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GEL CHROMATOGRAPHIC BEHAVIOUR OF TETRAALKYLAMMONIUM IONS ON SEPHADEX G-10

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(First received July 28th, 1980; revised manuscript received January 20th, 1981)

SUMMARY

The gel chromatographic behaviour of tetraalkylammonium (TAA) ions on Sephadex G-10 was studied chiefly by a thermodynamic and an extra-thermodynamic approach. The pH dependence of the distribution coefficients of TAA ions was observed, and compared with those of alkali metal and halide ions. The extra-thermodynamic approach showed that no enthalpy–entropy compensation was operative in the partitioning processes of TAA ions between the mobile and the gel phase. Examination of the effects of eluent concentration, organic eluent and temperature suggested that hydrophobic interaction and sieving effects contribute to various extents to the separation mechanism of individual TAA ions in the temperature range studied.

INTRODUCTION

In gel chromatography it is well known that chromatograms of relatively small molecules and ions on tightly cross-linked gels in aqueous systems are often adversely affected by several kinds of side effects. One of the important side effects is a hydrophobic interaction, which sometimes plays a dominant rôle in the gel chromatographic behaviour of organic and biological solutes[1].

Several workers[2–9] have used a thermodynamic approach to describe the hydrophobic interaction. We have recently studied the separation mechanism of aliphatic *n*-alcohols in aqueous dextran gel systems by a thermodynamic and an extra-thermodynamic approach, where the hydrophobic interaction between the solute and the gel matrix was interpreted principally by correlating ΔS°_{GC} values of the alcohols with their ΔS°_{HY} values[10]; ΔS°_{GC} denotes the standard entropy change upon transfer of the solute from the mobile phase outside the gel beads to the gel phase at infinite dilution, and ΔS°_{HY}, usually called the entropy of hydration, is the standard entropy change upon dissolution of the gaseous solute in water at infinite dilution.

However, no study on the behaviour of tetraalkylammonium (TAA) ions in aqueous dextran gel systems has been reported, although such ions are regarded as a homologous series of hydrophobic solutes[11,12]. The aim of the present work, therefore, was to study the behaviour of TAA ions on Sephadex G-10.

The pH dependence of the distribution coefficients, K_d, of TAA ions was investigated with 0.1 *M* sodium chloride solutions in order to find the pH range in which the electrostatic interaction can almost be suppressed. The effects of eluent concentration, organic eluent and temperature upon the K_d values of TAA ions were examined. Enthalpy–entropy compensation tests were applied to the partitioning processes of TAA ions to differentiate the separation mechanisms.

EXPERIMENTAL

Sample solutions

Tetramethylammonium (Me_4N^+), tetraethylammonium (Et_4N^+), tetra-*n*-propylammonium (n-Pr_4N^+) and tetra-*n*-butylammonium (n-Bu_4N^+) chlorides were of guaranteed reagent grade from Nakarai Chemicals (Kyoto, Japan). Tetra-*n*-pentylammonium (n-Pe_4N^+) bromide was purchased from Fluka (Buchs, Switzerland). Sample solutions were prepared by dissolving each TAA salt in the eluents at a concentration of 0.01 *M*, except for n-Pe_4NBr which because of its poor solubility was used as a saturated solution.

Standard materials with $K_d = 1$ and 0 were used as in a previous study[10].

Columns and eluents

Gel columns packed with Sephadex G-10 (dry particle size 40–120 μm; Pharmacia, Uppsala, Sweden) were prepared according to the procedure described previously[10].

The eluents used were 0.1 *M* sodium chloride solutions at various pH values, 95% (v/v) methanol and 0.1 *M* lithium chloride in 95% methanol. Sodium chloride solutions at concentrations of 0.01, 0.1, 0.3, 0.5 and 1.0 *M* at pH 2.3 were also employed to investigate the effect of eluent concentration on the K_d values of TAA ions.

Other experimental details and the procedures for calculating K_d values and thermodynamic functions were as reported previously[10].

RESULTS AND DISCUSSION

pH dependence of K_d values

The pH dependence of the K_d values or capacity factors of aromatic acids, amines, ampholytes and monomeric oxo acids of phosphorus have been reported on Sephadex G-10[13–15], LH-20[16], Bio-Gel P-2[17], polystyrene copolymers[18–24] and octadecylsilica[25]. In these cases, the pH dependence was interpreted primarily in terms of acid or base dissociation of the solutes. We recently observed[26] that the K_d values of alkali metal and halide ions on Sephadex G-10 also vary significantly with the pH of the eluent over the range 2–12, although these ions are not transformed into any other form or species over this pH range. TAA ions also do not suffer any molecular change over a wide pH range. Therefore, their K_d values on Sephadex G-10 with 0.1 *M* sodium chloride as eluent were examined over the pH range 1.5–12.5. The results are shown in Fig. 1.

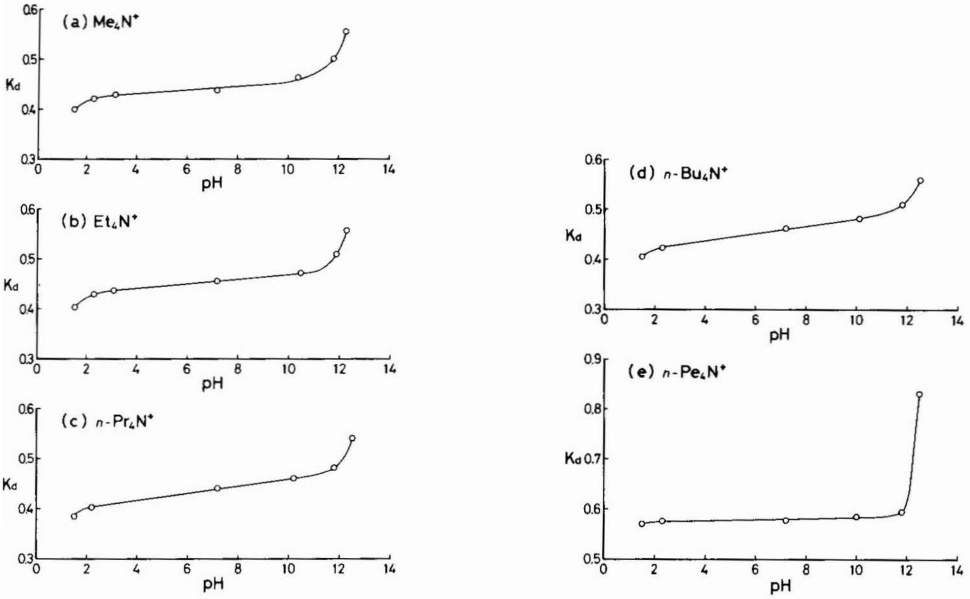

Fig. 1. pH dependence of K_d values of TAA ions on Sephadex G-10. Eluent: 0.1 *M* NaCl solutions at various pH values. Temperature: 20°C.

Each TAA ion showed a similar pH profile to those of the alkali metal ions. This behaviour may arise from the change in properties of the gel phase with the pH of the eluent. Namely, the slight increase in K_d value at pH <3 may be ascribed to the change of hydrogen ion concentration in the gel phase, and the rapid increase at pH >11.5 to the acid dissociation of alcoholic hydroxyl groups of the gel matrix.

In order to suppress the electrostatic interaction between the TAA ions and the charges of the gel matrix, the pH of the eluents used in subsequent experiments was adjusted to 2 or 2.3, except for 95% methanol systems.

Effect of eluent concentration

The salting-out of solutes from the mobile phase is a useful means of elucidating whether or not a hydrophobic interaction is operative in chromatographic processes[1].

Fig. 2 shows the effect of eluent concentration on the K_d values of TAA ions on Sephadex G-10 with sodium chloride eluents at pH 2.3. The K_d values of both sodium and chloride ion as the eluent are almost equal at pH 2.3[26].

As in the case of aliphatic *n*-alcohols[10], the K_d values of TAA ions increase gradually with increasing eluent concentration, especially above 0.1 *M*. This effect is more significant for $n\text{-}Pr_4N^+$, $n\text{-}Bu_4N^+$ and $n\text{-}Pe_4N^+$ than for Me_4N^+ and Et_4N^+. The relative increments of the K_d values with increasing eluent concentration from 0.01 to 1.0 *M* are 13% for Me_4N^+, 14% for Et_4N^+, 23% for $n\text{-}Pr_4N^+$, 37% for $n\text{-}Bu_4N^+$ and 68% for $n\text{-}Pe_4N^+$.

Deguchi[27] observed that differences between the K_{av} values of TAA ions on Sephadex G-15 are remarkable with sodium chloride, potassium sulphate and lithium sulphate as eluent, although those with water are negligibly small.

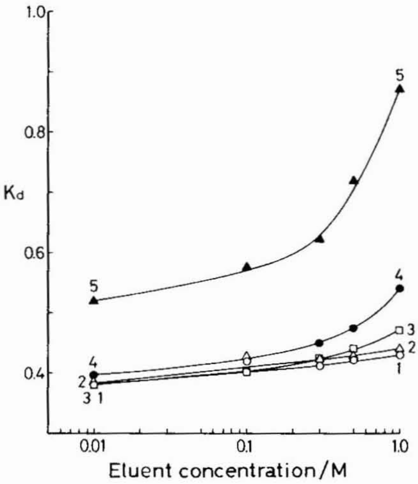

Fig. 2. Effect of eluent concentration on K_d values of TAA ions on Sephadex G-10. Eluent: NaCl solutions of various concentrations at pH 2.3. Temperature: 20°C. Cations: 1 = Me_4N^+; 2 = Et_4N^+; 3 = n-Pr_4N^+; 4 = n-Bu_4N^+; 5 = n-Pe_4N^+. The value of n-Bu_4N^+ (●) at 0.1 M NaCl is overlapped by those of Me_4N^+ (○) and Et_4N^+ (△).

These facts suggest that the hydrophobic interaction has an important rôle in the behaviour of TAA ions, at least of n-Pr_4N^+, n-Bu_4N^+ and n-Pe_4N^+, in aqueous dextran gel systems.

Effect of organic eluent

When only the molecular sieving effect depending on the solute size is operative in gel chromatography, the K_d values are linearly dependent on the logarithms of the molecular weights (log MW) or of the molar volumes of the solutes[1].

Fig. 3 shows the relationships between log MW of TAA ions and their K_d values on Sephadex G-10 with 0.1 M sodium chloride solution at pH 2.3, 95% (v/v) methanol and 0.1 M lithium chloride in 95% methanol. With 95% methanol the K_d values decrease with increasing log MW. This tendency is more significant with 0.1 M lithium chloride in 95% methanol. In both cases, the sieving effect must be predominant, but the non-linear dependence of the K_d values on log MW indicates probably that side effects occur even with those eluents which are almost free from water. The effect of addition of lithium chloride to 95% methanol may be attributable to the alteration of solvent properties in both the gel and the mobile phase rather than to the suppression of the electrostatic interaction, because carboxylic groups fixed to the gel matrix presumably do not dissociate in the methanol-rich solvents. With the aqueous eluent the K_d values of the TAA ions, except for n-Pe_4N^+, are almost equal. The notable increase of the K_d value of n-Pe_4N^+ conflicts with the sieving effect.

These facts support the hypothesis that hydrophobic adsorption is responsible, at least partly, for the anomalous behaviour of TAA ions with the aqueous eluent.

Effect of temperature

An endothermic temperature dependence of K_d values is the most important criterion for detection of hydrophobic interactions, because the hydrophobic affinity

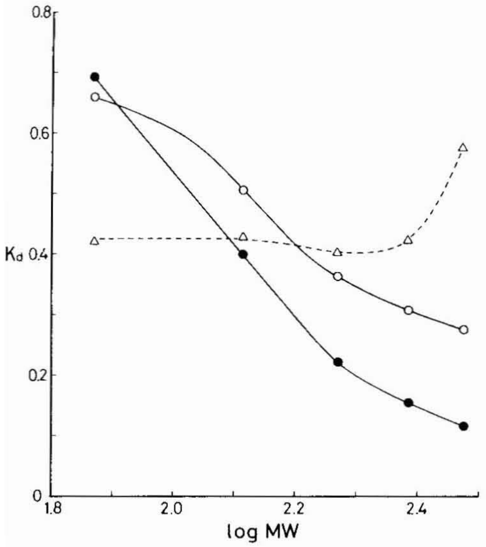

Fig. 3. Effect of organic eluent on K_d values of TAA ions on Sephadex G-10. Eluents: ○, 95% (v/v) methanol; ●, 0.1 *M* LiCl in 95% methanol; △, 0.1 *M* NaCl in water at pH 2.3. Temperature: 20°C.

of the solute for the gel matrix arises from an increase in entropy due to partial melting of an ice-like arrangement of the water structure around the solute[28]. The K_d values of TAA ions at various temperatures are summarized in Table I. The pH of the eluent was adjusted to 2 to suppress the electrostatic interaction as far as possible. It was found that the behaviour of TAA ions on Sephadex G-10 was considerably nfluenced by the previous usage of the gel column. Therefore, the K_d values at 20°C are somewhat different from those at pH 2 in Fig. 1 because different columns were used in each series of experiments.

The K_d values of all TAA ions increase with increasing temperature, but only slightly for those of Me_4N^+ and Et_4N^+. These data gave the thermodynamic funcions ΔH°_{GC} and ΔS°_{GC}, where ΔH°_{GC} is the standard enthalpy change for transfer of he solute from outside the gel beads to the gel phase at infinite dilution. Positive ΔH°_{GC} and ΔS°_{GC} values were obtained for all TAA ions except Me_4N^+, as shown in Fig. 4a. This again suggests that the hydrophobic interaction has an important rôle in he gel chromatography of TAA ions.

Enthalpy–entropy compensation test

The enthalpy–entropy compensation test is a diagnostic tool used to differeniate between mechanistically different separation processes. It has been successfully applied to the elucidation of the behaviour of organic and biological solutes in reversed-phase chromatography[29–31] and to that of aliphatic *n*-alcohols in gel chromaography[10].

Compensation behaviour had been tested by the linearity of a plot of ΔH° against ΔS° values for analogous substances, until Krug *et al.*[32,33] proposed a plot of ΔG° against ΔH° in order to minimize statistical compensation effects due to errors in ΔH° and ΔS° values. Fig. 4a shows a conventional plot of ΔH°_{GC} against ΔS°_{GC}, and Fig. 4b a plot of ln $K_{d(20)}$ against ΔH°_{GC} according to the procedure of Krug *et al.*; the

TABLE I

K_d VALUES OF TAA IONS AT VARIOUS TEMPERATURES

Gel: Sephadex G-10. Eluent: 0.1 *M* NaCl at pH 2.

Ion	*Temperature (°C)*	K_d
Me_4N^+	10.73	0.366
	16.05	0.371
	20.57	0.374
	25.66	0.377
	30.86	0.382
Et_4N^+	10.50	0.353
	15.95	0.360
	20.63	0.378
	25.46	0.393
	29.97	0.401
n-Pr_4N^+	10.70	0.300
	15.45	0.320
	19.50	0.352
	25.57	0.374
	29.93	0.402
n-Bu_4N^+	10.65	0.273
	15.50	0.323
	19.55	0.366
	25.57	0.419
	29.93	0.471
n-Pe_4N^+	10.70	0.327
	15.50	0.419
	19.25	0.481
	25.57	0.621
	29.95	0.706

quantity ln $K_{d(20)}$ was employed since it is proportional to ΔG_{GC}° and has the minimum statistical errors. The first plot gives an excellent linear relationship, in contrast to the second, indicating the validity of Krug's proposal. We conclude that the behaviour of the TAA ions did not obey a common physico-chemical mechanism under the present experimental conditions.

Separation mechanism

The electrostatic interaction is unlikely to be operative with the eluent at pH 2, because the charge of the gel matrix would be zero or negligibly small[26] and the surface charge densities of TAA ions would be much smaller than those of alkali metal and halide ions, because of their larger ionic radii[34].

The TAA ions did not exhibit common enthalpy–entropy compensation in their partitioning processes between the mobile and the gel phase. This suggests that the behaviour of individual TAA ions is governed by a number of factors whose individual importance may vary. One such factor is probably the hydrophobic interaction; this is strongly supported by the effects of eluent concentration, organic eluent and temperature on the K_d values. Further supporting evidence is provided by the fact

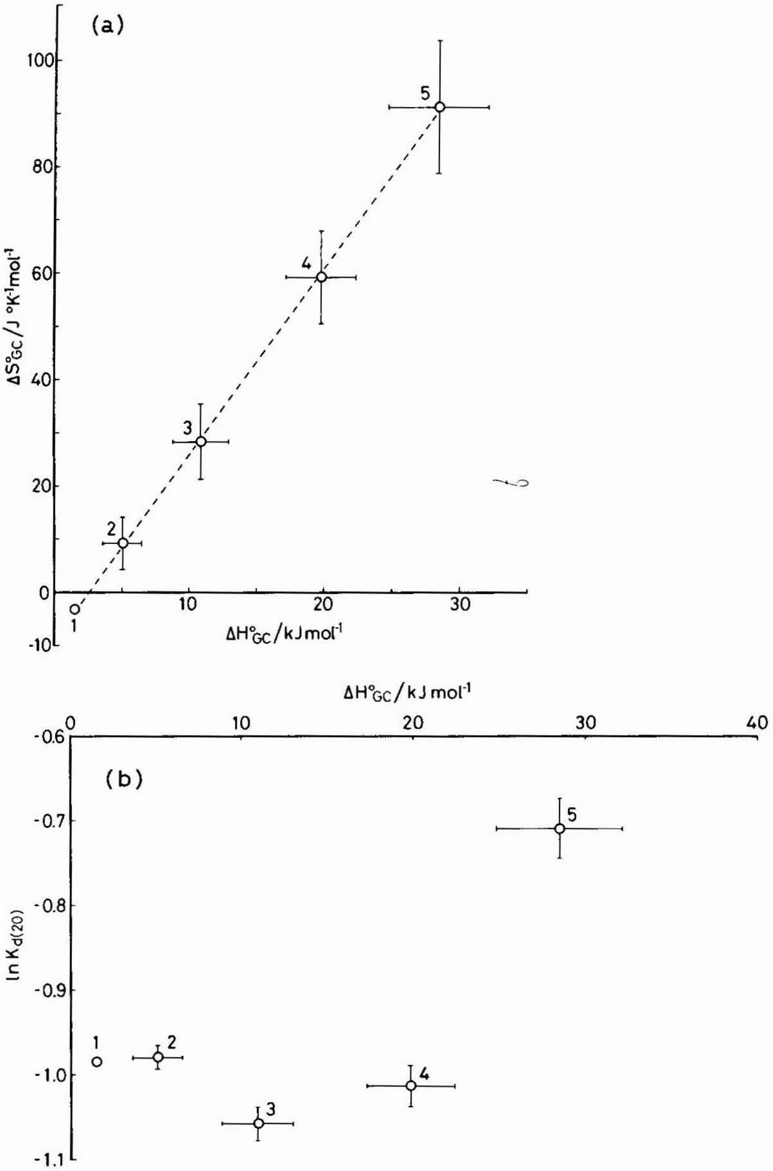

Fig. 4. Plots for enthalpy–entropy compensation test. Cations as in Fig. 2. The vertical and horizontal lines through the circles indicate the 0.95 confidence intervals of the thermodynamic functions. a, Conventional plot of ΔS°_{GC} against ΔH°_{GC}; b, Krug plot of ln $K_{d(20)}$ against ΔH°_{GC}.

that both the ΔH°_{GC} and ΔS°_{GC} values of all the TAA ions, except ΔS°_{GC} of Me_4N^+, are positive. This is similar to the case of aliphatic *n*-alcohols in aqueous dextran gel systems, where the linear correlations of the ΔS°_{GC} values of the alcohols with their ΔS°_{HY} values indicated the occurrence of hydrophobic interactions[10].

However, no ΔS°_{HY} values of TAA ions have been reported. We have proposed[35] the use of (B_η − 0.0025 $\bar{V}^{\circ}$) values as a substitute for the $\Delta S^{\circ}_{\mathrm{HY}}$ of hydrophobic solutes, where B_η is the second virial coefficient and $\bar{V}^{\circ}$ the partial molar volume of the solute in infinite dilute solution. Accordingly, the $\Delta S^{\circ}_{\mathrm{GC}}$ values of TAA ions are plotted against their (B_η − 0.0025 $\bar{V}^{\circ}$) values in Fig. 5. The excellent linear relationship obtained indicates that the separation mechanism of TAA ions can be interpreted in terms of a hydrophobic interaction.

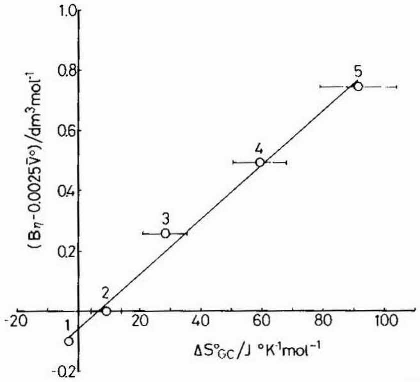

Fig. 5. Plot of $\Delta S^{\circ}_{\mathrm{GC}}$ against (B_η − 0.0025 $\bar{V}^{\circ}$) for TAA ions. Cations as in Fig. 2.

As discussed previously[10], the entropy of a system, including the gaseous hydrophobic solute and the solvent, decreases upon dissolution of the solute in water because of the formation of hydrophobic hydration shells, where the water structure is more ordered. This entropy loss will be linearly dependent on the degree of ordering of the water structure, which can be estimated from the (B_η − 0.0025 $\bar{V}^{\circ}$) value of the solute[35]. It may also be partly compensated by the permeation of the solute from the bulk to the gel phase and by the local sharing of the hydration shell of the solute with that of the hydrophobic site of the gel matrix, resulting in decrease in the degree of ordering of the water structure. The larger (B_η − 0.0025 $\bar{V}^{\circ}$) value of the solute results in the higher entropy compensation. This may account for the hydrophobic interaction in the separation mechanism of TAA ions, at least of n-$\mathrm{Pr_4N^+}$, n-$\mathrm{Bu_4N^+}$ and n-$\mathrm{Pe_4N^+}$, as discussed later.

Another important factor is probably the sieving effect. In order to confirm this, Van 't Hoff plots of the K_d values in Table I were carried out. On extrapolation of the straight lines in Fig. 6, it is found that the K_d values decrease in the order of $\mathrm{Me_4N^+}$, $\mathrm{Et_4N^+}$, n-$\mathrm{Pr_4N^+}$, n-$\mathrm{Bu_4N^+}$ and n-$\mathrm{Pe_4N^+}$ below 0°C, suggesting that the sieving effect may operate principally in this temperature range. In contrast, the K_d values increase in the same order above 30°C, where the hydrophobic interaction may be predominant.

According to Desnoyers and Perron[36], $\mathrm{Et_4N^+}$ having a (B_η − 0.0025 $\bar{V}^{\circ}$) value of zero is neither a structure-maker nor a structure-breaker for water and $\mathrm{Me_4N^+}$ having a negative (B_η − 0.0025 $\bar{V}^{\circ}$) value is a structure-breaker. Since hydrophobic solutes have been regarded as structure-makers, the behaviour of $\mathrm{Me_4N^+}$ and $\mathrm{Et_4N^+}$ can hardly be discussed in terms of the hydrophobic interaction. Nevertheless, TAA ions, including $\mathrm{Me_4N^+}$ and $\mathrm{Et_4N^+}$, yield an excellent linear correlation of $\Delta S^{\circ}_{\mathrm{GC}}$ with

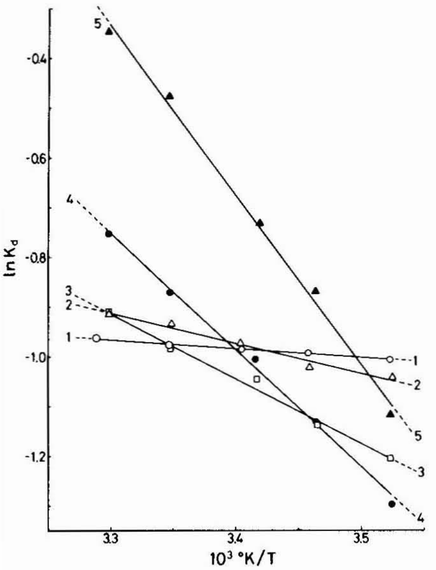

Fig. 6. Van 't Hoff plots of K_d values of TAA ions in Table I. Cations as in Fig. 2.

(B_η − 0.0025 $\bar{V}^\circ$) values, as shown in Fig. 5. The reason for this has not yet been ascertained.

In conclusion, both the hydrophobic interaction and the sieving effect may contribute to various extents to the behaviour of individual TAA ions in the temperature range studied.

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EFFECT OF THE COMPONENTS OF THE CHROMATOGRAPHIC SYSTEM ON THE ELECTROKINETIC STREAMING CURRENT GENERATED IN LIQUID CHROMATOGRAPHY COLUMNS

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SUMMARY

Electrokinetic streaming currents were studied in chromatographic systems comprising two different batches of silica gel and hexane–butanol mobile phases containing water, acetic acid or picric acid. The sign and the magnitude of the basic currents could be stabilized by rinsing the column with the mobile phase. The required volumes, 10^2 to 10^3 column dead volumes, depended on the history of the solid phase. Variations in the procedure for preparation of the silica gel influenced the basic currents, solutes retentions and responses. Irreversibly sorbed picric acid did not affect solute retention. However, it did change the response ratios in different mobile phases and increased the responses of some solutes to such an extent that they could be detected at concentrations of hundredths of ppm. The electrokinetic effects in liquid chromatographic systems are, therefore, influenced by alterations in the quality of the solid surface which need not be reflected in the mass equilibria.

INTRODUCTION

The measurement of electrokinetic streaming currents generated in a chromatographic column on passage of the mobile phase and at the separation of the components of the sample has been described[1–3] for liquid chromatography. However, attempts to apply this technique have drawn attention to certain problems associated with the long-term stability of the basic streaming current and response values.

Variations in the phase equilibria in the surface layer of the solid phase, which are most probably caused by variations in the composition of the mobile phase or by impurities in the injected samples, are manifested as an instability of the streaming current. Experiments have shown that the source of the instabilities may also lie in the differences in the properties of the solid phases caused by variations in their method of manufacture and in their subsequent rinsing with the mobile phase.

In order to examine the effects of these variations, two samples of silica gel, prepared from one technological batch but differing in the final stage of preparation, were used. The properties of the mobile phases were studied by varying the content of water and by adding compounds which exhibit different acidities. Simple organic compounds having different functional groups were used as solutes.

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EXPERIMENTAL

A home-made apparatus was used for the chromatographic measurements. An MC 300 plunger pump (Mikrotechna, N.E., Prague, Czechoslovakia) with a pressure-damping system according to Locke[4] was later replaced with a more suitable pulseless pump (Varian 8500). Streaming currents, from an isolated and electrically screened stainless-steel column[3], were led to a Vibron Model 33 C electrometric amplifier (Electronic Instrument, Richmond, Great Britain) with an input resistance of 10^9 ohms. A Siemens refractometer was used as a reference detector.

The viscosity variant of the filtration technique[5] was used to pack the columns (200 × 4 mm). Silica gel samples were suspended in cyclohexanol–methanol (95:5, v/v). Petrol-for-medical purposes was used to transfer the packing pressure of 25 MPa. Packed columns were conditioned directly with the mobile phase.

Both Silasorb 500 silica gel samples (Lachema, Brno, Czechoslovakia), with a specific surface area of $500 \pm 10\ \mathrm{m^2/g}$ and a particle diameter $d_p = 10\ \mu\mathrm{m}$, were from one technological batch. After grading, one sample was treated only with dilute hydrochloric acid (chloride-treated silica gel, "HCl"), and the other was rinsed additionally with phosphate buffer, pH = 7, until the reaction for chloride ions in the eluate was positive (phosphate-treated silica gel, "$\mathrm{PO_4^{n-}}$"). Both samples were then washed with a small volume of distilled water and dried. The samples supplied by the manufacturer were not treated prior to packing.

The fundamental mobile phase, butanol–hexane (95:5, v/v), was prepared from analytical grade chemicals. Mobile phases with higher water contents were obtained by saturating a portion of the mobile phase with water [a small excess of water above saturation (5–7 drops for 1 l) was ultrasonically stirred into a milk-like suspension which was sedimented freely overnight] and by subsequent mixing with non-saturated mobile phase in the ratio of 4:1 (v/v). The resulting water content was not determined. Acetic acid (HAc) of analytical grade was added to the mobile phases at a concentration of 0.1 % (v/v), picric acid (HPi) at concentrations of 1 and 10 ppm (w/v).

The relative permittivities and the conductivities of the mobile phases were measured by means of a Tesla BM 484 semiautomatic bridge (Tesla, N.E., Brno, Czechoslovakia). A capacitor with a volume of 142 μl and air capacitance of 401 pF (stray capacitance 10 pF) was a larger version of the detection cell of the capacitance detector[6]. The input filter of the capacitor was made of stainless steel and PTFE. The relative permittivities of the mobile phases varied from 2.000 to 2.014, and could not be correlated unambiguously with the contents of moderators (water and acids).

Solutes of different origin, mostly of analytical grade, were injected through a septum. Butanol (BuOH), acetic acid (HAc), benzyl cyanide (BC) and 2-bromopyridine (BP) were used without dilution. Lauric acid (HLa), *p*-nitrophenol (NP), 5,5-diethylbarbituric acid (barbital, HB), 5-ethyl-5-phenylbarbituric acid (phenobarbital, HPB) and 1,2-dibromo-1,2-dichloroethyl dimethyl phosphate (Naled, Nal) were injected as solutions in butanol. *o*-Nitroaniline (NA) was dissolved in nitrobenzene (NB).

RESULTS

Column equilibration

At the beginning of the measurements with chloride-treated silica gel, the degree of column adsorbent equilibration with the mobile phases was estimated by the usual manner in chromatography: constancy of the capacity factors of the eluted compounds. In the course of these measurements, variations in the basic streaming current and in the electrokinetic responses were observed, in agreement with experience. In some instances even a change in their sign was noted. This confirms that the usual procedure for the stabilization of sorption properties of a chromatographic system is not sufficient to stabilize the electrokinetic effects generated in them.

At first, rinsing volumes were measured only if a new component of the mobile phase was introduced for the first time or if its concentration was increased. It appeared (Table I) that under the conditions used the consumption of mobile phase never fell below hundreds of column dead volumes. Since the rinsing volumes varied markedly, it is obvious that the volume of the mobile phase passed cannot alone be a criterion for sufficient conditioning of the column from the viewpoint of the electrokinetic phenomena, despite the fact that this volume surpassed by one order of magnitude or more the volumes commonly considered as sufficient for the stabilization of sorption properties of chromatographic systems. From the differences in the required amounts of mobile phase it follows, however, that these volumes can serve as a source of information on phenomena associated with the sorbent equilibration.

In the final washing stage, the basic streaming current always changed very slowly. Therefore, towards the end of the measurements with chloride-treated silica gel and during the whole set of measurements with phosphate-treated silica gel the

TABLE I

CHARACTERISTICS OF THE INVESTIGATED SYSTEMS

Moderator	*Conductivity (μS)*	*Relaxation time (sec)*	*Rinsing volume (no. of column dead volumes)*		*Basic current (pA)*	
			HCl	*PO_4^{n-}*	*HCl*	*PO_4^{n-}*
–	0.0002	4.0	–	350	−1 to −4	1.1
H_2O	0.0005	1.6	135	225	2.3 to 6.3	1.9
–	0.0002	4.0	–	–	−2.3	–
–	0.0002	4.0	–	–	0 to −1.3	–
HAc	0.0007	1.1	275	825	51	0.8
HAc*	0.0007	1.1	–	–	31	–
H_2O, HAc	0.0009	0.9	–	875	6	5.7
H_2O	0.0005	1.6	–	655	−1.4	12.3
–	0.0002	4.0	–	375	−1.7	2.7
HPi (1)**	0.0011	0.7	600	150	5.3	6.0
H_2O, HPi (1)	0.0012	0.7	–	400	–	25
HPi (10)	0.0013	0.6	275	900	15.6	15.2
H_2O, HPi (10)	0.0031	0.3	500	750	27	30

* Measurement repeated after half a year and further rinsing of the column.

** Figure in parentheses gives the concentration of picric acid in ppm; similarly in Tables III–VII.

column was washed until the basic streaming current remained constant during the passage of at least 70 column dead volumes. After the stabilization in this way, constant responses were always obtained to all the solutes used. Instabilities of the basic streaming current were successfully obviated in all instances except the following two: when phosphate-treated silica gel was equilibrated with mobile phases to which water and acetic acid had been added, and in a next experiment where the acetic acid was omitted and the mobile phase contained an addition of water only. In these two instances the stepwise changes of the streaming current occur after the injection of solutes.

Responses generated by the injection of samples

In all the present chromatographic systems, positive exclusion responses[7] were recorded upon injection of any sample. A response is considered as positive if it results in an increase in the absolute magnitude of the positive streaming current or a decrease in the absolute magnitude of the negative streaming current.

The amounts of the injected samples varied roughly from 0.1 to 3 mg, the injected volumes from 2 to 5 μl (Tables V and VI). Since the electrokinetic responses were taken directly from the chromatographic column, the retention data had to be measured in a non-thermostatted column at room temperature. The retentions were characterized by the capacity factors. Butanol served as an unsorbed compound that provides an electrokinetic signal and was eluted, at the precision of these measurements, within the same volume as the unsaturated hydrocarbons in all the chromatographic systems investigated.

The magnitudes of the electrokinetic responses, measured at a volume flow-rate of 0.9 ml/min, were normalized at a concentration of 10 mg/ml and a volume flow-rate of 1.0 ml/min. The concentrations at the maxima of the eluted zones were calculated for the mean or for the most frequent values of the retentions, assuming that the variations in the shapes of the zones caused by deviations in the retention values or in the composition of the chromatographic system can be neglected to a first approximation. The capacity factors for which the concentrations were calculated are listed in Table II, and the retentions and the responses are summarized in Tables III–VI.

TABLE II

INJECTED AMOUNTS OF SOLUTES AND CONCENTRATIONS AT ZONE MAXIMA

Solute	*Injected amount (mg)*	*Concentration at maximum (mg/ml)*	*Typical retention value, k'*
Butanol	1.62	15.3	0
Acetic acid	2.10	3.68	2.3
Lauric acid	0.242	0.708	0.30
Benzyl cyanide	2.02	9.73	0.80
2-Bromopyridine	3.14	5.84	1.5
Nitrobenzene	2.32	18.4	0.24
o-Nitroaniline	0.0966	0.274	1.8
p-Nitrophenol	0.0974	0.301	1.2
Barbital	0.0855	0.119	5.6
Phenobarbital	0.2150	0.323	4.9
Naled	≈0.1	≈1	0.20

ABLE III

APACITY FACTORS OF SOLUTES ON SILICA GEL RINSED WITH HCl

oderator	*Basic current (pA)*	*HAc*	*HLa*	*BC*	*BP*	*NB*	*NA*	*NP*	*HB*	*HPB*	*NaI*
	−1 to −4	3.0	0.47	1.2	1.9	0.25	2.9	2.7		7.0	0.30
$_2$O	2.3–6.3	A* 2.9	0.41	0.82	1.2	0.23	2.2		–	–	–
	−2.3	1.8	0.31	0.93	1.6	0.26	1.9	1.2	–	2.7	0.21
	0–1.3	1.8	0.30	0.86	1.5	0.26	1.9	1.2	–	2.6	0.20
Ac	51	1.3	U**	0.87	1.5	0.25	1.8	1.2	–	2.4	0.21
Ac***	31	A, U	0.23	0.89	1.3	0.25	1.9	1.2	2.7	2.2	0.25
$_2$O, HAc	6.0	2.2	0.42	0.92	1.2	0.26	2.3	2.4	5.4	4.7	0.35
$_2$O	−1.4	2.3	0.46	0.87	1.2	0.29	2.5	2.2	5.7	4.8	0.38
	−1.7	1.7	0.42	0.84	1.4	0.24	1.8	1.2	2.7	2.7	0.19
Pi (1)	5.3	1.7	0.38	0.82	1.4	0.24	1.8	1.1	3.0	2.4	0.19
Pi (10)	15.6	2.6	0.30	0.82	1.4	0.25	1.7	1.1	2.8	2.8	0.19
$_2$O, HPi (10)	27.0	2.3	0.46	0.90	1.1	0.25	2.2	2.1	5.6	4.9	0.39

* Anomaly in electrokinetic response.

** The measurement cannot be evaluated.

*** Measurements repeated after half a year and further rinsing of the column.

DISCUSSION

Equilibration of the system; basic streaming current

Reference measurements of the relative permittivities and conductivities of the mobile phases showed that the former values were determined by the precision of addition of the butanol. The other components only affected the conductivity (Table I). The relaxation times of the mobile phases were always so long that their effect on the measured electrokinetic streaming currents could be neglected^3.

Irregular Silasorb sorbents are prepared from silicic acid of high purity. The samples of Silasorb 500 that were used here were not treated hydrothermally, nor did they come into contact with other substances which could chemically modify their surfaces. The HCl and the phosphate buffer were applied under conditions such that there was no reaction with the silica gel surface. Both silica gel samples studied can therefore be considered as relatively pure silicon dioxide, the surface of which should be contaminated only with residues of HCl or of the phosphate buffer and water, which are retained physically.

The concentrations of both water and acetic acid in the stationary phase could be varied reversibly by adjusting the composition of the mobile phase. However, picric acid was retained so strongly that, even after the passage of 2000 ml of the fundamental mobile phase (1000 dead volumes) through a column that had previously been equilibrated with a mobile phase containing 10 ppm of picric acid, no change was observed in the magnitude of the basic streaming current. In the hexane–butanol (95:5) mobile phase the sorption of picric acid can therefore be considered as irreversible.

Rapid elution of picric acid from silica gel can be achieved, for example, by using ethanol. Free inorganic acids, which are strongly ionized in aqueous solutions,

TABLE IV

ELECTROKINETIC RESPONSES OF SOLUTES ON SILICA GEL RINSED WITH HCl

Values are expressed in pA and normalized at concentrations of 10 mg/ml and flow-rates of 1 ml/min.

Moderator	*Basic current (pA)*	*BuOH*	*HAc*	*HLa*	*BC*	*BP*	*NB*	*NA*	*NP*	*HB*	*HPB*	*NaI*
–	−1 to −4	−2.9	−9.2	−157	−0.57	−0.95	−0.19	−41	26	–	U*	4
H_2O	2.3–6.3	A**	A −16	10.7	5.4	3.1	4.0	390	–	–	–	–
–	−2.3	−1.7	−26	−115	0.46	1.1	0.61	150	330	–	−38	6.6
–	0–1.3	0.26	−45	−47	2.3	U	2.0	145	330	–	−38	3.3
HAc	51	−1.1	16.8	U	−11.4	−16.5	−8.4	−240	350	–	−47	U
HAc***	31	A	A	−98	−7.3	−12.6	−4.0	−160	53	−120	−34	5.5
H_2O, HAc	6.0	U	−10.9	−100	−2.8	−10.0	−0.50	−32	86	−47	−17	4.4
H_2O	−1.4	U	−15	−31	−1.4	−2.7	0.37	49	92	−12	−4.4	3.3
–	−1.7	−1.8	−23	−68	−4.5	−5.2	1.6	41	110	U	45	14.5
HPi (1)	5.3	4.8	−47	−320	8.2	1.6	7.0	280	420	−29	−10	7.7
HPi (10)	15.6	7.5	−48	−370	11	4.3	11	470	620	U	U	14.5
Hpi (10), H_2O	27.0	13	−3.9	−110	9.1	12.8	6.8	490	590	U	21	14.5

* Response is not detectable.

** Anomaly in electrokinetic response.

*** Measurement repeated after half a year and further rinsing of the column.

TABLE V

CAPACITY FACTORS OF SOLUTES ON SILICA GEL RINSED WITH HCl AND PHOSPHATE BUFFER

Moderator	*Basic current (pA)*	*HAc*	*HLa*	*BC*	*BP*	*NB*	*NA*	*NP*	*HB*	*HPB*	*Nal*
–	1.1	A* 1.7	0	0.91	1.6	0.26	1.9	1.1	3.4	2.6	0.20
H_2O	1.9	A 2.6	0.49	A 0.96	A 1.3	0.30	2.4	2.5	6.7	5.7	0.40
HAc	0.8	A 2.6	0.52	1.26	A 0.72	A 0.28	A 2.9	3.1	8.8	7.5	0.40
H_2O, HAc	5.7	A 2.6	A 0.47	A 0.93	1.2	A 0.29	A 2.3	2.4	6.4	5.5	0.43
H_2O	12.3	A 3.9	A 0.5	A	A 0.84	A 0.23	A 1.8	2.3	7.7	6.3	0.33
–	2.7	1.9	0	A 0.92	1.5	0.27	1.9	1.4	4.0	3.6	0.24
HPi (1)	6.0	1.8	0	0.86	1.4	0.25	1.9	1.3	3.9	3.0	0.21
H_2O, HPi (1)	25	2.7	A 0.86	0.83	1.2	A 0.26	2.3	2.4	6.7	5.7	0.41
HPi (10)	15	A 1.5	0.25	0.87	1.5	0.25	1.8	1.1	2.7	2.2	0.22
H_2O, HPi (10)	30	A 0.43	A 0.45	A 0.94	1.2	0.24	2.2	2.2	5.5	4.5	0.34

* Anomaly in electrokinetic response.

