained with Fractogel PGM 2000, Sephasorb HP Ultrafine and Trisacryl GF 05 are comparable to the Fractogel HW 40 S, Sephadex G-10 and Bio-Gel P-2 profiles, respectively. It is clear that only the Fractogels and Sephadex G-10 allow a fair separation of tryptophan and that the peak obtained with Sephadex G-10 is far wider than that with Fractogel HW 40 S.

Table I illustrates the chromatographic parameters determined for all the supports. Two sets of gels can be identified when comparing the resolution coefficients: the gels with $R_s > 1.1$ give a good resolution (Fractogel HW 40 S, Fractogel PGM 2000, Sephasorb HP Ultrafine and Sephadex G-10), whereas the others are not able to separate tryptophan under these conditions. Nevertheless, the adsorption constants show that all the supports adsorb the tryptophan to various extents under pH and ionic strength conditions when no ion exchange is possible. Fractogel HW 40 S does not exhibit a greater K_{av} than the other gels giving a satisfactory resolution.

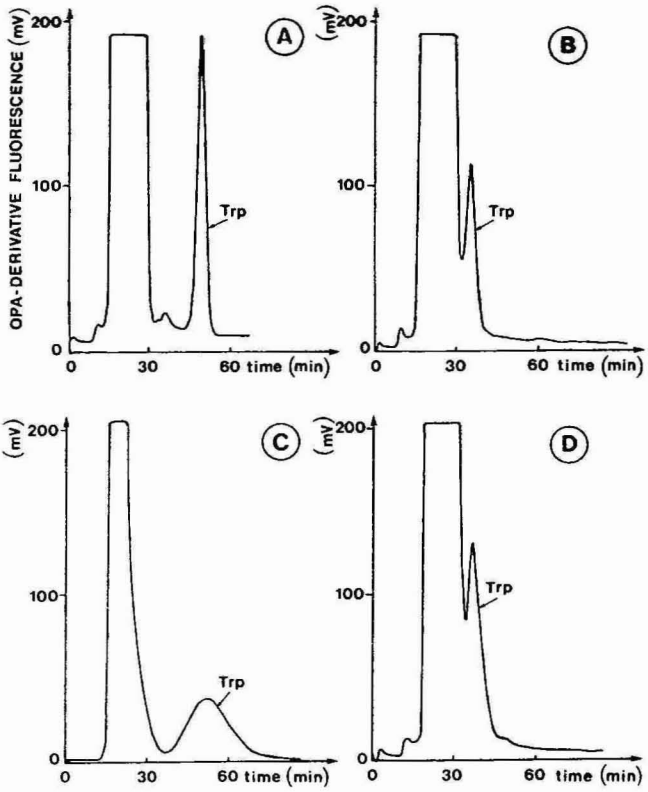

Fig. 1. Elution profiles of tryptophan after barytic hydrolysis of wheat flour. Separation on (A) Fractogel HW 40 S; (B) Bio-Gel P-2; (C) Sephadex G-10; (D) Sephadex G-25 Superfine. OPA fluorimetric detection (excitation wavelength 366 nm; emission wavelength >410 nm). The same flour sample was used with the four gels. Gel bed, 20 × 0.9 cm; flow-rate, 30 ml h^{-1}.

TABLE I

SEPARATION PARAMETERS OF TRYPTOPHAN USING DIFFERENT SOFT GELS

T = retention time; $W_{1/2}$ = peak width at half-height; K_{av} = adsorption constant; R_s = resolution coefficient; N = number of theoretical plates for a 20-cm column.

Gel	*T (min)*	$W_{1/2}$ *(min)*	K_{av}	R_s	*N*
Fractogel TSK HW 40 S	50	3.4	2.3	3.8	1200
Fractogel PGM 2000	46.5	4.3	2.1	3.0	660
Sephasorb HP Ultrafine	44	12	2.1	1.4	74
Sephadex G-10	52	16.3	2.3	1.2	56
Sephadex G-25 Superfine	35.5	5.5	1.4	1.0	230
Bio-Gel P-2 (200–400 mesh)	35.5	4.6	1.4	1.0	330
Trisacryl GF 05	36	5	2.3	0.9	290
Sephacryl S-200 Superfine	33	3.8	1.5	0.7	420
Ultrogel AcA-202	86	8.6	5	0.6	550

Owing to the peak width provided by this type of support, the efficiency of the column is far higher than with other gels, reaching 1200 theoretical plates per 20-cm column. All the gels that do not separate the tryptophan show a retention time less than 40 min, except for the peculiar case of Ultrogel AcA-202, which has a very low resolution coefficient. In contrast to the others, this gel does not absorb tryptophan specifically.

As regards the recovery of tryptophan from pure proteins and total wheat flour, we obtained 97.5 ± 1.1% (standard deviation for 14 assays) with a regression coefficient of 0.999 for lysozyme (using four different doses), and 94.1 ± 1.7% (standard deviation for 20 assays) with a regression coefficient of 0.997 for wheat flours to which were added the same lysozyme doses as the internal standard. Concerning the purified proteins, these results are in agreement with those from the literature[2,3] but are less scattered. As regards the determination of tryptophan in foodstuff protein, the recoveries we obtained with the internal protein standard are less scattered but comparable to results obtained with tryptophan adducts as internal standards[2,4].

Nevertheless, only the results obtained here by chromatographic means are reliable, in contrast to other methods[1,3] In addition, it is worth noting that under our hydrolytic conditions, tryptophan was stable for up to 2 months in the acidified hydrolysates.

CONCLUSION

The results show that Fractogel HW 40 S is a suitable support for the chromatographic determination of protein tryptophan after barytic hydrolysis. The advantage of Fractogel HW 40 S, like other good separating gels, is the moderate adsorption, but in addition it provides a very narrow tryptophan peak. This property may allow the ultraviolet detection of tryptophan instead of using fluorimetry of the OPA derivative. Nevertheless, it is important to emphasize that the ultraviolet detection of tryptophan is not specific, in contrast to the OPA reaction, which remains better for use in analyses of complex samples such as plant foodstuffs.

ACKNOWLEDGEMENTS

We are indebted to Drs. Jacques Mossé and Jacques Baudet for helpful advice. We particularly thank Anne-France de Jouvencel for her kind assistance, and are also grateful to Marie-Christine Aubrière and Monique Mansion for skillful technical aid.

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CHROM. 18 372

Note

Improved method for the sequential purification of polysaccharidases by affinity chromatography

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(Received November 26th, 1985)

Affinity chromatography is widely used for enzyme purification. The chromatographic support is generally an insoluble matrix on which a suitable ligand has been covalently linked. In the case of a polysaccharidase, the macromolecular substrate can be cross-linked leading to a matrix which is specifically recognized by the corresponding endopolysaccharidase. Rexová-Benková and Tibensky[1] introduced this affinity technique for the purification of *Aspergillus niger* polygalacturonase on cross-linked pectic acid. We have been able to generalize this method to other endopolygalacturonases[2,3] and to other types of endopolysaccharidases, mainly amylases[4] and cellulases[5,6].