TABLE VI

ELECTROKINETIC RESPONSES OF SOLUTES ON SILICA GEL RINSED WITH HCl AND PHOSPHATE BUFFER

Values expressed as in Table IV.

Moderator	*Basic current (pA)*	*BuOH*	*HAc*	*HLa*	*BC*	*BP*	*NB*	*NA*	*NP*	*HB*	*HPB*	*NaI*
–	1.1	1.4	A* 1.2	A, U**	1.8	0.58	1.4	69	330	U	U	A, U
H_2O	1.9	A 3.1	A −5.0	−19	A −7.3	A −15	0.36	64	280	−37	−20	2.8
HAc	0.8	A 1.2	A 0.39	2.0	A −0.22	A, U	A, U	A 12	74	U	U	U
H_2O, HAc	5.7	A 2.6	A −5.1	A 4.8	A 3.1	4.4	A 1.8	A 132	200	−66	−34	3.3
H_2O	12.3	A, U	A	A 8.0	A, U	A −2.9	A 1.8	A 29	210	−55	−34	U
–	2.7	5.5	−3.1	U	A −2.3	1.2	2.4	160	220	U	U	4.4
HPi (1)	6.0	4.1	−0.91	U	4.9	2.4	3.8	150	160	U	−18	6.6
H_2O, HPi (1)	25	3.9	0.91	A −4.8	5.3	6.4	2.5	120	140	−61	−10	3.3
HPi (10)	15	13	A 4.7	−43	18	5.3	14	630	850	−56	−31	6.6
H_2O, HPi (10)	30	8.0	A	A −55	A 8.0	14	6.3	340	360	U	U	2.8

* Anomaly in electrokinetic response.

** Response is not detectable.

TABLE VII

INFLUENCE OF PRE-ADSORPTION OF PICRIC ACID ON PHOSPHATE-TREATED SILICA GEL AND OF MOBILE PHASE MODERATORS ON RETENTION AND ELECTROKINETIC RESPONSE

Electrokinetic responses expressed as in Table IV.

Composition, stationary/mobile phase	*Basic current (pA)*	*Nitrobenzene*		*o-Nitroaniline*		*p-Nitrophenol*		*Benzyl cyanide*		*2-Bromopyridine*	
		k	*pA*	*k*	*pA*	*k*	*pA*	*k*	*pA*	*k*	*pA*
–/–	1.1	0.26	1.4	1.9	69	1.1	330	0.91	1.6	1.6	0.58
HPi/–	1.8	0.25	24	1.8	1160	1.1	2800	0.85	24	1.5	9.4
HPi/HPi	15	0.25	14	1.8	630	1.1	850	0.87	18	1.5	5.3
HPi/HPi, H_2O	30	0.24	6.3	2.2	340	2.2	3.60	0.94	8.0	1.2	14

can also be eluted with ethanol. They are eluted in the dead volume (H_3PO_4) or have negligible retentions ($HClO_4$, HNO_3, H_2SO_4). The sodium or potassium salts of phosphoric, boric or hydrofluoric acids could, however, not be eluted from silica gel with pure alcohol[8]. It follows that the ionization of free acids is strongly suppressed in alcoholic media.

With a mobile phase containing water, phosphoric acid and 10 ppm of picric acid it was possible to observe the elution of H_3PO_4 from the phosphate-treated silica gel by measuring the electrokinetic streaming current and even by refractometry.

It follows from the above experiments that a certain ability to elute free inorganic acids from silica gel can be ascribed to all the mobile phases used. This, together with previous knowledge of the origin of electrical double-layers on the surface of silicon dioxide, makes it possible to describe qualitatively some variations in the magnitude or in the sign of the charge of the electrical double-layer on the surfaces of the packings tested.

The conditions in the part of the liquid that is in direct contact with the solid phase are crucial insofar as the surface charge is concerned. With a multicomponent mobile phase containing a large amount of hydrocarbon and with silica gel as adsorbent, the liquid which is contiguous with the solid phase comprises the adsorbed layer of polar components; this may also contain compounds remaining on the surface from earlier treatments. It follows from the work of Scott and Kucera[9] that in all these systems a compact sorbed layer of butanol was formed on the surface of the solid phase. In the systems containing water or acids the butanol layer was enriched by these components[10].

The surface charge of adsorbents of the oxide type, determined by the concentrations of H^+ and OH^-, is dependent on the pH in aqueous media. The sign changes on passing through the isoelectric point[11]. pH values of 1.3–3.7 or 3–5 are observed[12,13] for the isoelectric point of silicon dioxide in water. In alcoholic media, SiO_2 gains surface charge by the same mechanism as in water[14]. Alcohols, however, possess greater electronegativity than water[14] and therefore the isoelectric point is expected to lie at higher pH values. This means that, at the same pH value, silica gel gains positive surface charge (or increases it) on passing from an aqueous to an alcoholic medium. When substances capable of ionizing via the elimination of a proton or hydroxyl group are present on the silica gel surface or in the adsorbed layer, it is necessary that these ionisation processes and their course in semi-aqueous or alcoholic media should be considered.

The surface of chloride-treated silica gel in a butanol medium gained a negative charge (Table I). The gradual decrease in the magnitude of this charge with increasing volume of the non-aqueous mobile phase passed can be explained by gradual washing out of HCl. On passage of a mobile phase enriched with water, the content of water in the sorbed phase increased. The establishment of the positive surface charge can be ascribed to dissociation of HCl and to the associated decrease in pH below the isoelectric point. The reason for the establishment of the negative charge on the surface of silicon dioxide in a butanol medium in the presence of HCl is not clear.

In the medium containing relatively electronegative acetic acid, silica gel gains a considerable positive charge. If water is added to the system, the content of acetic acid in the sorbed phase decreases with the simultaneous increase in the water content. Both these processes lead to reduction of the positive surface charge. The increase in the concentration of hydrogen ions caused by the dissociation of acetic acid in the presence of water is, bearing in mind the strength of acetic acid and pH of the isoelectric point for silica gel, obviously negligible.

Picric acid is known to ionize even in non-aqueous media. Thus its presence can so reduce the pH of the butanol phase that the silica gel surface gains a positive electric charge. On increasing the acid concentration, this charge increases. In the presence of water, the picric acid ionization increases, which appears as an even greater positive electric charge on the gel surface.

The systems containing phosphate-treated silica gel behave differently. On injection of samples, composite responses are often observed (Tables V and VI) similar to composite signals generated almost simultaneously by different sources[15]. Such responses cannot be ascribed to either the mobile phase or the manner of sensing the electrokinetic signals, which was selected so as to eliminate the generation of responses caused by the interaction of the effluent with the sensing element[15]. They must therefore be caused by the packing of the column. The variations in the magnitude of the basic streaming current after the injection of samples, observed, for example, in the system containing the mobile phase enriched with water and acetic acid, can also be ascribed to the sorbent. Rinsing of the silica gel with a small volume of distilled water after the application of phosphate buffer, used in the final stage of the preparation of the sample, does not ensure the quantitative removal of the phosphate.

Dobiáš[16] found that, with incompletely soluble solid phases of ionic character ($BaSO_4$, CaF_2), the potential-determining ions are the grid ions. The solubility of salts is very limited in alcoholic media. Moreover, the amount of the salt that can be transferred into the layer of the sorbed liquid is limited also by the volume of this liquid. It can therefore be assumed that solid residues of the buffer on the silica gel surface behave in the same way as an incompletely soluble ionic phase and may be an independent source of the streaming current.

A characteristic of the phosphate-treated silica gel is that it is positively charged in all the systems. From the increase in the positive charge after the addition of water to the mobile phase, which was always observed, it can be concluded that acidic components from the preparation, capable of ionizing easily, are present on the surface of the sample. The addition of acetic acid, which reduced the amount of water in the sorbed phase and suppressed, by its higher electronegativity, the ionization of these components, reduced the positive electric charge on the gel surface.

The addition of picric acid had the same effect as in the systems with chloride-treated silica gel. The similarity in behaviour of this compound and in the magnitudes of the basic streaming currents found in comparable systems suggest that of all the moderators tested picric acid possesses the strongest ability to affect the electric relationships at the phase interface. It can therefore be recommended for standardization of electrokinetic phenomena in comparable chromatographic systems or, at least, for the suppression of differences between them.

Retentions and responses of solutes

Model solutes were mostly selected to represent compounds of different types. It is seen from Tables III–VI that the differences in the mobile phase composition and in the history of the solid phase affect the values of both retention and electrokinetic response. Furthermore, the retentions and electrokinetic responses of different solutes are influenced by the variations in the properties of the chromatographic phases in different ways.

Regardless of the treatment of the silica gel, the addition of water to the mobile phase had a significant effect on the retentions of hydrophilic solutes or of compounds having markedly hydrophilic groups (acetic and lauric acids, barbiturates, *p*-nitrophenol), the retentions increasing by more than 100%. The retentions of *o*-nitroaniline, 2-bromopyridine, benzyl cyanide and naled changed by 20–60%, but there was almost no effect on the retention of nitrobenzene. In the majority of cases, the mode of silica gel treatment was reflected in the absolute values of the retentions. The relative changes in retention caused by the variations in water content in the mobile phase were, however, practically independent of the treatment.

The influence of acetic acid depended significantly on the treatment. In the chromatographic systems with chlorice-treated silica gel, acetic acid affected the retentions of only two solutes: acetic and lauric acids. In the systems with phosphate-treated silica gel, the addition of acetic acid caused multiple changes in the retentions of some compounds. The magnitude and the character of these changes also depended on the presence of water. Picric acid had a significant effect on the retentions only of acetic and lauric acids, this being more marked in the systems with phosphate-treated silica gel.

The differences in the retentions caused by the differences in the treatment of the solid phase are surprisingly large, amounting to ±80% for some solutes in comparable systems (*i.e.*, in systems with the same mobile phases). Despite this they usually do not play an important role in practice. Providing solutes of the same or related type are separated, the retention values can be adjusted by varying the mobile phase composition. The differences in retention which are sometimes found on silica gels of different type or on products of different origin are usually explained in terms of discrepancies between the declared and actual specific surface areas, differences in the structure and size of pores or variations in the degree of surface hydration. The results discussed above and those in ref. 8 show, however, that another possible reason may be differences in the mode of preparation of the silica gel.

The variability of the electrokinetic responses and their dependence on both components of the chromatographic system is greater than in the case of retention. It is useful to interpret in terms of electrokinetic responses the variations in the streaming currents caused by a reversible short-term change in the mobile phase composition[3]. Constancy of the basic streaming current can be considered as evidence of the stability of the relationships at the phase interface between consecutive injections of quantitatively eluted solutes. Two factors determine the value of the electrokinetic response: the relationships at the liquid–solid phase interface, and the short-term changes in these relationships caused by variations in the mobile phase composition (*i.e.*, by the solute concentration pulse).

The relationships at the phase interface (mass and charge balance equilibria) that are established in the course of the column equilibration evidently depend on the ability of the mobile phase to elute from the surface of the solid phase the components or their reaction products that were captured earlier, and on the ability of the solid phase to bind to its surface the components of the mobile phase. These equilibration processes and their dependence on the history of the solid phase and on the mobile phase composition can be deduced from the rinsing volumes and their variations (Table I).The influence of the quality of the solute on the electrokinetic response is obvious (see, for example, Tables III and IV), and the dependence of the response on the concentration of the solute, derived theoretically, was also verified experimentally[2].

Šlais and Krejčí[2] derived the following relationship for the value of the electrokinetic response

$$\frac{dI_{str}}{dc_i} = \frac{\kappa}{D} \cdot \frac{(\varphi_i - \varphi_0)}{c_{si}^0} \cdot K_d F_m \qquad (1)$$

where I_{str} is the streaming current (the measured electrokinetic response), c_i is the concentration of solute i in the mobile phase, κ is the conductivity of the mobile phase, D is the molar diffusivity of the charge, φ_i is the potential of the outer plane of the Stern layer comprising a monolayer of the molecules of compound i, φ_0 is the corresponding potential when the surface is not covered by compound i, K_d is the distribution constant of compound i and F is the volume flow-rate of the mobile phase. The mobile phase is thus characterized by the term F_m κ/D, the stationary phase and the solute by the factor $(\varphi_i - \varphi_0)$ K_d/c_{si}^0, where c_{si}^0 is the maximum interface concentration of component i. For a selected chromatographic system (and for the given solute) the right-hand side of the equation is constant.

It follows from this equation that if a column is rinsed consecutively with two mobile phases, the respective basic streaming currents and responses to any solute will be reproducible. However, after washing the column with a mobile phase containing picric acid, the values that were measured initially could not be reproduced on replacing this phase with the fundamental mobile phase.

It is seen from Table VII that the introduction of picric acid into the stationary phase does not measurably affect the mass equilibria of the solutes listed, expressed in terms of the capacity factors. (The changes that were observed are within the limits of experimental error.) There is essentially no difference between the mechanism of generation of the basic streaming current and that of the solute response. The differences in the magnitudes of the basic streaming currents in systems with the same mobile phases (lines 1 and 2 in Table VII) can therefore be explained, in terms of eqn. 1, by differences in φ_0 only, which is equivalent to the changes in quality of the surface of the solid phase. Thus, from electrokinetic phenomena in chromatographic systems, not only electrical relationships at the phase interface but also changes in the quality of the solid phase surface, which may not be revealed by means of adsorption measurements, can be evaluated.

Phosphate-treated silica gel when equilibrated by a mobile phase containing 10 ppm of picric acid can, with respect to the adsorption strength of picric acid, be considered as a solid phase of constant quality in all the cases listed in Table VII. The conductivities of butanol–hexane, butanol–hexane + 10 ppm of picric acid and butanol–hexane + 10 ppm of picric acid 80% saturated with water increase approximately in the ratio 1:7:15 (Table I). However, the responses of none of the solutes listed in Table VII follows this increase. On the contrary, with the exception of 2-bromopyridine, the measured responses decrease with increasing conductivity of the mobile phase. At the same time, the ratios of solute responses in different mobile phases vary.

The discrepancy between the response values found and the values expected from eqn. 1 cannot be explained by the changes in φ_0. As defined, the value of φ_i is only dependent on the charge of the sorbed particles of the solute and on their maximum surface density. The values of the retentions, K_d, and diffusivities, D, remained constant[3]. Sorption competition of polar components of the mobile phase (water, picric acid) or of their ions, which would be concentration dependent only, could not change the response ratios of the various pairs of solutes in different mobile phases so markedly. It is therefore necessary to consider the specific effects of minor polar components of the mobile phase on the ionization of solutes in non-aqueous media.

The response data in Table VII are also interesting from a practical standpoint. They show that by a suitable modification of the relationships at the phase interface and in the mobile phase not only the sensitivity but also the selectivity of the electrokinetic detection can be affected by orders of magnitude. Moreover, that the modification need not be achieved only by a change in the chemical composition of the stationary phase or its surface[2], but also by the addition of compounds that can, depending on the selection of mobile phase, be reversibly retained. The data also suggest that picric acid is a suitable modifying compound, and that it is advantageous to work with a mobile phase containing the lowest possible amount of highly polar components.

In this context, the effect observed with phosphate-treated silica gel and the fundamental mobile phase is worthy of note. Upon injection of naled the responses recorded exceeded by at least three orders of magnitude those obtained upon its injection into other chromatographic systems. Even though it was not possible to differentiate unambiguously the increase in the basic current from the response itself, this observation suggests that some other, and perhaps more efficient, way of controlling the sensitivity or selectivity of the response can be found than that which follows from, for example, eqn. 1 or by use of picric acid.

CONCLUSIONS

This work has shown that electrokinetic phenomena in a chromatographic system can be stabilized by passing a sufficiently large amount of the mobile phase. The volumes required were always at least an order of magnitude greater than those usually considered to be sufficient to stabilize the retention properties. In the systems tested, these volumes never decreased below hundreds of column dead volumes. The history of the solid phase, *i.e.*, the mode of its preparation, the individual operations associated with application of the sorbent and their sequence have a significant influence on the volume of the mobile phase required for stabilizing the electrokinetic phenomena.

However, the stability thus obtained does not ensure that the initial state can be reproduced precisely after executing any cycle of column operations. The experiments with picric acid show that the reasons for this lack of reproducibility should be found in the limited ability of the mobile phase to establish equilibria among all the components in both chromatographic phases. The standardization of electrokinetic phenomena in chromatographic systems thus remains an unsolved problem.

The variations in the streaming current, observed during the equilibration of silica gel samples with various mobile phases, have been explained qualitatively.

Although the explanation involves some assumptions that could not be verified in the present work, it is consistent for both silica gel samples and can be used to describe even the systems with picric acid.

The washing of silica gel with phosphate buffer led, in chromatographic systems with the same mobile phase, to variations in solute retentions of up to $\pm 80\%$. Together with previous results[8], this shows that even small differences in the silica gel preparation can result in considerable variations in the sorption selectivity of the final product, even when the specific surface area, pore volume and degree of surface hydration do not change.

When interpreting the responses, in addition to the quality of the surface of the solid phase, it is necessary to consider also the interactions of the solutes with the components of the mobile phase during the formation of ions in the solution and at the phase interface. The content of highly polar components, which ionize or can affect the ionization of the solute, has a significant influence on the resulting electrokinetic phenomena. Both these effects complicate the standardization of the electrokinetic responses and their application to detection purposes.

On the other hand, these dependences provide information on the quality of the solid phase surface and probably also the ionization phenomena in non-aqueous media that cannot be obtained by sorption measurements. The dependences of the electrokinetic phenomena on the quality (purity) of the solid phase surface and on the changes in the mobile phase composition, which are negligible from the viewpoint of retention, can also be used for detection purposes.

By the addition of picric acid, and apparently also of some other compounds, the sensitivity of the detection can be increased substantially and also its selectivity can be improved. For instance, for some tested solutes the increase in the response was so high that at the minimum detectable change in the current, $1 \cdot 10^{-14}$ A, which is easily accessible, these compounds could be detected in concentrations of hundredths of ppm.

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DIRECT SAMPLING METHOD FOR GAS CHROMATOGRAPHIC HEADSPACE ANALYSIS ON GLASS CAPILLARY COLUMNS

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SUMMARY

A method is described which allows a rapid, direct, gas chromatographic headspace analysis to be made on capillary columns. The procedure incorporates a simple concentration stage, on a conventional packed pre-column, such that minor components present in headspace vapours can be detected. The method is illustrated by reference to tobacco and other samples of plant origin.

INTRODUCTION

Gas chromatographic (GC) headspace analysis is now frequently used to examine the volatiles associated with a wide range of samples including foods[1], beverages[1], tobaccos[2,3], biological fluids[4,5] and polymers[6–8]. Such analyses may be used, in conjunction with other techniques such as mass spectroscopy, as a means of chemical identification or for obtaining characteristic "fingerprints" of samples in a procedure commonly known as "profile analysis". Whilst the use of chemical identification in flavour related studies is self evident, profile analysis[9] is used to correlate the "fingerprint" GC traces with the properties of the sample concerned. Such methods have been used in disease diagnosis[10,11], investigation of medical disorders[12,13], food quality evaluation[14,15] and air pollution studies[16,17].

Generally headspace vapours are complex mixtures containing chemicals over a wide range of concentrations. Satisfactory GC analysis requires good resolution to separate the components of the mixture and high sensitivity in order to detect those components that occur only at a low concentration. These requirements are generally met by using capillary column chromatography and employing a concentration stage prior to analysis. Several different methods of effecting a concentration of the headspace vapours have been reported[18] including the use of a transevaporator[19] and more commonly, carbonaceous[20,21] or porous polymeric adsorbents[22,23]. These methods may be called "indirect" as they involve a primary concentration of headspace vapours, usually carried out remote from the gas chromatograph prior to a desorption stage and finally chromatographic analysis. The tedious and sometimes lengthy procedures, often using large quantities of sample, involved in the concen-

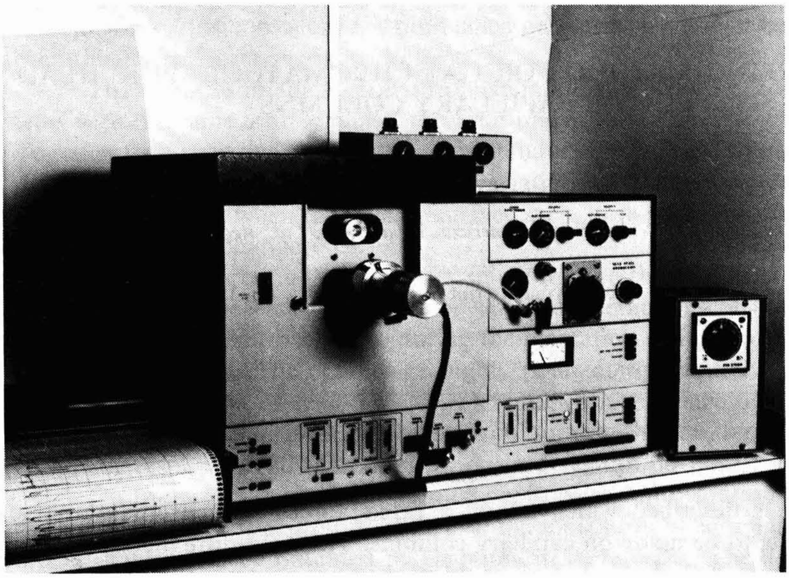

Fig. 1. Headspace sampling accessory assembled on a gas chromatograph.

tration stage led to the development of an essentially direct method. In this direct method, which uses the specially designed GC accessory[24] illustrated in Figs. 1 and 2, the headspace vapours are swept from the sample onto the head of a cooled chromatography column where the volatiles are trapped and therefore concentrated. Once sufficient sample has been collected on the column, GC analysis is initiated. This procedure however, in its originally reported form, was applicable only to packed columns; the analyses obtained therefore lacked the necessary resolution required for either successful mass spectroscopic identification of individual components or completely satisfactory profile analysis.

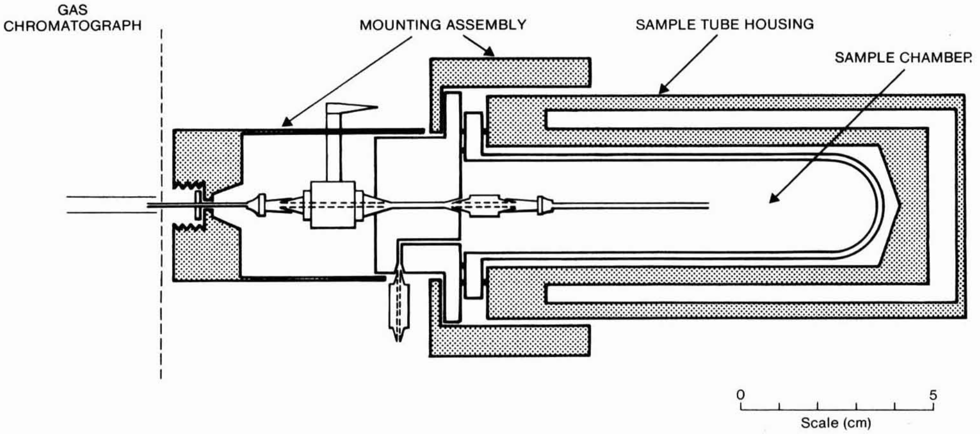

Fig. 2. Sectional diagram of sampling accessory.

Methods of obtaining direct headspace analyses using capillary column chromatography have been reported and suitable apparatus is commercially available. However, concentration stages, essential if minor components are to be detected, are not involved nor are they easily incorporated in these methods and, as a result, only the principal components of the headspace vapours figure in the results obtained.

The method described here allows a rapid, direct, GC headspace analysis to be made on capillary columns. The principle of this method involves using a short conventional packed column immediately before the capillary column. The sample capacity of the packed pre-column is used for collecting and concentrating the headspace vapours prior to the capillary column, with its superior resolving and separating power being used for the analysis.

EXPERIMENTAL

Apparatus

A Perkin Elmer gas chromatograph with a sub-ambient facility and a flame ionisation detector was used throughout this investigation. The specially designed, GC headspace sampling accessory, described elsewhere[24], was used in conjunction with the chromatograph and therefore no special capillary injection system is required.

A support-coated open tubular (SCOT) glass capillary column (61 m × 0.5 mm I.D.) coated with SP2250 and supplied by Scientific Glass Engineering (London,

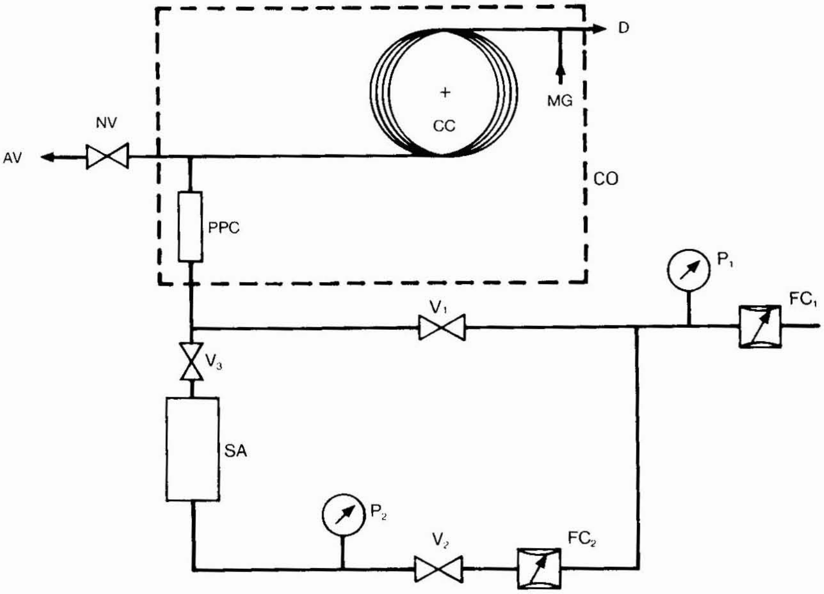

Fig. 3. Schematic representation of gas chromatographic apparatus to allow direct sampling of headspace vapours using capillary columns. AV = Atmospheric vent, CC = capillary column, CO = chromatograph oven, D = detector, FC_1/FC_2 = flow controllers, MG = make-up gas, NV = needle valve, P_1/P_2 = pressure gauges, PPC = packed pre-column, SA = sampling accessory (ref. 24) and $V_1/V_2/V_3$ = on/off valves.

Great Britain), was used and wherever possible glass lined stainless-steel tubing to connect pre-column to capillary and capillary to detector.

The pre-column (4 cm × 3 mm I.D.) was packed with 4% SP2250 on Gas-Chrom Q (80-100 mesh) and assembled between the injector block and the capillary column. A gas stream splitter, adjustable by means of a needle valve, is incorporated between the pre-column and the capillary column. This, together with the usual GC flow controls allows a suitable proportion of the sample to be presented to the capillary column (see *Transfer of sample from pre-column to capillary column*) whilst maintaining the optimum flow of carrier gas through the capillary column during the analysis.

High purity nitrogen was used as the carrier gas throughout these studies.

The apparatus is illustrated schematically in Fig. 3, the labelled components of which will be referred to in following sections.

Sampling procedure

The sample, typically between 0.5 and 3.0 g for solid samples, is placed in the sample chamber of the gas chromatographic headspace sampling accessory (SA) where it is equilibrated to the temperature required. With all the gas flows off (valves V_1, V_2 and V_3 closed) the pre-column (PPC) is cooled to −60°C with solid carbon dioxide or liquid nitrogen. If more convenient, the whole oven may be cooled to −60°C. A gas flow, controlled by using a flow controller (FC_2), is then initiated (valves V_2 and V_3 open) so as to sweep the headspace from the sample and onto the pre-column (PPC). A flow-rate of 25 cm^3 min^{-1} for 10 min usually results in sufficient volatiles from the headspace of the sample being condensed, as a discrete plug, on the initial millimetres of the pre-column to obtain a satisfactory GC trace. Alternative sampling times and flow-rates may be used if required for particular samples. The needle valve (NV) may be adjusted to allow practically all the sampling gas to pass out through the atmospheric vent (AV).

Transfer of sample from pre-column to capillary column

After the flow of carrier gas used to sweep the headspace vapours from the sample has been stopped (by closing valves V_2 and V_3) the oven is heated to the initial temperature of the chromatographic programme so as to vapourize the collected sample and prepare for the analysis. If only the pre-column has been cooled in order to trap the sample, with the oven kept at the initial temperature of the chromatographic programme, then clearly it is only the pre-column which needs to be heated. Once thermal equilibrium has been obtained in the oven the carrier gas is redirected along its usual course (valve V_1 open) using the inlet gas pressure (~650 kPa) to flush part of the collected sample rapidly through the pre-column and onto the capillary column. The fraction of sample presented to the capillary column can be controlled by operation of the needle valve (NV) which adjusts the split that allows the vapourized sample to be divided between the capillary column (CC) and the atmospheric vent (AV). This pressurized "injection", which occurs rapidly, (therefore introducing the sample to the capillary column over a very short time span) is followed by initiating the analytical run and adjusting, if necessary, the needle valve to a previously determined position in order to obtain the optimised flow through the capillary column for efficient separation.

RESULTS AND DISCUSSION

The method described above is direct in as much as there is no handling of the collected headspace volatiles between the concentration and analytical stages. This minimises the possibility of artefact formation.

The headspace vapours are collected on essentially the same material with which they come into contact during the gas chromatographic analysis, again this results in a minimisation of artefact formation.

The method is rapid requiring only a minimum of sample preparation and short sampling times.

The selectivity of some adsorbents commonly used in this type of analysis is well-known; the cooled pre-column used for concentrating the headspace vapours in this work results in complete retention of the volatiles. This has been shown by leading the atmospheric vent gas flow during the sampling time through a non-specific detector which resulted in no signal being recorded. Furthermore there was no evidence to suggest that the pre-column retained any of the trapped volatiles under the conditions of the pressurized "injections".

The apparatus described above has been used to examine a number of samples of plant origin where enrichment of the headspace vapours is essential if satisfactory chromatograms are to be obtained. The chromatographic details are given in Table I.

Three different tobaccos, each with its own post-harvest curing treatment (air curing, flue curing or fire curing[25]) were sampled in shredded form (3 g of each) and analysed according to the described procedure to obtain the fingerprint chromatograms given in Fig. 4. Visual inspection of the chromatograms shows qualitative and quantitative differences especially pronounced in the case of the Latakia tobacco which is cured in an atmosphere of smoke from which it adsorbs the volatiles.

The second application of the technique, illustrated here in Fig. 5, involves the

TABLE I

CHROMATOGRAPHIC DETAILS

Sampling details	
Amount of sample	≥3 g depending on sample
Sampling temperature	75°C
Sampling time	10 min
Flow of sampling gas	25 cm^3 min^{-1}
Temperature of pre-column	−60°C
Analytical details	
Instrument	Perkin Elmer F17 fitted with sub-ambient temperature facility
Pre-column	4 cm × 3 mm I.D. glass
Pre-column packing	4% SP2250 on Gas-Chrom Q (80–100 mesh)
Capillary column	SCOT, 61 m × 0.5 mm I.D. glass supplied by Scientific Glass Engineering
Capillary column coating	SP2250
Temperature programme	+60°C for 4 min then 3°C/min to 200°C
Carrier gas flow	2.5 cm^3 min^{-1}
Injection block temperature	250°C
Detector	Flame ionisation

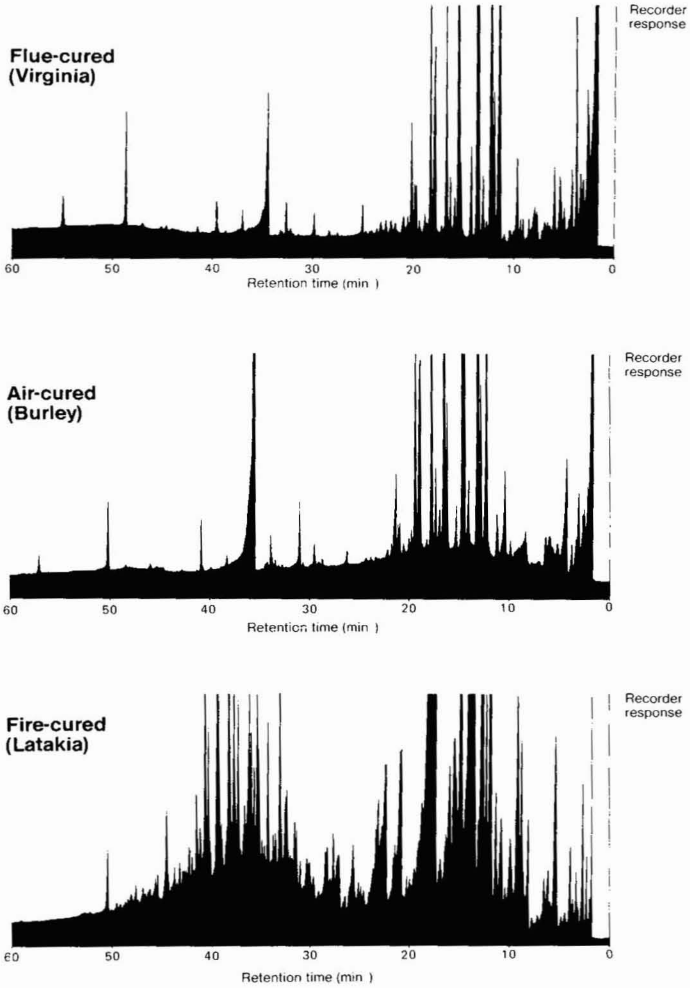

Fig. 4. Headspace chromatograms of cured tobacco samples.

headspace of three freshly-picked herbs: rosemary, sage and mint. In contrast to the tobacco samples, which contain only small amounts of moisture partly as a result of the drying that occurs during curing, the herbs contained substantial amounts of moisture. Nevertheless satisfactory chromatograms were obtained from samples of approximately 100 mg, being a single leaf in the case of sage and mint and a small sprig in the case of rosemary.

CONCLUSIONS

The method and apparatus described above provide a convenient route to obtaining headspace chromatograms of samples where an enrichment stage prior to

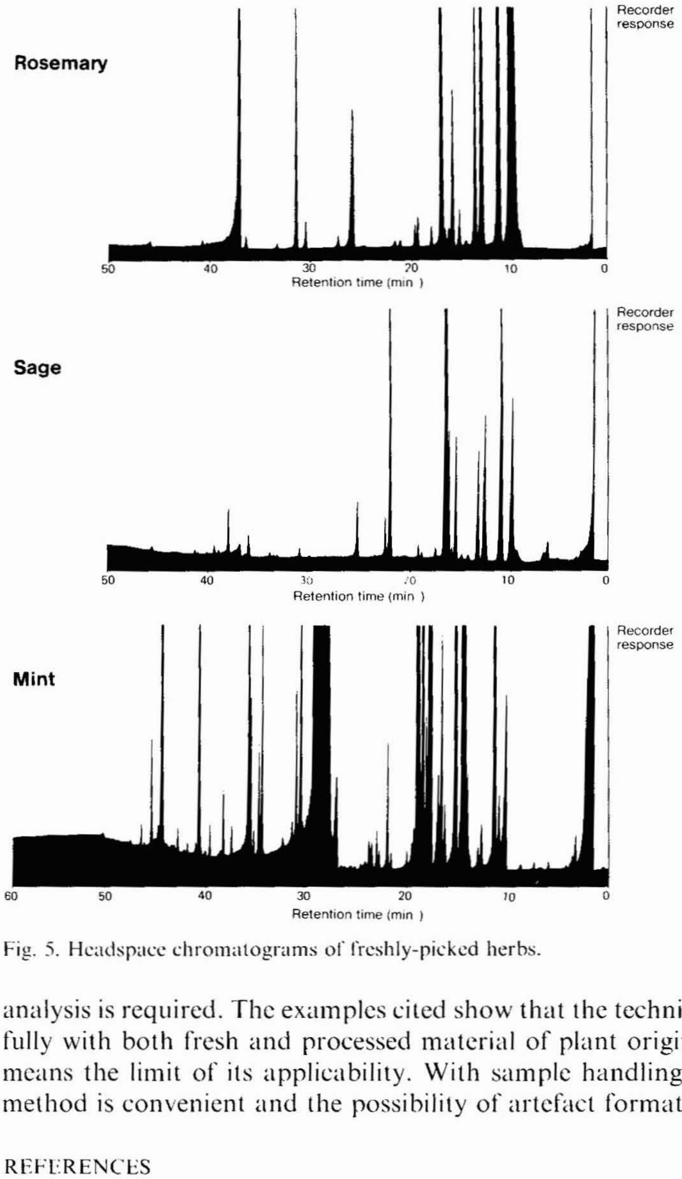

Fig. 5. Headspace chromatograms of freshly-picked herbs.

analysis is required. The examples cited show that the technique may be used successfully with both fresh and processed material of plant origin, although this is by no means the limit of its applicability. With sample handling kept to a minimum the method is convenient and the possibility of artefact formation is reduced.

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DESIGN AND APPLICATIONS OF A MICROPROCESSOR-CONTROLLED SYSTEM TO OPTIMIZE PREPARATIVE LIQUID CHROMATOGRAPHY

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SUMMARY

A microprocessor-controlled instrument, able to pilot pumps, solenoid valves, fraction collectors as well as any other electrical appliances, was designed to improve the efficiency of our preparative liquid chromatography equipment. Facilities to select buffers, load samples, generate linear gradients and modulate the programmed elution processes by positive feed-back interaction with up to eight on-line detectors were included in the software. As many as 50 consecutive instructions may be programmed as simple coded mnemonics and executed on the 16 parallel output lines with respect to actual time and/or external feed-back signals. Sets of instructions may be stored on tape cartridges using a normal tape-deck. The main improvements brought about by use of this technology are greater reproducibility of elution patterns and improved resolution of the detected peaks. An application to the fractionation of protein from human serum is described and differences between methods are discussed.

INTRODUCTION

In most of the laboratories where proteins are purified, liquid chromatography (LC) is often used. During the last decade, major improvements in the design of LC equipment have resulted in a new generation of sophisticated columns, applicators, fraction collectors and detectors having increased efficiency. LC gels are now available as beaded, cross-linked matrices and derived products for ion-exchange or affinity chromatography[1–3]. The strengthened structure of these gels insures stable hydrodynamic properties and prevents large volume variations upon pH or ionic strength changes[4]. Thus, they may be regenerated within the column. This is man-

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datory for full automation, which may now be considered for the preparation of most proteins.

Using programmable flow selectors to perform the sequential steps of LC, automatic loading of samples, elution with several buffers as well as regeneration processes may be achieved[5,6]. However, some events cannot be programmed in advance, for example those actions taken consequent upon the results obtained, such as the modification of an elution process with respect to the current elution pattern. Such feed-back interactions can more easily be directed by a microprocessor since full-time survey of long lasting experiments by laboratory staff is uneconomic and tedious.

The aim of this work is to improve the management (automation of systems) and the overall efficiency of LC techniques (modulation of elution processes by feed-back). To this end we have designed and built BIDULE 1, the first prototype of a "Basic Instrument Directing Unattended Laboratory Equipment".

MATERIALS

LC equipment

The liquid chromatography columns were obtained from Pharmacia (Uppsala, Sweden), Cheminert connectors and valves from Laboratory Data Control (Riviera Beach, FL, U.S.A.), the Type 311 and 332 miniature electrovalves from Huba Control (Würenlos, Argovie, Switzerland), the UA-5 UV detector from ISCO (Lincoln, NE, U.S.A.) and the COM2e conductivity meter from Radiometer (Copenhagen, Denmark). The DE-52 cellulose was a product from Whatman (Maidstone, Great Britain), chemicals were reagent grade from E. Merck (Darmstadt, G.F.R.) and distilled, pyrogen-free water was supplied by the Hôpital Cantonal Universitaire. Human sera for routine analysis were collected in our laboratory, then pooled and stored at −25°C.

Electronic components

The MCS-85 System Design Kit and corresponding integrated circuits were purchased from Intel Corp. (Santa Clara, CA, U.S.A.), the DL-1416 four-digit, sixteen-segment alphanumeric displays from Litronix (Hitchin, Great Britain), the HCBB 75-W power supply from Power-One (Camarillo, CA, U.S.A.), the cases from Elma Electronic (Wetzikon, Zürich, Switzerland), the F-104 relays from Siemens-Albis (Dietikon, Zürich, Switzerland), and optocouplers and other electronic components in common use from local dealers.

METHODS

Chromatography of pooled normal human sera

DE-52 cellulose was recycled as described by the manufacturer and equilibrated in 20 m*M* Tris–HCl buffer, pH 8.2, degassed under vacuum. It was then poured into a column (70 × 1.6 cm) equipped with reservoir and sedimented at 32 ml/h overnight, to yield a 63-cm bed of packed anion exchanger. An adaptor was then fitted to the top of the bed and the flow-rate adjusted to 16 ml/h. A pool of sera was dialysed three times against ten volumes of 20 m*M* Tris–HCl buffer, pH 8.2, and centrifuged for 1 h at 15,000 *g*. An aliquot (10 ml) of the dialysate was loaded on the top of the column. The column was washed with equilibration buffer to recover unadsorbed proteins, a linear gradient from 0 to 0.3 *M* NaCl in 20 m*M* Tris–HCl buffer, pH 8.2, was applied using either the standard two-vessels, siphon and magnetic stirrer method[7] or by the gradient generator of the process controller, as described below.

Design and construction of the process controller

Hardware. BIDULE 1 (Fig. 1) is a modular control unit for any combination of up to sixteen independent electrical appliances. It works like an array of programmable switches piloted by a quartz clock. The choice of the microprocessor was very limited since our development facilities included only an Intel 8080 based computer terminal and a cross-assembler for this family of processors. Basically, BIDULE 1 comprises an Intel development kit for the microprocessor 8085, as shown in Fig. 2. This kit was chosen since it includes almost all the elements needed: central processing unit (CPU); 2-kbytes erasable programmable read-only memory (EPROM) for the internal control program; 512-bytes random access memory (RAM) for user's instructions; parallel input/output ports for sixteen output and eight feed-back input lines and a timer to control the clock. In addition, it was one of the few kits which contain a 24-key keyboard and a display directly controlled by a dedicated circuit.

This choice allowed us to minimize hardware development. Minor modifications of the kit include the relocation of the six-digit, seven-segment display (clock) and keyboard upon the front and keyboard panels of the case, the addition of a twelve-digit, fourteen-segment alphanumeric display for the listings of the user's program and flags, as well as the implantation of optocouplers in the output and input

Fig. 1. BIDULE 1 prototype as currently used in our laboratory.

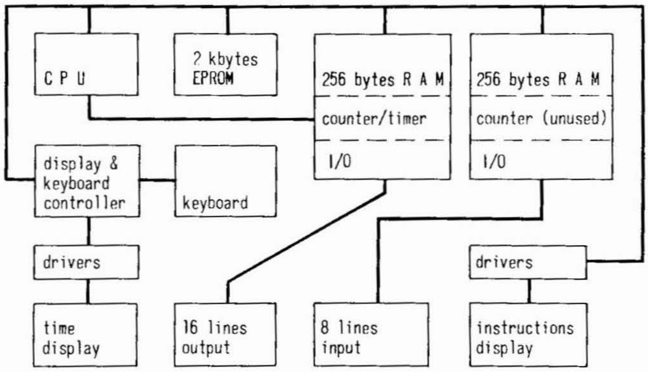

Fig. 2. Block diagram of hardware.

ports to protect the electronic components from external damage. The building and modifications of the kit were performed according to user's manuals[8,9]. A remote relay-box contains the power supply to satisfy the requirements of the electrical appliances to be used and is connected to BIDULE 1 through up to 25 m of multiple pole cable. The state of the input/output ports is continuously monitored by a row of light-emitting diodes (LED) on the front panel of both BIDULE 1 and the relay-box. Facilities offered by BIDULE 1 include on/off functions, generation of impulses and gradients as well as feed-back control of the processor by internal or external events.

Software. The internal software is written in ASSEMBLER[10], and is divided in two distinct parts.

(1) A kind of editor specially designed for BIDULE 1 which allows the user to give task-oriented instructions to be either immediately executed or stored in RAM memories for delayed execution. Short instructions control the state of BIDULE 1 and are used to start or stop the clock, to enable or disable the feed-back system and delayed execution and to read/write the user's program from/to a conventional tape-deck. The other instructions describe the state of the output ports with respect to time and/or changes in the state of the feed-back input lines. These instructions have the same twelve-character format, which may exceptionally be truncated on the right-hand side. The first two characters define the type of the instruction and are used to enter a set of instructions, to select feed-back registers, to modify immediately the state of the output ports or to clear all or part of the RAM memory. The next six characters are reserved to set the time at which the instruction has to be executed or to update the clock. These are followed by two characters used to define functions and their parameters which control the state of the output ports. These are usually on/off functions, generation of impulses or gradients. The last two characters indicate which of the sixteen output lines is concerned with the instruction. Extended error checks are provided to avoid the execution of illegal orders and the corresponding flags are displayed as descriptive words for convenience. Edit facilities such as "clear", "list" and "back-space" insure ease of correction, insertion or deletion of instructions within a program or of characters within an instruction.

(2) Internal software controlling the delayed execution of the instructions described above, with respect to the actual time displayed by the clock and the feed-back signals. Every time a new instruction is entered through the keyboard, BIDULE

1 checks for immediate or delayed execution. In the latter case, the instruction is stored in the RAM memory. If the delayed execution mode is enabled, BIDULE 1 scans the stored instructions ten times a second, checks for conditions of execution and finally executes, or continues the execution of these instructions. The immediate execution of any instruction is always possible.

APPLICATIONS TO CHROMATOGRAPHY

The facilities offered by BIDULE 1 in the management of liquid chromatography systems may be placed in the following categories.

On/off selectors. The programmable on/off functions are used to start or stop pumps, detectors, chart recorders, fraction collectors as required, at any time within the range of the clock (99 h, 59 min, 59 sec) or according to external events. Two-way solenoid valves are also piloted to open or close different buffer reservoirs and to inject large samples, via T-shaped flow connectors. Three-way solenoid valves are used to divert flows from the main line, or to select one from two streams of solvents.

Impulses. The generation of single impulses from 0.1 to 25 sec allows one to pilot rotating valves and fraction collectors, and to inject very small samples without having to use sample loops and dedicated sample injection valves.

Feed-back. Up to eight parallel analogue feed-back input lines are used to connect external detectors such as a photometer, conductivity meter or pH meter. The signals are compared to a preselected threshold level (Fig. 3). If the signal potential crosses the threshold the programmed instruction is executed. This instruction

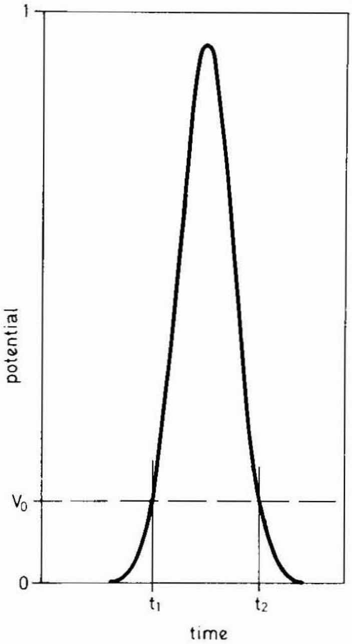

Fig. 3. Generation of feed-back signals. The threshold potential, V_0 (broken line), is chosen at a given level between 0 and 100% of the full scale. As the input signal (heavy solid line) crosses the threshold at times t_1 (activation) and t_2 (base return) the feed-back instructions programmed and stored in registers A and B are executed, respectively.

can be a jump of the clock to any given new time and/or any other standard instruction described above. Two independent feed-back signals are recognized on each of the input ports: these are "activation" (increasing potential across the threshold) or "base return" (decreasing potential across the threshold) signals which respectively detect the beginning and end of peaks during chromatography. In addition, an internal software loop simulates external feed-back signals. This allows recycling of programs or jumps of the clock to avoid execution of a segment of program.

Gradients. Linear gradients are generated by pumping increasing amounts, y, of solvent Y in proportionally decreasing amounts, x, of solvent X, step by step as shown in Fig. 4. The electric pulses applied to the solenoid valves generate a stair-like gradient resulting in a continuous linear inclined plane of the same slope beyond an appropriate mixing chamber. This stair climbs from the initial level, N_0, to the final level, N_n, through $n - 1$ intermediate levels, N_i (n-step staircase, $0 \leqslant i \leqslant n$, Fig. 4A). The length, L, of each step is a multiple of the length, C, of the programmed repetitive cycle generating the impulses (Fig. 4B)

$$L = mC \tag{1}$$

where m is a positive integer ($1 \leqslant m \leqslant 250$) chosen from a table of 30 selected values. The length, C, of a cycle depends on the number, n, of steps chosen, and on the length of the base time, z:

$$C = nz \tag{2}$$

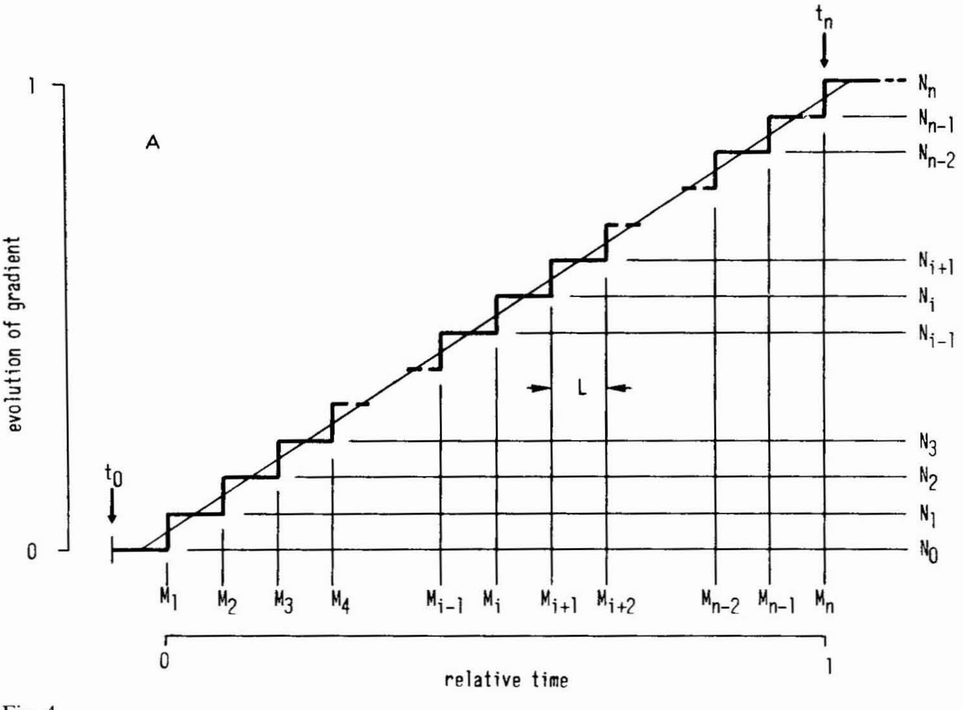

Fig. 4.

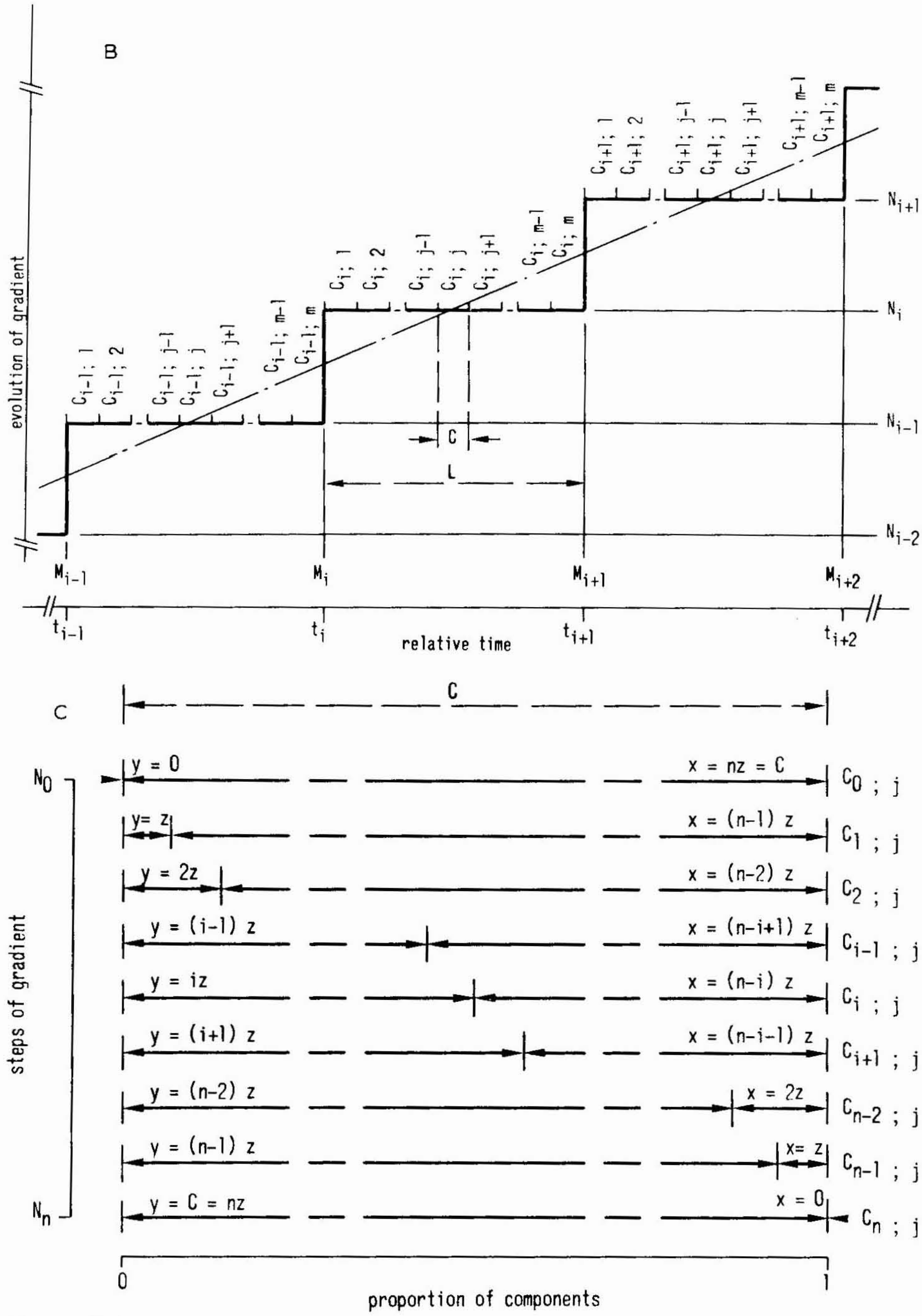

Fig. 4. Principle of generation of linear gradients. A, Stair-like gradient showing steps, M_i, of length L, levels N_i and duration from initiation (t_0) to achievement (t_n), as well as the mean slope obtained beyond the mixing chamber. B, Modulation of the slope through repetition of cycles C_i. n = Number of steps; m = number of cycles C_i per step; $C_{i;j}$ = single cycles of impulses, $0 \leqslant i \leqslant n$, $0 \leqslant j \leqslant m$; C = length of a single cycle $C_{i;j}$. C, Generation of sequential cycles by the processor. z = Smallest increment of time (base time = 0.1 sec, see text); i = number of the cycle within the sequence, $0 \leqslant i \leqslant n$; x_i, y_i = proportions of solutions X and Y, respectively, delivered within cycle $C_{i;j}$.

Since the response time of electromechanical components piloted by BIDULE 1 to generate gradients (electrical relays, solenoid valves) is the limiting factor of the system, the base time was fixed to the lowest possible value allowing reproducibility, *i.e.*, $z = 0.1$ sec. This value determines the smallest useful increment (Fig. 4C). The composition of each cycle with respect to both of the gradient constituents X and Y is given at any time, t_i, by the relations

$$C = x_i + y_i \tag{3}$$

$$y_i = iz \tag{4}$$

$$x_i = (n - i)\, z \tag{5}$$

where x_i and y_i are the respective amounts injected during the cycle C_i as illustrated in Fig. 4C. The conditions for the initial and final levels are then given by $y_0 = 0$ and $x_n = 0$ respectively. From Fig. 4A, we find that the duration of the gradient is determined by:

$$t_n - t_0 = nL \tag{6}$$

Substituting the L value from eqn. 1 and the C value from eqn. 2 into eqn. 6 we obtain the duration of the gradient with respect to the directly programmable parameters only:

$$t_n - t_0 = mn^2\, z \tag{7}$$

Both parameters m and n can be selected through the keyboard. The number of steps, n, is chosen before the gradient starts and cannot be changed during gradient generation ($n = 25\,k$, $1 \leqslant k \leqslant 10$). The repetition factor, m, of each cycle is selected as a part of the instruction setting the gradient, and can be modified at any time. This factor allows the modulation of the slope of the gradient, either by standard instruction or by feed-back control. The shortest gradient possible lasts 62.5 sec ($n = 25$, $m = 1$), the longest lasts 18 days, 2 h, 1 min and 40 sec ($n = m = 250$)! Non-linear concave or convex as well as other special gradients may be built from multiple consecutive modifications of the slope and/or juxtaposition of several consecutive gradients. Arbitrarily, the value $m = 0$ indicates an instruction which suspends the evolution of the gradient (slope = 0), until another value is selected. Such an instruction allows one to modulate the development of a gradient with respect to the actual elution pattern, using the feed-back signals from an on-line detector connected to BIDULE 1, as shown in Fig. 5.

EXAMPLES OF APPLICATION AND DISCUSSION

As an example, the fractionation of proteins from pooled human sera on DEAE-cellulose is discussed for two types of elution processes: a conventional two-vessel, syphon and magnetic stirrer gradient mixing chamber (Fig. 6A), and the gradient generated by BIDULE 1 and modulated by the UV detector through the

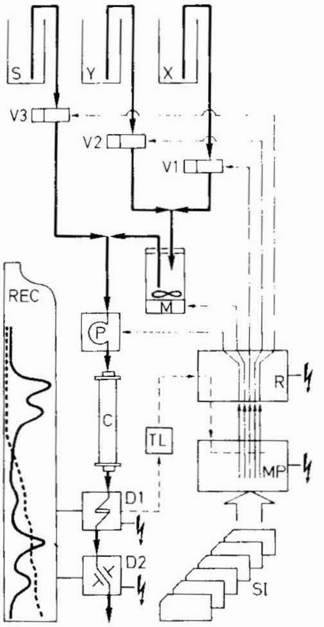

Fig. 5. Schematic diagram of the equipment used for the generation of the gradients by the microprocessor, with modulation by feed-back signals from a UV detector. S = Sample vessel; X, Y = buffer reservoirs for first and second gradient components, respectively; V1, V2 = solenoid valves; V3 = sample injection valve; M = mixing chamber (1 ml) with magnetic stirrer and bubble trap; P = pump; C = column; D1 = detector 1 (UV monitor); D2 = detector 2 (conductivity meter); REC = recorder coupled to detectors; R = relays; MP = microprocessor (BIDULE 1); TL = threshold level adjustment; SI = set of instructions (program) to the microprocessor.

feed-back facilities (Fig. 6B). Using a peak separator able to detect the minima of the absorbance curve ($\delta A/\delta t = 0$), the number of fractions collected increases from 7 to 13. Their degree of separation can be adjusted to individual requirements by selecting an optimum threshold potential for the feed-back system. Generally, this optimum value should be estimated from the type of column to be eluted (size, ratio of length to section, type of packing, flow-rate) and from the complexity of the mixture to be purified and results expected (number of components in the sample, number of peaks to separate, degree of similarity between single components to be eluted in discrete fractions, behaviour of single components with respect to the column packing). Too high a threshold will decrease the resolution, since the gradient will be incremented before the elution of the preceding peak is completed. Lowering the threshold produces some longer intermediate levels, an elution at a lower ionic strength and, thus, a concomitant spreading and dilution of the fractions. This is best understood by considering Fig. 6B, where most of the well separated, sharp peaks are eluted just after an increase of the ionic strength, in contrast to the shoulders and broader peaks which are eluted at the end of the intermediate levels of the gradient. For a given column at a constant flow-rate, the shift between the programmed gradient injected on the column (Fig. 6B, solid line) and the resulting gradient flowing out through the detector (Fig. 6B, dotted line) is a direct measure of the liquid phase volume, V_1,

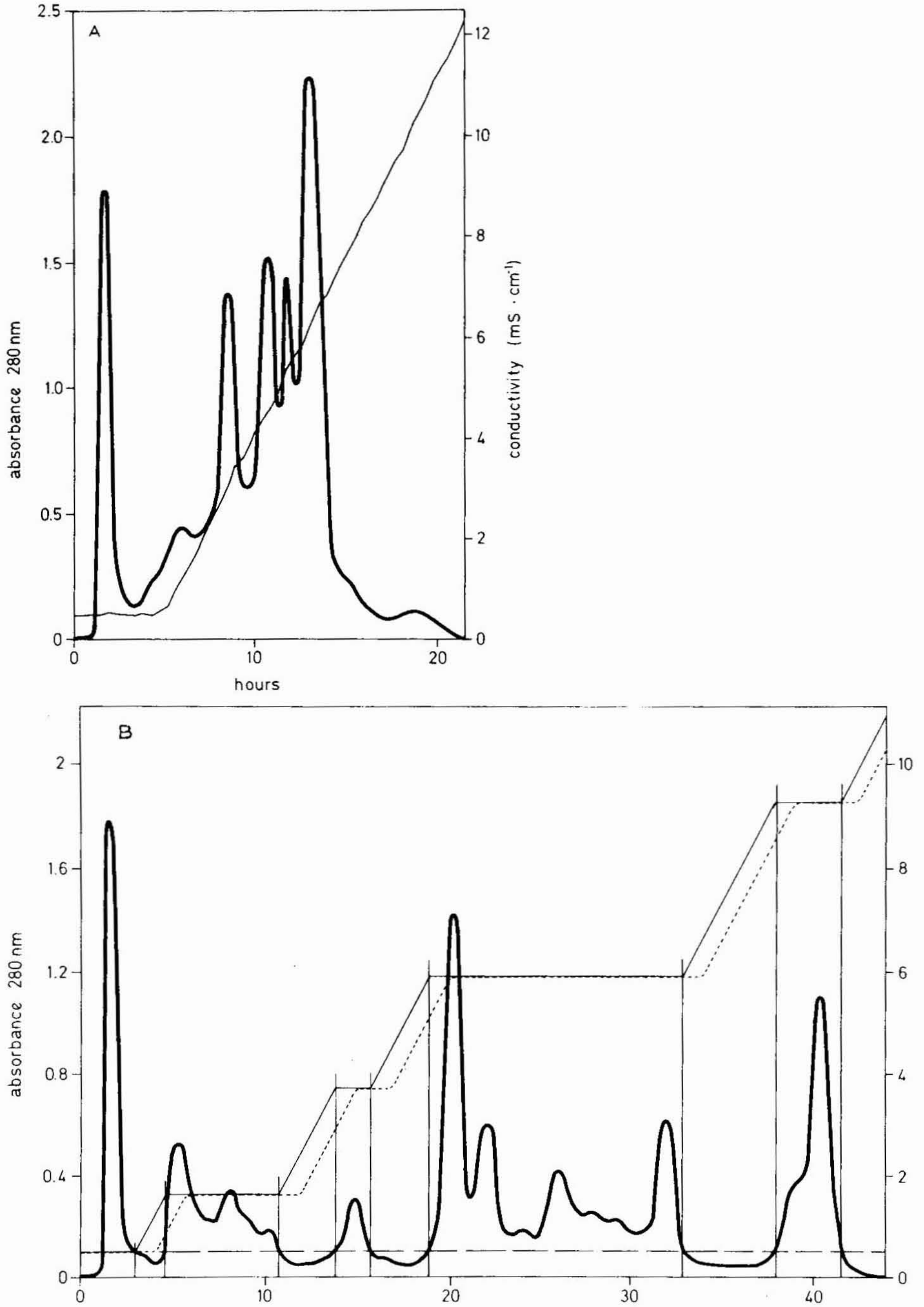

Fig. 6. Chromatography of proteins from pooled human sera on DE-52 cellulose. Column: 63 × 1.6 cm. Flow-rate: 16 ml/h. Buffer: 20 m*M* Tris–HCl, pH 8.2. Gradient: 0 to 0.3 *M* NaCl in buffer. A, Manual operation. Sample loaded through the pump, gradient formation by two-vessel, siphon and magnetic stirrer technique. B, Microprocessor-controlled operation. Sample loaded by sample injection valve, gradient generated by microprocessor and modulated as shown by feed-back signals from the UV detector. Solid line: generated gradient as injected onto the column. Dotted line: gradient as measured in the exit liquid stream. Broken line: threshold level. Heavy solid curve: absorbance at 280 nm.

within the column as a function of time. Thus, the optimum length, L, for each step of the gradient should be $L = V_1$. However, such a value for L may yield poor results if the column is large and/or the flow-rate is low, since both these parameters will contribute to flattening the slope of the gradient.

Among the facilities offered by BIDULE 1, the feed-back system provides many opportunities of dealing with automation problems and with events which are expected but which cannot be scheduled. Beside modulation of the eluting gradient discussed above, other possible uses of the feed-back system are briefly presented here to illustrate the broad range of potential applications in chromatography. When connected to temperature and pressure detectors, buffer level gauges or any other sensors installed within the chromatography set-up, the microprocessor may be programmed to modify these conditions, switch off some defective apparatus, stop the experiment and/or trigger alarm devices (crash programs). When connected to an on-line peak detector and piloting a three-way solenoid valve, BIDULE 1 is a perfect tool for the direction of recycling chromatography[11]: bleeding and recycling sequences may automatically be alternated with respect to the actual position and degree of separation of peaks (dynamic evolution of programmed events). With an additional pulse-piloted rotating valve to divert the flow stream from the main line, BIDULE 1 may be used instead of a fraction collector for several discrete fractions during repetitive chromatography with multiple injections of sample.

CONCLUSIONS

Over a year of continuous tests, we have demonstrated the ability of BIDULE 1 to successfully control the operation of up to three peristaltic pumps and twelve solenoid valves delivering samples and buffers to three distinct parallel flow streams. The main improvements achieved in our laboratory by this simple process controller are simplified management of LC equipment, better reproducibility of results and higher resolution of ion-exchange chromatography. Each of our LC columns may now be used 24 h a day, since loading of samples, changing buffers or eluting and recycling processes require no human intervention. This saves time and valuable reagents, and produces more results, both quantitatively and qualitatively. Furthermore, the reproducibility is improved since, once given the parameters of chromatography, the processor excludes even minor changes in the conditions of experimentation. Finally, coupling of a UV detector to the processor (feed-back) in order to modulate the slopes of gradients according to the development of the elution considerably increases the resolution of a given separation, as demonstrated in Fig. 6.

Some applications of BIDULE 1 to specific purification procedures of proteins will be described in separate papers. We are currently testing an automatic multi-column LC system designed to yield weekly highly purified single protein fractions from human plasma.

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SEPARATION OF CARBOHYDRATES AND POLYOLS BY A RADIALLY COMPRESSED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SILICA COLUMN MODIFIED WITH TETRAETHYLENEPENTAMINE

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SUMMARY

Optimization of separation of carbohydrates and polyhydric alcohols on a silica column modified with tetraethylenepentamine is described. Eluent tetraethylenepentamine concentration, pH, solvent concentration and flow-rate were optimized with respect to compound separation and baseline stability. This method offers advantages over existing techniques, including room temperature operation, low operating pressure, low cost, simplicity, high resolution, relatively lengthy column life, and high linear sample capacity.

INTRODUCTION

The simultaneous analysis of carbohydrates and polyols by high-performance liquid chromatography (HPLC) has received considerable attention in recent years[1–7], including methods employing amine-modified silica as the stationary phase[8–12]. Of various amine-modified systems considered by Wheals and White[12], tetraethylenepentamine (TEPA) was found to provide the best separation of fructose, glucose, sucrose, maltose and lactose as well as long term column stability. The addition of TEPA and amines of similar characteristics increases the retention of carbohydrates by silica columns[1] and thus allows the resolution of carbohydrates of biochemical interest which are not resolvable by a number of HPLC techniques[9]. However, the list of carbohydrates and polyols investigated in such separation systems has been limited and the variables of pH, amine concentration in the eluent, solvent flow-rate and composition have not been optimized previously.

The recently developed radial compression cartridge[13,14] offers a number of advantages over previously available HPLC techniques. The relatively short, large-

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diameter column provides a closer approximation to "infinite diameter[15]." Further, the pressurized flexible column walls produce an ability to compensate for packing material non-homogeneity[13]. This allows for a wider range of eluent pH than with uncompensated columns since compression compensates for silica dissolution at high pH. We have used a single Radial-Pak silica cartridge (Waters Assoc., Milford, MA, U.S.A.) and basic eluent pH (9.2) for several months without noticable degradation of column performance. Further, employment of solvent recirculation and the relatively low cost of the Radial-Pak silica cartridges results in a considerable decrease on cost per assay.

EXPERIMENTAL

Apparatus

A Waters Model R401 differential refractometer was used in conjunction with a Waters Model 6000A pump and WISP 710B automated injection system. The output from this system was recorded on a Waters data module. A Radial-Pak silica cartridge (10 cm × 8 mm I.D.) was employed in a Waters RCM-100 radial compression module to effect separation of these compounds. A Waters guard column filled with AX/Corasil was placed in line with the Radial-Pak B column to protect the column and saturate the eluent with SiO_2.

Chemicals and reagents

Acetonitrile used for solvent preparation was Fisher HPLC grade (Pittsburgh, PA, U.S.A.), technical grade TEPA was obtained from Eastman (Rochester, NY, U.S.A.) and carbohydrates and polyols from Sigma (St. Louis, MO, U.S.A.). Water used in solvent preparation was deionized and glass distilled. The pH of elution solvents was adjusted with glacial acetic acid. Eluents were degassed by vacuum filtration through Millipore 0.2-μm filter (Bedford, MA, U.S.A.).

Column pretreatment

Waters Radial-Pak silica cartridges were initially conditioned by pumping 50 of acetonitrile–water (70:30) containing 0.1% (v/v) TEPA (pH 9.2) through the column. The final elution solvent was then introduced [nominally, pH 8.9 acetonitrile–water (81:19) containing 0.02% TEPA] and the column stabilized by recirculating this solvent overnight before column usage.

Operating conditions

Solvent effluent was returned to a continuously stirred 1-l pump reservoir for recirculation. Columns were nominally operated at *ca.* 26°C and a flow-rate of 2 ml/min which produced a backpressure of approximately 2.75 MPa (400 p.s.i.). Carbohydrate and polyol standards were dissolved in distilled water (5 mg/ml); nominal injection volume was 50 μl.

RESULTS AND DISCUSSION

Separation of polyols, mono-, di-, and trisaccharides

Initial separation of polyol and carbohydrate mixtures employing a pH 9.2

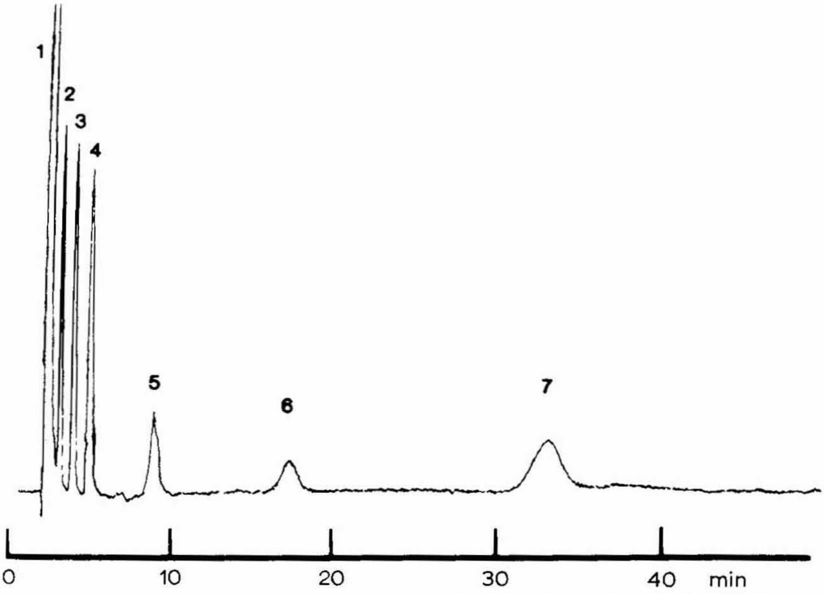


Fig. 1. Chromatogram of elution of polyol–saccharide mixture by a hydraulically compressed Radial-Pak silica column (10 cm × 8 mm I.D.) modified with TEPA. Elution solvent: acetonitrile–water (81:19), pH 8.9, containing 0.02% TEPA. Eluent flow-rate, 2 ml/min, pressure, 2.34 MPa (340 p.s.i.), 26°C. Injected sample contained, in 50 μl, 250 μg of each of the following: 1 = solvent "front"; 2 = ethylene glycol; 3 = glycerol; 4 = erythritol; 5 = glucose; 6 = maltose; and 7 = raffinose.

mobile phase containing acetonitrile–water (81:19) produced results (Fig. 1) similar to those published for other amine-modified silica columns[1,4,10,12] with respect to the order of carbohydrate elution and to produce excellent separation of components in the test mixture. Capacity factors (k') of these compounds were found to be a function of the composition of the mobile phase (Fig. 2), particularly for the di- and trisaccharides. Changing the acetonitrile–water ratio did not change the elution order of these compounds but did have an effect upon the k' of all compounds in the test mixture. Capacity factors were found to be independent of flow-rate over the range of 2 to 10 ml/min (not shown); however, below 2 ml/min, k' was found to decrease with increasing mobile phase velocity.

To test the suitability of this method for the separation of a wide range of carbohydrates and polyols, additional compounds were chromatographed under identical conditions (Table I). Whereas most compounds eluted in the approximate order of molecular size, certain exceptions were noted. For example, sucrose elutes before inositol and sedoheptulose anhydride was found to elute prior to a number of hexoses. Separation in this system may, therefore, be due to factors other than molecular size, such as the number and geometry of hydroxyl groups, as suggested by D'Ambroise *et al.*[9] for a similar system employing LiChrosorb-NH_2 as the stationary phase. This may also explain why ribose, galactose, arabinose and mannose were not detectable with this system, while very similar compounds were detected.

The order of elution and approximate number of theoretical plates calculated (N) for these compounds are consistent with data from similar investigations[1,4,9,12].

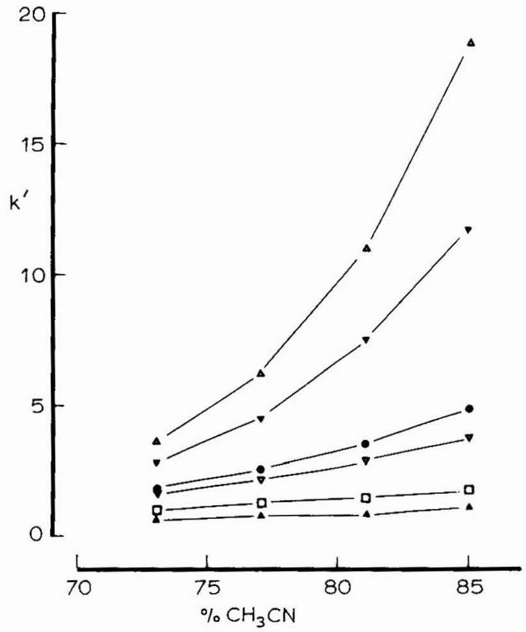


Fig. 2. Effect of eluent acetonitrile content upon k' of a standard polyol-saccharide mixture. △ = Trehalose; ▼ = sucrose; ● = sorbitol; ▽ = fructose; □ = erythritol; ▲ = glycerol. Other conditions as in Fig. 1.

TABLE I

ELUTION OF CARBOHYDRATES AND POLYOLS FROM A RADIAL-PAK SILICA COLUMN

Conditions of elution as in Fig. 1.

Compound	*Molecular weight*	k'	*N/10 cm*	R_s*
Ethylene glycol	62.07	0.497	634	1.889
Glycerol	92.09	1.017	657	1.355
Rhamnose	164.16	1.451	903	0.417
Erythritol	122.12	1.594	834	0.136
Threitol	122.12	1.640	1125	0.610
Fucose	164.16	1.880	596	0.515
Xylose	150.13	2.091	1259	0.480
Ribitol (adonitol)	152.15	2.286	812	0.010
Sedoheptulose anhydride	210.20	2.331	924	0.355
Arabitol	152.15	2.497	800	0.321
Fructose	180.16	2.903	837	1.038
Sorbitol	180.16	3.451	1185	0.270
Dulcitol (galactitol)	182.16	3.594	1160	0.035
Mannitol	182.16	3.617	583	0.620
Glucose	182.16	4.046	1066	3.100
Sucrose	342.30	6.520	946	1.680
Inositol	180.16	8.257	1153	0.131
Cellobiose	324.30	8.400	1189	0.100
Maltose	360.31	8.617	1270	0.581
Trehalose	378.33	9.286	1132	0.424
Lactose	342.30	9.829	1049	5.091
Raffinose	594.52	17.640	1470	

* Calculated resolution between adjacent members of the table.

The maximal number of theoretical plates we observed for this column (14,700 plates/m) is high in comparison to similar calculations for other HPLC carbohydrate columns[4,10,12].

If we consider a peak resolution (R_s) of 0.8 to be the value of least acceptable resolution[16], we see that a number of compounds in Table I are acceptably resolved, some are marginally resolved (*e.g.*, R_s glucose:mannitol = 0.62) and a number are too poorly resolved to be separated by this system (*e.g.*, R_s sedoheptulose:ribitol = 0.01). For those showing marginal resolution, increased separation may be achieved by increasing the percentage of acetonitrile in the eluent (Fig. 2). For example, resolution of sorbitol and fructose (*cf.* Fig. 2) is very poor with acetonitrile–water (73:27) ($R_s = 0.45$) but increases to virtual complete separation ($R_s = 1.57$) with acetonitrile–water (85:15) as the eluent. Such a strategy would be expected to have a greater chance of success for carbohydrates and polyols with a molecular weight greater than approximately 150, due to the roughly exponential effect of acetonitrile concentration upon k' mentioned earlier (Fig. 2). Since increased resolution gained by increasing acetonitrile concentration in the eluent is achieved at the expense of extended elution times, minimally acceptable component resolution will have to be balanced against maximal allowable elution times.

Maximization of separation and baseline stability

In order to maximize flow-rate for greatest efficiency of separation, data taken from the elution of glucose were evaluated (Fig. 3). A plot of reduced velocity (v) versus reduced plate height (h), a Knox plot[15–17], theoretically passes through a minimum at the reduced velocity of maximal separation efficiency. Data gathered over the flow-rate range of 0.4 to 9.9 ml/min shows an apparent decrease in slope at lowest flow-rates but significant scatter was noted in data corresponding to flow-rates under 2 ml/min. Nonetheless, this column appears to be most efficient at flow-rates of approximately 2 ml/min and diffusion does not interfere with efficiency of separation even at the lowest flow-rates investigated[16,17]. Unlike a similar system employing silica modified with γ-aminopropyltriethyloxysilane[10], we find an almost direct proportionality between plate height and elution velocity (Fig. 3), reinforcing our conclusion that low elution velocities lead to the greatest separation efficiency in this system.

Further, the low back pressure generated by the unusual geometry of this column allows the use of flow-rates as high as 10 ml/min, rates which would create unacceptably high pressures with many other HPLC columns. This low pressure response to flow-rates is reflected in an unusually low flow resistance parameter[16–19] value ($\varnothing = 390$) for the elution of glucose from this column, which is much less than that for other commercially available HPLC carbohydrate systems. The unusual column geometry is also apparent in the exceptional column sample capacity we find with this system (Fig. 3). Most silica HPLC columns have linear sample capacity values which lie between 10^{-4} and 10^{-3} g/g column packing[18], but we find no significant decrease in k' even with sample loads of $2 \cdot 10^{-3}$ g/g. The response of this system is linear with respect to sample size and it produces symmetrical, reproducible peaks for injected glucose samples ranging in size from 20 to 20,000 μg.