In this report we describe the use of this affinity technique for the sequential purification of some polysaccharidases (amylases, polygalacturonases, cellulases) from a complex biological extract rich in each of these enzymes, *i.e.*, an extract from the larvae of a xylophagous insect, *Phoracantha semipunctata*.

MATERIALS AND METHODS

The polysaccharides comprised wheat starch, soluble starch (Merck, Darmstadt), insoluble cellulose (Whatman), carboxymethylcellulose (Blanose R 195, Novacel) and sodium polygalacturonate (Sigma).

The crude enzyme extract was obtained from *Phoracantha semipunctata* larvae as described previously[7].

Protein concentrations were estimated by the method of Bradford[8] using bovine serum albumin as standard.

Chromatographic supports

Polysaccharides were cross-linked by epichlorhydrin under alkaline conditions. The experimental conditions were adapted to each polysaccharide–polysaccharidase pair.

Starch and cellulose. A modification of the technique of Kuniak and Marchessault[9] was used. Each polysaccharide was treated with a 10% aqueous solution of sodium hydroxide and epichlorhydrin. The molar ratio sodium hydroxide/poly-

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saccharide (expressed in anhydroglucose units) was 1 for starch and 5 for cellulose. The molar ratio epichlorhydrin/polysaccharide was 1.25 for starch and 0.6 for cellulose.

After continuous stirring, first at room temperature and then at 50°C, the mixture was neutralized by acetic acid, after which the polysaccharide was washed, dried and ground to fine particles (Table I).

Polygalacturonic acid. This was cross-linked according to the technique of Rombouts *et al.*[10]. Sodium polygalacturonate was treated in an ethanolic medium (95°) with epichlorhydrin and 5 *M* sodium hydroxide. The molar ratio sodium hydroxide/polysaccharide (expressed in anhydrogalacturonic acid) was 1 and the molar ratio epichlorhydrin/polysaccharide was 1.5. After continuous stirring at 40°C, the mixture was washed with distilled water and neutralized with acetic acid. After filtration, the cross-linked polysaccharide was washed with ethanol–water (3:1) and with ethanol. It was then air dried (Table I).

The experimental conditions used for the cross-linking of each polysaccharide are summarized in Table I.

Enzyme assays

All enzyme assays were performed at 37°C.

α-Amylase. The amylolytic activity in column effluents was located using the iodine–potassium iodide reagent[11].

For all other assays, the Nelson–Somogyi colorimetric method was used as previously described[12,13] to determine the liberated reducing groups using wheat starch as a substrate.

Polygalacturonase. The polygalacturonase activity was estimated by the Nelson–Somogyi method as previously described[3] using sodium polygalacturonate as a substrate.

Cellulase. The cellulolytic activity was estimated by the Nelson–Somogyi method as previously described[6] using insoluble cellulose or carboxymethylcellulose as substrates.

In column effluents, the cellulolytic activity was located using agarose gels containing carboxymethylcellulose (1%). An aliquot (10 μl) of each effluent was

TABLE I

EXPERIMENTAL CONDITIONS FOR CROSS-LINKING OF POLYSACCHARIDES

Polysaccharide	*Molar ratio*		*Continuous stirring*
	*Sodium hydroxide/ polysaccharide**	*Epichlorhydrin/ polysacharide*	
Starch	1	1.25	Room temperature: 90 min 50°C: 2 h
Cellulose	5	0.6	Room temperature: 15 min 50°C: 1 h
Sodium polygalacturonate	1	1.5	40°C: 4 h

* Expressed in anhydrohexose units.

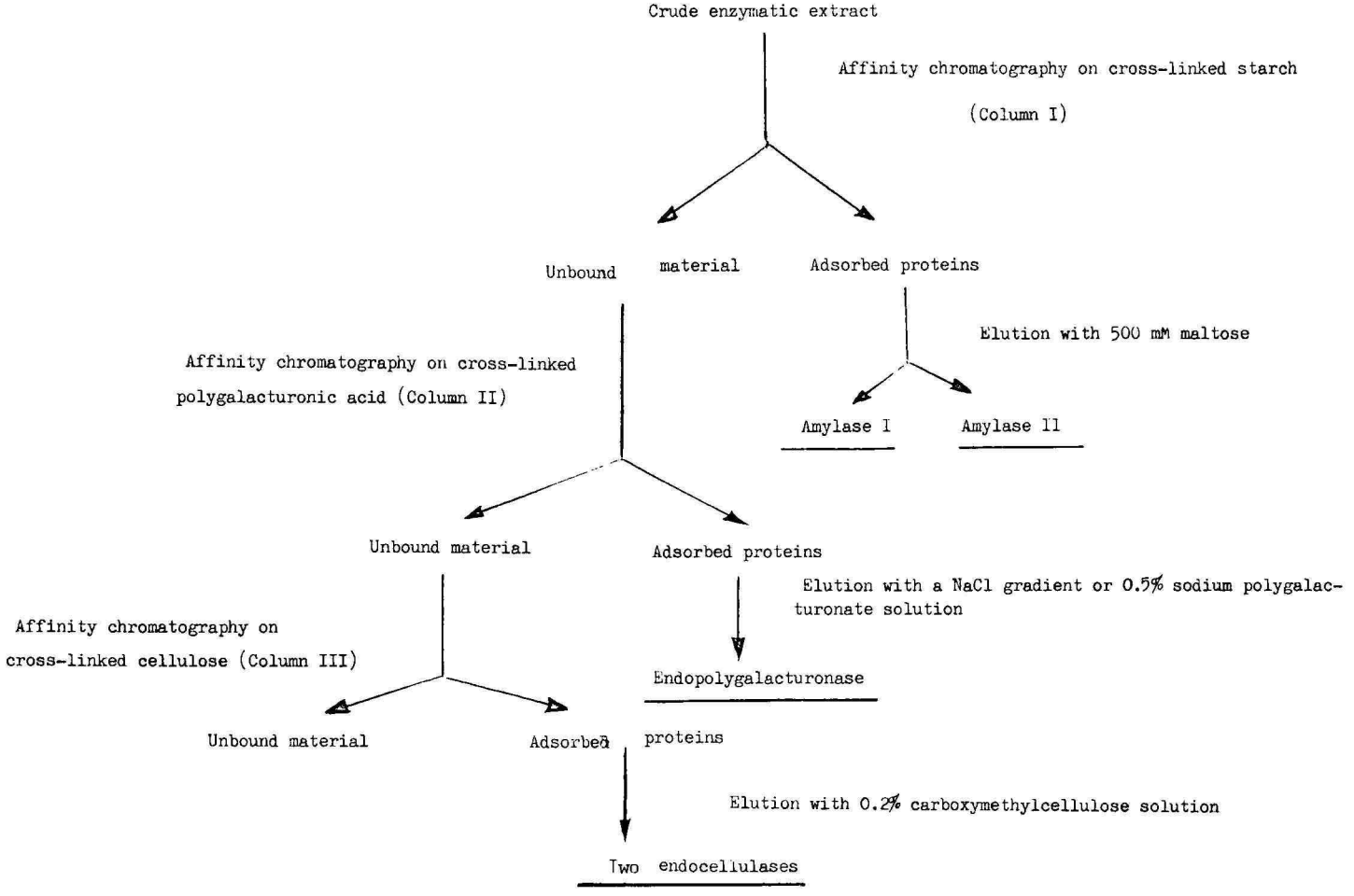

Fig. 1. Scheme for the sequential purification of polysaccharidases from *Phoracantha semipunctata* larvae.

placed in a well. After incubation for 1 h at 37°C and washing with 0.9% sodium chloride the gels were stained with an aqueous solution of Congo Red (0.1%). Activities were revealed as colourless zones on the red plate.