Carbohydrate and polyol separation in this system were found to be a function of eluent pH (Fig. 4). Elution mixtures with acidic pH values strongly decrease k' but not elution order for all compounds tested, by compressing the elution pattern with

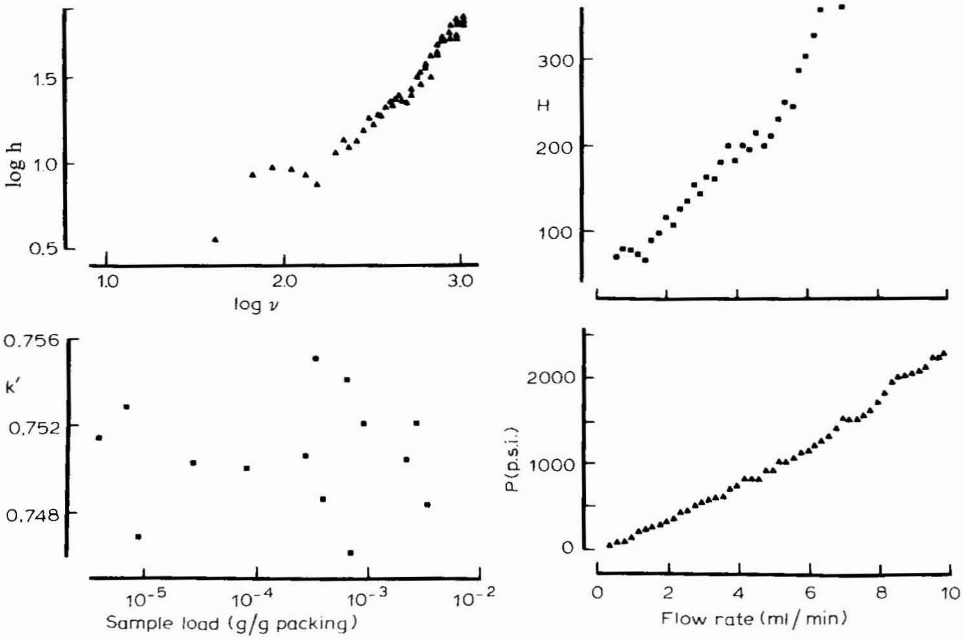
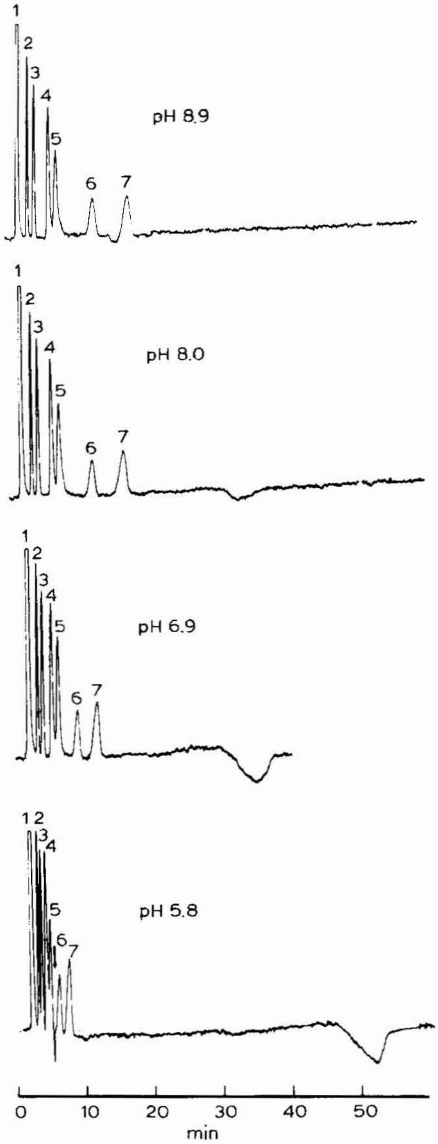


Fig. 3. Effect of eluent velocity upon glucose elution. Theoretical plate height (H) and pressure (P) plotted as a function of flow-rate. Reduced plate height (h) and reduced velocity (ν) plotted as a Knox plot from which, in theory, the least slope represents the elution velocity corresponding to maximal separation efficiency[15–17]. For this calculation, the effective spherical diameter of the packing was taken as 8.5 μm and the diffusivity coefficient of acetonitrile–water (81:19) was taken to be $2.1 \cdot 10^{-9}$ m^2/sec. Linear sample capacity plot (k' *vs.* g/g) is plotted for samples varying from 20 to 20,000 μg glucose. Other conditions as in Fig. 1.

respect to time. However, such acidic eluents introduce a strong negative peak following trisaccharide elution (Fig. 4). In addition, a second (but shorter) baseline deflection was also noted during the elution of the polyol–saccharide test mixture (arrow). The later negative peak decreases the apparent amount of monosaccharides with similar retention times. Both of these baseline deflections were found to decrease with increasing pH (Fig. 4), the larger deviation completely disappearing at pH 8.9. Elution of solvents with basic pH values also increases the k' of the mixture, but does not alter the order of elution of these compounds.

A series of injections of water, TEPA and various acetonitrile–water mixtures was employed to clarify the negative peaks noted in Fig. 5. The concentration of TEPA in the sample was found to have a relatively minor effect upon these negative deflections and no effect upon the k' of components in the test mixture. Of those concentrations tested (Fig. 5), the addition of 0.02% TEPA was found to produce the greatest baseline stability. Higher concentrations of TEPA introduced a perturbation (arrow) approximately 12 min following injection.

The negative peaks were found to be primarily a function of injected sample water (Fig. 6), being proportional in magnitude to injected water mass (Fig. 6). These negative peaks were also determined to be a function of eluent pH. Higher pH values eliminated the major perturbation and decreased the minor dip (arrow) which occurs



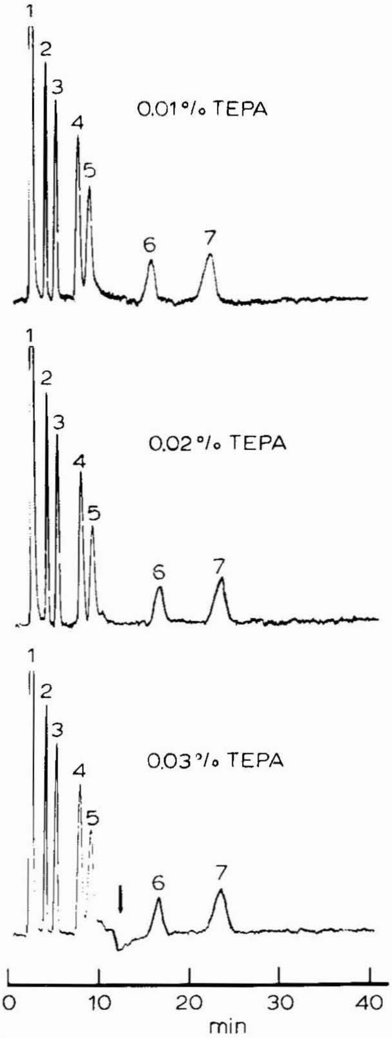


Fig. 4. Effect of pH of eluent upon elution of a polyol–saccharide mixture. Injected sample contained 250 μg of each of the following: 1 = solvent "front"; 2 = glycerol; 3 = erythritol; 4 = fructose; 5 = sorbitol; 6 = sucrose; and 7 = trehalose. Eluent pH from top to bottom: 8.9, 8.0, 6.9, 5.8. Other conditions as in Fig. 1.

Fig. 5. Effect of varying TEPA concentration in eluent upon chromatograms of standard polyol–saccharide mixture. Elution conditions and identification of compounds as in Fig. 4.

during monosaccharide elution. The problems associated with water injection could be eliminated by injection of materials dissolved in the elution mixture, but the superior solubility of many carbohydrates and polyols in water suggests that water would serve best for injection of unknown biological samples. The major negative peak

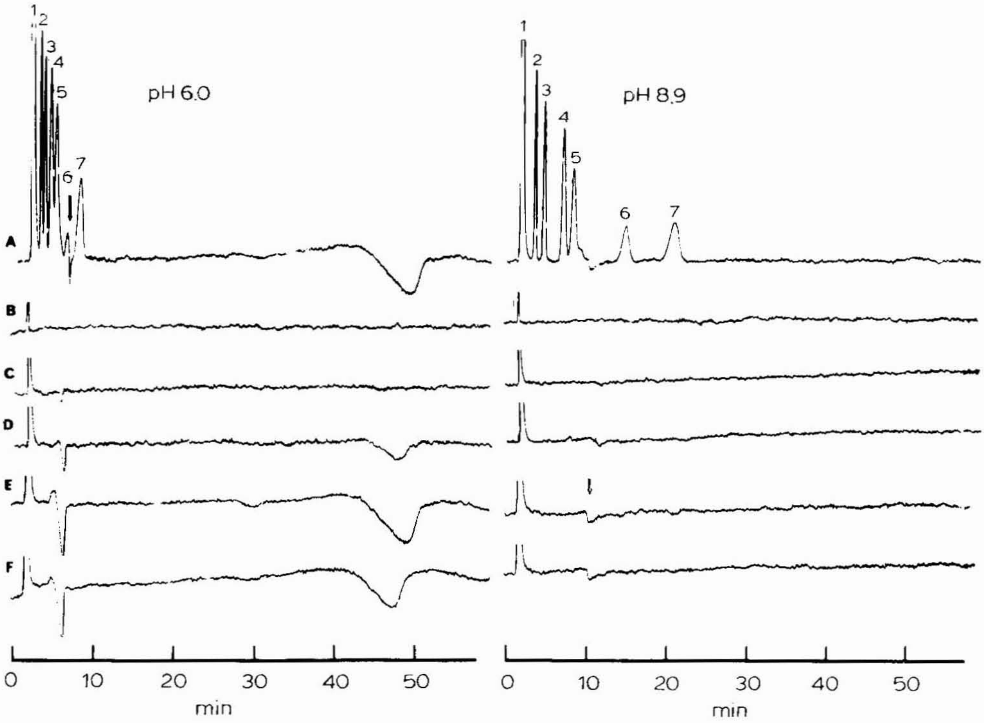


Fig. 6. Effect of sample water content on negative peaks produced in elution solvents at pH 6.0 and 8.9. Elution conditions and identification of compounds as in Fig. 4. B corresponds to an injection of 50 μl of eluent; C, D and E correspond to injections of 10, 25 and 50 μl of water, respectively; F corresponds to an injection of 50 μl 0.1% TEPA in water. Note the shift in size and k' in the smaller water-induced negative peak (arrow) with pH and the elimination of the larger negative peak at pH 8.9.

occurs after the elution of most carbohydrates and polyols, but would prove very unfavorable in automated injection systems or in situations where the number of analysis per time must be maximized since the baseline would require a significant period (nearly an hour) to restabilize before subsequent injections could be made.

CONCLUSION

Hydraulically compressed silica columns modified with TEPA have been shown to provide a simple, rapid and inexpensive method for the analysis of carbohydrates and polyols. Separation of these materials occurs over a wide range of pH, but basic eluents have been found to eliminate negative peaks found with acidic eluents. Both the k' and compound resolution of polyols and carbohydrates was found to be a function of the percentage of acetonitrile in the eluent. The k' of di- and trisaccharides were found to be especially sensitive to the eluent composition.

Unlike other HPLC carbohydrate columns, relatively low elution rates were found to produce the maximal separation efficiency in this system, due to the almost direct proportionality between plate height and flow-rate. The unusual geometry of this column produces a very low flow resistance ($\varnothing = 390$) and an exceptionally high linear sample capacity ($>2 \cdot 10^{-3}$ g/g column packing).

Carbohydrate and polyol separation in this system was found to be a function of eluent pH, but the relation between pH and elution was not as significant as that between elution rate and eluent acetonitrile content.

Several negative peaks were detected which were determined to be largely a function of injected water (in the sample). These peaks were also determined to be, to a lesser extent, a function of eluent pH.

The conditions determined for maximal separation efficiency were found to include a relatively high pH (9), which must normally be avoided with non-compressed silica columns due to shortened column life from silica etching at high pH. The radial compression design employed in the present paper tends to compensate for such silica dissolution and, combined with the addition of TEPA to the eluent[12], provides a relatively long column life, even at pH 9.2.

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AFFINITY ADSORBENTS WITH POLYSACCHARIDE SPACERS

PREPARATION AND PROPERTIES

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SUMMARY

Soluble branched and neutral polysaccharides may be used as polymeric hydrophilic and inert spacers in affinity adsorbents. A series of methods for preparation of such adsorbents were developed. These methods involve introduction of a definite number of reactive functional groups into a polysaccharide molecule with subsequent coupling of the modified polysaccharide to a solid matrix, activation of the polysaccharide spacer and, finally, covalent binding of ligands of various chemical nature. The conditions are specified for the preparation of biospecific adsorbents containing hemoglobin, ribonuclease, poly(U), uridine, hexamethylenediamine and L-lysine as ligands and dextran, glycogen and amylopectin derivatives as spacers. The adsorbents having polysaccharide spacers were characterized by higher ligand concentrations and stability than analogous adsorbents without such spacers.

INTRODUCTION

Considerable progress has recently been achieved in the isolation and purification of biopolymers by affinity chromatography[1,2]. At the same time, however, certain disadvantages of this method can now be seen more clearly. The most important complications accompanying the biospecific purification are non-specific (hydrophobic, electrostatic) biopolymer–adsorbent interactions[3] and a gradual leakage of ligand from the support during chromatography[4].

The first phenomenon may be due to the use of hydrophobic spacers in biospecific adsorbents, while the second is inherent in the activation of polysaccharide supports by BrCN. The latter disadvantage becomes critical when very small amounts of protein are purified, for instance, in the case of protein receptors. Tesser *et al.*[5] have suggested that the leakage of ligands from the carriers can be prevented by the use of polymeric spacers. Such affinity adsorbents were first prepared by Wilchek[6]. Polypeptides and proteins were used as the spacers[7]. Such spacers would render the adsorbent highly stable due to a large number of binding points between the spacer and the solid support. Disruption of one or several spacer–support linkages would not lead to the release of the whole spacer and, consequently, of the coupled ligand. However, the multifunctionality of the protein spacers imparts undesirable ion-exchange properties to the adsorbent and leads to non-specific binding of biopolymers.

We have used branched water-soluble and neutral polysaccharides as polymeric hydrophilic and inert spacers in affinity adsorbents[8]. The non-specific binding during biochromatography was considered to be due to the combined action of hydrophobic and charged groups of the adsorbent[9]. Therefore, the polysaccharide spacers were expected to increase the biospecificity of the purification process, *i.e.*, to exclude or minimize hydrophobic and electrostatic interactions. In addition, such spacers should retain other advantages of polymeric spacers: (1) enable the location of a ligand far from the surface of a solid matrix; (2) increase the stability of the adsorbent; (3) provide a high ligand concentration on the adsorbent and possibly increase the adsorbent capacity. The use of polysaccharide spacers also provides a wider choice of chemical methods for subsequent covalent attachment of a ligand.

The present paper describes a series of chemical methods for attachment of water-soluble polysaccharides to solid supports and for subsequent ligand immobilization on the polysaccharide spacer. The syntheses of several new affinity adsorbents having polysaccharide spacers are presented, and some properties of the adsorbents are compared with analogous adsorbents without such spacers.

EXPERIMENTAL

Materials and methods

Sepharose 4B, Sephadex G-50 and Dextran T20 were obtained from Pharmacia (Uppsala, Sweden), Bio-Gel P-300 from Bio-Rad Labs. (Richmond, CA, U.S.A.), pancreatic ribonuclease, human hemoglobin and poly(U) from Reanal (Budapest, Hungary), dicyclohexylcarbodiimide (DCC) from Chemapol (Prague, Czechoslovakia), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) from Sigma (St. Louis, MO, U.S.A.), BrCN from Serva (Heidelberg, G.F.R.) and hexamethylenediamine from Koch-Light (Colnbrook, Great Britain). All other chemicals were from Soyuzreactiv (Moscow, U.S.S.R.).

Acetonitrile was distilled several times over P_2O_5 and then over K_2CO_3. Carboxymethyldextran (substitution degree, $\gamma = 5$, *i.e.*, 5 carboxymethyl groups per 100 anhydroglucose residues) was prepared as described[10]. Glycogen was isolated from rabbit liver as described in ref. 11.

UV spectra were recorded on SF-4 or Specord spectrophotometers. The chromatography was carried out on a Mini-Rac collector and a Uvicord-2 densitometer (LKB, Stockholm, Sweden). The BrCN activation of Sepharose was based on previous methods[12,13].

Ribonuclease was assayed as described previously[14]. Protein concentrations were determined by the method of Lowry *et al.*[15] or spectrophotometrically at 280 and 400 (for hemoglobin) nm.

Estimation of ligand contents in the adsorbents

The concentration of immobilized ligands was determined by: (a) a differential method (measuring the difference between the ligand content in the starting solution and in the filtrate and washings after coupling of the ligand to the support); (b) a solubilization method[16] (measuring the UV absorption of gel aliquots solubilized by warming at 30°C in 80% acetic acid for 2 h); (c) a suspension method in gel[12] (measuring the UV absorption of a suspension of trinitrophenylated derivatives of NH$_2$-containing adsorbents in 0.05% agarose solution).

In accordance with method c, a slurry of 1 g (wet weight) of a washed NH$_2$-containing Sepharose, 4 ml of water, 10 ml of 2 *M* potassium borate buffer, pH 9.2, and 5 ml of a 0.1% solution of trinitrobenzenesulphonic acid (TNBS) containing 0.1% NaHCO$_3$ were stirred for 2 h at 37°C. The mixture was then filtered, the gel was thoroughly washed with water and dried at a water pump for 3 min. The samples of the TNBS derivatives obtained (0.15–0.90 g wet weight) were suspended in 1.5 ml of 0.1% agarose solution and 1.5 ml of 2 *M* potassium borate buffer, pH 9.2, containing 0.1% NaHCO$_3$. The absorption at 420 nm was then measured against an analogous suspension containing the corresponding amount of unsubstituted Sepharose. The calculation of the NH$_2$-group content was carried out as previously described[17].

Aminoethyl-Sepharose (1)

Sepharose (20 ml) was washed with water (200 ml) and transferred to a vessel containing 20 ml of 5 *M* potassium phosphate buffer, pH 11.9. A BrCN solution (prepared from 5 g of BrCN in 2.5 ml acetonitrile and subsequently diluted with 10 ml water) was added to the suspension for 2 min at 4°C with constant stirring. The mixture was vigorously stirred for another 2 min at 4°C, and the gel was then transferred to a glass filter and rapidly washed with 200 ml of cold water and 200 ml of cold 0.1 *M* NaHCO$_3$. The BrCN-activated Sepharose thus prepared was then stirred with 20 ml of an aqueous solution of ethylenediamine (2 mmoles per ml of packed volume), which had previously been adjusted to pH 10 with 6 *N* HCl. The gel was separated and washed with 200 ml of 0.1 *M* NaHCO$_3$, water, 200 ml of 0.1 *N* HCl and water. As shown by method c, adsorbent 1 contained 2.5 μequiv. of NH$_2$-groups per g wet weight.

Hydrazidosuccinyl-Sepharose (2)

BrCN-activated Sepharose (20 ml) (described above) was stirred for 5 h at 20°C with 20 ml of a saturated aqueous solution of succinic acid dihydrazide, adjusted to pH 8.0–8.5 with 6 *N* HCl. On completion of the reaction the gel was washed with water until the washings gave a negative reaction with TNBS. Finally the gel was washed with 200 ml of 0.2 *M* NaCl and 200 ml water.

Hemoglobin–dextran–Sepharose (6)

Step A. A 1.48-ml volume of 50% aqueous ethylenediamine, water (2 ml) and pyridine (5 ml) were added to a cooled solution (4°C) of carboxymethyldextran (3) (2 g, $\gamma = 5$)[10] in 6 ml water. DCC (1.91 g) in 5 ml pyridine was then added to the stirred solution, and the reaction mixture was stirred for 48 h at 20°C. The mixture was then diluted in three volumes of water, a precipitate was separated and the filtrate was extracted with diethyl ether (3 × 30 ml). The aqueous phase was evaporated, and the residue was dissolved in 20 ml water and applied to a column (volume 100 ml) packed with Sephadex G-50 and equilibrated with water. The excluded fractions were evaporated to 30 ml, the pH value was adjusted to 8–9 and the polymer was precipitated by two volumes of ethanol. The precipitate was separated and triturated first with 96% ethanol and then with absolute ethanol. The product was dried over P$_2$O$_5$ at

80°C/0.1 mmHg for 5 h, yielding 1.2 g (60.1%) of aminoethylamidocarboxymethyldextran (4). Found: N 1.03%.

Step B. A suspension of BrCN-activated Sepharose (8 ml) in 8 ml of 0.1 *M* $NaHCO_3$ containing 0.5 *M* NaCl was stirred with 150 mg of polysaccharide 4. The Sepharose was then washed with water (100 ml). Measurement of the nitrogen content in washings by Kjeldahl's method indicated that 114 mg of the dextran derivative 4 were covalently bound to Sepharose. The gel was washed and then stirred in water with one drop of acetic acid and 2 mg of EDC at 20°C for 16 h. The "dextran–Sepharose" (5) thus prepared was used in the next step.

Step C. Seven millilitres of 2 *M* Na_2CO_3 and a solution of BrCN in acetonitrile (2 mg of BrCN per ml) were added to a cooled suspension of 7 ml "dextran–Sepharose" (5) in 7 ml water. The suspension was vigorously stirred at 4°C for 2 min and then filtered. The gel was washed with 100 ml of 0.1 *M* $NaHCO_3$ containing 0.5 *M* NaCl and immediately added to a solution of hemoglobin (40 mg) in 8 ml of 0.1 *M* $NaHCO_3$ containing 0.5 *M* NaCl. The mixture was stirred for 20 h at 4°C, after which the bioadsorbent (6) was separated and washed with 250 ml $NaHCO_3$ + 0.5 *M* NaCl, then with 50 ml of 0.1 *M* sodium acetate buffer + 1 *M* NaCl (pH 4) and 50 ml of 0.1 *M* sodium-borate buffer + 1 *M* NaCl (pH 8) (the last two washings were repeated three times). As shown by analytical data, adsorbent 6 contained 5.2 mg of protein per ml of gel. Direct coupling of hemoglobin with BrCN-activated Sepharose was carried out by the standard method[18]. The dark brown adsorbents were stored as aqueous suspensions in the presence of 0.02% NaN_3.

Uridine–dextran–Sepharose (7)

A 10-ml volume of "dextran–Sepharose" (5) prepared as described above (B) was activated with 2.5 g BrCN and washed with 50 ml of cold 0.1 *M* phosphate buffer (pH 7.0). The gel was mixed with a solution of uridine (242 mg) in 10 ml of the same buffer, stirred for 16 h at 4°C, washed with 100 ml of water and then stirred with 10 ml of 1 *M* ethanolamine (pH 9) for 2 h at 20°C. The adsorbent was washed with water (50 ml), 0.2 *M* NaCl (50 ml) and water (50 ml). The UV spectrum of the solubilized gel showed that the adsorbent 7 contained 12 μmoles of uridine per ml of gel.

Ribonuclease–dextran–Sepharose (9)

Step A. Aminoethyl-Sepharose (1) (6 ml) was added to a solution of carboxymethyldextran (3) (0.5 g) in 6 ml water, the pH was adjusted to 4.7–5.0 with 1 *N* HCl and a solution of EDC (20 mg) in 3 ml water was gradually added. The mixture was stirred for 48 h at 20°C in the range pH 4.7–5.0, pH adjustments being made with 1 *N* HCl during the first hour. The gel was filtered off, and washed with water (100 ml), 0.1 *M* $NaHCO_3$ (100 ml) and water again (100 ml). A suspension of the gel in 6 ml water was stirred with a drop of acetic acid and 2 mg EDC for 16 h at 20°C. The residual carboxy groups were blocked with ethanolamine in the presence of EDC. The adsorbent (8) was washed with 1 l water and used in the next step.

Step B. A solution of $NaIO_4$ (130 mg) in 2 ml water was gradually added to a suspension of "dextran–Sepharose" (8) in 5.5 ml of sodium phosphate buffer, pH 6.0. The mixture was stirred in the dark for 1 h at 20°C. The activated gel was washed with 100 ml water, and a solution of ribonuclease (10 mg) in 10 ml of 0.1 *M* $NaHCO_3$ containing 0.5 *M* NaCl was added. The suspension was stirred for 20 h at 20°C and

100 mg of $NaBH_4$ in 5 ml water were then added gradually. Stirring was continued for 1 h at 20°C, at which point the gel was washed as described for sorbent 6. The adsorbent (9) contained 6 mg protein per ml of gel as revealed by the differential spectrophotometric method as well as by enzyme activity measurements in the starting protein solution and in the washings after protein coupling. For comparative purposes, "ribonuclease–Sepharose" was also prepared by the standard method. Adsorbent 9 was stored for a long time at 4°C as an aqueous suspension in the presence of 0.02% NaN_3.

Glycogen–hydrazidosuccinyl-Sepharose (10)

Step A. A solution of $NaIO_4$ (331 mg) in 3 ml water was gradually added to a solution of glycogen (2.41 g) in 15 ml water, with stirring and cooling in the dark. The mixture was stirred for 1.5 h at 20°C in the dark, the pH (6.5) being adjusted with 0.1 *M* NaOH. The mixture was then diluted to 35 ml with 0.1 *M* sodium acetate buffer, pH 4.8, and used in the next step.

Step B. Hydrazidosuccinyl-Sepharose (2) (30 ml) was washed with 150 ml of 0.1 *M* sodium acetate buffer, pH 4.8, and stirred with 35 ml of the solution of periodate-oxidized glycogen (see *Step A*) for 16 h at 4°C. The gel was then washed with 5 l water, 200 ml of 2 *M* NaCl and 200 ml water and suspended in 30 ml of 0.1 *M* $NaHCO_3$. Solid $NaBH_4$ (600 mg) was added to the suspension at 4°C, and the mixture was stirred for 3 h at 4°C. The gel was then washed with 1 l of water. The washings after glycogen coupling were concentrated *in vacuo* at 40°C and used for determination of the amount of unbound ligand by the phenol sulphate method. The gel was stirred with 30 ml of 0.2 *M* acetaldehyde in 0.1 *M* sodium acetate buffer for 2 h at 4°C, washed with water and stirred with 100 mg of $NaBH_4$ for 2 h at 4°C. The adsorbent prepared (10) was washed with 1 l of water.

Aminohexyl-hydrazidoadipinyl–glycogen–hydrazidosuccinyl-Sepharose (11)

Step A. Glycogen–hydrazidosuccinyl-Sepharose (10) (10 ml) was activated with 2.5 g BrCN as described above. The activated gel was washed with 100 ml of cold water and then stirred with 10 ml of a saturated solution of adipinic acid dihydrazide in water (pH 8.0–8.5) for 16 h at 4°C. The gel was washed with 100 ml water, 100 ml of 0.2 *M* NaCl and 100 ml water. It became bright red after treatment with TNBS in potassium borate buffer (pH 9.2)[19].

Step B. The adsorbent prepared as above (10 ml) was washed with 50 ml of 0.5 *N* HCl and stirred with 10 ml of 0.1 *M* $NaNO_2$ in water for 7 min at 4°C. The gel was rapidly washed with cold water and stirred with 10 ml of 0.2 *M* hexamethylenediamine in 0.2 *M* Na_2CO_3 for 8 h at 4°C and pH 9.0–9.2. After washing with 100 ml water, the gel was stirred with 10 ml of ammonium buffer (2 *M* NH_4Cl and 1 *M* NH_4OH, pH 8.8) and then washed with water. The adsorbent (11) contained 3.77 μequiv. of NH_2 groups per g of gel (wet weight).

L-*Lysine hydrazide (12)*

Hydrazine hydrate (8.75 ml, 0.174 moles) was gradually added to a suspension of 2.8 g (0.0174 moles) of the hydrochloride of L-lysine methyl ester[20] in 30 ml methanol. The mixture was stirred for 50 h at 20°C, the reaction being monitored by thin-layer chromatography (TLC) on Silufol [solvent, ethanol–ammonia (8:2)]. The mixture was evaporated and the residue was treated by 4 *M* hydrogen chloride in dioxan with cooling; the precipitate was collected, washed with diethyl ether and recrystallized from ethanol. After drying the crystals at 60°C/0.1 mmHg, the yield was 1.53 g (54%); m.p. 225°C (lit.[21] 225–228°C), R_F 0.32.

L-*Lysine–amylopectin–hydrazidosuccinyl-Sepharose (13)*

Amylopectin–hydrazidosuccinyl-Sepharose was prepared as described for glycogen–hydrazidosuccinyl-Sepharose. This sorbent (10 ml) in 10 ml of potassium phosphate buffer, pH 6.0, was activated with a solution of $NaIO_4$ (140 mg) in 1 ml water. The suspension was stirred for 1 h at 20°C in the dark, after which the gel was thoroughly washed with water. It was then stirred with a solution of 1 g of L-lysine hydrazide (12) in 10 ml of 0.1 *M* potassium phosphate buffer, pH 6.0, for 1.5–2.0 h at 20°C and washed with water until the filtrate gave a negative reaction with TNBS. The gel was then suspended in 10 ml water and stirred with 100 mg of $NaBH_4$ in 2 ml water for 0.5 h at 20°C. After washing with water, the adsorbent (13) containing 2 μequiv. of NH_2 groups per g of gel (wet weight) was obtained.

L-*Lysine–glutaraldehyde–hydrazidosuccinyl-Sepharose (14)*

A suspension of hydrazidosuccinyl-Sepharose (2) (10 ml) in 4 ml water was stirred with 6 ml of 25% aqueous glutaraldehyde for 30 min at 20°C; the gel was washed with 100 ml water and transferred to a solution of 1 g of L-lysine hydrazide (12) (100-fold excess) in 10 ml of potassium phosphate buffer, pH 6. The mixture was stirred for 30 min at 20°C, the pH being maintained at 6.0 with 6 *N* HCl. The gel was filtered off and washed with the same buffer (100 ml) and water (100 ml). The gel was treated with $NaBH_4$ (100 mg) in 10 ml water for 30 min at 20°C and washed with water. The adsorbent (14) contained 0.8 μequiv. of NH_2 groups per g wet weight (the suspension method).

Poly(U)–glycogen–Bio-Gel P-300 (15)

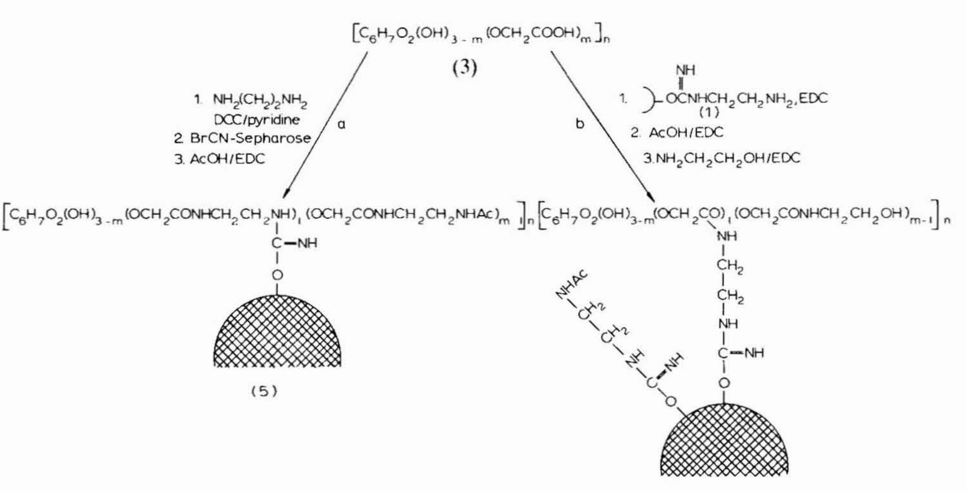
Periodate-oxidized glycogen (2.41 g) was coupled to a hydrazide derivative of Bio-Gel P-300[22] under the conditions described for sorbent 10. After the stabilization of bonds by treatment with $NaBH_4$, followed by blocking the excess of hydrazide groups with acetaldehyde–$NaBH_4$ and activation of a glycogen spacer by BrCN (4 g), the coupling of poly(U) (45 mg) with the gel was carried out in 0.1 *M* potassium phosphate buffer, pH 7.5 (16 h, 4°C). The gel was washed with the same buffer and then treated with ethanolamine to remove the excess of active groups. The adsorbent (15) contained 2 mg of poly(U) per ml of gel (the differential method).

RESULTS AND DISCUSSION

A number of methods have been described for the coupling of polysaccharides to solid supports[3]. The polysaccharides were used mainly as ligands. The general use of water-soluble polysaccharides as spacers in affinity adsorbents has not yet been reported. The methods described here consist in the introduction of reactive functional groups into a water-soluble polysaccharide and subsequent covalent binding of the modified polysaccharide to a support using these reactive groups. Activation of a polysaccharide spacer enabled the immobilization of ligands of different chemical nature.

Affinity adsorbents with dextran spacers

Dextran was chosen as a model water-soluble polysaccharide for the preparation of hemoglobin–dextran–Sepharose (6). The degree of branching of the dextran molecule was due to the presence of $\approx 5\%$ of α-(1–3) bonds. Dextran was converted into carboxymethyldextran containing 5 carboxymethyl groups per 100 anhydroglucose residues ($\gamma = 5$)[10]. Immobilization of carboxymethyldextran on Sepharose may be carried out by either (a) condensation with an excess of ethylenediamine in aqueous pyridine in the presence of DCC and subsequent coupling with BrCN-activated Sepharose, or (b) condensation with aminoethyl-Sepharose (1) in the presence of the water-soluble carbodiimide, subsequent acetylation of residual free NH_2 groups by acetic acid in the presence of EDC and blocking of unreacted carboxy groups with ethanolamine–EDC (Scheme 1).



Scheme 1.

In the synthesis of adsorbent 6, the introduction of a polysaccharide spacer was carried out by method a. The modified dextran (4) contained 1.03% N, suggesting amidation of all the carboxymethyl groups in the starting compound (3). Polysaccharide 4 was coupled to BrCN-activated Sepharose by the usual method. The amount of dextran coupled to the gel (14.2 mg per ml of gel) was determined by the differential method from the nitrogen content (by Kjeldahl's method). Blocking of residual active groups on Sepharose was carried out by treatment with ethanolamine, and the free NH_2 groups on the polysaccharide spacer were acetylated with acetic acid in the presence of EDC. Attachment of hemoglobin to dextran-Sepharose (5) was performed after activation of the polysaccharide spacer by the BrCN method. Adsorbent 6 contained *ca.* 5.5 mg of protein per ml of gel (the differential method). It may be assumed that the ligand is mainly localized on the polysaccharide spacer; repeated activation of an agarose support by BrCN should proceed to only insignificant degree. The bioadsorbent hemoglobin–dextran–Sepharose (6) may be used for purification of proteolytic enzymes[23]. It was very stable: no leakage of hemoglobin was visible during the storage of the adsorbent in water in the presence of 0.02% NaN_3 for 1 year at 4°C.

The adsorbent uridine–dextran–Sepharose (7) was prepared by a similar method. Dextran–Sepharose (5) was activated with BrCN and stirred with uridine at pH 7.0. Although the chemical structure of adsorbent 7 was not strictly uniform, it may be assumed that uridine couples to a polysaccharide spacer via position 4 of the pyrimidine ring, as is the case in the preparation of polyU–Sepharose[24]. Adsorbent 7 (12 μmoles of uridine per ml of gel) may be used for the purification of enzymes posessing an affinity for the immobilized nucleoside, *e.g.*, uridine kinase[25].

An adsorbent may lose its stability if a low-molecular-weight ligand is singly coupled to a polysaccharide spacer by the BrCN method. In this case gradual leakage of the ligand would take place in accordance with the general mechanism for BrCN-activated polysaccharides[4]. Therefore, we tested another method for the polysaccharide spacer activation, namely periodate oxidation[26], followed by coupling with a ligand containing primary amino groups and stabilization of a ligand–spacer bond by $NaBH_4$. (In the case of a protein ligand the treatment with $NaBH_4$ could be omitted without a decrease in the adsorbent stability.)

Aldehyde matrices prepared by periodate oxidation of polysaccharide supports or glass beads covered with dextran have recently been used[27] for enzyme immobilization by the method of reductive alkylation. Junowicz and Charm[28] described further modifications of periodate-treated polysaccharide supports designed for introduction of reactive hydrazide and azide groups into the matrix. The polysaccharide spacers permitted introduction of a large number of aldehyde groups into an adsorbent by a simple and convenient treatment with periodate.

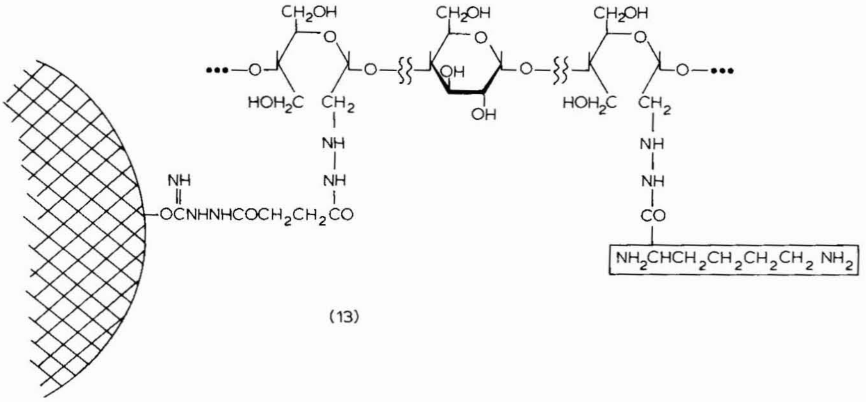
The biospecific adsorbent ribonuclease–dextran–Sepharose (9), which may be used for purification of enzyme inhibitors, was prepared. In this case the introduction of a polysaccharide spacer into the solid support was achieved by the method b (Scheme 1). Dextran-Sepharose (8) was then treated with sodium periodate, condensed with ribonuclease and the protein–spacer bonds were stabilized with $NaBH_4$. The adsorbent (9) thus prepared containing 6 mg of the enzyme per ml of gel.

Affinity adsorbents with glycogen and amylopectin spacers

Attachment of ligands or spacers containing NH_2 groups to BrCN-activated polysaccharide supports results in the introduction of a positive charge because of ionization of the isourea groups formed (pK 9.6–10.4)[29,30]. This charge imparts ion-exchange properties to the adsorbent and may interfere with the biospecificity of the purification procedure. In order to avoid the introduction of charged groups we used α,ω-dicarbonic acid dihydrazides for binding water-soluble polysaccharides to the agarose matrix. Thus the treatment of BrCN-activated Sepharose with an excess of succinic acid dihydrazide leads to hydrazide–Sepharose which is uncharged at the physiological pH (pK 4.2)[29]. The polysaccharide spacers can be introduced into this sorbent after water-soluble polysaccharide activation with BrCN or periodate.

This approach was applied to the preparation of aminohexylhydrazidoadipinylglycogen–hydrazidosuccinyl-Sepharose (11). A similar adsorbent without the polysaccharide spacer has been successfully used for purification of pig kidney diamine oxidase[31]. Hydrazidosuccinyl-Sepharose (2) was incubated with glycogen which had previously been oxidized by treatment with periodate. The oxidation was carried out for 5% of the anhydroglucose residues of the polysaccharide. After the coupling glycogen, the gel was treated with $NaBH_4$, and the residual free hydrazido groups were blocked by treatment with acetaldehyde and then with $NaBH_4$. The content of glycogen in the gel was calculated to be 25–30 mg per ml of gel. Then, adipinic acid dihydrazide was coupled to the BrCN-activated polysaccharide spacer. Subsequent attachment of hexamethylenediamine to the hydrazide-adsorbent was carried out via the corresponding azide as described by Klimova *et al.*[31]. At the physiological pH, adsorbent 11 did not contain any additional cationic groups with the exception of the terminal NH_2 groups. The amount of bound hexamethylenediamine (3.77 μmoles/g wet weight) was determined by the suspension method[17].

Various natural polysaccharides as well as synthetic and natural oligosaccharides may be used as spacers in the affinity adsorbents. One of the most readily available branched water-soluble polysaccharides, amylopectin, was used as a spacer for preparation of L-lysine–amylopectin–hydrazidosuccinyl-Sepharose (13). Amylopectin was immobilized on hydrazidosuccinyl-Sepharose (2) as described for immobilization of glycogen on the same sorbent (2). After activation of the amylopectin spacer with sodium periodate, the aldehyde matrix prepared was stirred for 1.5–2.0 h at 20°C and pH 6.0 with L-lysine hydrazide (12) and then treated with $NaBH_4$. It may be assumed that the ligand (12) is mainly attached through the hydrazide group. This immobilization procedure results in the formation of an adsorbent containing 1,5-diaminopentane, substrate of diamine oxidase, as ligand (Scheme 2). Therefore adsorbent 13 could be used for purification of this enzyme.



(13)

Scheme 2.

Adsorbent 13 contained 2 μequiv. of amino groups per g of gel (wet weight). A similar adsorbent having a monomeric spacer, L-lysine–glutaraldehyde–hydrazidosuccinyl-Sepharose (14), was also synthesized for comparison.

Affinity adsorbents having polysaccharide spacers may be synthesized on the basis of various supports including polyacrylamide or glass beads, porous silica gel, Spheron, etc. The preparation of poly(U)–glycogen–Bio-Gel P-300 (15) was considered as an example of such possibilities. Bio-Gel P-300 was converted into the hydrazido-derivative[22]. Then, periodate-oxidized glycogen was coupled to the hydrazido matrix as described for adsorbent 10. Activation of the glycogen spacer by BrCN and subsequent coupling of poly(U) resulted in formation of adsorbent 15 containing 2 mg of poly(U) per ml of gel (the differential method).