Identification of products of enzymatic hydrolysis

Hydrolysis products were separated by thin-layer chromatography on silica gel plates.

For the separation of oligosaccharides from starch and cellulose hydrolysates, the chromatograms were developed with propanol-1–ethyl acetate–ethanol–acetic acid–pyridine–water (7:3:3:2:2;4, v/v). For the separation of oligogalacturonates, the solvent system was butanol-1–formic acid–water (2:3:1, v/v).

In all cases, spots were revealed by heating the plates after spraying with a solution of sulphuric acid in acetone (5%).

Isoelectric focusing

Amylase and polygalacturonase. Analytical isoelectric focusing was performed using 5% acrylamide plates (LKB Ampholine PAG plates) containing 2.4% ampholytes (pH 3.5–9.5). Electrofocusing was performed as previously described[7].

Cellulose. An agarose gel I.E.F. (Pharmacia) aqueous solution (1%, w/v) containing 2.4% ampholytes (pH 3.5–9.5) was bedded on a gel-bound plate (LKB).

The anodic and cathodic solutions were 0.05 *M* sulphuric acid and 1 *M* sodium hydroxide, respectively. The electric power was maintained constant at 7 W for 90 min. Electrofocusing was performed at 4°C.

RESULTS AND DISCUSSION

The crude enzymatic extract from *Phoracantha semipunctata* larvae contains high levels of different polysaccharidase activities, mainly amylase, polygalacturonase and cellulase. So it can be used as a model for the sequential isolation of endo-polysaccharidases.

The purification of each polysaccharidase was performed sequentially by affinity chromatography on the corresponding cross-linked polysaccharide as described in Fig. 1.

Column I (cross-linked starch)

The first step was the purification of amylase on cross-linked starch according to a modification of the procedure previously described[4] (Fig. 2).

The buffered crude enzyme extract (1 ml, 20 mg proteins) was applied on a column (25 × 1 cm) containing an homogeneous mixture of cross-linked starch (4 g) and Sephadex G-10 (4 g) equilibrated with 10 m*M* malate buffer pH 6.5 containing 10 m*M* calcium acetate and 20 m*M* sodium chloride. Protein contaminants and all enzyme activities except that of α-amylases were eluted by the equilibrating buffer. The two amylasic activities (amylases I and II) were then eluted by a 500 m*M* buffered maltose solution.

The homogeneity of the two fractions and their mode of action were studied on the lyophilizates obtained after maltose elimination (Sephadex G-25 chromatography). Homogeneity was tested by isoelectric focusing. Amylase I showed a major

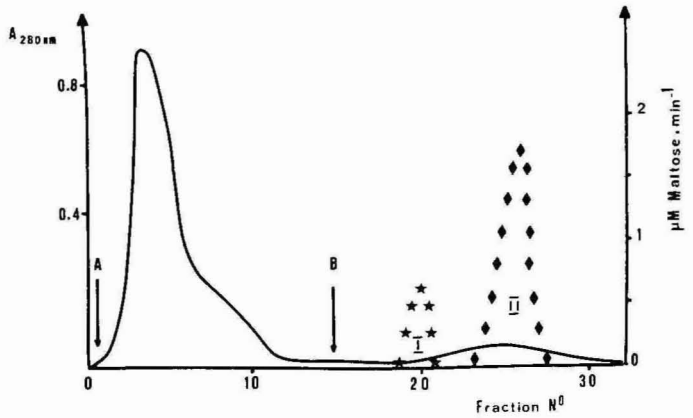

Fig. 2. Affinity chromatography of the crude extract of *Phoracantha semipunctata* larvae on cross-linked starch (column I). The column (25 × 1 cm) was equilibrated with 10 m*M* malate pH 6.5 containing 10 m*M* calcium acetate and 20 m*M* sodium chloride. Elution was carried out with (A) equilibrating buffer and (B) 500 m*M* maltose solution in the equilibrating buffer. The flow-rate was 10 cm/h and 2.5-ml fractions were collected. Proteins were measured estimated from the absorbance at 280 nm (———). Enzyme activity was estimated by the Nelson–Somogyi colorimetric method. Amylasic activity was eluted in two peaks: amylase I (★) and amylase II (◆).

protein band at p*I* 4.2. In addition, a faint band was detected but no amylase activity was revealed at this position. The enzyme activity was located only in the major protein band. Amylase II showed a single protein band at p*I* 4 with which was associated the amylasic activity. These two enzymes differed with respect to their optimum pH (6 for amylase I and 5.3 for amylase II).

The mode of action of the two enzymes was investigated as previously described[7]. Amylase I behaves as a maltotetraose-forming exoamylase, while amylase II is a typical endoamylase (α-amylase).

Column II (cross-linked polygalacturonic acid)

After dialysis against distilled water and lyophilization, the unbound material from column I was chromatographed on a cross-linked polygalacturonic acid column (Fig. 3). The buffered enzyme solution (1 ml) was applied on a column (20 × 1.2 cm) containing an homogeneous mixture of cross-linked polygalacturonic acid (5 g) and Sephadex G-10 (5 g) equilibrated with 100 m*M* acetate buffer pH 4.8.

Protein contaminants and all enzyme activities except that of endopolygalacturonase were eluted by the equilibrating buffer. The polygalacturonase was then eluted out by a linear gradient of 0–0.5 *M* sodium chloride in the equilibrating buffer. It can also be specifically eluted by a buffered 0.5% sodium polygalacturonate solution. The first type of elution was chosen to avoid chromatographic elimination of the excess of polygalacturonate and of oligogalacturonates present in the effluent.

The homogeneity and the mode of action of the enzyme were tested on the lyophilizates obtained after dialysis against distilled water. A single protein band at p*I* 5.8, which was associated with the polygalacturonasic activity, was revealed by isoelectric focusing. The optimum pH was 5. When the enzyme was incubated with sodium polygalacturonate, oligogalacturonates of various degrees of polymerization

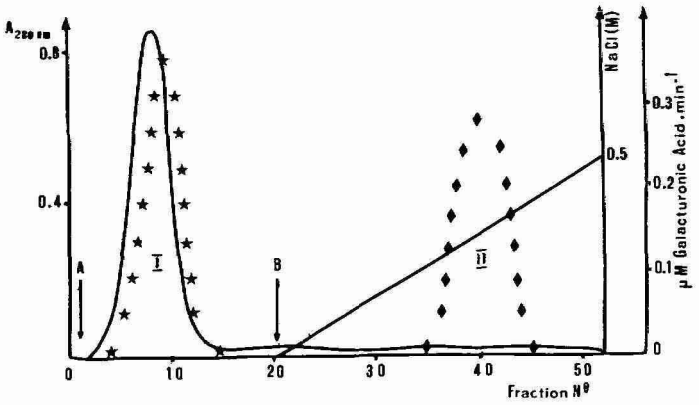

Fig. 3. Affinity chromatography on cross-linked polygalacturonic acid (column II). The column (20 × 1.2 cm) was equilibrated with 0.1 *M* acetate buffer pH 4.8. Elution was carried out with the equilibrating buffer (A) and with a linear gradient of 0–0.5 *M* sodium chloride (B). The flow-rate was 13 ml/h and 3-ml fractions were collected. Proteins were estimated from the absorbance at 280 nm (———). Enzyme activity was estimated by the Nelson–Somogyi colorimetric method: exopolygalacturonase (I) (★) and endopolygalacturonase (II) (◆).

(DP) were produced in the early stages of hydrolysis. The final product of the degradation was digalacturonate. This pattern is typical of an endopolygalacturonase which produces oligogalacturonates with various DPs.

Column III (cross-linked cellulose)

After dialysis against distilled water and lyophilization, the unbound material from column II was chromatographed on a cross-linked cellulose column (Fig. 4). The buffered enzyme solution (1 ml) was applied on a column (25 × 1.5 cm) con-

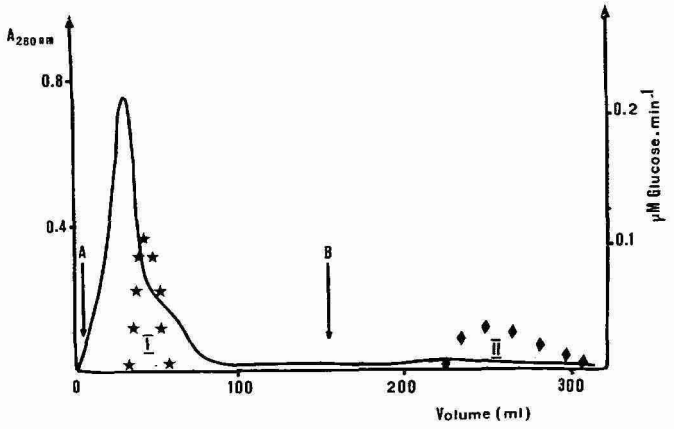

Fig. 4. Affinity chromatography on cross-linked cellulose (column III). The column (25 × 1.5 cm) was equilibrated with 50 m*M* acetate buffer pH 4.8. Elution was carried out with the equilibrating buffer (A) and a 0.2% carboxymethylcellulose solution (B) in the equilibrating buffer. The flow-rates were 2 ml/h (A) and 5 ml/h (B); 2-ml fractions were collected. Proteins were estimated from the absorbance at 280 nm (———) and enzymatic activity by the Nelson–Somogyi colorimetric method: exoglucanase (I) (★) and endoglucanase (II) (◆).

taining cross-linked cellulose (10 g) equilibrated with 50 m*M* acetate buffer pH 4.8. Protein contaminants and all enzyme activities except that of the endocellulase were eluted by the equilibrating buffer. The endocellulase activity was then specifically eluted by a 0.2% carboxymethylcellulose buffered solution.

Two protein bands at $pI \approx 4$ were revealed by isoelectric focusing. Each of them corresponds to an endocellulasic activity leading to the liberation of cellodextrins of various DPs after incubation with crystalline cellulose.

Assays for differential elution of these two endocellulases were attempted with carboxymethylcellulose solutions of different concentrations, as previously described for *Trichoderma viride* endocellulases[5]. A good resolution cannot be achieved because of the poor stability of the cellulolytic activities of *Phoracantha semipunctata*. However, this chromatographic step allows the separation of endoglucanasic activities from exoglucanase eluted by the equilibrating buffer as shown previously[6].

CONCLUSIONS

Sequential affinity chromatography on cross-linked polysaccharidses allowed the isolation of the corresponding polysaccharidases.

Successive chromatography on cross-linked starch, cross-linked polygalacturonic acid and cross-linked cellulose led respectively to the purification of two amylases, one endopolygalacturonase and to the separation of two endocellulases from the exocellulasic activities contained in the crude extract of *Phoracantha semipunctata* larvae.

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CHROM. 18 368

Letter to the Editor

Reusability of copper(II) sulphate-impregnated Chromarods

Sir,

Recently Ritchie[1] reported that impregnation of Chromarods with copper(II) sulphate, as proposed by us[2], offers no advantages over the unimpregnated rods in the thin-layer chromatography–flame ionization detection (Iatroscan) analysis of lipids. We had stated that impregnation provides (i) uniform response for various lipid classes (barring sterols and phospholipids), dispensing with the need to determine response factors, (ii) uniform chromatographic behaviour of the treated rods, eliminating the need for time-consuming regrouping of the rods, (iii) clear visibility of the solvent front, avoiding the over-development that occurs during development by time, and (iv) better peak shape for polar compounds such as free fatty acids, fatty alcohols and phospholipids, which normally tail to some extent. Without refuting any of the above facts, Ritchie states that our method does not offer any real advantage. His main criticism centres on the reusability of the copper(II) sulphate-impregnated Chromarods. Apparently, Ritchie has cleaned the impregnated rods and reimpregnated them after each run. This is absolutely unnecessary. The Chromarods, once impregnated, can be repeatedly used at least 30 times before cleaning and reimpregnation. In between runs, the Chromarods should be stored on its frame in a desiccator in a humid atmosphere.

We had not ascertained how many cleaning–reimpregnation cycles the rods could take. However, Ritchie has found that signal enhancement was not affected until three or four cleaning–reimpregnation cycles were completed, which means, the impregnated Chromarods would be useful for a minimum of 90 runs. We had determined (though not included in our paper) the copper content of the freshly impregnated Chromarods, and of those that had been used 30 times consecutively, by atomic absorption spectroscopy; the values were 195 and 145 μg per Chromarod, respectively. Though there was some reduction in the copper content with repeated use, the content of copper is more than adequate to impart the desired effect since the sample load rarely exceeds 10 μg. If, after ten cleaning–reimpregnation cycles, the Chromarods are unusable, as stated by Ritchie, the reason has to lie with the damage caused to the silica gel layer, which in turn could affect the impregnation with copper sulphate. The discrepancies observed in signal enhancement by Ritchie after three or four cleaning operations could be due to the same reason. Also, Ritchie has not compared the number of similar cleaning operations the untreated Chromarods could take. Further, a smaller number of runs yielding reliable information is preferable to a larger number of runs generating unreliable data.