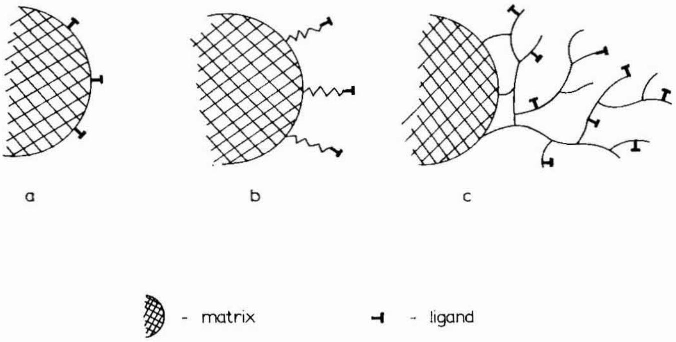


Fig. 1. The different types of affinity adsorbents: a, without a spacer; b, with a monomeric spacer; c, with a polysaccharide spacer.

The experiments described enable the preparation of stable biospecific adsorbents. The amount of the ligands coupled via the polysaccharide spacers (Fig. 1) was considerably higher than that for adsorbents without the polymeric spacer (Table I).

TABLE I

COMPARISON OF THE LIGAND CONTENTS IN THE ADSORBENTS WITH OR WITHOUT POLYSACCHARIDE SPACERS

Adsorbent	*Ligand content*
Hemoglobin–dextran–Sepharose (6)	5.2 mg/ml
Hemoglobin–Sepharose	1.5 mg/ml
Ribonuclease–dextran–Sepharose (9)	6.0 mg/ml
Ribonuclease–Sepharose	2.0–2.5 mg/ml
Uridine–dextran–Sepharose (7)	12 μmoles/ml
Uridine–Sepharose	8 μmoles/ml
L-Lysine–amylopectin–hydrazidosuccinyl-Sepharose (13)	2 μequiv./g wet weight
L-Lysine–glutaraldehyde–hydrazidosuccinyl-Sepharose (14)	0.8 μequiv./g wet weight

The use of polysaccharide spacers in affinity chromatography offers an additional possibility for elution of adsorbed biopolymers. The elution procedure is frequently the most complicated step in biospecific purification. The use of high concentrations of denaturating agents, solutions having extreme pH values or organic compounds are sometimes required for successful desorption. In these cases denaturation of biopolymers, especially enzymes, may occur. In the presence of polysaccharide spacers this complication may be overcome by destruction of the spacer by means of the corresponding enzyme (for instance, dextranase or amylase). A similar technique has been applied for elution of cells from adsorbents based on Sephadex[32] or gelatin–Sepharose[33].

Affinity chromatography on adsorbents having polysaccharide spacers is complicated if protein impurities possessing affinity for the spacer itself are present in a crude enzyme preparation. In this case preliminary chromatography on a column of a "polysaccharide spacer–support" adsorbent is recommended. This adsorbent would selectively bind the biopolymers possessing the affinity for the spacer. The "polysaccharide spacer–support" system may be used as a biospecific adsorbent for purification of proteins with affinity for a spacer (for instance, glycogen–Sepharose for purification of α-amylase[34], glycogen phosphorylase[35], etc.).

In this paper we have described some methods for introducing polysaccharide spacers into various supports. The development of new methods for immobilization of biologically active compounds on polysaccharides provides ample opportunities for the attachment of various organic compounds to polysaccharide spacers. In the case of protein ligands the method can also be used for enzyme immobilization.

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USE OF BIOSPECIFIC ADSORBENTS WITH POLYSACCHARIDE SPACERS IN AFFINITY CHROMATOGRAPHY

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SUMMARY

The isolation of mouse liver poly(A)-containing mRNA [poly(A)–mRNA] and purification of trypsin and kallikrein using new biospecific adsorbents having dextran, glycogen or amylopectin spacers are described as the first examples of affinity chromatography on such adsorbents. A comparative study of these adsorbents and similar adsorbents without spacers was carried out. The optimum conditions for affinity chromatography of poly(A)–mRNA were considered.

INTRODUCTION

In the preceding paper[1] we described the preparation and properties of a number of affinity adsorbents having polysaccharide spacers. It seemed expedient to demonstrate the advantages of such adsorbents by concrete examples of the isolation and purification of biopolymers of different chemical nature, particularly polyadenylated mRNA (polynucleotide biopolymer) and proteolytic enzymes (protein biopolymers).

The isolation of poly(A)–mRNA on poly(U)–Sepharose (1)[2] or oligo(dT)–cellulose[3] is based on the hybridization of complementary bases. Some non-specific effects of a different nature were involved in the chromatography of poly(A)–mRNA on oligo(dT)–cellulose[4]. The disadvantages of chromatography on poly(U)–Sepharose (1) are the non-quantitative recovery of poly(A)–mRNA and insufficient adsorbent capacity.

At the present time, the immobilized soy bean trypsin inhibitor (SBTI) is widely used for purification of serine proteinases[5]. Highly purified trypsin[6,7] and human serum kallikrein[8,9] have been prepared by means of affinity chromatography on SBTI–Sepharose (2). Attachment of SBTI to Sepharose is generally achieved by the BrCN method[10]. However, direct binding of a high-molecular-weight ligand to a solid support may result in steric hindrance to subsequent biospecific interaction of immobilized ligand with the active centre of the enzyme to be purified. Consequently, a decrease in adsorbent capacity may take place. We believe that the use of a spacer to separate the ligand from the surface of the solid matrix would improve some properties of the adsorbent. A recently published paper[11] described the immobilization of SBTI via a monomeric spacer using a glutaraldehyde-activated amino derivative of Sepharose.

In the present work, we describe the first applications in affinity chromatography of biospecific adsorbents having polysaccharide spacers. The isolation of poly(A)–mRNA on poly(U) immobilized on Sepharose via dextran or glycogen spacers as well as the purification of trypsin and human serum kallikrein on SBTI bound to Sepharose via an amylopectin spacer have been performed. The properties of adsorbents with and without polysaccharide spacers are compared.

EXPERIMENTAL

Materials and methods

Most of the materials used in the present study were as specified in the preceding paper[1]. Poly(U) and SBTI were obtained from Reanal (Budapest, Hungary), pig pancreatic trypsin from Spofa (Prague, Czechoslovakia). Human serum kallikrein was partially purified by chromatography on DEAE-Sephadex A-50 and SP-Sephadex C-50 (the method will be published in detail elsewhere). N-Benzoylarginine ethyl ester (BAEE) was obtained from Koch-Light (Colnbrook, Great Britain). O-[N-2-(Aminoethyl)carbamoyl]dextran (AED) (γ^{NH_2} = 12) was prepared as described previously[12]. Nuclear and cytoplasmic RNAs from mouse liver cells were isolated by the phenol–temperature fractionation procedure[13].

Some of the methods used in this work were as described in the preceding paper[1]. DNA-like RNAs were selectively labelled with [^{14}C]orotic acid (30 μCi per mouse) for 60–90 min by the use of the actinomycin D-induced block of rRNA synthesis. For the determination of radioactivity, an RNA carrier (100 μg/ml) and trichloroacetic acid (final concentration 5%) were added to the samples. The precipitates were then collected on HUFS filters, and after drying were measured in a Nuclear Chicago Mark 2 liquid scintillation spectrometer. Sedimentation coefficients were calculated as described previously[14].

Estimation of the ligand content in the adsorbents

The concentration of the protein ligand was determined by the differential method. In the case of a polynucleotide ligand the solubilization method[15] was used. A mixture of 0.2 ml of a Sepharose derivative and 12 ml of 1 *N* NaOH containing 0.1% $NaBH_4$ was heated for 2 h at 75–80°C. The mixture was then centrifuged for 15 min at 7000 rpm (3000 *g*). The absorbance of the supernatant was measured at 260 nm.

AED–Sepharose (3)

A suspension of 18 ml of BrCN-activated Sepharose (2.5 g of BrCN per 10 ml gel)[1] in 18 ml of 0.1 *M* $NaHCO_3$ containing 0.5 *M* NaCl, pH 9, and 2.7 g AED (γ^{NH_2} = 12)[12] was stirred for 16 h at 4°C. The gel was then washed with 1 l water, 200 ml of 0.2 *M* NaCl and 200 ml water and stirred with 20 ml of 1 *M* ethanolamine (pH 9) for 2 h at 20°C. The gel was washed with water (200 ml), suspended in an equal volume of water and the mixture was stirred with two or three drops of acetic acid and 20 mg EDC for 72 h at 20°C. After washing with 200 ml of 0.1 *N* HCl and 500 ml of water, the AED–Sepharose (3) was stored as an aqueous suspension at 4°C in the presence of 0.02% NaN_3.

Poly(U)–AED–Sepharose (4)

A solution of 2 g BrCN in 1 ml acetonitrile was diluted to 5 ml with water and added to a suspension of 10 ml "AED–Sepharose" (3) in 10 ml of 5 *M* potassium phosphate buffer, pH 11.9. The suspension was vigorously stirred for 5 min at 4°C. Then the gel was quickly washed with 100 ml of 0.1 *M* $NaHCO_3$, 100 ml water and 100 ml of 0.1 *M* potassium phosphate buffer, pH 7.5. The gel was immediately suspended at 4°C in a solution of 15 ml poly(U) in 10 ml of the same buffer and stirred for 16 h at 4°C. The sorbent was washed with 500 ml of 0.1 *M* potassium phosphate buffer, pH 7.5, and 100 ml water and then stirred with 1 *M* ethanolamine (10 ml) for 2 h at 20°C and pH 9.0. Finally, the gel was washed with water.

Poly(U)–glycogen–hydrazidosuccinyl-Sepharose (5)

Glycogen–hydrazidosuccinyl-Sepharose (10 ml) prepared as described previously[1] was activated with 2 g BrCN and then stirred with a solution of 15 mg poly(U) in 10 ml of 0.1 *M* potassium phosphate buffer, pH 7.5, for 16 h at 4°C. The gel was washed with 500 ml of the same buffer and 100 ml water, stirred with 3 mg acetic acid hydrazide in 10 ml water for 2 h at 20°C and washed with 500 ml water. The adsorbent (5) was stored in 0.1 *M* potassium phosphate buffer (pH 7.5) in the presence of 0.02% NaN_3.

Affinity chromatography of poly(A), poly(U) and poly(A)–mRNA

The chromatography of different samples on the adsorbents 1, 4 and 5 was carried out using a glass column (7.0 × 0.6 cm) at a flow-rate of 15 ml/h. The buffers used for sample application and elution were: a, hybridization buffer, 0.02 *M* Tris–HCl, pH 7.0, 0.3 *M* NaCl, 0.001 *M* ethylenediaminetetraacetate (EDTA); b, 0.02 *M* Tris–HCl, pH 7.6, 0.001 *M* EDTA. Fractions were assayed for absorbance at 260 nm or radioactivity.

SBTI–amylopectin–hydrazidosuccinyl-Sepharose (6)

Adsorbent 6 was prepared by means of activation of amylopectin–hydrazidosuccinyl-Sepharose[1] (10 ml) with 2 g BrCN as described above and subsequent incubation with 100 mg SBTI in 10 ml of 0.1 *M* $NaHCO_3$ containing 0.5 *M* NaCl. Washing of the adsorbent and blocking of the excess of active groups were performed as described in the preceding paper[1] for protein immobilization. SBTI–Sepharose (2) was synthesized as described[10]. Adsorbents 2 and 6 contained 7–9 mg SBTI per ml of gel (the differential method).

Determination of antitryptic activity of immobilized SBTI

Aliquots (0.01–0.1 ml) of adsorbents 2 and 6 were adjusted to 1.4 ml with 0.05 *M* Tris–HCl buffer, pH 8.0, and 0.1 ml of 0.01% trypsin in 2.5 m*M* HCl was then added. The mixture was kept for 5 min, and 0.5 ml of 0.1% agarose solution and 1 ml of 1.5 m*M* BAEE were added. The mixture was thoroughly stirred, and the absorbance at 253 nm was measured against a corresponding amount of unsubstituted Sepharose. The antitryptic activity was expressed as the number of inhibitor units per mg of immobilized SBTI: 1 IU = one unit of inhibitory activity, corresponding to the amount of SBTI which inhibits the hydrolysis of 1 μmole of substrate under the conditions described above.

Proteinase assay

Assays for trypsin and kallikrein activities were carried out in 0.05 *M* Tris–HCl buffer (pH 8.0) at 25°C by measurement of the absorption at 253 nm with BAEE as substrate. One unit of activity, E, is defined as the amount of enzyme which hydrolyses 1 μmole of BAEE per min under the standard conditions.

Affinity chromatography of kallikrein and trypsin

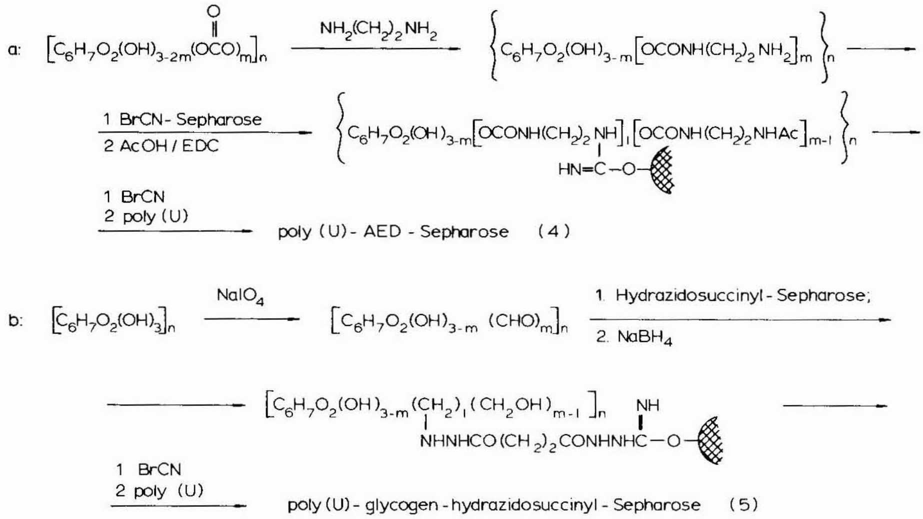
All procedures were performed at 4°C. A solution of partially purified human serum kallikrein was applied to a column (4.0 × 0.8 cm) packed with adsorbent 2 or 6. The column was equilibrated with 0.1 *M* sodium phosphate buffer (pH 6.2) containing 0.2 *M* NaCl. The flow-rate was 12 ml/h. Protein contaminants were eluted from the columns with the starting buffer (eluate I). Kallikrein was eluted with 0.01 *M* HCl containing 1 *M* NaCl (eluate II). Fractions (2.5 ml) were collected at a flow-rate of 48 ml/h. Each fraction was immediately adjusted to pH 8.0 with 3.5 *M* NaOH, and the BAEE-esterase activity was determined.

Affinity chromatography of pig pancreas trypsin was performed under similar conditions.

RESULTS AND DISCUSSION

The adsorbents having polysaccharide spacers (4–6) were prepared according to the methods described in the preceding paper[1], but with some modifications.

In the synthesis of adsorbent 4 (Scheme 1a) AED containing 12 N-2-(aminoethyl)carbamoyl groups per 100 anhydroglucose residues was used for introduction of the polysaccharide spacer into the adsorbent. AED was prepared by condensation of dextran cyclic carbonate with ethylenediamine[12]. The use of AED prevents the presence of unreacted carboxyl groups on the adsorbent[16], in contrast to the method using carboxymethyldextran[1]. The amount of bound dextran (15–20 mg per ml gel)



Scheme 1.

was determined from the difference in nitrogen content (by Kjeldahl's method) of the starting solution of the polysaccharide and of the washings (after dialysis). The active groups on Sepharose were blocked with ethanolamine, and the unreacted amino groups on the polysaccharide spacer were acetylated with acetic acid in the presence of EDC. Acetylation was complete in 72 h, the reaction being monitored by the colour test with trinitrobenzenesulphonic acid.

Attachment of poly(U) to adsorbent 3 was performed after activation of the dextran spacer with BrCN[2]. The content of poly(U) in the adsorbent (1.47 mg per ml gel) was determined by measurement of the absorbance at 260 nm of the starting solution and that of the washings. The solubilization method[15] was also used. Fig. 1 shows the UV spectrum of the solubilized adsorbent (4). It should be noted that complete solubilization of adsorbents with polysaccharide spacers was not achieved under the standard conditions[15]. Centrifugation of the reaction mixture before adsorption measurements was found to be necessary. The increase in stability of agarose beads may be due to the presence of the polysaccharide spacer and to the occurrence of cross-linking during its attachment. However, this did not affect the accuracy of determination of the poly(U) content since the immobilized poly(U) was completely hydrolysed under the conditions used.

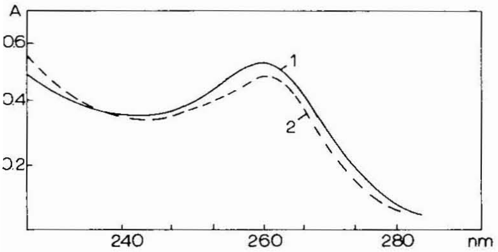


Fig. 1. UV spectra of solubilized adsorbents: 1 = adsorbent 4; 2 = adsorbent 5. The conditions of solubilization are given in the text.

The method used for synthesis of poly(U)–glycogen–hydrazidosuccinyl-Sepharose (5) (Scheme 1b) minimizes the amount of charged groups on the adsorbent, through the use of Sepharose hydrazide. As shown by UV spectrophotometric analysis of the solubilized gel (Fig. 1), adsorbent 5 contained 1.45 mg poly(U) per ml gel. Poly(U)–Sepharose (1) was prepared as described[2], and contained 0.5 mg poly(U) per ml gel.

The chromatographic behaviour of poly(A) and poly(U) on adsorbents 4 and 5 was studied in order to evaluate the capacity and biospecificity of the adsorbents. Efficient adsorption of poly(A) was observed after application of the polynucleotide to the columns in the hybridization buffer of high ionic strength. Columns of the adsorbents 4 and 5 were saturated with poly(A), and the capacity of the adsorbents was estimated by measuring the absorbance at 260 nm of the polynucleotide fraction eluted by the buffer of low ionic strength. Adsorbents 4 and 5 possessed poly(A)-binding capacities of 1.44 and 1.48 mg poly(A) per ml gel, respectively. These values significantly exceed that of adsorbent 1 (0.4 mg/ml). However, a large part of the bound poly(A) (61.1% for adsorbent 4 and 16.8% for adsorbent 5) could not be desorbed from the column under the conditions generally used for elution of poly(A)–

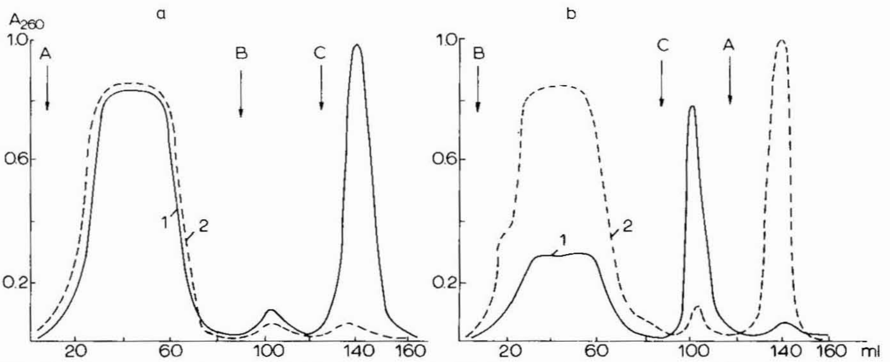


Fig. 2. Chromatography of poly(A) (1) and poly(U) (2) on adsorbent 4 (a and b). Arrows indicate change of the conditions: A, hybridization buffer; B, elution buffer at 20°C; C, elution buffer at 50°C. The polynucleotides (40–60 μg/ml) were applied to the columns in the corresponding buffer.

mRNA[17]. Nevertheless, the initial adsorption of poly(A) was biospecific since poly(U) was not adsorbed on the columns under the same conditions (Fig. 2a).

To achieve a quantitative yield of poly(A) during the elution step, the adsorbents were washed with 90% formamide in the elution buffer in the presence of 0.5% sodium dodecyl sulphate (SDS) at 50°C. The formamide was then washed off, and an aliquot of the gel was solubilized and tested as above. The results show that desorption of poly(A) was quantitative under these conditions.

The behaviour of poly(A) and poly(U) on adsorbents 4 and 5 equilibrated with the elution buffer of low ionic strength was also studied. These conditions were unfavourable for specific base pairing between the poly(A) and poly(U) chains, but, on the other hand, they promoted the non-specific ionic interactions. Considerable adsorption of the polynucleotides was observed on both adsorbents. Moreover, the quantitative desorption of poly(U) was achieved by means of the hybridization buffer (Fig. 2b).

The results of the model experiments (Table I) suggest that electrostatic interactions between the polynucleotide phosphate groups and cationic groups of the adsorbents occur when buffer of low ionic strength is used. These interactions seem to be multivalent since the non-specific binding of adenosine 5′-phosphate to the adsorbents was not observed under the same conditions. The content of poly(A) bound non-specifically to adsorbents 4 and 5 (Table I) may be correlated with the number of cationic groups of these adsorbents. Indeed, some positively charged groups were definitely introduced into adsorbent 4 during the multivalent attachment of AED to BrCN–Sepharose via the primary amino groups. On the other hand, the adsorbent formed after the attachment of succinic acid dihydrazide to BrCN–Sepharose was uncharged at the physiological pH. For this reason, the blocking of residual active groups in adsorbent 5 was carried out by use of acetic acid hydrazide, not ethanolamine.

The undesirable processes of ligand release and of increasing cationic charge during storage of adsorbents based on BrCN-activated polysaccharides have recently been recognized[18]. We found that the recovery of poly(A) from freshly prepared adsorbent 1 was 90%, while after storage of the adsorbent for 1 year at 4°C and pH 7

TABLE I

PROPERTIES OF THE AFFINITY ADSORBENTS 4 AND 5

Capacity of adsorbent 1 was 360 µg poly(A) per ml gel.

Adsorbent	*Capacity*		*Non-specific adsorption of poly(A)* (% of bound polynucleotide)*	*Adsorption of poly(U) under conditions of high ionic strength (µg per ml gel)*	*Adsorption of poly(A) under conditions of low ionic strength (µg per ml gel)*	*Adsorption of poly(U)** under conditions of low ionic strength (µg per ml gel)*
	µg poly(A) per ml adsorbent	*mg poly(A) per mg immobilized poly(U)*				
Poly(U)–AED–Sepharose (4)	1440	0.98	61	0	760	650
Poly(U)–glycogen–hydrazido-succinyl-Sepharose (5)	1480	1.02	16.2	0	440	480

* The fraction not desorbed with the elution buffer at 50°C.

** The bound poly(U) was quantitatively eluted with the hybridization buffer.

the recovery was only *ca.* 50 %. Repeated usage of the adsorbent also resulted in deterioration of its properties.

Adsorbent 5 yielded better results than adsorbent 4, and optimal recovery of poly(A) was achieved by desorption of the bound polynucleotide with 90 % formamide in the elution buffer containing 0.5 % SDS, at 50°C. Under these conditions, the biospecific A–U hybridization was interrupted and also the ion-exchange interactions were diminished.

Isolation of mouse liver cytoplasmic and nuclear poly(A)–RNAs was performed using adsorbent 5 under optimum conditions. Approximately 2 % of the total cytoplasmic RNA and 30 % of nuclear RNA were bound to the adsorbent. The total RNA content in the fractions was estimated by UV spectrophotometry, and that of non-ribosomal RNA was calculated on the basis of the radioactive label. The desorption of cytoplasmic RNA was carried out with the elution buffer at 20 and 50°C and, finally, with 90 % formamide in the same buffer containing 0.5 % SDS. The results obtained suggest that quantitative recovery of the bound RNA was achieved when the formamide-containing buffer was used. The eluted cytoplasmic and nuclear poly(A)–RNAs were precipitated by two volumes of ethanol containing 0.2 % sodium acetate (final concentration). The labelled material gave a typical pattern for high-molecular-weight RNA under the conditions of centrifugation in a sucrose gradient 10–35 %) at 23,000 rpm (64,000 *g*) (rotor SW 25.2) and 4°C.

Quantitative recovery of poly(A)–mRNA from a biospecific adsorbent is of great importance for the study of mRNA metabolism. Non-specific effects, in particular ion-exchange ones, interfere with the affinity chromatography of poly(A)–RNAs. Ion-exchange interactions are of little importance under adsorption conditions (the hybridization buffer of high ionic strength). However, part of the material may be retained on the column as a result of multipoint interactions between the ionized phosphate groups of RNA and cationic groups of the adsorbent. Part of the electrostatically bound material would again interact biospecifically with the immobilized poly(U) during repeated use of the adsorbent or its regeneration with buffers of high ionic strength. Contamination of the poly(A)–RNA preparations with labelled material from previous experiments may take place during the subsequent elution. Therefore, minimization of the content of positively charged groups on the biospecific adsorbent is especially important for affinity chromatography of poly(A)–mRNA.

In order to demonstrate further the advantages of the adsorbents having polysaccharide spacers, we carried out a comparative study of two bioadsorbents designed for the purification of proteolytic enzymes: SBTI–amylopectin–hydrazidosuccinyl-Sepharose (6) and a similar adsorbent without the polysaccharide spacer (2).

Adsorbent 6 was prepared by coupling of SBTI to amylopectin–hydrazidosuccinyl-Sepharose[1] after activation of the polysaccharide spacer with BrCN. Adsorbent 2 was synthesized by the direct attachment of SBTI to BrCN-activated Sepharose. As shown by the differential method, these adsorbents contained 7–9 mg SBTI per ml of gel. The antitryptic activity of the immobilized SBTI was then estimated by spectrophotometric assay of the BAEE hydrolysis in the gel suspension stabilized by addition of 0.1 % agarose solution. The use of the agarose solution ensured accurate measurements of the absorption. The specific antitryptic activity of SBTI immobilized via the amylopectin spacer was almost twice that of SBTI immobilized directly on Sepharose (Table II). Thus, the use of the polysaccharide spacer preserved 20 % of the activity of immobilized SBTI with respect to that in solution. The presence of the spacer results in a considerable distance between the ligand and the matrix surface, and the native protein conformation is apparently preserved to a greater extent than can be achieved by direct attachment of SBTI to Sepharose.

The capacity of adsorbents 2 and 6 for trypsin and kallikrein was also determined. These enzymes exhibited very similar affinities for the native soy bean inhibitor (inhibition constant *ca.* 10^{-10} $M^{19,20}$). The proteinases were applied to the column in 0.1 *M* sodium phosphate buffer (pH 6.2) containing 0.2 *M* NaCl until the column was saturated with the enzyme. After washing the column with the starting buffer, the bound proteinase was eluted with 0.01 *M* HCl containing 1 *M* NaCl. The capacity for both enzymes of the adsorbents having the amylopectin spacer was three

TABLE II

PROPERTIES OF THE AFFINITY ADSORBENTS CONTAINING SBTI

Adsorbent	*Antitryptic activity (IU per mg immobilized SBTI)*	*Capacity for trypsin*		*Capacity for kallikrein*	
		mg per ml gel	*µmoles per µmoles immobilized SBTI*	*mg per ml gel*	*µmoles per µmoles immobilized SBTI*
SBTI–amylopectin–hydrazidosuccinyl-Sepharose (6)	4.4	2.5	0.25	2.8	0.07
SBTI–Sepharose (2)	2.4	0.75	0.08	1.0	0.025

TABLE III

AFFINITY CHROMATOGRAPHY OF PIG PANCREATIC TRYPSIN AND HUMAN SERUM KALLIKREIN ON ADSORBENTS 2 AND 6

Adsorbent	*Fraction*	*Trypsin*						*Kallikrein*					
		Protein		*Activity*		*Yield (%)*	*Purification factor*	*Protein*		*Activity*		*Yield (%)*	*Purification factor*
		mg/ml	*mg*	*Specific (E/mg)*	*Total (E)*			*mg/ml*	*mg*	*Specific (E/mg)*	*Total (E)*		
SBTI–amylopectin–hydrazidosuccinyl-Sepharose (6)	Starting preparation	0.093	14.0	18.0	250.0	–	1	1.04	836.0	0.17	142.0	–	1
	Filtrate + eluate I	0.047	8.2	3.3	27.1	10.8	–	0.94	800.0	0.0	0.0	–	–
	Eluate II (0.01 *N* HCl + 1 *M* NaCl)	0.49	5.3	38.0	202.0	80.8	2.1	0.57	6.4	20.0	128.0	90.0	117
SBTI–Sepharose (2)	Starting preparation	0.093	4.6	18.0	82.8	–	1	1.04	420.0	0.17	71.4	–	1
	Filtrate + eluate I	0.036	2.4	4.2	11.0	13.0	–	0.91	410.0	0.015	6.2	8.6	–
	Eluate II (0.01 *N* HCl + 1 *M* NaCl)	0.137	1.6	38.0	61.0	74.8	2.1	0.26	3.2	20.0	64.0	89.6	117

times that of SBTI–Sepharose (2) (Table II). The increase in the capacity of adsorbent 6 may be due to better accessibility of the reactive centre of the immobilized SBTI to the active centre of the enzyme.

The results of purification of the commercial pig pancreas trypsin and partially purified human serum kallikrein under the conditions described are presented in Table III. The yields, specific activities and purification factors were practically identical for the two adsorbents. The essential advantage of adsorbent 6 over the adsorbent without a spacer lies in the use of much smaller volumes of buffer for elution of the bound enzymes. The increase in the concentrations of trypsin or kallikrein eluted from the adsorbent having the amylopectin spacer (6) is of importance for future studies of these enzymes.

Adsorbent 6 may be used repeatedly (five or six times) without loss of binding capacity or inhibitory activity. The results presented here demonstrate that the introduction of a polysaccharide spacer into a solid matrix improves the properties of a biospecific adsorbent and enables highly efficient purification of biopolymers.

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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC RESOLUTION OF NON-ESTERIFIED ENANTIOMERIC AMINO ACIDS BY DERIVATIZATION WITH 2,3,4,6-TETRA-O-ACETYL-β-D-GLUCOPYRANOSYL ISOTHIOCYANATE AND 2,3,4-TRI-O-ACETYL-α-D-ARABINOPYRANOSYL ISOTHIOCYANATE

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SUMMARY

Novel methods for reversed-phase high-performance liquid chromatographic resolution of non-esterified amino acid enantiomers by the formation of diastereomers using two chiral reagents, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate and 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate, are described. These compounds react readily with enantiomeric free amino acids at room temperature and the reaction mixture can be injected directly into the chromatograph. The separation of the enantiomers was monitored spectrophotometrically at 250 nm. Complete resolutions were observed for all enantiomers examined on a reversed-phase column eluted with methanol–10 mM potassium phosphate (pH 2.8).

INTRODUCTION

Many attempts have been made to resolve optical isomers by liquid chromatographic techniques, using chiral derivatization reagents[1–3], chiral eluents or chiral stationary phases[4]. In particular, various pre-column chiral derivatization methods[5–8] have recently been developed for the resolution of enantiomeric amino acids by high-performance liquid chromatography (HPLC). However, these methods require concomitant protection of free amino or carboxyl residues not involved in the chiral derivatization reaction.

The present paper describes the chiral derivatization of amino acid enantiomers using either 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) or 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC). The derivatives thus prepared could effectively be resolved by reversed-phase chromatography without further protection of the free carboxyl residue. Simple and rapid separation of enantiomeric amino acids was accomplished.

MATERIALS AND METHODS

Amino acids and other reagents were obtained from Wako (Osaka, Japan) and Tokyo Chemical Industry Co. (Tokyo, Japan). All the reagents were of analytical reagent grade. Methanol and water were distilled before use. GITC and AITC were prepared by treatment of α-acetobromoglucose and β-acetobromoarabinose with silver thiocyanate as described previously[9]. The 10 m*M* phosphate buffer was prepared from monobasic potassium phosphate and was adjusted to pH 2.8 with perchloric acid.

Equipment

The chromatographic system consisted of a high pressure pump equipped with a universal valve injector (Sanuki Industry Co., Tokyo, Japan), a Develosil ODS-5 column (15 cm × 4.6 mm I.D., particle size 5 μm; Nomura Chemical, Seto, Japan) and an SPD-2A spectrophotometric detector (Shimadzu Seisakusho, Kyoto, Japan).

Derivatization and separation

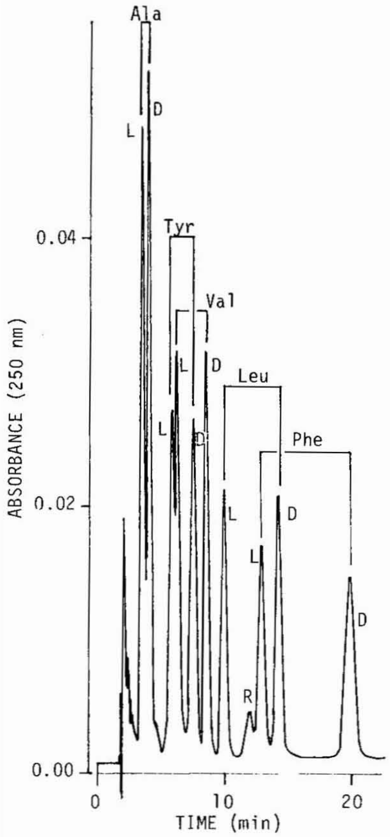
A 5-mg amount of each amino acid was dissolved in 50% (v/v) aqueous acetonitrile containing 0.4% (w/v) triethylamine to give a final volume of 10 ml. To a 50-μl aliquot of this stock solution were added 50 μl of 0.2% (w/v) chiral reagent, either GITC or AITC, in acetonitrile. The resulting mixture was allowed to stand at room temperature for 30 min and a 2-μl aliquot was injected directly into the chromatograph. The column was eluted at room temperature and at a flow-rate of 0.9 ml/min with a mobile phase prepared by mixing methanol and 10 m*M* phosphate buffer, pH 2.8, in an appropriate ratio.

For the resolution of phenylalanine diastereomers, the reagent peak was removed as follows. To the reaction mixture described above were added 10 μl of 0.25% (w/v) monoethanolamine in acetonitrile and the resulting mixture was allowed to stand for 10 min at room temperature prior to the injection.

RESULTS

GITC and AITC react readily with free amino acids under mild conditions without the formation of by-products. The resulting mixture can be injected directly into the chromatograph. The diastereomers resolved were detected spectrophotometrically at 250 nm; the molar extinction coefficients of the thiourea derivatives were around 12,000 $l \cdot mol^{-1} \cdot cm^{-1}$, whereas those of the reagents were around 1000 $l \cdot mol^{-1} \cdot cm^{-1}$.

Figs. 1 and 2 show the chromatograms of several sets of isomeric amino acids when GITC and AITC respectively are used for the derivatization. The reagent peaks were well separated from those of most of the amino acid derivatives and do not interfere with the detection. The limit of the detection for amino acids was 5 ng. However, a slight overlapping was observed between the peaks of the reagent(s) and phenylalanine diastereomers, 50 ng phenylalanine being required for unambiguous detection. This interference could be avoided by addition of monoethanolamine to the reaction mixture, since monoethanolamine reacts with the excess of reagent and the reaction products are eluted faster than any of the amino acid



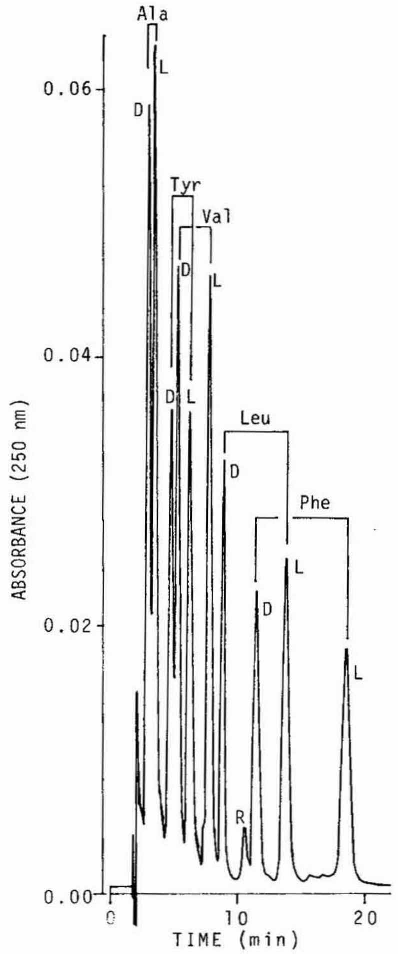


Fig. 1. Separation of diastereomeric thiourea derivatives formed from amino acids with GITC. Mobile phase: methanol–10 mM phosphate buffer (pH 2.8) (55:45); flow-rate 0.9 ml/min. About 250 ng of each derivative were injected. R = Reagent (GITC).

Fig. 2. Separation of diastereomeric thiourea derivatives formed from amino acids with AITC. Mobile phase: methanol–10 mM phosphate buffer (pH 2.8) (50:50); flow-rate 0.9 ml/min. About 250 ng of each derivative were injected. R = Reagent (AITC).

derivatives. This procedure did not affect the detection of other amino acids and 5 ng phenylalanine could be detected.

The retentions and resolutions of the diastereomeric GITC and AITC derivatives are listed in Table I; k', α and R_s refer to the capacity ratio, separation factor and resolution respectively for a pair of diastereomers. The resolution of diastereomeric GITC and AITC derivatives of non-esterified amino acids on the reversed-phase column, Develosil ODS, with methanol–10 mM phosphate buffer (pH 2.8) as mobile phase, was excellent.

TABLE I

SEPARATION OF DIASTEREOMERIC THIOUREA DERIVATIVES FORMED FROM FREE AMINO ACIDS WITH GITC AND AITC

t_0 = 2.0 min. Column, Develosil ODS-5 (15 cm × 4.6 mm I.D.). Mobile phase: methanol–10 m*M* phosphate buffer (pH 2.8) (40:60) (A), (45:55) (B), (50:50) (C), (55:45) (D); flow-rate 0.9 ml/min. k', α and R_s are defined in the text.

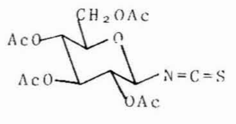
Amino acid	*GITC*					*AITC*				
	k'		α	R_s	*Mobile phase*	k'		α	R_s	*Mobile phase*
	L	*D*				*D*	*L*			
Glutamic acid	2.70	3.15	1.17	1.80	B	1.20	1.40	1.17	1.14	A
Aspartic acid	3.80	4.35	1.14	1.69	B	1.40	1.65	1.18	1.43	A
Proline	2.80	3.70	1.32	3.00	B	1.55	2.15	1.39	3.00	A
Alanine	3.85	5.50	1.43	4.40	B	1.60	2.45	1.53	3.78	A
Tyrosine	1.75	2.55	1.46	3.20	D	1.65	2.45	1.48	3.56	C
Valine	2.00	3.10	1.55	4.00	D	1.95	3.45	1.77	4.96	C
Phenylglycine	2.10	2.80	1.33	2.80	D	2.15	3.25	1.51	4.40	C
Isoleucine	3.55	5.50	1.55	5.01	D	4.05	7.25	1.79	6.94	C
Leucine	3.75	5.65	1.51	5.43	D	4.25	7.35	1.73	7.75	C
Tryptophan	4.95	7.25	1.46	5.11	D	6.55	9.85	1.50	6.00	C
Phenylalanine	5.00	8.10	1.62	6.89	D	6.25	11.10	1.78	8.43	C

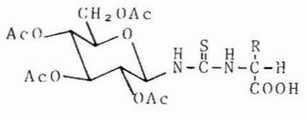
DISCUSSION

Chiral reagents for pre-column derivatization have been developed for the liquid chromatographic resolution of enantiomers[1–8]. Among these reagents, the terpene isothiocyanates which have recently been synthesized by Nambara *et al.*[6] were suitable for HPLC separation of enantiomeric amino acids on normal phases but not on reversed phases. Scott *et al.*[1] and Nambara and co-workers[6,7] have pointed out that the degree of separation of diastereomers on a normal-phase column should be dependent on the rigidity of the conformation. Introduction of a bulky group into the amino acids increases the conformational rigidity. Nambara *et al.*[6] reported that *tert.*-butyldimethylsilyl groups improve the resolution through their stereochemical effect.

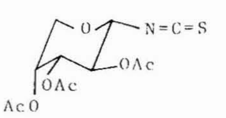
We have recently reported[9] the reversed-phase chromatographic resolution of enantiomeric amino acid ethyl esters using GITC for the pre-column derivatization. The GITC derivatives were well resolved compared with terpene thiourea derivatives of *tert.*-butyldimethylsilyl esters. This fact was assumed to be due to the bulkiness of the tetraacetylglucosyl moiety[9].