Ritchie confirms that the signals are enhanced with the impregnated Chromarods, but to a lesser extent than that we had observed. Unfortunately, no mention was made either of the operating conditions of the instrument or of the sample load-

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ing. The impregnated rods should be initially scanned (before use) at a speed of 3.14 mm/s (using the speed gear No. 30) twice to char the rods uniformly. Actual analysis is carried out at a scan speed of 4.17 mm/s (using the speed gear No. 40). These two conditions are critical. The scan speed affects the response most.

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1 A. S. Ritchie, *J. Chromatogr.*, 346 (1985) 468.
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Author Index

Errata

J. Chromatogr., 288 (1984) 365–376

Page 366, Fig. 1, structure of compound X (nogalarene) and XI (nogarene): at position 9 a methyl group should be added.

J. Chromatogr., 349 (1985) 405–414

Page 409, the caption to the figure should read: "Fig. 1. Response contours to methane."

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Vol. 355, No. 2

journal of chromatography news section

SYMPOSIUM PROGRAMME

HPLC '86, 10th INTERNATIONAL SYMPOSIUM ON COLUMN LIQUID CHROMATOGRAPHY, SAN FRANCISCO, CA, U.S.A., MAY 18–23, 1986

HPLC '86 HAS RECORD NUMBER OF PAPERS

HPLC '86, the 10th International Symposium on Column Liquid Chromatography (ISCLC), will be held in San Francisco, CA, U.S.A., May 18–23, 1986. According to Dr. Ronald E. Majors, Varian's Life Science Marketing Manager and HPLC '86 Symposium Chairman, the number of abstracts submitted, over 500, is the largest ever submitted to a conference devoted solely to liquid chromatography. This prestigeous series of symposia, which alternate between the U.S.A. and Europe, was last held in Edinburgh, Scotland. HPLC '84 was held in New York and attracted over 1000 attendees.

The ISCLC Symposia have attracted the top HPLC papers by scientists from all over the world. This year's symposium will be no exception. Presentations will be in the form of Oral, Poster, and a new experimental Scientific Roundtable. Discussion sessions on more general topics such as trace analysis, new detection possibilities, micro LC, ion separations, and silica *vs.* polymeric phases are planned.

The Scientific Roundtable, new for HPLC '86, will allow those scientists sharing common interests to have more in-depth coverage on a focussed topic (*e.g.*, HPLC of antibiotics, separations of monoclonal antibodies, catecholamines in serum), than would ordinarily be available at a poster or lecture session. The Scientific Roundtable will be conducted in a more informal environment and will have a chairman leader. Each of several scientists will give a short oral presentation of his work followed by an in-depth discussion where all can explore common problems and successes in more detail. "We can provide such focussed sessions due to the fact that we have received such a large number of submitted papers" reports Majors. "The Roundtables shall be very beneficial for those scientists working on such problems on a daily basis."

Topics of more general interest will be covered by oral or poster presentations. Among the scientific sessions that are planned are those pertaining to column switching and multidimensional chromatography, microbore and micro LC techniques, and new stationary phases and column materials with a major focus on the separation of enantiomers using chiral columns and mobile phases. There will be coverage on new detection principles such as enzyme amplification, capillary viscosity, photothermal refraction, redox chemiluminescence, and quenched phosphorescence. Papers on the application of hyphenated techniques of LC–MS, LC–electrochemical, LC–GC, and LC–FTIR will be featured.

Sample preparation in chromatography is of major interest. Special sessions are planned on the advances in solid phase extraction and robotics. Supercritical fluid chromatography has some unique overlap with HPLC and GC. A number of contributions on this technique are planned.

In the life science area, a special symposium on the separation of cells is planned. Although not all the techniques used are HPLC, a comparison of the various approaches by noted experts in the field will be given. Preparative chromatography interest is high; undoubtedly driven by the recent emphasis

on biotechnology. Both oral and poster papers are planned. Applications in the determination of drugs and endogenous compounds in biological fluids will be a major area of coverage. Separation and purification applications for proteins, polypeptides, and nucleic acid constituents are numerous.

Applications papers in the areas of food, agriculture, and industrial round out the more practical aspects of the symposium. A full social programme has been planned including trips to the Napa Valley wine country, the California Redwoods, and other San Francisco highlights including a Bay Cruise.

In conjunction with the symposium, an exhibition of the latest LC instrumentation, columns, and accessories is planned. For further information of the symposium and exhibition as well as registration materials and a preliminary scientific programme please contact Ms. Shirley Schlessinger, HPLC '86 Symposium Manager, 400 E. Randolph Dr., Chicago, IL 60601, U.S.A. Tel.: (312) 527-2011.

HPLC '86 PRELIMINARY SCIENTIFIC PROGRAMME

MONDAY, MAY 19, 1986

RECENT ADVANCES IN SEPARATIONS

An objective method for the comparison of the kinetic performance of LC columns of widely different shape and size – H. Poppe and J.C. Kraak, University of Amsterdam, Amsterdam, The Netherlands.

Automated instrument for capillary electrophoresis of proteins – J.W. Jorgenson, J. Green, D. Rose and Y. Walbroehl, University of North Carolina, Chapel Hill, NC, U.S.A.

New generation of laser-based detectors for LC – Edward S. Young, Iowa State University, Ames, IA, U.S.A.

RECENT ADVANCES IN PROTEIN SEPARATIONS

Movement of components in columns packed with mixed stationary phases for use of biopolymer HPLC with gradient elution – Ziad El Rassi, Yih-Fen Maa, Firos Antia and Csaba Horváth, Yale University, New Haven, CT, U.S.A.

Electrostatic and hydrophobic interaction chromatography: conformational changes as a probe of interfacial phenomena – B.L. Karger, S.L. Wu, N. Grinberg, R.E. Shansky and A. Figueroa, Northeastern University, Boston, MA, U.S.A.

The influence of three dimensional structure on retention in ion exchange chromatography of proteins – F.E. Regnier and R.R. Drager, Purdue University, W. Lafayette, IN, U.S.A.

SUPERCRITICAL FLUID CHROMATOGRAPHY

Correlation of solvatochromic effects with retention in supercritical fluid chromatography – C.R. Yonker, R.W. Gale and R.D. Smith, Batelle Pac Northwest, Richland, WA, U.S.A.

Chromatographic behavior of various pure eluents in the liquid and in the supercritical state – Dietger Leyendecker, Franz P. Schmitz and Ernst Klesper, Lehrstuhl f. Makro. Chem., Aachen, F.R.G.

Development of super-critical fluid chromatographic separation and ion mobility spectrometric detection of carboxylic acid esters – S. Rokushika, H.H. Hill Jr. and H. Hatano, Kyoto University, Kyoto, Japan.

CHIRAL SEPARATIONS

Extrapolation of chromatographically-derived chiral recognition models to transition state models for asymmetric synthesis – William H. Pirkle and Daniel Reno, University of Illinois, Urbana, IL, U.S.A.

Covalently or ionically bonded tartaric acid amides as chiral selectors for HPLC enantioseparations – W. Lindner, Ch. Leitner, W. Schned and F. Reiter, University of Graz, Graz, Austria.

Chiral recognition model for the resolution of enantiomeric amides on a commercially available HPLC chiral stationary phase – Irving W. Wainer and Marc C. Alembik, US FDA, Washington, DC, U.S.A.