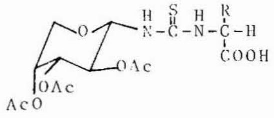
The present study has demonstrated that esterification of the carboxyl group of GITC or AITC is not necessary for the reversed-phase resolution. The excellent resolution obtained may be due to the lipophilic nature of the acetylglycosyl residues as well as their conformational rigidity. This greatly simplifies the pre-column derivatization. An examination using the Corey–Pauling Koltun molecular model (Ealing Corp., MA, U.S.A.) suggests that the conformations of the GITC- and


GITC


GITC-Amino acid

Ac = $-COCH_3$


AITC


AITC-Amino acid

AITC-amino acids are rigidly fixed owing to the bulky acetylglycosyl residues. Interestingly, ennantiomeric pairs were generally eluted in the order L before D when GITC was used, but in the opposite order when AITC was used. The model revealed that both amino acid derivatives have hydrophobic surfaces which may interact with the hydrophobic ODS residues and thus account for the resolution observed. The conformational difference between the two acetylglycosyl residues, especially at the anomeric carbon atom which affects the surface structure, may be responsible for the difference in the order of elution. This also suggests that pre-column labelling using sugar derivatives may result in specific separation in liquid chromatography.

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CHROM. 13,654

GAS–LIQUID CHROMATOGRAPHY OF SELECTED BENZIMIDAZOLE FUNGICIDES BY FLASH-HEATER METHYLATION WITH TRIMETHYLANILINIUM HYDROXIDE

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SUMMARY

A new method of methylation of benomyl, thiabendazole and fuberidazole (systemic benzimidazole fungicides) was established, based upon the flash-heater reaction with trimethylanilinium hydroxide. The chromatographic response was observed to be linear with the concentration of synthetic standards and the flash-heater products of the examined fungicides. The methyl derivatives were identified spectroscopically.

INTRODUCTION

Benzimidazole pesticides have high systemic fungicidal activity and a wide application. They are commonly used in plant protection. A number of chromatographic methods have been employed in the determination of the active substances in these formulations and of the residues from the plant material. Quantitative thin-layer chromatography (TLC) was used to evaluate the residue of methyl 2-benzimidazole carbamate (carbendazim) and methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate (bemomyl), applying a biological method of detection, *i.e.*, bioautography[1–5]. For the analysis of residues of benomyl[6–9] and 2-(4′-thiazolyl)benzimidazole (thiabendazole)[9] one can also employ high-performance liquid chromatography (HPLC). However, there are inherent difficulties in the analysis of these compounds, especially when using gas–liquid chromatography (GLC). Nevertheless, these fungicides have been analysed by GLC after transforming them into methyl[10] or pentafluorobenzoyl[11,12] derivatives. The methods of identification and determination employed have been described both for the compounds and for their residues.

Derivatization by flash-heater reaction with trimethylanilinium hydroxide (TMAH) has recently been developed for the analysis of carbendazim[13]. This paper describes the application of this methylation technique to the GLC analysis of selected benzimidazole fungicides.

EXPERIMENTAL

Materials

Pesticide standards were obtained from commercial sources and purified by recrystallization from *n*-hexane–dimethylformamide: 2-(2′-furyl)benzimidazole (fuberidazole) (Bayer, Leverkusen, G.F.R.), thiabendazole (Celamerck, Ingelheim, G.F.R.) and benomyl (DuPont, Wilmington, DE, U.S.A.). Trimethylanilinium hydroxide (Methelute®) was purchased as a 0.02 *M* methanolic solution from Pierce (Rockford, IL, U.S.A.).

Preparation of methyl derivatives of benzimidazole fungicides. Methyl fuberidazole [1-methyl-2-(2′-furyl)benzimidazole] and methyl thiabendazole [1-methyl-2-(4′-thiazolyl)benzimidazole] were obtained in a conventional way from fuberidazole and thiabendazole, using a 0.1 *M* methanolic solution of sodium hydroxide and methyl iodide. Dimethyl carbendazim (2-methoxycarbonyl-N-methylamino-1-methylbenzimidazole) was synthesized from carbendazim by a previously described sodium hydride–methyl iodide procedure[14].

Methyl derivatives of benzimidazole fungicides were purified by preparative TLC on glass plates (E. Merck, Darmstadt, G.F.R.) covered with silica gel 60 F_{254}. The mobile phase was *n*-hexane–benzene–acetone (1:1:1).

The melting points of the methyl derivatives were: methyl thiabendazole, 152°C; methyl fuberidazole, 74°C; dimethyl carbendazim, 65°C.

Apparatus

For identification of the methyl derivatives, several types of equipment were used. For the m.p. measurements, an electrothermal capillary apparatus was employed. The UV and IR spectra were run on Pye Unicam SP-1800 and SP-200 spectrophotometers, respectively. The mass spectra were recorded with a LKB-9000 s mass spectrometer, and the nuclear magnetic resonance (NMR) spectra were measured at 60 Hz with a Jeol spectrometer (tetramethylsilane as internal standard).

Gas–liquid chromatography

A Perkin-Elmer 900 chromatograph with a flame ionization detector was used, equipped with a glass column (6 ft. × 3 mm I.D.). The stationary phase was 3% OV-25 on Gas-Chrom Q (100–120 mesh) (Applied Science Labs., State College, PA, U.S.A.). Gas chromatographic analysis of benzimidazole fungicides after flash-heater methylation was performed at a detector temperature of 280°C and an injection chamber temperature of 260°C; hydrogen flow-rate, 40 ml/min; air flow-rate, 360 ml/min; nitrogen flow-rate, 40 ml/min. The isothermal analysis was performed at 240°C.

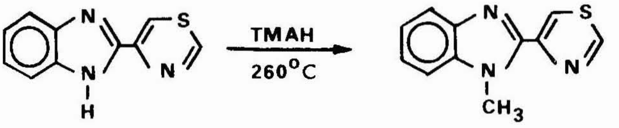
Optimization of methylation reaction

Samples containing 0.02 mmole of thiabendazole and fuberidazole in 10 ml of methanol, and 0.02 mmole of benomyl in 10 ml of dimethylformamide, were treated with TMAH, at molar ratios (THAH: fungicide) ranging from 1:1 to 8:1. For each determination, 2 μl (0.04 μmole) of the fungicide–TMAH solution were injected into the gas chromatograph. The quantities of TMAH required for the maximum product yields were estimated by the peak area method, employing a standard curve prepared from authentic samples of the methyl derivative of a pesticide.

Standard methanol solutions of the fungicide methyl derivatives ranging in concentration from 10 ng/μl to 100 ng/μl were prepared to determine the linear behaviour of the response. The linearity of the response with respect to the flash-heater methylation was then estimated by use of methanol or dimethylformamide solutions containing 5.0, 10.0, 20.0, 40.0 and 80.0 ng/μl of a pesticide with an approximate molar ratio TMAH:fungicide of 4:1.

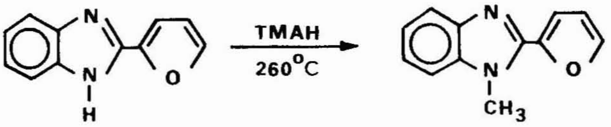
RESULTS AND DISCUSSION

Methylation of the examined fungicides by TMAH at the injection chamber temperature of 260°C takes place at the position 1 of the imidazole ring. In the methylation of thiabendazole and fuberidazole, a molar ratio of TMAH to fungicide of 2:1 gives the maximum yield of the methyl derivatives, 98.2% and *ca.* 99%, respectively.



Thiabendazole

Methyl thiabendazole

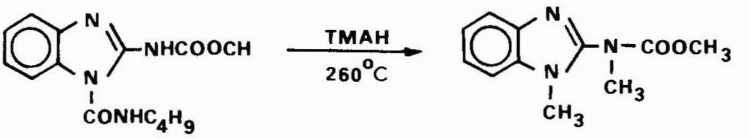


Fuberidazole

Methyl fuberidazole

Methylation of benomyl occurs both at the position 1 of the imidazole ring and at the carbamate chain. It is accompanied by transformation of benomyl to carbendazim and by activation of the nitrogen atom at position 1 of the imidazole ring. Eventually the dimethyl carbendazim derivative is obtained. The fungicidal activity of benomyl depends on its hydrolysis to carbendazim, *i.e.*, the biologically active metabolite[15]. Activation of the nitrogen atom in the imidazole ring of benomyl during the flash-heater reaction with TMAH gives a 98% yield of the dimethyl derivative at a molar ratio of TMAH to fungicide of 4:1. In the case of flash-heater methylation of carbendazim the minimum molar ratio is 8:1[13].

Fig. 1 shows the detector response curves (calibration graphs) for the benzimidazole fungicide methyl derivatives prepared from standards. The flash-heater



Benomyl

Dimethyl carbendazim

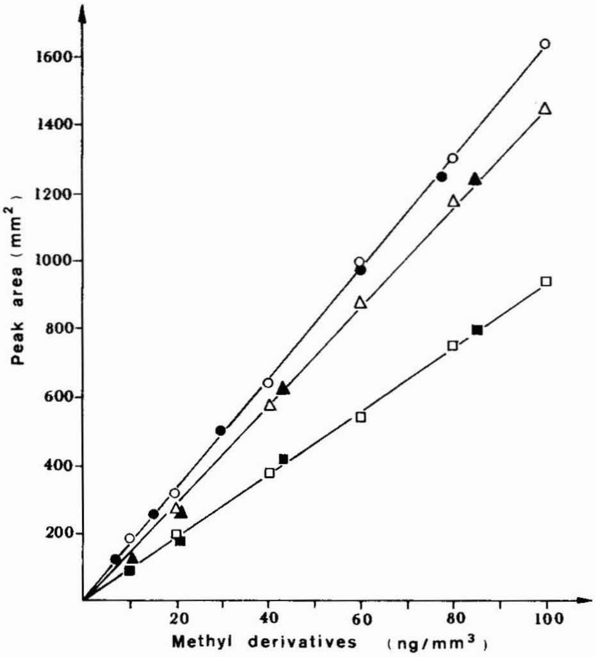


Fig. 1. Calibration graphs of the methyl derivatives of benzimidazole fungicides: ○, dimethyl carbendazim; ●, TMAH + benomyl (4:1); □, methyl fuberidazole; ■, TMAH + fuberidazole (2:1); △, methyl thiabendazole; ▲, TMAH + thiabendazole (2:1).

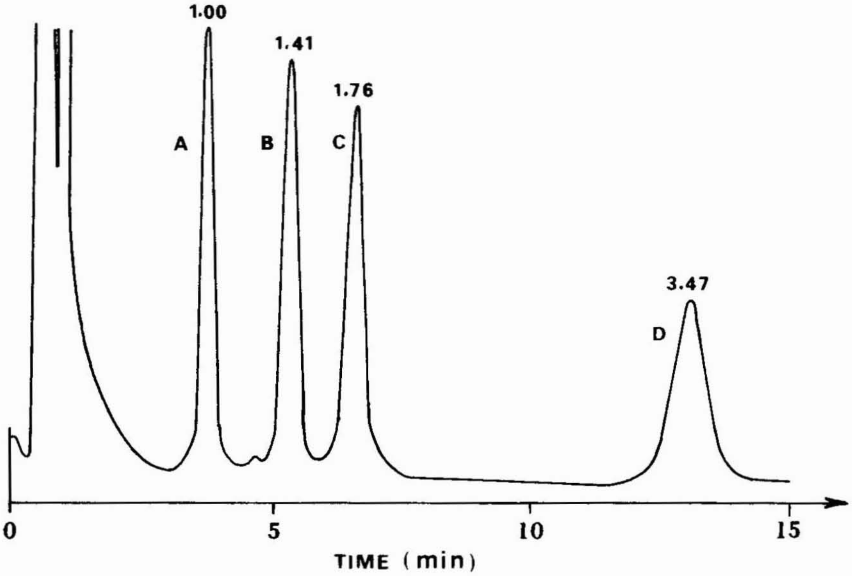


Fig. 2. Separation of a mixture of the flash-heater products of benzimidazole fungicides using a 3% OV-25 column. Peaks: A = Internal standard (9-fluorenone); B = dimethyl carbendazim; C = methyl fuberidazole; D = methyl thiabendazole.

method was then tested to determine whether a linear response could also be obtained with increased concentrations of the methyl derivative. The chromatographic response of the compounds was observed to be linear with concentration of the methyl benzimidazole derivatives. The curves also passed through zero, indicating that no measurable degradation took place on the column.

The chromatographic separation and the retention times, relative to the internal standard 9-fluorenone, for the flash-heater methylated products of benzimidazole fungicides are given in Fig. 2.

Identification of the methyl derivatives

The obtained standards, *i.e.*, the dimethyl derivative of carbendazim and the methyl derivatives of thiabendazole and fuberidazole, were identified by ultraviolet (UV) and infrared (IR) spectroscopy, and also by means of nuclear magnetic resonance (NMR) spectroscopy and mass spectral (MS) analysis.

TABLE I

CHARACTERISTICS OF THE UV AND IR SPECTRA OF THE METHYL DERIVATIVES

Derivative	*Spectroscopic characteristics*			
	UV (nm)		*IR (cm^{-1})*	
Dimethyl carbendazim	$\lambda_{max.}$	250	ν_{N-CH_3}	2960
		254	$\nu_{C=O}$	1715
		276		
		284		
Methyl thiabendazole	$\lambda_{max.}$	236	ν_{N-CH_3}	2940
		244		
		272		
		298		
Methyl fuberidazole	$\lambda_{max.}$	246	ν_{N-CH_3}	2920
		250		
		308		
		322		

As shown in Table I, a methanol solution (10 ppm) of the dimethyl derivative of carbendazim has a characteristic UV spectrum. The UV spectrum of benomyl is dentical with that of carbendazim, having absorption maxima at 244, 280 and 286 nm. Introduction of a methyl group into the imidazole ring and also into the carbamate chain results in both red and blue shifts. The wavelengths of maximum absorption for the methyl derivatives of thiabendazole and fuberidazole are also given in Table I, together with the IR spectra (KBr technique). None of the analyzed methyl derivatives exhibited the N–H imine group absorption from the imidazole ring at 3500–3300 cm^{-1}). The intense absorption band at 1715 cm^{-1}, characteristic of C=O vibration of the dimethyl carbendazim derivative, was observed. This demonstrates the preservation of the carbamate chain in this derivative.

In the NMR spectrum of the dimethyl carbendazim derivative dissolved in deuterochloroform (Fig. 3), signals appear at $\delta = 3.45$, 3.75 and 3.84 ppm (singlets due to the N–CH_3 group of the carbamate chain, to the ester group and to the methyl

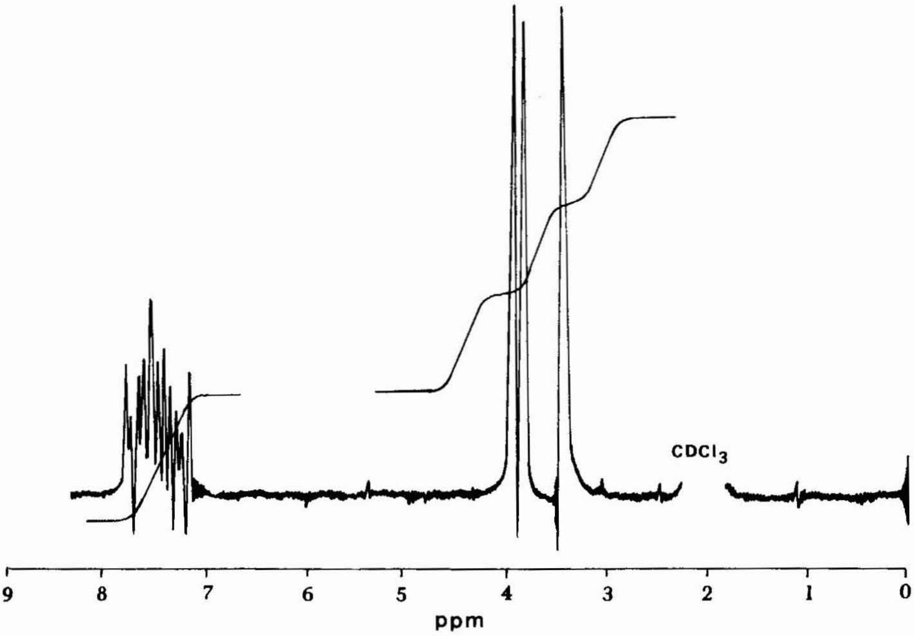


Fig. 3. NMR spectrum of the dimethyl carbendazim derivative at 60 Hz.

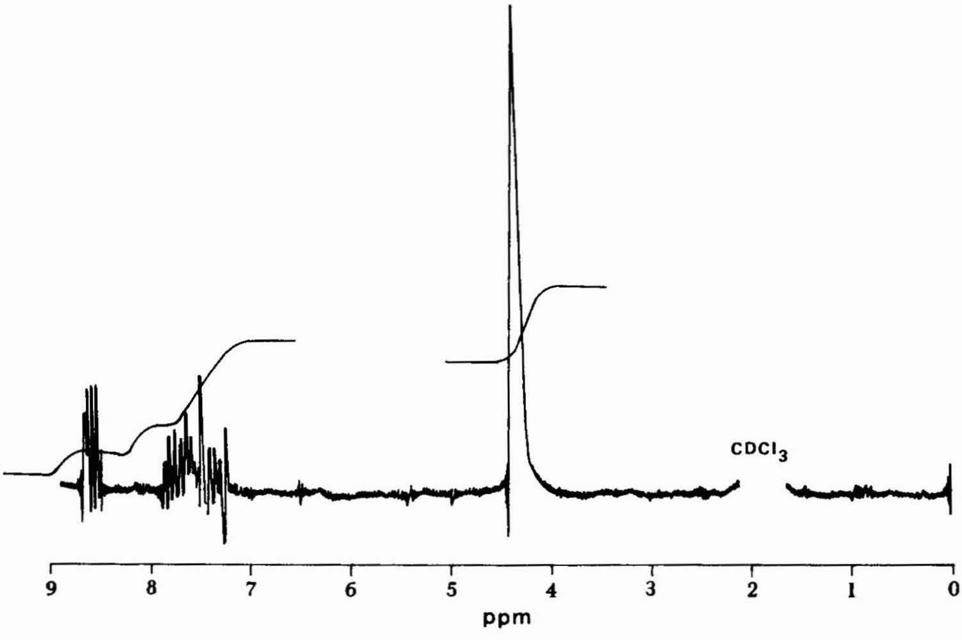


Fig. 4. NMR spectrum of the methyl thiabendazole derivative at 60 Hz.

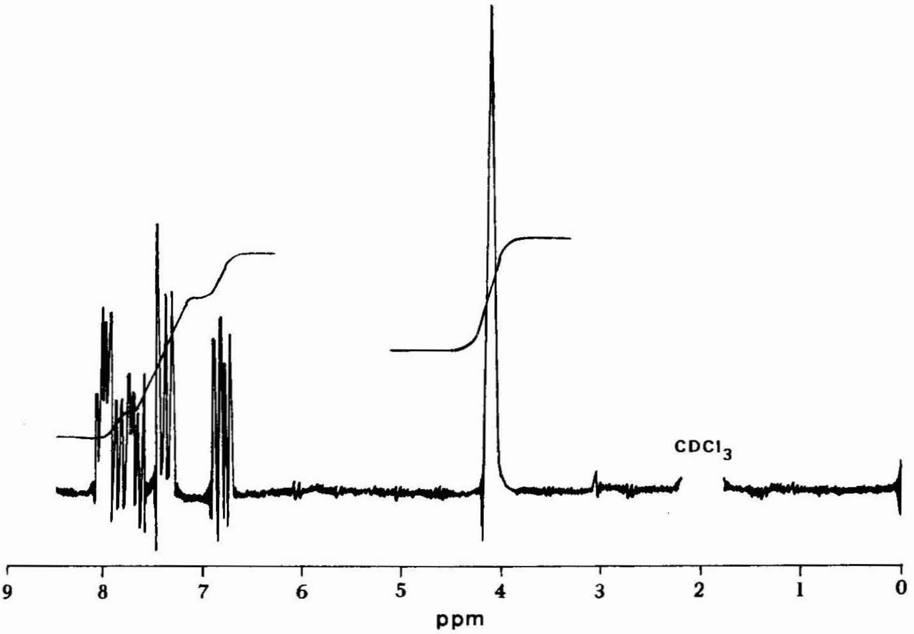


Fig. 5. NMR spectrum of the methyl fuberidazole derivative at 60 Hz.

group in the imidazole ring, respectively). As shown in Figs. 4 and 5, the NMR spectra of the methyl derivatives of thiabendazole and fuberidazole, dissolved in the same solvent, show singlets at δ = 4.32 ppm (3H) and δ = 4.15 ppm (3H), respectively, most probably due to the methyl group of the imidazole ring.

The mass spectrum of benomyl (Fig. 6) lacks the molecular peak at *m/e* 290 (M^+). However, a molecular ion peak is observed for (*m/e* 191). The fragmentation pattern of benomyl is as follows: *m/e* 191 (M^+), 159 (M^+ − CH_3OH), 131 (− CO),

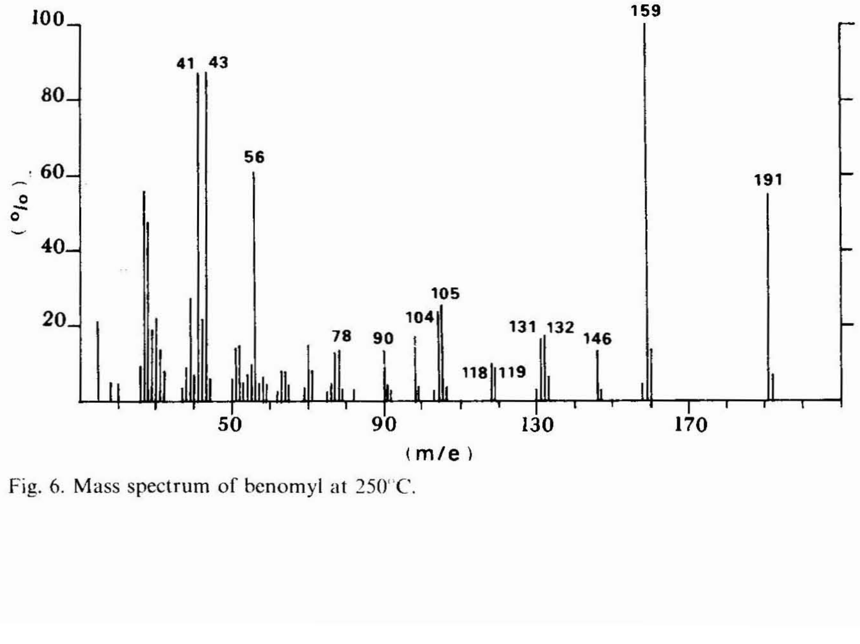


Fig. 6. Mass spectrum of benomyl at 250°C.

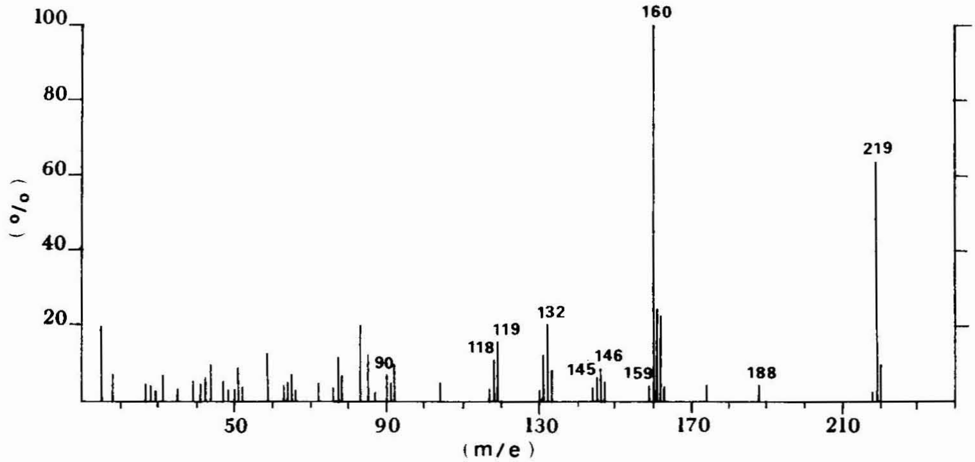


Fig. 7. Mass spectrum of the dimethyl carbendazim derivative at 250°C.

104 (− HCN), 78 (− CN); *m/e* 191 (M^+), 132 (− CH_3COO), 105 (− HCN); *m/e* 191 (M^+), 146 (− H − CO_2), 119 (− HCN), 118 (− H) and 90 (− H − HCN). The peak at *m/e* 56, 43, 41 are connected with fragmentation of butyl isocyanate, which is a product of the transformation of benomyl to carbendazim.

The mass spectrum of the dimethyl carbendazim derivative (Fig. 7) exhibited ion peaks at *m/e* 219 (M^+), 188 (− CH_3O), 160 (− CO), 145 (− CH_3) and *m/e* 219 (M^+), 160 (− CH_3COO), 159 (− H), 132 (− HCN), 118 (− CH_2) and 90 (− H − HCN). Three fragmentation paths are observed in the benomyl mass spectrum (Fig. 6) and two for its dimethyl derivative (Fig. 7).

The fragmentation patterns of the methyl derivatives of thiabendazole and fuberidazole are shown in Figs. 8 and 9. The mass spectrum of methyl thiabendazole exhibited ion peaks at *m/e* 215 (M^+), 188 (− HCN), 155 (− SH), 143 (− C), 131 (−

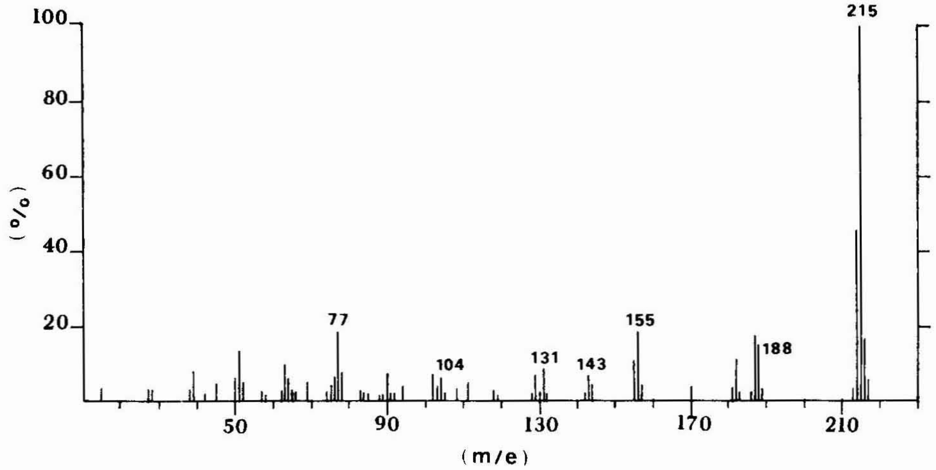
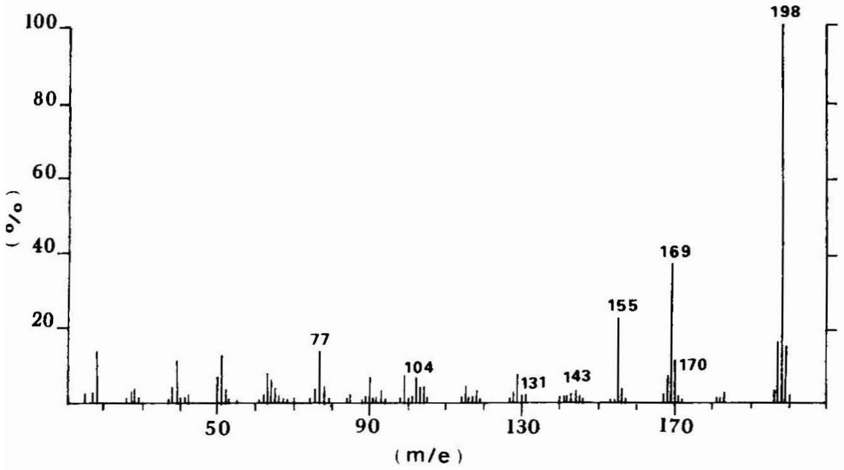


Fig. 8. Mass spectrum of the methyl thiabendazole derivative at 250°C.



ig. 9. Mass spectrum of the methyl fuberidazole derivative at 250 °C.

), 104 (− H − C_2H_2) and 77 (− HCN). The mass spectrum of methyl fuberidazole xhibited ion peaks at *m/e* 198 (M^+), 170 (− CO), 169 (− H), 155 (− CH_2), 143 (−), 131 (− C), 104 (− H − C_2H_2) and 77 (− HCN).

The parent peaks for dimethyl carbendazim (*m/e* 219) methyl thiabendazole *m/e* 215) and methyl fuberidazole (*m/e* 198) correspond with the molecular weight of ach compound. Shift of the peak at *m/e* 219 to 132 for dimethyl carbendazim (Fig. 7) an be attributed to initial degradation of the carbamate chain, and the subsequent hift from *m/e* 132 to 90 for all the methyl benzimidazole derivatives can be ascribed o N-demethylation and to degradation of the imidazole ring. Shift of the peak at *m/e* 215 to 132 for methyl thiabendazole and that at *m/e* 198 to 132 for methyl fuberidazole can be attributed to initial degradation of the thiazolyl and furyl rings.

The results concerning the identification of dimethyl carbendazim and methyl hiabendazole supplement those from previous work[10,13].

CONCLUSION

The possibility of application of the flash-heater methylation with trimethylanilinium hydroxide to the selected benzimidazole fungicides and their qualitative identification and GLC determination has been established. The selectivity of this reaction is preserved when the molar ratio of TMAH to fungicide is 2:1 (fuberidazole and thiabendazole) or 4:1 (benomyl), and the injection chamber temperature is 260 °C, which gives almost quantitative yields of the methyl derivatives. The linear behaviour of the detector (FID) response for nanogram amounts of benzimidazole fungicides with TMAH in the flash-heater reaction allows one to apply this method to the GLC determination of the residues of these fungicides.

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CHROM. 13,703

IDENTIFICATION OF RESIDUAL ORGANIC COMPOUNDS IN FOOD PACKAGES

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SUMMARY

Residual organic compounds in laminated plastic material used in food packages have been identified through gas chromatographic and gas chromatographic–mass spectrometric analyses of solid samples using a simple apparatus and direct headspace sampling of vapors from heated samples. Preliminary studies show no decomposition of components at evolution temperatures between 30 and 125°C. Although recovery of residual components was much less than 100% at all temperatures, reproducibility based on peak areas was 19–53% relative standard deviation with 10–30 μg/g with no sample pretreatment. Various volatile organic compounds including methanol, 1-ethoxy-2-propanol, 1-propanol, 2-(2-hydroxypropoxy)-1-propanol, *n*-propyl acetate, 2-methyl-2-propanol, *tert.*-butanol and a C_9H_{20} hydrocarbon were identified in samples of laminated plastic material.

Using a Tenax trap and the above technique these components were also detected in the vapor phase inside sealed bags made from the same material. Application of this technique to analysis of vapors in commercial potato chip packages revealed the presence of benzene as a major constituent.

INTRODUCTION

Organic compounds which promote stability and appeal of food products are often added to foods during various stages of processing. Occasionally other organic compounds may be present inadvertantly as residues from the manufacturing process. Such compounds include methylene chloride and trichloroethylene in coffee[1] and fluorocarbons in frozen seafood[2]. Similar volatile organic compounds may also be present originally in the packaging material, but these contaminants may later migrate into the packaged food. Examples of this type of contamination include vinyl chloride in corn oil[3], styrene in meats[4] and plasticizers in yogurt[5].

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Although the determination of these and other volatile contaminants in actual food samples is difficult, analyses of packaging materials and the vapor phase inside sealed packages may be useful in the quality control of such contaminants in plastics and also for preliminary screening of offensive odors and tastes in foods. Since packaging materials often contain a mixture of volatile organic compounds, most methods for the determination of residual contaminants have been based on gas chromatographic techniques. Most methods of analysis involve headspace sampling, although various techniques differ in sample preparation. Typically, a portion of the sample is dissolved in solvents such as dimethyl formamide, isopropanol or diethyl ether, and the solution is sealed in a glass container which is equipped with a septum. The container is placed in a heated bath at 70–80°C for 1–2 h and a portion of the headspace is then removed and analyzed using gas chromatography (GC). Examples of this method include methylene chloride in polycarbonates[6] and plasticizer in 2,4-diacetate cellulose[7].

Wilks and Gilbert[8] described a method for the determination of residual volatile solvents in packaging materials using GC analysis of headspace samples of solid materials. However, only samples which were spiked with known amounts of certain compounds were analyzed. Headspace analysis using solid samples has also been applied to synthetic rubbers[9], vinyl chloride in polyvinyl chloride[10] and residual hydrocarbon solvent in polyacrylic acid[11].

Laminated packages which consist of two sheets of plastic held together with an adhesive or glue have been widely used in the snack food industry. These materials have excellent properties for retention of odor and flavor, protection from moisture and adaptation to automated packaging. However, if the laminated material is not properly dried, residual solvents may be present in the final food packages. This paper describes results from a simple and rapid headspace method which has been developed for the determination of trace concentration levels of volatile organic compounds in laminated and other packaging materials and in vapor samples from inside sealed packages.

EXPERIMENTAL

Instrumentation

A Hewlett-Packard Model 5830A gas chromatograph was equipped with a 2 m × 2 mm I.D. glass column containing 2% OV-7 on Aue packing[12], a flame ionization detector (FID), a heated four-port, gas-switching valve (Valco, Houston, TX, U.S.A.) and a flash desorption oven. Sample tubes were attached to the four-port valve using 0.635–0.159-cm Swagelok reducing unions (Niagara Valve and Fitting, Hamilton, Canada) and 0.635-cm I.D. Vespel ferrules (Chromatographic Specialties, Brockville, Canada). The aluminium flash desorption oven was heated using two 500-W heating cartridges (Watlow, St. Louis, MO, U.S.A.). The final design is shown in Fig. 1. Conditions of analysis were: initial temperature, 0°C; temperature program rate, 10°C/min; final temperature, 150°C; injection port temperature, 250°C; detector temperature, 275°C; slope sensitivity 0.1 mV/sec; area reject, 1000; attenuation, 64 or 128; carrier gas, helium; flow-rate of carrier gas, 30 ml/min; temperature of gas valve, 200°C and temperature of transfer lines, 180°C. Conditions for headspace sampling are specified when necessary.

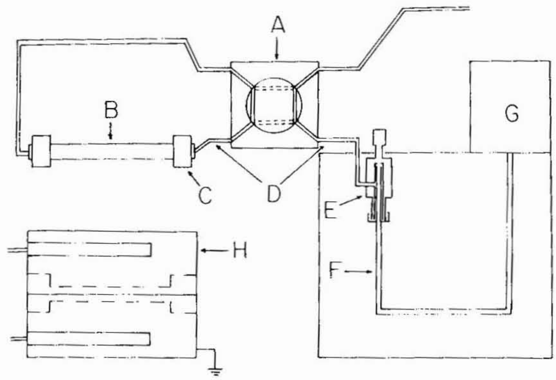

Fig. 1. Final design of desorption apparatus for solid headspace analysis. Details of the apparatus include; A = heated gas-switching valve; B = steel sample-containing tube; C = Swagelok fittings; D = 1.58-mm O.D. stainless-steel tubing wrapped in heating tape; E = GC inlet; F = GC column; G = FID or MS; H = thermal desorption oven.

A Model 5992A gas chromatograph–mass spectrometer was equipped with a single flexible disc, *x*–*y* plotter, membrane separator and thermal desorption system as described above. The chromatographic column was the same as described above. Conditions for analysis by GC–MS were: initial temperature, −30°C; temperature program rate, 10°C/min; final temperature, 150°C; lower mass range, 25 a.m.u.; upper mass range, 400 a.m.u.; scan rate, 330 a.m.u./sec; electron multiplier voltage, 1800 V; mass peak detection threshold, 2; and GC peak detection threshold, 300. The temperature of the gas-switching lines and transfer lines was approximately 60 ± 10°C and the temperature of the flash desorption oven was 80°C. The total time of heating samples was 3 min including a 2-min period for preheating. Software used in these GC–MS analyses was supplied by the manufacturer and modified by Dickson[13].

Preparation and analysis of samples

Packaging material. Plastic samples (approx. 7 cm^2) were cut using scissors and weighed to ±0.001 g. Various samples were taken from center sections of unused sheeting, sealed but unfilled bags and bags which contained food products. A square of sample was then rolled and inserted into an empty stainless-steel tube with dimensions 7.6 × 0.64 cm O.D. × 0.41 cm I.D. The sample-containing tube was attached to the switching valve while the valve was in the by-pass position (position 1). In GC analyses of the packaging materials, the tube was heated with the flash desorption oven for a time while the valve was still in position 1. After this preheating period the valve was switched to divert the carrier gas through the tube and into the inlet of the gas chromatograph (position 2). After an additional 1 min the valve was returned to position 1 and the oven was removed from the tube. When the valve was switched in this last step the temperature program was started.

In GC–MS analysis a similar procedure was followed except residual air in the transfer lines and the tube was minimized by a 15-sec flush of the desorption system while the tube containing the sample was at ambient temperature and the GC column was at 50–80°C. Following this step to remove air from the desorption system, the analysis was continued as described above.

Vapor samples. Vapor samples were prepared by drawing the headspace inside sealed packages through tubes containing Tenax-GC (Applied Science Labs., State College, PA, U.S.A.) sorbent material. Both empty and filled packages were used in these analyses. Each tube contained approximately 100 mg of Tenax-GC. A gentle vacuum was applied to one end of the trap using an aspirator while a needle attached at the other end of the trap was used to puncture the bag. A sharp No. 11 gauge needle fitted in a 0.635–0.318-cm Swagelok reducing union was used in this step, and the seal between the bag and needle was surprisingly good under these conditions. The traps were analyzed by GC and GC–MS using procedures described above.

Reproducibility and effects of time and temperature

The effect of temperature on the amounts of components released from samples of laminated plastic sheeting was examined using five desorption temperatures. These temperatures were: 30, 55, 77, 95 and 125°C. The total time of desorption was 3 min including a 2-min time for preheating. Peak areas were converted to concentrations using integration values, sample weights and an average response factor of 400 area counts/ng for the FID. In a similar study the effect of preheating time on amounts of components released was studied at a desorption temperature of 85°C using four values for preheating time. These values, exclusive of the 1-min period for sweeping organic compounds into the inlet of the gas chromatograph were: 1, 2, 5 and 10 min.

Triplicate analyses of samples of laminated material which were cut from similar sections of unused plastic sheeting were used to determine the reproducibility of the technique. The temperature of the desorption oven was 80°C while the time of desorption was 3 min.

RESULTS AND DISCUSSION

Analysis by GC

Results from studies on the effect of temperature on amounts of components which are released from laminated material are shown in Figs. 2 and 3. Gas chromatograms are presented as bar plots[14] from five analyses at different temperatures using thermal desorption techniques. A full-scale value of 30,000 area counts was chosen for use in each plot of Fig. 2 for convenient visual comparison. The plot in Fig. 2A shows that the vapor-phase above the solid laminated plastic contains at least 18 major and minor components. No detectable amounts of volatile organic components were found in chromatograms of blank analyses. In the blank analyses, identical conditions were used except the sample was omitted. The plots in Fig. 2A–E illustrate the magnitude of differences in absolute concentration of individual components among analyses. Actual concentrations of all organic compounds for the different temperatures were: 30°C, 1.9 μg/g; 55°C, 11.5 μg/g; 77°C, 21.9 μg/g; 95°C, 85.8 μg/g; and 125°C, 491.1 μg/g. Although these results show considerable quantitative differences, the distribution of components in each plot was similar and no major new components were detected at higher temperatures. Therefore, no thermal decomposition of the laminated materials was evident using the conditions of this study.

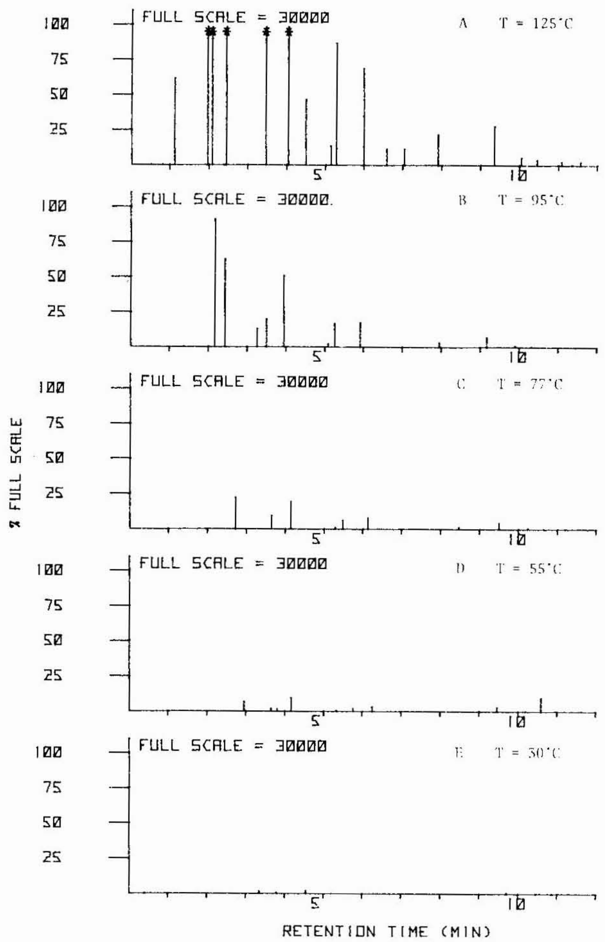

Fig. 2. Bar plots from GC analyses of laminated plastic material using five desorption temperatures.