Chiral recognition mechanisms in non-aqueous liquid and supercritical fluid chromatography – Shoji Hara, Tokyo College Pharmacy, Tokyo, Japan.

LIQUID CHROMATOGRAPHY PACKINGS

Non-porous *vs.* porous 1-micrometer reversed-phase silica packings in high-performance liquid chromatography (HPLC) – K.K. Unger, G. Jilge and B. Eray, Johannes Gutenberg University, Mainz, F.R.G. and I. Novak, Polymer Institute CCR, Bratislava, Czechoslovakia.

Characterization of ultra-micro wide-pore spherical silica column packings for the HPLC of peptides and proteins – N.D. Danielson and J.J. Kirkland, E.I. duPont de Nemours, Wilmington, DE, U.S.A.

Hydroxyapatite HPLC – Tsutomu Kawasaki, Japan Biomed Research Center, Tokyo, Japan.

New polymer-based stationary phases for HPLC – E. Bayer, Universität Tübingen, Tübingen, F.R.G.

POSTER SESSIONS

Recent advances in protein separations
Supercritical fluid chromatography
Chiral separations
LC packings
Chromatographic theory/optimization
Drugs in biological matrices
Endogenous compounds in biological matrices

DISCUSSIONS

Protein separation/purification – is there a best way?
New column packings
Chiral separations

SCIENTIFIC ROUNDTABLES

Catecholamines in serum and plasma
HPLC of steroids

TUESDAY, MAY 20, 1986

POLYMER SEPARATIONS/GEL PERMEATION CHROMATOGRAPHY

High-performance size exclusion chromatography of polymers – John V. Dawkins, Loughborough University of Tech, Loughborough, Leics, U.K.

New developments in size exclusion chromatographic detectors – Howard G. Barth and Syhchang S. Huang, Hercules, Inc., Wilmington, DE, U.S.A.

Hydrodynamic chromatography of Macromolecules in open microcapillary tubes – R. Tijssen, J. Bos and M.E. van Kreveld, Koninklijke Shell-Labor., Amsterdam, The Netherlands.

SAMPLE PREPARATION

Solid state extraction in organic analysis – U.A.Th. Brinkman and R.W. Frei, Free University of Amsterdam, Amsterdam, The Netherlands.

Multimodal bonded phase extraction for sample preparation – D.D. Blevins and M.L. Stolowitz, Analytichem Intl., Harbor City, CA, U.S.A.

Determination of erythrocyte haem biosynthetic enzyme activities by HPLC coupled to an advanced sample processor (AASP) – C.K. Lim, F. Li, D.J. Wright, J.M. Rideout and T.J. Peters, MRC Clinical Research Centre, Harrow, Middlesex, U.K.

PROTEIN SEPARATIONS

Control of selectivity behavior by co- and counter-ions in preparative high-performance ion exchange chromatography of proteins: a re-appraisal – A.N. Hodder, M.I. Aguilar and M.T.W. Hearn, St. Vincent's Inst. Med., Melbourne, Vic., Australia.

The contribution of the base silica and pore size upon protein separations in reversed-phase LC – Brian A. Bidlingmeyer, Carmen T. Santasania and F. Vincent Warren Jr., Millipore Corp., Milford, MA, U.S.A.

A model of protein conformation in the reversed-phase separation of Interleukin-2 muteins – M.G. Kunitani and D.J. Johnson, Cetus Corp., Emeryville, CA, U.S.A. and L.R. Snyder, L.C. Resources, Orinda, CA, U.S.A.

SAMPLE PREPARATION/ROBOTICS

Laboratory robotics applied to HPLC analyses – James N. Little, Zymark Corp., Hopkinton, MA, U.S.A.

Separation of drugs from serum by direct injection onto high-performance internal surface reversed-phase (ISRP) polypeptide columns – Thomas C. Pinkerton, Steve E. Cook, Terry M. Miller and Carla P. Desilets, Purdue University, West Lafayette, IN, U.S.A.

On-line precolumn chemistry for HPLC. An assessment of the method – Gerard P. Rozino, Rainer Schuster and James A. Apffel, Hewlett-Packard GmbH, Waldbronn, F.R.G.

SELECTIVITY IN LIQUID CHROMATOGRAPHY

Metal ion selectivity on sulfonated cation-exchange resins of low capacity – Gregory J. Sevenich and James S. Fritz, Iowa State University, Ames, IA, U.S.A.

Metal chelate interaction chromatography in HPLC of proteins – Zial El Rassi, Guhan Subramian, Abraham Liao and Csaba Horváth, Yale University, New Haven, CT, U.S.A.

Electrokinetic chromatography with micellar solution. Application to the separation of closely related compounds – Shigeru Terabe, Koji Otsuka and Teiichi Ando, Kyoto University, Kyoto, Japan.

IONIC SEPARATIONS

Electrical double layer sorption model for ions and for "ion pair" chromatography on ODS-bonded phases – Hanjiu Liu and Frederick F. Cantwell, University of Alberta, Edmonton, Alberta, Canada.

A simultaneous liquid chromatographic separation of common inorganic cations and anions using a single column and eluent – D.J. Pietrzyk and D. Brown, The University of Iowa, Iowa City, IA, U.S.A.

Effects of injection induced eluting zones on analytes in ion-pair reversed-phase LC – Douglas Westerlund, Uppsala University Biomedical Ctr., Uppsala, Sweden.

POSTER SESSIONS

Polymer separations/GPC
Sample preparation/robotics
Protein separations
Ionic separations
Detectors/new principles
Detectors/diode array and mass spectrometric
Drugs in biological matrices
Affinity chromatography
Drug analysis
Automation/instrumentation
Analysis of hydrocarbons
Derivatization

DISCUSSIONS

Silica *vs.* polymeric phases
Directions in sample preparation technology (robotics, on-line approaches, etc.)
Ion separations

SCIENTIFIC ROUNDTABLES

Separation of monoclonal antibodies
Precolumn derivatization of amino acids

WEDNESDAY, MAY 21, 1986

CHROMATOGRAPHIC THEORY/OPTIMIZATION

A model for overloaded HPLC separations. Computer simulations based on Craig-distributions and a single solute – J.E. Eble and P.E. Antle, DuPont, Wilmington, DE, U.S.A., R.L. Grob, Villanova University, Villanova, PA, U.S.A. and L.R. Snyder, LC Resources, Inc., Orinda, CA, U.S.A.

Micropreparative separations under the conditions of low dispersion liquid chromatography – Klaus-Peter Hupe, Hewlett-Packard GmbH, Waldbronn, F.R.G.

High-performance affinity chromatographic measurement of desorption rate constants by a peak-decay method – R.M. Moore, S.C. Crowley and R.R. Walters, Iowa State University, Ames, IA, U.S.A.

The kinetics of sorption–desorption in liquid chromatography – David B. Marshall and James W. Burns, University of Idaho, Moscow, ID, U.S.A.