The relative quantitative differences for organic compounds in each analysis are shown by plots in Fig. 3A–E. Although the data used in Fig. 3A–E are the same as in Fig. 2A–E, the full-scale values have been normalized to the area count for the most abundant component in each plot. With a thermal desorption temperature of 30°C, the major component eluted near the middle (4.6 min) of the chromatogram. A later eluting component (9.6 min) was approximately 70 % in abundance of the major component. The abundance ratios of various components differed at each desorption temperature. At a 125°C desorption temperature the major component was an early eluting (1.9 min) component. Furthermore, the compound which was the major component at 30°C was only 25 % of the new major component (3.5 min) at 125°C. Another large change in relative concentration was seen for a later eluting component which decreased from 68 % (30°C) to 5 % (125°C) of the most abundant compound. While other differences are discernable in these plots, three features characterize the results: the presence of volatile organic compounds in the vapor phase, even at 30°C;

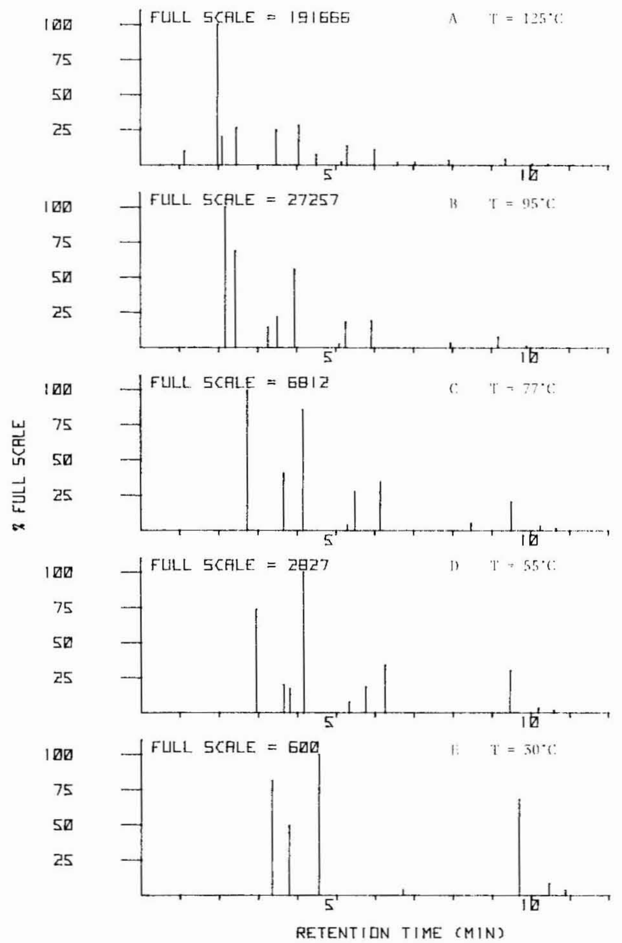

Fig. 3. Bar plots from Fig. 2 with full-scale values normalized to largest component in each plot.

large amounts of organic compounds in the laminated material; and the shift at higher desorption temperatures to lower-retention-time components.

The presence of organic compounds in the vapor phase at 30°C was not unexpected since odors had been associated with the packaging material. However, the concentration of these components showed that the composition of the sample was changing daily and that the drying process was incomplete. Clearly, when characterizing a particular batch of material, long storage times may alter the quantitative results.

Variations in retention times as large as 1.5 min for the early eluting components were observed. Since the components are condensed at the inlet of the column, events which occur before this thermal focusing step are not expected to influence retention times.

Although the causes for these differences have not been determined, large concentration changes which exceed the linear range of the distribution isotherms may result in reduced retention times. This behavior is particularly evident when low capacity ($\leqslant 2\%$ liquid phase loading) columns are employed.

When the total concentration of components in the headspace is plotted *versus* desorption temperature using the above data, the curve shows no evidence that the system was reaching an equilibrium value. This was supported by a second study where times for preheating as long as 10 min were used with desorption temperatures of 85°C. The total amounts of material which were released during these analyses were: 1 min, 22.0 μg/g; 2 min, 32.0 μg/g; 5 min, 97.0 μg/g; and 10 min, 210 μg/g. The results from these experiments also showed that total recovery of the volatile compounds from laminated material was incomplete using the above conditions. However, the reproducibility of quantification for a specific preheating time and desorption temperature based on peak areas had a range for a relative standard deviation of 15–53% with a median value of 22%. These data from replicate analyses are given in Table I and include the total measure of precision from sample handling, headspace sampling and GC analysis with thermal focusing. Although numerous aspects of quantification such as preparation of standards, storage of samples and calibration of response for particular analyses still require development, some progress has been made in quantitating similar methods. Kalb[15] has used a "discontinuous gas extraction" procedure and found that his procedure was useful in the determination of solvents in printed foil, but was too time consuming for routine analyses. Others[16] have measured amounts of volatile components in solids through the use of Henry's constant which was determined for experimental conditions. Another alternative reported by Kalb[15] is the use of reference materials with known compositions that were predetermined using dissolution methods.

TABLE I

VALUES FOR REPRODUCIBILITY OF GC HEADSPACE ANALYSIS OF LAMINATED MATERIAL

Retention time (min)		*Estimated concentration (ng/g)*	
Average	*Standard deviation*	*Average*	*Standard deviation*
2.43	–	15091	2891
3.33	0.04	4340	710
3.80	0.06	8538	1814
4.95	0.04	395	90
5.13	0.04	2892	599
5.82	0.02	3371	1237
7.87	0.08	695	366
9.24	0.05	2891	447
9.97	0.05	338	131
10.38	0.04	189	46

Analysis by GC–MS

Samples of both laminated plastic material from a food package and the vapor phase inside the same package were analyzed using GC–MS techniques. Results from these analyses are shown in Figs. 4 and 5. In Fig. 4, the total ion chromatogram (TIC) is shown from an analysis of a 0.138-g sample of packaging material. The number and

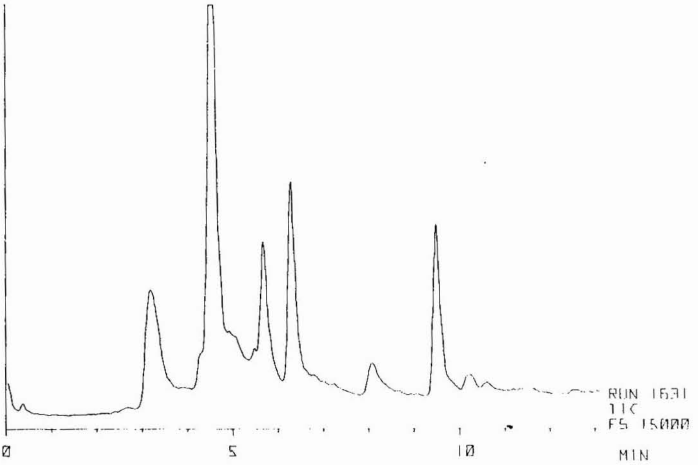

Fig. 4. Plot of TIC from GC–MS analysis of solid laminated material. Conditions of analysis given in text.

distribution of components in this TIC compare favorably with patterns which are shown in Figs. 2 and 3 from analysis of the same material using GC. Furthermore, the relative abundances of the various components were also very similar to those found by GC analyses. The TIC consists of six major components with another seven minor components or shoulders on other peaks. These components were identified using mass spectra which were then compared to reference spectra from the Environmental Protection Agency/National Institute of Health reference volumes[17]. The first four major components were esters or alcohols while the last major component was a C_9H_{20} branched hydrocarbon. The identity of these and other components are listed in Table II, although an exact structure for this C_9H_{20} was not determined. In Fig. 4 a very small amount of air was detected at an elution time of 0.4 min. This shows that switching the valve for 15 sec to force carrier gas through the sample tube and carrier

TABLE II

COMPONENTS IDENTIFIED USING GC–MS ANALYSIS OF LAMINATED PACKAGE SYSTEMS

From Figs. 4 and 5.

Retention time (min)	*Component*	*Package material*	*Vapor phase*
2.7	Methanol	×	×
3.2	1-Ethoxy-2-propanol + second unidentified component	×	×
3.8	Methyl ethyl ketone		×
4.3	1-Propanol	×	
4.5	2-(2-Hydroxypropoxy)-1-propanol	×	×
5.6	*n*-Propyl acetate	×	×
6.3	2-Methyl-2-propanol + trace of toluene	×	×
8.1	*tert.*-Butanol	×	×
9.5	C_9H_{20}	×	×
10.2	C_9H_{20}	×	×
10.6	C_9H_{20}	×	×

lines was sufficient to sweep most of the residual air from the desorption system and to prevent excessive amounts of air to enter the mass spectrometer. The initial temperature of -30°C resulted in a separation time of about 2.5 min between air and methanol.

Results from GC–MS analysis of the vapor phase inside two packages used in the above analysis are shown as a TIC in Fig. 5. The pattern of this TIC is nearly qualitatively identical with the TIC shown in Fig. 4. Most components in the gas phase were the same as in the laminated material and retention times were close or identical matches. Also the relative abundances of components in the gas phase were similar to those from GC headspace analysis of the plastic material. A single major difference between Figs. 4 and 5 is a sizable component which eluted at 3.8 min in Fig. 5. This component was identified as methyl ethyl ketone using mass spectra.

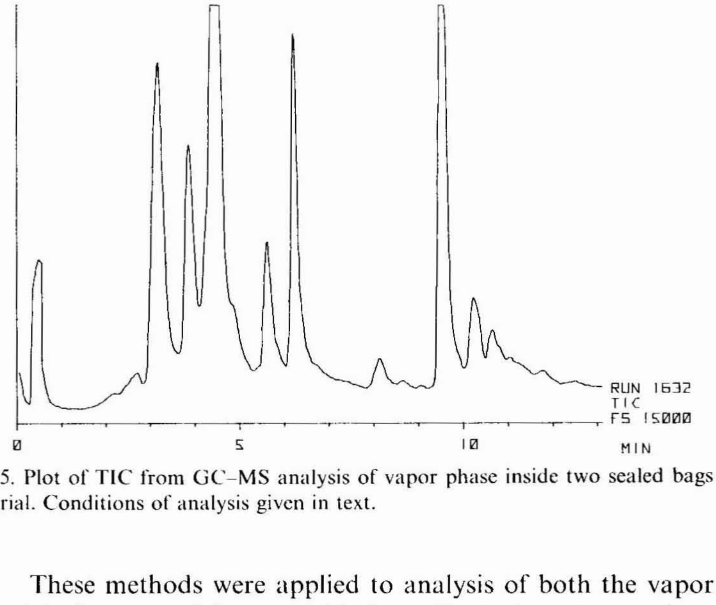

Fig. 5. Plot of TIC from GC–MS analysis of vapor phase inside two sealed bags made from laminated material. Conditions of analysis given in text.

These methods were applied to analysis of both the vapor space and plastic material of commercial potato chip bags. Typical results are shown in Figs. 6 and 7. The packaging material used in these analyses was a smooth white flexible plastic, perhaps polyethylene or polypropylene, which was used as a package for potato chips. Results from GC–MS analysis of the white plastic are shown in Fig. 6 as a TIC. Very few components were detected in this analysis and concentrations were estimated to be less than 10 μg/g. These components were mostly branched or unsaturated hydrocarbons. However, GC–MS analysis of the vapor phase inside these bags showed a very different composition. Only a single major component was detected in the vapors, and the component was clearly identified as benzene. Varying amounts of benzene were found in replicate analyses of similar packages. The amount of benzene present in the vapor phase of two bags was more than sufficient to overload the GC–MS. In Fig. 7 the distortion of the peak is a result of the mass spectrometer being automatically turned off when excessive amounts ($>$ *ca.* 10 μg) of components enter the mass spectrometer.

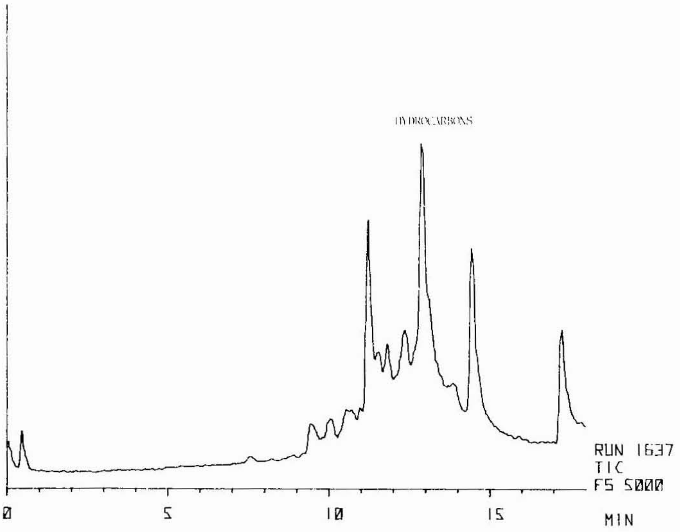

Fig. 6. Plot of TIC from GC–MS analysis of white homogeneous solid material used in potato chip bags.

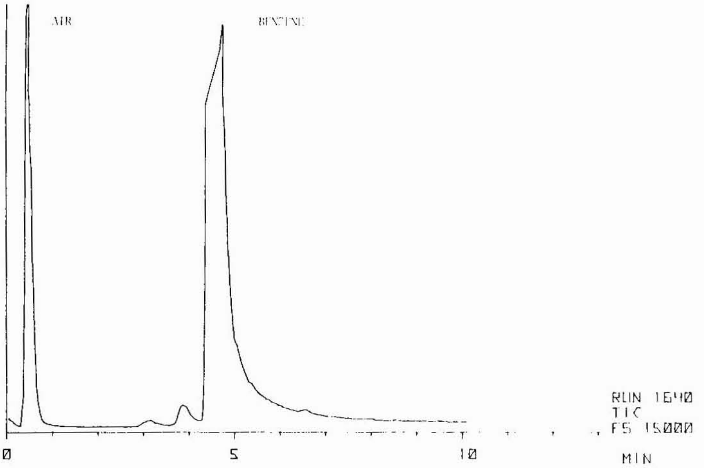

Fig. 7. Plot of TIC from GC–MS analysis of vapor phase inside two potato chip bags.

The origin of the benzene is unknown, since it could have been introduced at any point in the product preparation or in pressurization of the bag. A more complete study is underway to determine the extent and concentration of benzene and other components in this type of packaging system.

ACKNOWLEDGEMENT

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SEPARATION OF BOVINE CASEINS USING HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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SUMMARY

All of the casein components could be adsorbed onto Octyl-Sepharose CL-4B from a phosphate buffer solution and separated chromatographically using a gradient of 0–6 *M* urea. β-Casein, despite its higher average hydrophobicity, was eluted first. The large peptides α_{s1}-I and β-I (derived from α_{s1}- and β-casein by chymosin action) were readily separated from their precursor proteins using this technique, and the rare A variant of α_{s1}-casein was well resolved from the commoner B variant.

INTRODUCTION

The caseins of bovine milk constitute nearly 80% of the total milk proteins and one of their predominant characteristics is their strong association with one another. This association is partly caused by hydrophobic interactions between the proteins[1,2], and it might be expected that the caseins could be separated, one from another, by hydrophobic interaction chromatography[3,4]. Hydrophilic Sepharose gels substituted with hydrophobic groups, such as *n*-octyl groups, have been shown to interact with the surface hydrophobic regions of globular proteins, and these gels have been used to isolate and purify otherwise recalcitrant proteins[5] and for the surface characterization of the surface hydrophobicity of bacteria[6] and proteins[7]. Hydrophobic interaction chromatography was therefore explored as a possible technique for the separation of the bovine caseins from one another.

EXPERIMENTAL

Materials

Phenyl- and Octyl-Sepharose CL-4B were purchased from Pharmacia, Uppsala, Sweden. All other chemicals were reagent grade, and the water was purified with a Milli-Q ion-exchange and filter system (Millipore, Bedford, MA, U.S.A.).

Method

Column chromatography. The Octyl-Sepharose CL-4B beads were packed into a 1.6-cm diameter column to a depth of 25–30 cm in a 0.1 *M* solution of Na_2HPO_4

adjusted to pH 7.0. The sample (0.1–1.0 g) was applied in 5–10 ml of 0.1 *M* Na_2HPO_4 pH 7.0 buffer. The column was eluted with 200 ml of 0.1 *M* Na_2HPO_4 pH 7.0 buffer at a flow-rate of 160 ml/h maintained with a Pharmacia P-3 peristaltic pump, followed by a linear gradient of 0–6 *M* urea (300 ml each solution) in 0.1 *M* Na_2HPO_4, then 200 ml of 6 *M* urea in 0.1 *M* Na_2HPO_4 and finally 200–400 ml of 0.1 *M* Na_2HPO_4 buffer. The column effluent was fractionated into 20.0-ml fractions using an LKB Ultrorac fraction collector. The absorbance of each fraction was determined at 280 nm, and its casein composition determined by disc gel electrophoresis followed by densitometry using the procedure of Creamer and Berry[8]. Para-κ-casein was determined with the aid of an sodium dodecyl sulfate (SDS) gel system[9].

Chymosin degradation of whole casein. A sample of whole casein[8] was dissolved using 0.1 *M* NaOH to give a 5% solution at pH 6.3. This solution was held at 30°C and mixed with commercial rennet (N.Z. Co-operative Rennet Company, Eltham, New Zealand) to give a rennet concentration of 0.1%. At selected times between 2 min and 16 h, 20-ml samples of the mixture were heated in a boiling-water bath to denature the enzyme. Gel electrophoresis was used to determine the extent of chymosin hydrolysis of the caseins.

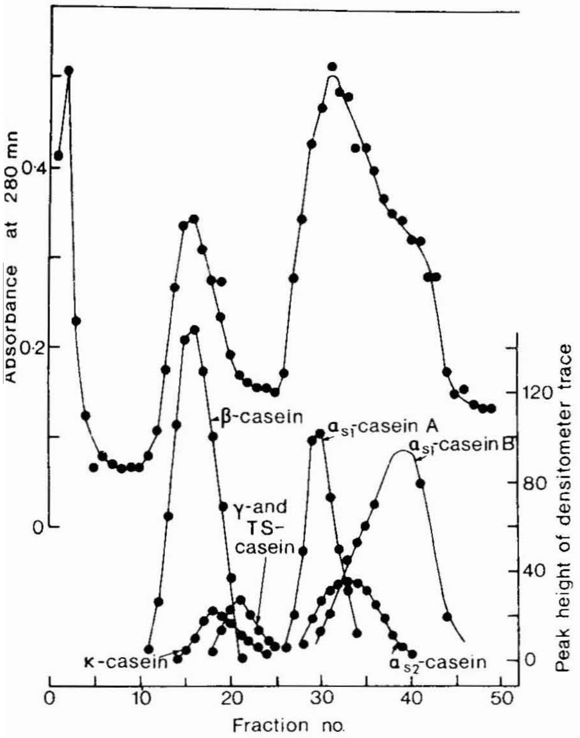
Hydrophobicity estimations. The average hydrophobicity per residue was determined using the procedure of Bigelow[10]. The distribution of hydrophobicity along the protein sequence was determined from the sequences of the bovine caseins[11] by a moving-average smoothing technique in which the hydrophobicity of each and every nonapeptide was calculated using the binomial coefficients (1:8:28:56:70:56:28:8:1) as the weighting factors for the nine residues of the nonapeptide. Five phantom residues of zero hydrophobicity were appended to each end of the sequence so that the hydrophobicity plots did not start or end abruptly. Although the hydrophobicity of each of the nine residues of the nonapeptide contributes to each of the calculated points, the major contribution is from the central three residues. These smoothed curves were calculated and plotted using a Hewlett-Packard Model 9830A calculator fitted with a Model 9862A plotter.

RESULTS

Preliminary results showed that whole casein, of which α_{s1}-, α_{s2}-, β- and κ-casein are the major components, adsorbed onto Octyl- or Phenyl-Sepharose CL-4B from dilute phosphate buffers and could be totally desorbed with 6 *M* urea or 40% (v/v) ethanol. A gradient of 0–40% (v/v) ethylene glycol only desorbed a portion of the protein while the remainder was desorbed with 6 *M* solution. In every case, β-casein was eluted more readily than α_{s1}-casein.

Optimum separation was obtained when an increasing gradient of urea concentration was used in 0.1 *M* phosphate buffer. The maximum quantity of casein that could be chromatographed with good resolution of its components was about 0.25 g (3 mg/ml of Octyl-Sepharose). Thus large columns would be required if hydrophobic interaction chromatography were to be used to prepare large quantities of casein components.

Fig. 1 shows a typical chromatogram of whole casein containing equal quantities of the A and B variants of α_{s1}-casein. The initial peak of 280 nm absorbance which eluted at the void volume did not contain any protein as determined by gel



ig. 1. Chromatography of whole casein on Octyl-Sepharose CL-4B. Upper curve: the absorbance at 280 m *versus* fraction number is shown; lower curve: the densitometer peak heights for each component on isc gels are shown.

lectrophoresis. The first major peak eluting near fraction 16 (320 ml of buffer, 120 ml nto the urea gradient) was found by gel electrophoresis to be predominantly β-casein. he second larger peak of 280 nm absorbance was found to be a mixture of α_s-caseins y gel electrophoresis, α_{s1}-caseins A and B being well resolved from one another. Vhen purified α_{s1}- and κ-caseins were chromatographed individually, they were luted as symmetrical peaks at the peak positions (centered on fractions 40 and 18, espectively) expected on the basis of chromatography of whole casein. Changing the emperature of the system from 20–22°C to 1 or 40°C did not alter the order of elution f the components from the column, although all components eluted at higher urea oncentrations at the higher temperature.

The pH of the system at 1°C determined both the order of elution and the verall tenacity of retention. In general the casein components were retained more trongly at the lower pH values (Fig. 2). The concentrations of $\mathrm{Na^+}$, $\mathrm{PO_4^{3-}}$, $\mathrm{HPO_4^{2-}}$, $\mathrm{H_2PO_4^-}$, etc., that were present in the buffer changed with pH, and the lution positions may have been influenced by the modified concentrations of these onic buffer components.

hromatography of chymosin-treated casein

The elution pattern from a sample of whole casein that had been partially

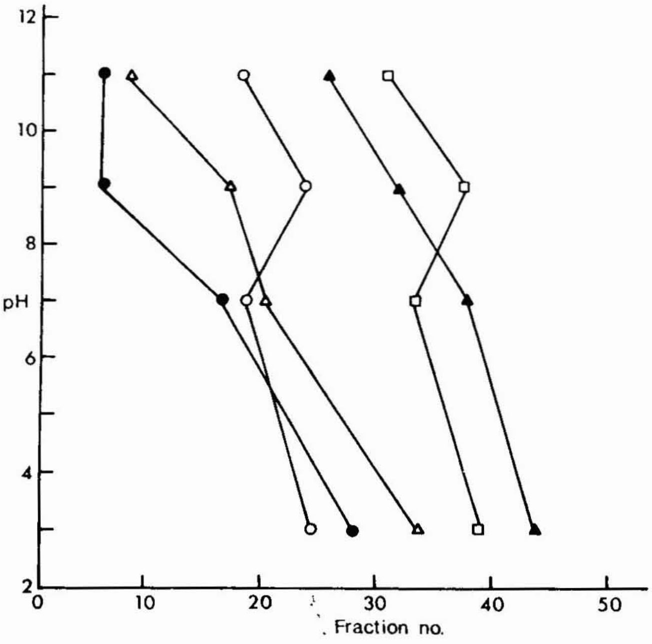


Fig. 2. Effect of pH on the chromatographic elution position of the casein components of whole casein. ●, β-Casein; △, γ-casein; ○, κ-casein; ▲, α_{s1}-casein; □, α_{s2}-casein.

degraded with chymosin is shown in Fig. 3. It is clear that the elution order is β-I, β-casein, para-κ-casein, α_{s1}-I, α_{s2}-casein and α_{s1}-casein.

An attempt to determine the elution position of para-κ-casein in isolation was not successful because it could not be dissolved and adsorbed to the column in the absence of α_{s1}- or β-casein.

Calculated hydrophobicities

Table I shows the calculated average hydrophobicities for a selection of casein components and derived peptides, while Fig. 4 shows the smoothed distribution of hydrophobicity along sequences of these proteins and peptides. It can be seen that the hydrophobicity of each casein or casein peptide is not evenly spread along its sequence and regions of higher-than-average hydrophobicity show as peaks in the curves. (The horizontal lines denote the average hydrophobicity of each protein or peptide.)

Varying the parameters in the smoothing calculation did not alter the shape of the curves greatly. However, smoothed curves are easier to comprehend than the jagged plots that are obtained when the hydrophobicity of each residue is plotted *versus* residue number.

Comparison of the hydrophobicity plots (Fig. 4) of α_{s1}-casein A and α_{s1}-I with that of α_{s1}-casein B shows that a peak of hydrophobicity near residue 25 of α_{s1}-casein B has diminished and that a small peak near residue 5 of α_{s1}-casein B is not present in α_{s1}-I. Overall, α_{s1}-casein B has only a few peaks of hydrophobicity, while β-casein A^2

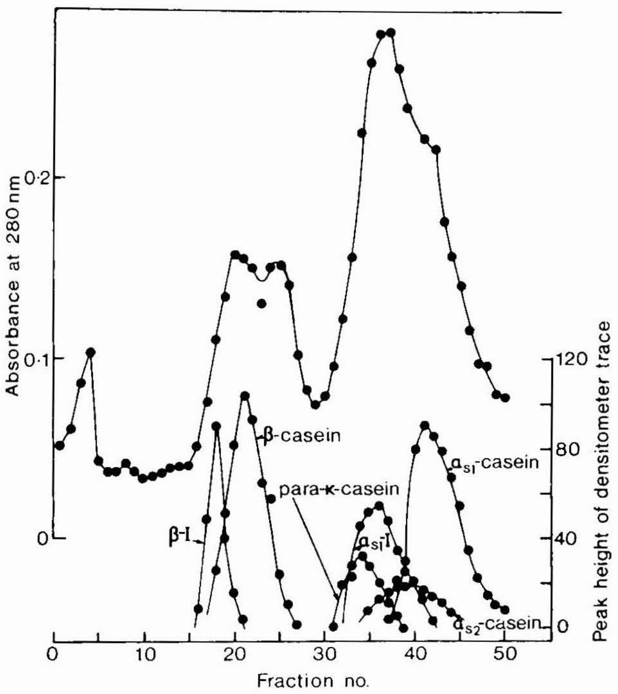


Fig. 3. Chromatography of chymosin-treated whole casein on Octyl-Sepharose CL-4B. The upper and lower curves are 280 nm absorbance and densitometer readings, respectively. However, the γ- and TS-casein curves are not shown and the para-κ-casein curve was derived from SDS disc gel densitometry.

TABLE I

AVERAGE HYDROPHOBICITIES OF BOVINE CASEINS AND CASEIN PEPTIDES

Protein or peptide	*Relationship to parent casein*	$H\varphi_{av}$* *(kJ/mole/residue)*
α_{s1}-Casein B		4.90
α_{s1}-Casein A	α_{s1}-Casein B (1–13, 27–199)	4.85
α_{s1}-I	α_{s1}-Casein B (25–199)	4.81
β-Casein A^2		5.59
β-I	β-Casein A^2 (1–189)	5.35

* After Bigelow[10].

has a large number of peaks between residues 60 and 209. One of these peaks is absent from β-I, but none is absent from γ-casein (residues 29–209 of β-casein).

DISCUSSION

The present results show that the large casein peptides α_{s1}-I and β-I can be separated from their precursor proteins, α_{s1}- and β-casein. The rare A variant of α_{s1}-

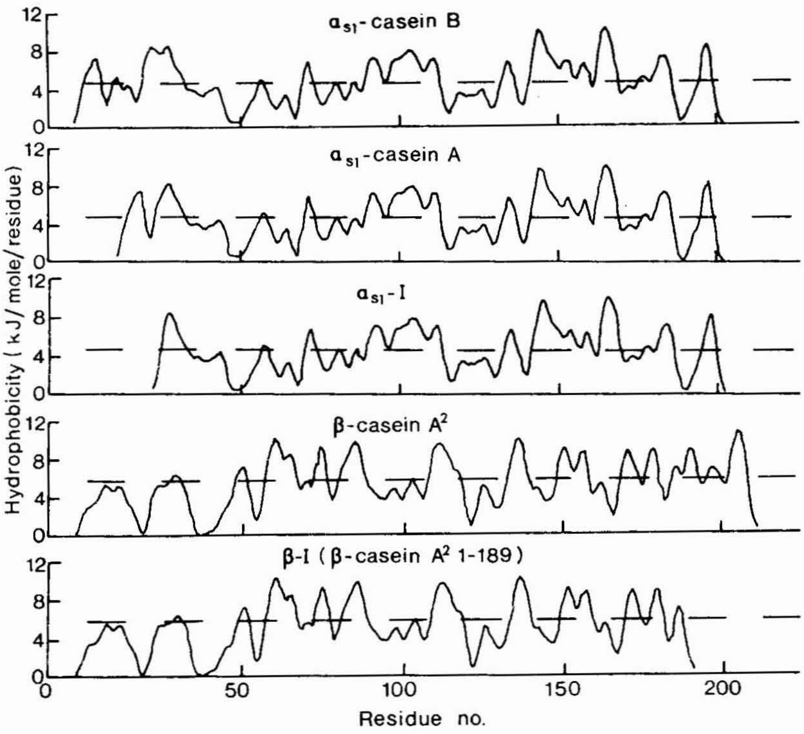


Fig. 4. Plots of the smoothed hydrophobicities of α_{s1}- and β-caseins. The calculations were carried out as described in the text. The horizontal lines are the average hydrophobicity for each peptide or protein. In some cases the plots are displaced to align the C-terminal residues.

casein is also readily separated from the common B variant, with which it often occurs in equimolar quantities. None of these separations is easily achieved using the standard methods of gel filtration[12] or cellulose ion-exchange chromatography[13,14]. Apart from these specific applications, the relatively low resolving power and capacity of the gels make their widespread use for routine isolation of casein components from whole casein less attractive.

The present results suggest a mechanism for the well-known method of preparing casein components[15] in which whole casein at its isoelectric point (approx. pH 4.6) is dissolved in 6 *M* urea and differentially precipitated as α_s- and β-casein by the step-wise addition of water; *i.e.*, dilution of the urea. Interpolation of the data shown in Fig. 2 to pH 4.6 indicates that α_{s1}-casein would interact more readily with hydrophobic groups than β- or κ-casein, and the self-interaction of α_{s1}-casein is thus likely to be hydrophobic. The present results also suggest that a similar mechanism may exist for the Zittle and Custer[16] method of preparing κ-casein by the acidification of whole casein dissolved in urea solution to precipitate α_s- and β-casein.

The most unexpected result from the present study was the early elution of β-casein compared with α_{s1}-casein when their calculated hydrophobicities (Table I) were in the opposite direction.

Kashavarz and Nakai[17] found that a series of globular proteins were not eluted from a column of hexyloxy-Sepharose with an ethylene glycol gradient in the order of increasing average hydrophobicity, but that the order was closely correlated to the order of the distribution coefficients of those proteins between two immiscible phases of differing polarity. They concluded that both the distribution coefficients and the order of elution in hydrophobic interaction chromatography were governed by the size and structure of the surface "patches" of hydrophobic residues on the protein molecules. The caseins cannot be considered as globular proteins because they have a low degree of order in their structure[1,2,18,19], and yet their structures must be such that α_{s1}-casein has a region or regions that can interact strongly with the octyl groups attached to the Sepharose, while β-casein has no such region. Examination of the smoothed hydrophobicity *versus* residue number plots (Fig. 4) does not suggest that there is any difference between these proteins in their distribution and/or clustering of hydrophobic residues that might account for their observed elution behavior. It seems likely that the small amount of secondary structure that probably exists[18] in α_{s1}-casein is such that one or more regions of the molecule adopt a conformation that can react strongly with the octyl groups of the Octyl-Sepharose. The elution of both α_{s1}-casein A and α_{s1}-I prior to α_{s1}-casein B suggests that one such region may involve residues that are close to the sequence that is missing from both α_{s1}-casein A and α_{s1}-I, *viz.*, residues 14–24 of α_{s1}-caseins.

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ION-PAIR EXTRACTION AND ION-PAIR ADSORPTION THIN-LAYER CHROMATOGRAPHY FOR RAPID IDENTIFICATION OF IONIC FOOD DYES

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SUMMARY

An easy, rapid and accurate method for routine analysis of seventeen commonly used ionic food dyes by means of ion-pair extraction and ion-pair adsorption thin-layer chromatography on silica gel plates has been developed. Cetyltrimethylammonium bromide was selected as the counter ion for both isolation and separation. Its concentration in the sample before extraction with methylene chloride should be around 0.0125 *M*. A pH value of 2.5 for the extraction gives an acceptable recovery of most of the dyes. The thin-layer plates were impregnated with the counter ion which was also present in the eluent. Sharp spot application yields a very good separation, enabling satisfactory identification of the dyes.

INTRODUCTION

General features of the existing methods for analysis of dyes are that they are time-consuming, laborious and do not always yield a satisfactory separation. In most cases, identification is achieved by paper or thin-layer chromatography, although the number of high-performance liquid chromatographic (HPLC) methods is steadily increasing[1,2]. A thorough procedure for the systematic identification of 49 synthetic food dyes has been elaborated by Hoodless *et al.*[3].

Drevon and Laur[4] were the first to employ quaternary ammonium salts to isolate food dyes. Several workers have subsequently applied this method[5–8] but none has demonstrated the potential of the combination of ion-pair extraction and ion-pair chromatography. In the work presented here seventeen common food dyes, all containing sulphonate or carboxylate groups, are both isolated and chromatographed as their ion pairs with the cetyltrimethylammonium ion. The main advantage of this procedure is that it allows a rapid identification with a minimum of sample treatment and within a reasonable time, and is highly reliable.

Both permitted and non-permitted additives were considered. However, since no two countries in the world have identical lists of permitted colour additives, no distinction is made between the two categories.

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EXPERIMENTAL

Reagents

All compounds were used as purchased. Analytical grade chemicals were employed where available. The dye standards (Table I) were supplied by local distributors and were of "Food Grade" quality. They all contain sulphonate groups except erythrosine which has a carboxylate function. Cetyltrimethylammonium bromide (CTMA) was from UCB (Drogenbos, Belgium). Appropriate dilutions were made in distilled water. Dye solutions were prepared in buffer, except for erythrosine which was dissolved in buffer–methanol (1:1). Four standard solutions, each containing all

TABLE I

THE SULPHONATED DYES

Common name	*Colour index no.**	*EEC no.*	*Synonyms*
Yellow			
Tartrazine	19140	E102	FD & C Yellow No. 5; C.I. Food Yellow 4
Chrysoin S	14270	E103	C.I. Food Yellow 8; Resorcin Yellow
Quinoline yellow	47005	E104	C.I. Acid Food Yellow 3; D & C Yellow No. 10
Acid yellow	13015	E105	C.I. Food Yellow 2; Fast Yellow AB
Orange			
Sunset yellow FCF	15985	E110	FD & C Yellow No. 6; C.I. Food Yellow 3
Orange GGN	15980	E111	C.I. Food Orange 2
Red			
Azorubine	14720	E122	C.I. Food Red 3; Fast Red G; Carmoisine
Amaranth	16185	E123	FD & C Red No. 2; C.I. Food Red 9
Cochineal red A	16255	E124	C.I. Food Red 7; Ponceau 4R
Scarlet GN	14815	E125	C.I. Food Red 2
Ponceau 6R	16290	E126	C.I. Food Red 8, Scarlet 6R
Erythrosine**	45430	E127	FD & C Red No. 3; C.I. Food Red 14
Blue and black			
Patent blue V	42051	E131	C.I. Food Blue 3
Indigo carmine	73015	E132	FD & C Blue No. 2; C.I. Food Blue 1; Indigotine
Green S***	44090	E142	C.I. Food Green 4; Wool Green BS
Brilliant black BN	28440	E151	C.I. Food Black 1
Black 7984	27775	E152	

* *Rowe Colour Index*, 2nd ed., Bradford, Great Britain, 1956.

** Although not a sulphonated dye, this substance is included because of its widespread use among water-soluble food dyes.

*** Has a bluish shade at certain concentrations.

dyes of the same colour group (yellow, orange, red, blue + black) at a concentration of 0.025 % in water–methanol (1:1), were used as references for thin-layer chromatography (TLC). Buffers of different pH values were prepared by mixing known amounts of two stock solutions and appropriate dilution. The pH values were measured with a glass electrode and carefully adjusted where necessary. Elution solvents were freshly prepared before each chromatographic run. The thin-layer plates were pre-coated silica gel plates (Merck No. 5721, 20 × 20 cm, layer thickness 0.25 mm).

Isolation of the dyes

As the isolation from the samples was to be undertaken by batch extraction, the influence of several parameters on the efficiency of the procedure had first to be established.

pH value. A 0.01 % solution of each dye was prepared in buffers of different pH value (2, 2.5, 3, 3.5, 4, 5, 6). One millilitre of each dye solution, 1 ml of a 0.01 *M* CTMA solution and 2 ml methylene chloride were mixed in a glass-stoppered tube on a Vortex for 30 sec. After separation of the phases, the extraction efficiency was evaluated visually.

Counter-ion concentration. It is known from the theory of ion-pair extraction that the distribution ratio can be varied within certain limits by changing the nature and the concentration of the counter ion. As the nature of the counter ion selected was based upon empirical observations, the influence of the concentration remained to be examined. Therefore, 1 ml of the 0.01 % dye solutions in buffer (pH 2.5), 1 ml of aqueous CTMA solutions, having concentrations ranging from 0.1 *M* to 0.001 *M* and 2 ml methylene chloride were treated as described above. The dye concentration in both layers was evaluated visually.

Dye concentration. One millilitre of each of a series of dye solutions in buffer (pH 2.5) having concentrations ranging from 0.002 % to 0.1 %, 1 ml 0.025 *M* CTMA solution and 2 ml methylene chloride were treated as described above. The results were evaluated visually.

Sample extraction

Surface coloured sweets were shaken with buffer (pH 2.5) until the coloured substance had dissolved. The phases were immediately separated to avoid dissolution of the sugar and other substances which complicate the separation of the immiscible liquid layers in the next stage of the analysis.

Other samples were used as such after adjustment to pH 2.5 (*e.g.*, beverages) or macerated in methanol–water (1:1) slightly alkalinized with ammonium hydroxide (*e.g.*, preserved fruits, instant desserts, caviar). After filtration and evaporation to dryness, the residue was taken up in buffer (pH 2.5) solution.

Jellies, containing large amounts of gelatine, were treated as follows. A quantity of jelly was dissolved in the minimum volume of water. Two volumes of methanol containing 0.1 % ammonium hydroxide were added and the mixture cooled in a refrigerator at 4°C for 2 h to precipitate the proteins. After centrifugation the supernatant was evaporated, the residue taken up in 20 ml of water–methanol (1:4), alkalinized with 0.1 % ammonium hydroxide, and the entire procedure repeated. The final dry residue was dissolved in buffer (pH 2.5).

One millilitre of a coloured solution or of an extract, 1 ml of a 0.025 *M*

TABLE II

EXTRACTION EFFICIENCY OF A CONSTANT AMOUNT OF DYE (0.01 %) AS A FUNCTION OF CONCENTRATION OF COUNTER IONS AT pH 2.5

Code: −, no dye extracted; =, both layers coloured; +, complete extraction.

*Concentration of counter ion (M)**	*E102*	*E103*	*E104*	*E105*	*E110*	*E111*	*E122*	*E123*	*E124***	*E125*	*E126*	*E127****	*E131*	*E132*	*E142*	*E151*	*E152*
0.1	−	−	−	−	−	−	−	−	−	−	−	(+)	−	−	−	−	−
0.05	+	+	=	+	+	+	−	−	−	−	+	(+)	+	−	+	=	+
0.025	=	+	=	=	+	+	+	+	+	+	+	(+)	+	=	+	+	+
0.01	−	=	=	=	=	=	+	=	=	+	=	(+)	+	=	+	+	+
0.005	−	=	=	−	=	=	+	=	=	+	−	(+)	+	−	=	=	−
0.0025	−	−	−	−	−	−	=	−	−	=	−	(+)	+	−	=	−	−
0.001	−	−	−	−	−	−	=	−	−	=	−	(+)	=	−	=	−	−

* Of the solution added to the dye solution (actual dilution 1:2).