The effect of model inaccuracy on selectivity optimization procedures in RPLC – Peter J. Schoenmakers and Thomas Blaffert, Philips Res. Laboratory, Hamburg, F.R.G.

DETECTORS/ NEW PRINCIPLES

A micronebulizer interface for HPLC and gas phase detectors – J.F. Kamicky and Sj. van der Wal, Varian Instrument Group, Walnut Creek, CA, U.S.A.

Simple laser refractive index detector for capillary liquid chromatography – Darryl J. Bornhop and Norman J. Dovichi, University of Wyoming, Laramie, WY, U.S.A.

HPLC analysis of naphthalene dialdehyde derivatives of amines, amino acids and dipeptides with chemiluminescence detection – B. Matuszewski, R. Givens, K. Srinivasachar and R.G. Carlson, University of Kansas, Lawrence, KS, U.S.A.

Design and performance of new thermospray detectors for HPLC – C.H. Vestal, G.J. Fergusson and C.R. Blakley, Vestec Corp., Houston, TX, U.S.A, and M.L. Verstal, University of Houston, Houston, TX, U.S.A.

A quantitative model for indirect detection in reversed-phase chromatography – Jacques Crommen, University of Liege, Liege, Belgium and Goran Schill, University of Uppsala Biomedical Ctr., Uppsala, Sweden.

CELL SEPARATIONS

Steric field-flow fractionation: a tool for cell separation – Karin D. Caldwell and John F. Ash, University of Utah, Salt Lake City, UT, U.S.A.

Countercurrent chromatography: its application to the separation of cells and sub-cellular particles by aqueous two-phase partition – I.A. Sutherland and D. Heywood-Waddington, Natl. Inst. Medical Res., London, U.K. and Y. Ito, Natl. Heart, Lung & Blood, Bethesda, MD, U.S.A.

Countercurrent distribution of cell and cell organelles – Per-Åke Albertsson, Chemical Center, Lund, Sweden.

Separation of cells by partitioning in two-polymer aqueous phase systems and the information on surface properties conveyed by the cells' partitioning behavior – Harry Walter, VA Medical Center, Long Beach, CA, U.S.A.

Centrifugal elutriation and fractionation of wheat leaf subcellular particles – Jiann-Tysh Lin and Joseph W. Corse, USDA, Agric. Res. Serv., Albany, CA, U.S.A. and Owen M. Griffin, Beckman Instruments, Palo Alto, CA, U.S.A.

LIFE SCIENCE APPLICATIONS

Microscale purification of estrogen receptor isoforms by multidimensional HPLC analyses – James L. Wittliff, Salman M. Hyder, Nobuko Sato and Denis M. Boyle, University of Louisville, Louisville, KY, U.S.A.

Preparative HPLC of proteins on an anion-exchanger, using unfractionated carboxymethyldextran displacers – A.R. Torres and S.C. Edberg, Yale University, New Haven, CT, U.S.A. and E.A. Peterson, NIH, Natl. Cancer Inst., Bethesda, MD, U.S.A.

HPLC determination of nucleosides and bases in serum with a porous polymer gel column and micellar liquid phase – Yong-Nam Kim and Phyllis R. Brown, University of Rhode Island, Kingston, RI, U.S.A.

Separation and structural characterization of complex oligosaccharides by HPLC and LSIMS – James W. Webb, Illinois State University, Normal, IL, U.S.A. and Beth Gillece-Castro, Ke Jiang, A.L. Burlingame, University of California, SF, San Francisco, CA, U.S.A.

Pre-column (laser)fluorescence labelling of glucuronides – H. Lingeman and U.R. Tjaden, Leiden University, Leiden, The Netherlands and A. Hulshoff, State University of Utrecht, Utrecht, The Netherlands.

CHROMATOGRAPHIC THEORY/OPTIMIZATION

Peak compression in HPLC: theory, practice and application – Josef F.K. Huber, Robert Hirz and Claudia Hischenhuber-Fioresi, University of Vienna, Vienna, Austria.

Solvatochromic solvent polarity measurements, retention, and selectivity in reversed-phase liquid chromatography – Bruce P. Johnson, Morteza G. Khaledi, Bruce P. J Johnson and Mortezza G. Khaledi, University of Florida, Gainesville, FL, U.S.A.

Theoretical investigation of the potentiality of non-porous particles in liquid chromatography – Michel Martin, Ecole Polytechnique, Palaiseau, France.

Void column liquid chromatography reactor studies to determine reaction rates in mobile and stationary phases – Alexander H.T. Chu and Stanley H. Langer, University of Wisconsin-Madison, Madison, WI, U.S.A.

Retention processes and complexing equilibria in liquid chromatography as studied through system peaks – Shulamit Levin and Eli Grushka, The Hebrew University, Jerusalem, Israel.

Elution behavior of peropyrene type polycyclic aromatic hydrocarbons on various chemically bonded stationary phases in non-aqueous reversed-phase liquid chromatography – K. Jinno and T. Nagoshi, Toyohashi University of Tech., Toyohashi, Japan, N. Tanaka, Kyoto Technical University, Kyoto, Japan, M. Okamoto, Prefectual Tajimi Hosp., Tajimi, Japan and J.C. Fetzer and W.R. Biggs, Chevron Research Co., Richmond, CA, U.S.A.

Retention indices and drug identification by HPLC – Roger M. Smith and Tony G. Hurdley, Loughborough University Tech., Loughborough Leics., U.K. and Richard Gill and Anthony C. Moffat, Home Off. Forensic Sci., Aldermaston, Read., U.K.

THURSDAY, MAY 22, 1986

STATIONARY PHASE CHARACTERIZATION

Studies of the interfacial region in HPLC using surface immobilized probes – R.K. Gilpin, A. Kasturi and S.S. Yang, Kent State University, Kent, OH, U.S.A.

Fluorescence studies of molecular interactions at reversed-phase silica surfaces – J.M. Harris, M.L. Hunnicutt and J.W. Carr, University of Utah, Salt Lake City, UT, U.S.A.

Probing the reversed-phase chromatography surface using fluorescence spectroscopy – James B. Callis and Cathleen Webb, University of Washington, Seattle, WA, U.S.A.

Physico-chemical studies of hydrocarbon bonded phases – C.H. Lochmuller, Duke University, Durham, NC, U.S.A.

DETECTORS

A new capillary viscometer detector for size exclusion chromatography – Wallace W. Yau, E.I. duPont de Nemours, Wilmington, DE, U.S.A.

Cascade chromatographic enzyme amplification – Roger W. Giese, Markus Ehrat and Douglas Cecchini, Northeastern University, Boston, MA, U.S.A.

Radiometric detection in LC of ^{14}C-labelled polar compounds using an effluent storage principle – R.W. Frei and U.A.Th. Brinkman, Vrije Universiteit, Amsterdam, The Netherlands and H.J. van Nieuwkerk, A.C. Veltkamp and H. Das, ECN, Petten, The Netherlands.

Redox chemiluminescence detectors and prospects for adaptation to HPLC – R.E. Sievers, S.S. Banning, J.J. DeAngelis, S. Barak, R.L. Shearer, S.A. Montzka, N. Pourreza and R.M. Barkley, University of Colorado, Boulder, CO, U.S.A.

STATIONARY PHASE CHARACTERIZATION

A new family of silane surface modifiers and their use in probing steric effects in the surface deactivation of porous silica – R.D. Golding, A.J. Barry and M.F. Burke, University of Arizona, Tucson, AZ, U.S.A.

Characterization of bonded phase sorbents by small angle neutron scattering – Lane C. Sander, Stephen A. Wise and Charles J. Glinka, Nat. Bureau Standards, Gaithersburg, MD, U.S.A.

DETECTORS

A new universal detector for HPLC – the density detector – Bernd Trathnigg and Christian Jorde, University of Graz, Graz, Austria.

Whole column detection chromatography: computer simulations – David G. Gelderloos, Kathy L. Rowlen, John W. Birks and James P. Avery, University of Colorado, Boulder, CO, U.S.A. and Christie G. Enke, Michigan State University, East Lansing, MI, U.S.A.

FIELD FLOW FRACTIONATION

Recent advances in field-flow fractionation – J. Calvin Giddings, University of Utah, Salt Lake City, UT, U.S.A.

Theoretical and experimental aspects of focusing field-flow fractionation – Josef Janca, Czech. Academy of Sci., Brno, Czechoslovakia.

Examination of corn root membranes by SED-FFF, HPLC, and electron microscopy – S.M. Mozersky, R.A. Barford, B.E. Maleff and S.B. Jones, USDA/ARS, Philadelphia, PA, U.S.A. and K.D. Caldwell, University of Utah, Salt Lake City, UT, U.S.A.

DETECTORS/ELECTROCHEMISTRY

Common misconceptions about liquid chromatography–electrochemistry – Peter T. Kissinger, Bioanalytical Systems, West Lafayette, IN, U.S.A.

On-column pyrolitic-carbon detector for micro-column separations – Ross A. Wallingford and Andrew G. Ewing, Pennsylvania State University, University Park, PA, U.S.A.

Electrochemical detection in liquid chromatography – G. Horvai, J. Fekete, Zs. Niegreisz, L. Szucs and E. Pungor, Inst. Gen. & Anal. Chem., Budapest, Hungary.

POSTER SESSIONS

Detectors
Micro liquid chromatography
Biomedical applications
Column switching/multidimensional LC
Mobile phase techniques in LC
Industrial and environmental applications
Monoclonal antibodies
Amino acids and peptides
Nucleic acids
Food/agricultural applications
Preparative LC

DISCUSSION SESSION

"Small needles in big haystack" – general approaches to separations of trace substances in biological matrices
New detection possibilities
Micro LC – packed *vs.* open tubular – is either worth it?

SCIENTIFIC ROUNDTABLE

HPLC of antibiotics

FRIDAY, MAY 23, 1986

MICRO LIQUID CHROMATOGRAPHY

Microcolumn liquid chromatography and its related techniques: a unique approach to identifications and measurements in complex biological and environmentally important mixtures – Milos Novotny, Indiana University, Bloomington, IN, U.S.A.

The status of multicapillary liquid chromatography – James L. Powers, John Ellis, Michael Buchin and Richard Mead, Collimated Holes, Inc., Campbell, CA, U.S.A.

Open-bore capillary liquid chromatography – Hermann Bauer, University of Tübingen, Tübingen, F.R.G.

Methods for the application of liquid stationary phases on open tubular columns of 5–20 micrometer inner diameter – J.C. Kraak, H. Poppe, Olga van Berkel and Renee Peerboom, University of Amsterdam, Amsterdam, The Netherlands.

BIOMEDICAL APPLICATIONS

High-performance ion-exchange chromatography of proteins on agarose columns – Stellan Hjerten and Kunquan Yao, University of Uppsala, Uppsala, Sweden.

Role of HPLC with diodearray detector and column switching in laboratory diagnosis of human metabolic diseases – Egil Jellum and Odd Thoresen, Rikshospitalet, Oslo, Norway.

An integrated biological/physico-chemical system for the identification of antitumor compounds from fermentation broths – Derek J. Hook, Carolee F. More, Joseph J. Yacobucci, George R. Dubay and Sean O'Connor, Bristol-Myers Co., Syracuse, NY, U.S.A.

Drug screening by solvent gradient HPLC using a narrow bore column and a high-speed diode array detector – David Demorest, Central Pathology Lab, Santa Rosa, CA, U.S.A.

LIQUID CHROMATOGRAPHY PACKINGS

Stationary phases for HPLC – M. Verzele, State University of Gent, B-9000 Gent, Belgium.

Chemically bonded and polymer coated reversed-phase materials – polar stationary phases – G. Schomburg, H. Czisch, A. Deege and J. Kohler, Max-Planck-Inst., Mulheim/Ruhr, F.R.G.

Specification of reversed-phase diversity – H. Engelhardt, M. Jungheim and M. Czok, Universität des Saarlandes, Saarbrücken, F.R.G.

Improved silica-based column packings for HPLC – J. Kohler and J.J. Kirkland, E.I. duPont de Nemours, Wilmington, DE, U.S.A.

Eluotropic relationships for porous graphite carbon – John H. Knox and Bulvinder Kaur, University of Edinburgh, Edinburgh, U.K.

DETECTORS/DIODE ARRAY AND MASS SPECTROMETRIC

Generalized rank annihilation to LC–UV data – Eugenio Sanchez, M.L. Scott Ramos and Bruce R. Kowalski, University of Washington, Seattle, WA, U.S.A.

An evaluation and combination of several methods of peak-recognition in liquid chromatography using a photodiode array detector – J.K. Strasters, H.A.H. Billiet and L. de Galan, TH-Delft, 2628 BX Delft, The Netherlands

Application of LC–MS to the analysis of biological material – K. Schellenberg, M. Lindner, A. Groeppelin and F. Erni, Sandoz Ltd., Basle, Switzerland.

Direct coupling of micro high-performance liquid chromatography with fast atom bombardment mass spectrometry: improvement of the ionization system – D. Ishii, Y. Ito, T. Takeuchi and M. Goto, Nagoya University, Nagoya, Japan.

Techniques for increased use of thermospray LC–MS – S.A. Lammert and W.H. McFadden, Finnigan MAT, San Jose, CA, U.S.A.

PUBLICATION SCHEDULE FOR 1986

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	O 1985	N 1985	D 1985	J	F	M	A	M	J	
Journal of Chromatography	346 347/1	347/2 347/3 348/1	348/2 349/1 349/2 350/1 350/2	351/1 351/2 351/3	352 353 354	355/1 355/2 356/1	356/2 356/3 357/1	357/2 357/3 358/1 358/2 359		The publication schedule for further issues will be published later
Chromatographic Reviews								373/1		
Bibliography Section					372/1		372/2		372/3	
Biomedical Applications				374/1 374/2	375/1	375/2	376 377	378/1	378/2 379	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 329, No. 3, pp. 449–452. A free reprint can be obtained by application to the publisher.)

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.

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