** Experiments carried out at pH 3.

*** Due to the instability of the dye in an acidic medium, the aqueous layer always becomes discoloured after centrifugation, erroneously indicating complete recovery.

TABLE III

EXTRACTION EFFICIENCY OF VARIOUS AMOUNTS OF DYE AT CONSTANT pH (2.5) AND COUNTER-ION CONCENTRATION (0.025 *M* CTMA)

Code as in Table II.

*Dye concentration** *(%)*	*E102*	*E103*	*E104*	*E105*	*E110*	*E111*	*E122*	*E123*	*E124***	*E125*	*E126*	*E127****	*E131*	*E132*	*E142*	*E151*	*E152*
0.1	=	=	=	=	=	=	=	=	=	+	=	(+)	+	=	+	+	=
0.08	=	=	=	=	=	=	=	=	=	+	=	(+)	+	=	+	+	=
0.06	=	=	=	=	=	=	+	+	=	+	+	(+)	+	=	+	+	+
0.04	=	=	=	=	=	=	+	+	=	+	+	(+)	+	=	+	+	+
0.02	=	=	=	=	+	+	+	+	=	+	+	(+)	+	=	+	+	+
0.01	=	+	=	+	+	+	+	+	+	+	+	(+)	+	=	+	+	+
0.008	=	+	+	+	+	+	+	+	+	+	+	(+)	+	=	+	+	+
0.006	=	+	+	+	+	+	+	+	+	+	+	(+)	+	=	+	+	+
0.004	=	+	+	+	+	+	+	+	+	+	+	(+)	+	=	+	+	+
0.002	=	+	+	+	+	+	+	+	+	+	+	(+)	+	=	+	+	+

* Of the sample.

,* See footnotes to Table II.

cetyltrimethylammonium bromide solution and 2 ml of methylene chloride were mixed in a glass-stoppered tube on a Vortex for 30 sec. In most cases, centrifugation was applied to speed up phase separation. Concentration of the organic layer, where indispensable, was carried out in a stream of nitrogen.

Thin-layer chromatography

Preliminary work had shown that adsorption chromatography of sulphonated dyes on impregnated layers was highly promising on silica gel plates. Therefore, attention was concentrated on the potential of silica gel as the sorbent. It soon became obvious that the counter ion should be present both in the eluent system and in the sorbent layer. This was achieved either by dipping the pre-coated plates in a dilute solution of the counter ion (0.1 *M* in methanol) followed by air drying or by developing them twice with a 0.1 *M* solution of the counter ion in methanol with careful air drying after each run. Aliquots (1–5 μl) of the extracts were spotted on thin-layer plates 2.0 cm from the bottom and 1.0 cm apart using a capillary pipette or a syringe. Reference standard solutions were similarly applied (2 μl), each spot corresponding to 0.5 μg of the individual dye. The mobile phases were: A, methanol–acetone (9:1) + 1% glacial acetic acid, 0.1 *M* counter ion; B, methanol–acetone (1:1), 0.1 *M* counter ion. After solvent development in a saturated chamber to a distance of 15 cm, the plate was removed, air dried and examined both visually and under UV light of 366 nm.

RESULTS AND DISCUSSION

Isolation of the dyes

The highest extraction efficiency for most of the 0.01% dye solutions was achieved with the buffer of pH 2.5 (50 ml 0.1 *M* potassium hydrogen phthalate + 38.8 ml 0.1 *M* HCl, diluted to 100 ml), although for some the yield was apparently higher with the buffer of pH 3 (50 ml 0.1 *M* potassium hydrogen phthalate + 22.3 ml 0.1 *M* HCl, diluted to 100 ml). For each of the dyes a certain amount of the ion pair formed was extracted into the organic layer. That ion-pair extraction had occurred was demonstrated by the fact that when the counter-ion solution was substituted by water no colour could be observed in the methylene chloride layer. The influence of counter-ion concentration is summarized in Table II. It can be seen that, with few exceptions, the concentration of the counter-ion solution should lie between 0.01 and 0.05 *M*. Higher concentrations of 0.1 *M* prevent extraction. Although no clear explanation of this behaviour can be given, Knox and Laird[9] suggested that, above a certain concentration, adsorption or micelle formation may be relevant. The poor extraction efficiency with less concentrated CTMA solutions results from poor ion-pair formation. The results in Table III show that the extraction efficiency is little or not affected by the dye concentration in most instances, in agreement with the theory of ion-pair extraction which assumes that any excess of counter ion should be present for ion-pair formation.

Thin-layer chromatography

For the chromatographic separation several counter ions with different lipophilic character and of various sizes were examined. The best results with regard to separation efficiency were obtained with CTMA, so that this was selected for general use. The poor discriminating ability of many other ions was reflected in a moderate mobility of the ion pair or in a very pronounced tailing of the spots.

The two modes of plate impregnation did not yield identical results. Although both generally gave satisfactory results, the quality of the pre-developed plates was sometimes difficult to reproduce, considerable run-to-run differences in separation efficiency being encountered. An acceptable reproducibility was achieved when the plates were immersed for exactly 1 min in a fresh 0.1 *M* solution of the counter ion in methanol, followed by careful air drying in hot air.

A typical chromatogram required 35–45 min which is a relatively short time compared with the elution time of one to several hours with conventional butanol–water mixtures. Usually, well defined circular spots were obtained, an advantage of the present method compared to many others, where peak tailing considerably decreases separation efficiency and identification possibilities.

Identification in foodstuffs was achieved by comparison of the R_F values and colours of the unknowns with those of the constituents of the reference mixtures. UV detection at 366 nm was shown to be useful in corroborating the identity of azorubine (red fluorescence) or erythrosine (pale orange fluorescence) on chromatograms of a somewhat poorer quality.

A satisfactory separation of all dyes within each colour group was not possible with only one eluent system. Table IV shows the R_F values in the two selected mobile phases. The orange dyes may be developed in any of the solvent systems, but they were not separated. This means that when a spot with a given R_F value is detected one

TABLE IV

R_F VALUES OF SOME SULPHONATED DYES SEPARATED BY ION-PAIR THIN-LAYER CHROMATOGRAPHY ON IMPREGNATED SILICA GEL LAYERS

Solvents: A, methanol–acetone (9:1) + 1% glacial acetic acid, 0.1 *M* CTMA; B, methanol–acetone (1:1), 0.1 *M* CTMA.

Dye	*A*	*B*
Tartrazine	0.14	0.07
Chrysoin S	0.48	0.33
Quinoline yellow	0.33	0.28
Acid yellow	0.37	0.31
Sunset yellow FCF	0.32	0.25
Orange GGN	0.32	0.25
Azorubine	0.28	0.25
Amaranth	0.26	0.19
Cochineal red A	0.07	0.03
Scarlet GN	0.46	0.40
Ponceau 6R	0.17	0.07
Erythrosine	0.34	0.20
Patent blue V	0.36	0.34
Indigo carmine	0.38	0.29
Green S	0.44	0.39
Brillant black BN	0.12	0.09
Black 7984	0.16	0.12

should proceed to a further differentiation by other chromatographic procedures such as high-performance thin-layer chromatography (HPTLC)[10] or on conventional cellulose plates with water–concentrated HCl–*n*-butanol (62:28:10) as the eluent[11]. It was also shown that chromatographic spot resolution is especially dependent on the sharpness of the spots applied.

Low extraction efficiency may be overcome by adjusting the pH and/or counter-ion concentration. A practical example is quinoline yellow which over the whole dye concentration range was completely recovered at pH 4 upon addition of a 0.005 *M* CTMA solution. The determination of optimum conditions for quantitative extraction of each dye was beyond the scope of this paper, which was directed to rapid identification. Further research on the former is being undertaken.

Samples heavily loaded with interfering materials should be subjected to a pre-extraction with methylene chloride. After removal of the organic layer, the counter-ion solution is added and another portion of methylene chloride which will now extract the dye–CTMA ion pair. The appropriateness of the method was fully demonstrated with the various kinds of sample, all the dyes being correctly identified.

CONCLUSION

The method for ion-pair extraction and ion-pair adsorption TLC presented here requires only limited sample treatment and is very easy to apply. TLC development is much faster than conventional methods with butanol- and/or water-containing eluents. The high separation efficiency, due to the sharpness of the spots, offers a highly reliable means of identification.

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FLUORODENSITOMETRIC DETERMINATION OF POTATO GLYCOALKALOIDS ON THIN-LAYER CHROMATOGRAMS

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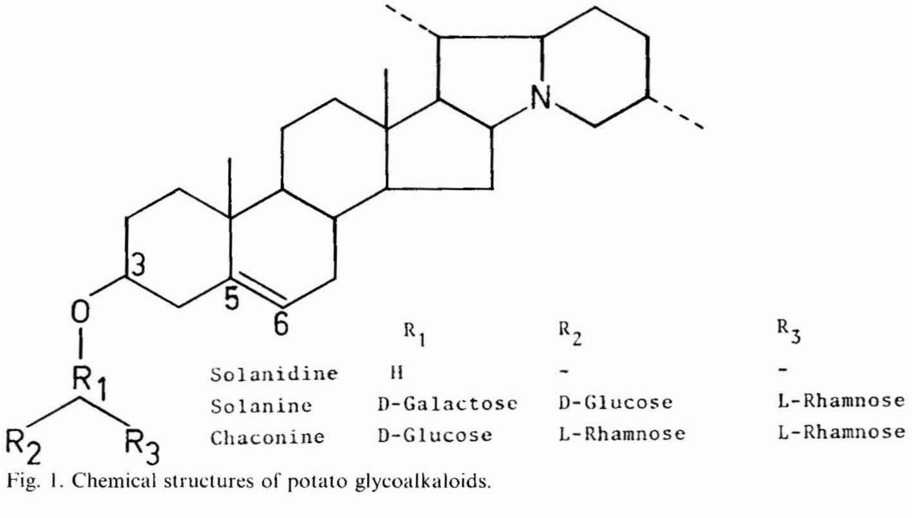
SUMMARY

Separation and analysis of solanine and chaconine has been achieved by thin-layer chromatography using an optical brightener as detection reagent. The conditions for optimal measurement *in situ* have been established. The method has been applied to the examination of leaf and tuber extracts of *Solanum tuberosum* cultivars. The errors in sample preparation and quantitative analysis range from 2.5 to 5.5%.

INTRODUCTION

The glycoalkaloids are a group of natural substances comprising a steroid base to which are attached one to four sugar molecules. The potato glycoalkaloids solanine and chaconine possess the same aglycone solanidine, but differ in their sugar moiety (Fig. 1). The sugars of solanine are galactose, glucose and rhamnose, which together form the trisaccharide β-solatriose. The trisaccharide part of chaconine is β-chacotriose, which comprises two molecules of rhamnose and one of glucose[1].



	R_1	R_2	R_3
Solanidine	H	-	-
Solanine	D-Galactose	D-Glucose	L-Rhamnose
Chaconine	D-Glucose	L-Rhamnose	L-Rhamnose

Fig. 1. Chemical structures of potato glycoalkaloids.

Glycoalkaloids are, like saponins, hemolytically active, decrease the surface tension of water and are fungitoxic[2]. Solanine and chaconine also inhibit human plasma cholinesterase[3,4] and have a positive inotropic effect on the heart[5]. The suggestion that glycoalkaloids are teratogenic[6] is not supported by other evidence[7–9], although solanine induces changes in the mitotic cycle of human fibroblast cultures[10].

Potato glycoalkaloids are lethal to animals after intravenous or intraperitoneal administration. Death resulting from solanine or chaconine intoxication is caused by depression of the central nervous system[9,11]. These compounds are also toxic to humans, several cases of solanine intoxication being mentioned in the literature[12–16]. The consumption of damaged potatoes is especially dangerous, because in wounded or green potatoes the glycoalkaloid content is raised[17]. Tubers containing up to 20 mg of glycoalkaloids per 100 g fresh weight are considered to be safe for human consumption.

Several assays are available for the glycoalkaloids. The wet-chemical methods (titrimetry, colorimetry)[18–20] give the total glycoalkaloid content. These assays are also used for the determination of other glycoalkaloids, *e.g.*, solasonine and tomatine. If the content of the individual glycoalkaloids is required, *e.g.*, in breeding programs, screening of *Solanum* species or the evaluation of extraction procedures, then a chromatographic separation is needed before the quantitative analysis. Herb *et al.*[21] separated and determined glycoalkaloids after permethylation by gas–liquid chromatography. The determination by high-performance liquid chromatography avoids the need for derivatization[22,23]. Owing to their poor UV-absorbing properties, the glycoalkaloids are detected at low wavelength (210 nm) or with a RI-detector. This makes the method insensitive and unspecific.

Thin-layer chromatography (TLC) offers the advantage of using a specific and sensitive detection reagent. Cadle *et al.*[24] detected glycoalkaloids using antimony trichloride, which reacts with the $\Delta 5,6$ double bond of solanidine. Dragendorff reagent has also been used for the detection[25]. In both methods the glycoalkaloids are determined by *in situ* measurement of the remission of the spots. Because fluorescence determinations *in situ* are more sensitive and have a more linear response–concentration relation than remission determinations *in situ*[26], we have investigated the application of detection reagents which give fluorescing spots with glycoalkaloids.

PRELIMINARY EXPERIMENTS

Choice of solvent system

For the measurements *in situ* the spots have to be well separated and sharp. We investigated several solvent systems (Table I) comprising mixtures of three or four solvents, which can be divided into three groups: I, organic solvents immiscible with water; II, organic solvents miscible with water; III, water containing an acid or a base. The water content of the mixture determines the migration rate of the compounds[27]. These mixtures are comparable with those used for the separation of saponins or oligosaccharides, which, like the glycoalkaloids, are polar compounds. The hR_F values show that solanine and chaconine are separated by all the mixtures, but sharp spots were obtained only with systems A1 and D1. To avoid the use of two-layer systems, systems A1 and D1 were modified to A2 and D2, respectively.

For qualitative TLC we used systems A1 and 2[28]. In order to remove the

TABLE I

SOLVENT SYSTEMS FOR THE TLC OF GLYCOALKALOIDS

o.l. = Organic layer. hR_F = 100 × R_F. Distance from start to solvent front = 10 cm. All TLC was carried out on pre-coated silica gel plates in unsaturated tanks.

	I	*II*	*III*	*Composition*	hR_F	
					Solanine	*Chaconine*
A1	*n*-Butanol	Formic acid	Water	40:10:50; o.l.	9	15
A2	*n*-Butanol	Formic acid–methanol	Water	75:5:15:7	12	22
B	Chloroform	Methanol	Water	50:50:10	28	42
C	Chloroform	Methanol–glacial acetic acid	—	50:45:5	24	65
D1	Chloroform	Methanol	2% Ammonia	20:20:10; o.l.	16	25
D2	Chloroform	Methanol	2% Ammonia	70:30:5	24	36
E	Ethyl acetate	Pyridine	Water	30:10:30	17	38
F	—	Ethanol	95% Acetic acid	20:60	49	63

solvents after development, the plates were heated for 30 min at 120°C. Under the nfluence of the formic acid and the heat the glycoalkaloids partly decomposed. In two-dimensional TLC the decomposition products did not react with the detection reagents in the same way as the glycoalkaloids. Therefore we used system D2 for the quantitative TLC.

Choice of detection reagent

Since glycoalkaloids show no absorbance or fluorescence in the visible and UV ranges, reagents are necessary to convert the glycoalkaloids into absorbing or fluorescng substances. The detection reagents which give fluorescing spots are preferred, because the response of such spots is a linear function of the concentration and the response is less dependent on the form of the spots than is the response of absorbing spots. With remission measurements *in situ* the relation between response and concentration is more complex and has to be determined empirically. Also the sensitivity is lower.

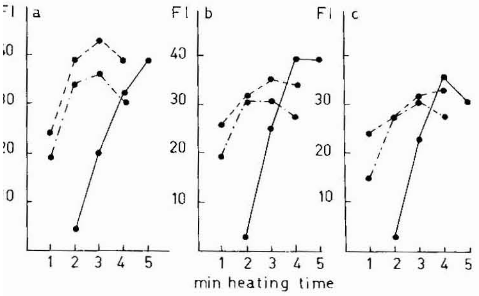


Fig. 2. Fluorescence response of 2-μg solanine spots with 5% sulphuric acid in diethyl ether as detection reagent. a, Measurement directly after cooling of the plate; b, measured after 30 min; c, measured after 60 min. Conditions: Zeiss PMQ II TLC densitometer; excitation, 365 nm (mercury lamp); emission wavelength, 424 nm. ●–●, Plate heated at 80°C; ●- -●, plate heated at 100°C; ●-·-·-●, plate heated at 120°C. FI = Fluorescence response in arbitrary peak area units.

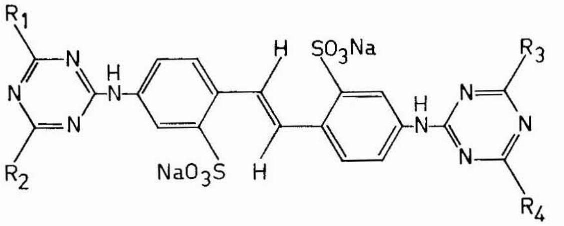


Fig. 3. Structure of the optical brighteners of the stilbenedisulphonic acid type, used as TLC detection reagents for glycoalkaloids. R_1, R_2, R_3 and R_4 are substituents.

First we tried sulphuric acid, a general reagent for steroids. The plates with the glycoalkaloid spots were dipped in a solution of 5% sulphuric acid in diethyl ether, and heated. The glycoalkaloid spots gave a blue fluorescence under long-wavelength UV light (365 nm). The response was dependent on the time and the temperature of the heating, and also on the time between heating and measurement (Fig. 2a, b, c). Maximum fluorescence was obtained with heating at 100°C for 3 min. However, the fluorescence was not stable enough for quantitative work.

Therefore we investigated the application of optical brighteners (OBs), which we have used previously[28,29]. The OBs of the stilbenedisulphonic acid type (Fig. 3) are sensitive and specific detection reagents for glycoalkaloids. In TLC they give blue fluorescing spots with the glycoalkaloids. Fig. 4 shows the fluorescence spectrum. Maximum fluorescence is obtained with an excitation wavelength of 380 nm and an emission filter of 450 nm.

These OBs have *cis–trans* isomers owing to the ethylene bond in the stilbene nucleus[30]. Under influence of light the fluorescing *trans*-form changes into the non-fluorescing *cis*-form. The fluorescence of glycoalkaloid spots was stronger on a plate dipped in an OB solution, kept in the dark, then on a similar plate kept in daylight (Fig. 5). For quantitative work however, the fluorescence was again not stable enough. When the plates were dried for 15 min at 105°C after dipping, the fluorescence was stable for several hours. For quantitative work, all manipulations of the plates between drying after development and placing in the TLC scanner were carried out in the dark. Plates treated in this way and kept in the dark showed the blue fluorescing glycoalkaloid spots even after some months.

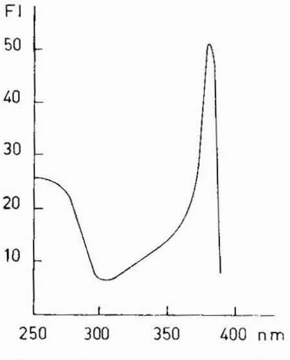


Fig. 4. Fluorescence excitation spectrum of 0.1-μg solanine spot on TLC, dipped in a 0.02% methanolic solution of Blankophor® BA 267%. Emission filter, 450 nm; slit 5 × 1 mm.

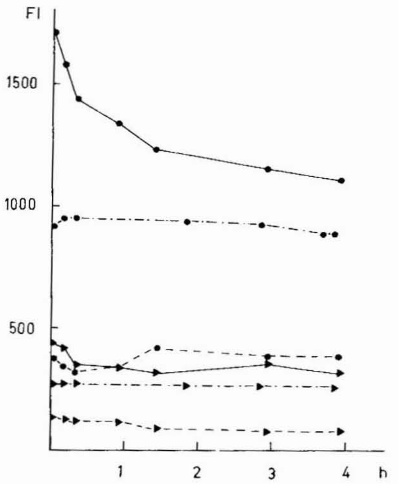


Fig. 5. Variation of fluorescence response with time of 0.5- (●) and 0.1-μg (▲) solanine spots detected with Blankophor® BA 267% dip solution. ——, Dip solution kept in dark; – – –, dip solution kept in daylight; –·····–, dip solution kept in dark; after dipping the plate was dried for 15 min at 105°C.

EXPERIMENTAL

Reference compounds

A purified glycoalkaloid extract from potato sprouts was separated by open column chromatography (stationary phase, aluminium oxide; mobile phase, water-saturated *n*-butanol) into solanine and chaconine. After recrystallization from aqueous methanol, the identity of the glycoalkaloids was confirmed by TLC and mass spectrometry. The purity was determined by water-free potentiometric titration with perchloric acid. For quantitative TLC, standard solutions in methanol were prepared containing 50, 150 and 250 μg/ml of each compound.

Extraction

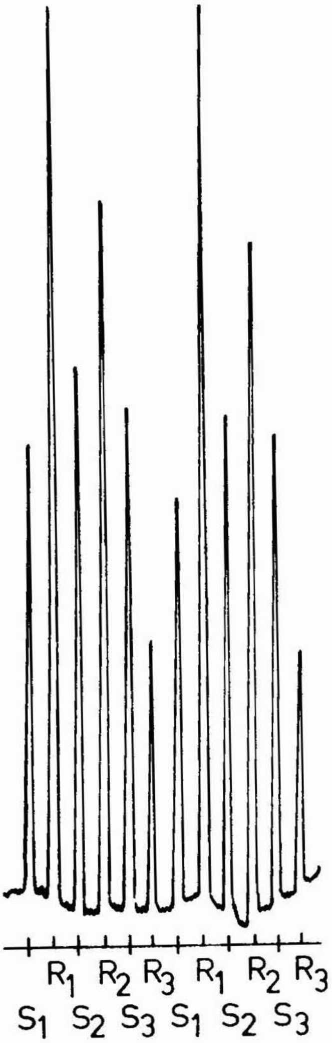
Fifty grams of mashed and frozen potatoes or 10 g of dried and pulverized leaves were refluxed three times with 250 ml methanol, first for 30 min and then twice for 15 min. The methanol extracts were filtered while hot and the methanol was evaporated on a rotary evaporator at <40°C. The residue was dissolved in 5 × 5 ml 5% acetic acid.

This solution was centrifuged (20 min, 3000 *g*), the supernatant decanted and the residue in the centrifuge-tube was washed twice with 5 ml 5% acetic acid and centrifuged. The combined acid solutions were shaken three times with 30 ml diethyl ether. The combined ether extracts were shaken once with 10 ml 5% acetic acid; the resulting acid extract was added to the previous combined acid extracts. All combined acid extracts were made alkaline with concentrated ammonia (25%), and warmed at 70°C for 30 min. After cooling for at least 6 h at 4°C, the precipitated glycoalkaloids were filtered off on a G-3 glass filter, provided with a layer of 1 g Hyflo Super Cel®.

The precipitate was washed with 6 × 5 ml 1% cold ammonia, and dissolved in 100 ml methanol. The methanol was evaporated on a rotary evaporator at <40°C.

The residue was dissolved in methanol to give a concentration of each glycoalkaloid between 50 and 250 μg/ml.

All TLC was carried out on precoated silica gel plates, layer thickness 250 μm (Cat. no. 5721; E. Merck, Darmstadt, G.F.R.). The solvents were of analytical grade. The sample and standard solutions were applied to the plate in duplicate, using the same 2-μl capillary for all solutions. Of the duplicates, one was spotted on the left part of the plate, the other on the right part (Fig. 6). The plate was developed with solvent system D2 over 10 cm, dried for 15 min at 105°C and cooled for another 15 min at room temperature. Subsequent manipulations were carried out in the dark.



```
C-1
                                 1 SC
                                63 MD
                      AR
                   30123
                   74474
                   36905
                   48218
                   34374
                   17053
                   27221
                   77519
                   32642
                   45482
                   31002
                   15062

                  460030 T

slope              1498 x 10^2
intercept          13.5 x 10^2
r                  0.9998
```

Fig. 6. Fluorescence location curve of chromatogram of solanine spots with integration print-out and coefficients of the calibration curve. R_1, R_2 and R_3 = Calibration points of 0.1, 0.3 and 0.5 μg, respectively; S_1, S_2 and S_3 = samples 1, 2 and 3, respectively. SC = Scan number; MD = method, giving peak area; AR = area; T = total area.

Detection

The cooled plate was dipped in a 0.02% methanolic solution of Blankophor® BA 267% (Bayer, Leverkusen, G.F.R.), dried for 15 min at 105°C and cooled for at least 15 min at room temperature.

Densitometry

The fluorescence intensity of the glycoalkaloid spots was measured with a Shimadzu CS 910 TLC scanner with fluorescence attachment. The plates were scanned perpendicular to the direction of development. Instrumental conditions: excitation wavelength, 380 nm; slit, 8 × 0.25 mm; emission filter, 450 nm; scan speed, 20 mm/min. The fluorescence signal was recorded with a Kipp BD 8 flat-bed recorder: sensitivity, 5 mV full scale; paper speed, 10 mm/min. The integrated values of the signal were obtained with a Shimadzu Chromatopec E1A integrator. For each plate the sensitivity of the densitometer was adjusted to full scale deflection of the recorder-pen for the 0.5-μg spot.

RESULTS AND DISCUSSION

With fluorescence measurements *in situ* the relationship between response and concentration is linear only within a limited range of low concentrations. To determine this concentration range we calculated the regression lines for three ranges. Table II shows that for both glycoalkaloids the best fit was obtained for the 0.1–0.5 μg range (correlation coefficient, $r > 0.9994$). The intercept of this line with the ordinate was slightly above the origin. At higher concentrations the calibration graph curved downwards with increasing concentration. The low slope for the 1.0–5.0 μg range was caused by a lower setting of the densitometer sensitivity.

TABLE II

REGRESSION-LINE COEFFICIENTS FOR THREE CONCENTRATION RANGES OF SOLANINE AND CHACONINE

r = Correlation coefficient; res. s.d. = residual standard deviation; n = number of calibration points.

	Concentration range (μg)	*Slope*	*Intercept*	*r*	*res. s.d.*	*n*
Solanine	1.0–5.0	128.1	252.3	0.9798	47.8	5
Solanine	0.2–1.0	896.2	74.0	0.9990	14.8	10
Chaconine	0.2–1.0	441.0	13.2	0.9968	14.7	10
Solanine	0.1–0.5	820.2	5.1	0.9994	7.8	6
Chaconine	0.1–0.5	648.2	1.5	0.9998	3.1	6

To optimize the method we investigated several sources of errors, which influence the reproducibility of the results. Apart from the errors in preparing the sample and the calibration solutions, other errors arise from: 1, application of the spot; 2, the TLC (plate development and detection); 3, the instruments (densitometer and integrator). To avoid errors due to differences in volume between capillaries, all solutions for one plate were applied with the same capillary. Error type 1 is also influenced by the volume of the spot. When spots were applied with a 5-μl capillary, the relative standard deviation (r.s.d.) of the chaconine peak areas was 3.6%. The same amount of chaconine, applied with a 2-μl capillary, gave a r.s.d. of 2.3%. For solanine, having a lower R_F value, no difference was found. Therefore all spots were applied with a 2-μl capillary.

The large r.s.d.s of the slopes of calibration curves, which were calculated from several plates, indicated that, even under standardized conditions, the plates, development and application of the detection reagent were not reproducible. For solanine (mean slope 1175; $n = 10$) the r.s.d. was 12.7%; for chaconine (mean slope 914; $n = 10$) it was 15.7%. Therefore the fluorescence responses of spots on different plates are not comparable and a calibration graph must be constructed for each plate.

When several spots with the same glycoalkaloid concentration were measured on one plate, the r.s.d. of the peak areas for both glycoalkaloids was 1.3–4.7%. Apart from the error in the spot application, this is caused by irregularities in layer thickness. By applying the spots to the plate according to the data-pair technique[31] the r.s.d. for both glycoalkaloids is reduced to 0.8–2.5%.

The instrumental error was determined by measuring one spot 30 times. The r.s.d. of the peak areas was 0.4–0.7%.

Table III shows the results of the application of the method to three samples. Six extracts were made from each sample and analysed. The r.s.d. contains the error in the extraction, and the values obtained are comparable with those from HPLC and GC methods[21–23] (3.4–10.0%). Studies on these and other potato varieties indicate that the method is sensitive and precise. Because the applied OB is a specific and sensitive TLC detection reagent for glycoalkaloids[28], this method can also be used for the determination of solasodine glycosides, which are important raw materials for steroid drug manufacture, and of tomatine, a fungitoxic glycoalkaloid of *Solanum lycopersicum*, the tomato.

TABLE III

SOLANINE AND CHACONINE CONTENTS OF THREE SAMPLES

r.s.d. = Relative standard deviation.

Sample	*Part*	*Solanine (mg/100 g)*	*r.s.d. (%)*	*Chaconine (mg/100 g)*	*r.s.d. (%)*
Element	Tubers	4.2	3.0	6.7	2.6
Element	Leaves	31.7	4.6	169.5	3.5
Mara	Tubers	14.6	3.3	12.3	5.9

By using transferable calibration factors[32], five instead of three samples can be determined on one plate.

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Note

Simple model of liquid–solid chromatography involving solute–solvent and solvent–solvent interactions

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In previous papers[1,2], the solute–solvent and solvent–solvent interactions in the mobile and surface phases have been discussed in terms of Snyder's theory of liquid–solid chromatography (LSC)[3]. These interactions may also be described in terms of the formation of associates between molecules of solute and solvent in the mobile and surface phases[4]. This approach is more suitable for the investigation of specific molecular interactions.

Recently, Jaroniec and Piotrowska[4] considered theoretically the solvent–solvent and solute–solvent association in LSC with a binary mobile phase by assuming energetic homogeneity of the adsorbent surface and the same molecular size for all components. They discussed three models involving the formation of double associates BS, BB or AB, where A and B denote the solvents or single molecules of the solvents and S denotes the solute or a single molecule of the solute. From the theoretical discussion[4] and experimental studies it is seen that the mixed associates BS and BB play an important rôle in the chromatographic process.

In this note, we discuss the simple model involving the simultaneous formation of double associates BB and BS in the mobile phase. However, in the surface phase the silanol groups of the silica surface can compete with BS and BB associates in the adsorbed phase. Thus, stronger interactions of S and B molecules with the silica silanols can preclude BB and BS interactions in the surface phase. Taking into account the possibility of destruction of the associates BS and BB in the surface phase by the silica silanols, we assume that these associates form only in the mobile phase. The above model may be represented by the following quasichemical reactions

$$B_{(s)} + S_{(m)} \leftrightarrows B_{(m)} + S_{(s)};\ K = (y_S x_B)/(y_B x_S) \tag{1}$$

$$B_{(m)} + B_{(m)} \leftrightarrows BB_{(m)};\ L = x_{BB}/(x_B)^2 \tag{2}$$

$$B_{(m)} + S_{(m)} \leftrightarrows BS_{(m)};\ M = x_{BS}/(x_B x_S) \tag{3}$$

where the subscripts (m) and (s) refer to the mobile and surface phases, respectively, K, L and M are the equilibrium constants of reactions 1–3 and x_i and y_i (i = B, S, BB, BS) are the mole fractions of the ith individual in the mobile and surface phases,

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respectively. The total mole fractions of B and S in the mobile and surface phases may be expressed as:

$$x_B^0 = x_B + x_{BS} + 2x_{BB} \approx x_B + 2Lx_B^2 \qquad (4)$$

$$x_S^0 = x_S + x_{BS} = x_S + Mx_B x_S \qquad (5)$$

$$y_B^0 = y_B \quad \text{and} \quad y_S^0 = y_s \qquad (6)$$

The distribution coefficient, $k_{(AB)S}$, for solute S and mobile phase A–B is defined as:

$$k_{(AB)S} = y_S^0/x_S^0 \qquad (7)$$

Combining eqns. 1, 5, 6 and 7 we have:

$$k_{(AB)S} = Ky_B/[x_B(1 + Mx_B)] \qquad (8)$$

Solution of eqn. 4 with respect to x_B gives:

$$x_B = [(1 + 8Lx_B^0)^{1/2} - 1]/(4L) = X_B/(4L) \qquad (9)$$

Finally, eqns. 8 and 9 lead to the general expression:

$$k_{(AB)S} = 16L^2Ky_B/[X_B(4L + MX_B)] \qquad (10)$$

Eqn. 10 may be simplified by applying the following assumptions: (a) when the difference between the elution strengths of both solvents is large and at high values of x_B^0, the mole fraction y_B may be approximated by unity, and (b) for small values of L the variable X_B may be approximated by

$$X_B = 4Lx_B^0\,(1 - 2Lx_B^0) \qquad (11)$$

Taking into account eqn. 10, the assumptions (a) and (b) and neglecting terms involving third and higher powers of x_B^0, we have:

$$1/(k_{(AB)S}x_B^0) = 1/K + [(M - 2L)/K]\,x_B^0 \qquad (12)$$

The constant K is always greater than zero, therefore, the sign of the slope of eqn. 12 is dependent upon the sign of $(M - 2L)$ only. If the magnitude of the equilibrium constant M, corresponding to the solute–solvent association, is greater than that of the constant $2L$, corresponding to association of solvent B, then the slope is positive. However, if $2L > M$ a negative slope is observed.

Eqn. 10 and especially its simplified form, eqn. 12, may be useful in the determination of the effects of solute–solvent and solvent–solvent interactions in LSC with mixed mobile phases.

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Note

Effects of hydrogen treatment on typical gas chromatographic supports*

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Iron found on the surface of gas chromatographic (GC) supports is considered detrimental[1]; hence the higher price one pays for acid-washed (AW) materials. Iron is not the only deleterious element (aluminium is another major one[1,2]) but, because of the color of its compounds, it is the most obvious. One may formally ascribe the negative effects of any contaminating metal to two factors: The general inhomogeneity of the surface it produces, and the specific interactions it undergoes with the solute and/or the liquid phase.

In contrast to iron contamination of a silicic surface, pure iron oxide — whether in supported[2] or unsupported[3,4] form — is not a bad separation medium; especially when it is covered by a polar liquid phase[5,6]. "Reduced" iron is even better[2].

Why this should be so is not immediately clear. But it has been established that a reduced iron layer of several ångstroms thickness, carrying a bonded layer derived from Carbowax 20M, yields gas chromatograms of the same quality as a similarly covered pure silica surface would have done[2].

If iron oxides present on the surface of typical diatomaceous supports are indeed to blame for inferior chromatographic performance, and if a layer of "reduced" iron behaves indeed better in chromatography than the iron oxide layer from which it originated, then it may be possible to improve regular supports by changing the iron (and perhaps other elements) on the surface from an "oxide" to a "reduced" state. In this case the surface would still be heterogeneous in an elemental sense, but some highly active surface sites might then have lost some of that activity and become more similar, chromatographically speaking, to other adsorption centers.

Perhaps the easiest and most often pursued path in reducing surfaces is to expose them to hydrogen at elevated temperature. This approach is common to a variety of disciplines. In chromatography, however, it is relatively rare: The best known example here is the treatment of graphitized carbon to render it non-polar[7]. In some of our studies, we attempted to reduce bonded ferric oxide and did observe the expected color change from brick-red to grey[2]. Now, if one treats typical GC supports such as Chromosorb W (W for white) and Chromosorb P (P for pink) with hydrogen at 700°C, both also turn grey. If these grey materials are oxidized, *e.g.* by

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* Taken from thesis research of P.P.W.

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exposure to air at 700°C, both take on again their former white and pink appearances.

Though photoelectron spectra (of plain and reduced iron oxide layers) were equivocal on the question, it would be reasonable to presume that the observed color change from pink to grey reflected a change in the valence state of iron. In addition, changes may have occurred that were not immediately visible. Since hydrogen treatments are cheap and easy to perform, it may be worthwhile to use them routinely on conventional GC supports, provided one could demonstrate some beneficial effects.

Just of what kind such potential effects may be, is open to speculation. For instance: Oxidizing —but not reducing— conditions are generally avoided in GC. Often an analyst fights ppm levels of oxygen in his gas supply while, on other occasions, he employs pure hydrogen as carrier. It is not unusual to hear the premature degradation of both stationary liquid phases and GC solutes being attributed to the unwanted presence of an oxidizing agent. By using a "reduced" support, one could perhaps counteract such deterioration in chromatographic performance.

Other speculative effects concern the solid surface: It is often blamed for tailing and "irreversible adsorption" of solutes. Reducing it could change its adsorptive and catalytic properties. It may also alter the solid–liquid interface region by re-arranging the orientation of liquid phase molecules in respect to the surface.

A final bit of speculation deals with "bonded" phases: Any attempt at bonding a liquid phase depends critically, and in ways not well understood, on the chemical state of the surface. The better these reactions function the better "deactivated" is the surface and the more satisfying will be its chromatographic performance.

Speculations aside now, it is this "chromatographic performance" that decides whether or not there is an advantage in the hydrogen treatment of supports. To show any potential effect as clearly as possible, we started out with *non*-acid-washed (NAW) materials. These were used (as received) for the synthesis of thin, bonded layers based on Carbowax 20M (ref. 8), as well as for the preparation of conventional gas–liquid chromatographic (GLC) packings from the popular liquid phases Apiezon L, OV-17 and Carbowax 20M. We wanted to compare hydrogen-treated with non-treated materials in two respects: Whether bonded phases showed any differences in chromatography, and whether regular GLC phases showed any change in bleed rates.

EXPERIMENTAL

Chromosorbs W and P, NAW, 45–60 and 60–80 mesh, respectively, were filled into a cylindrical quartz reservoir and, after flushing with nitrogen, were heated for 4 h at 700°C in a tubular furnace, while a *ca.* 10 ml/min hydrogen stream passed through. Hydrogen was shut off only when the supports had again reached room temperature.

The "bonding" with Carbowax 20M was carried out in boiling hexadecane[8], and non-bonded material was removed by extracting for 10 h with methanol at boiling point temperature in a continuous extractor[9].

Regular GLC phases were prepared in 5% load by conventional rotary evaporation. All materials were packed into 100 × 0.2 cm I.D. borosilicate glass U-tubes and tested with mixtures of *n*-alkanes and *n*-alkanols in an 8°C/min temperature program. "Bleed" was evaluated —after conditioning the columns at 220°C for 48 h— by measuring the rise in the baseline reflecting a 40 to 220°C, 8°C/min temperature program followed by an isothermal hold at the upper temperature (until the baseline current had become constant).

Elemental analysis of bonded phases was done by Guelph Chemical Labs., Guelph, Canada.

RESULTS AND DISCUSSION

Table I lists the carbon content of bonded phases and the relative bleed rate of regular phases. Elemental analysis shows the amount of bonded material to be higher on hydrogen-treated than on untreated supports: Slightly so on Chromosorb W, decidedly so on Chromosorb P.

TABLE I

CARBON CONTENT OF BONDED PHASES AND BLEED RATE OF REGULAR PHASES

Chromosorb		*Bonded phase: % C*	*Regular phase: bleed**		
			Apiezon L	*Carbowax 20M*	*OV-17*
W, NAW,	untreated	0.18	100	100	100
	hydrogen-treated	0.23	77	38	77
P, NAW,	untreated	0.22	100	100	100
	hydrogen-treated	0.76	42	44	88

* At 220°C, relative (untreated = 100).

The chromatograms of alkanols demonstrate that the bonded phases based on hydrogen-treated supports are better "deactivated"; *i.e.* they give rise to larger, sharper, and more symmetrical peaks. Part of this effect on Chromosorb P is caused, no doubt, by the higher organic load. But it is also obvious, at least in the case of Chromosorb W where loads are comparable, that the hydrogen treatment itself had done some good. Fig. 1 shows some typical chromatograms. (It must be noted, however, that the chromatographic performance shown here is still not as good as that obtainable from a scrupulously cleaned Chromosorb W (ref. 8) or P (ref. 10) taken through the same reaction with Carbowax 20M.)

Chromatographic performance was also routinely checked on the regular GLC phases but, as expected, their comparatively heavy liquid loads prevented any significant differences from showing up. Rather, the noteworthy aspect of regular phases is their bleed rate. The data of Table I show that hydrogen treatment of the support reduces the bleed rate of each of the three representative liquid phases coated on it.

Interestingly enough, reduction in bleed appears to correlate roughly with the phases' susceptibility to oxidation. (Carbowax 20M consists of two polyethyleneglycol chains linked by a diepoxide; Apiezon L can be characterized as a molecular distillate of hydrocarbons with some unsaturation present; and OV-17 is a linear methylphenylpolysiloxane.)

It would be interesting to know how long the reduction in bleed lasts through conventional chromatographic usage and, should the effect vanish after some time,

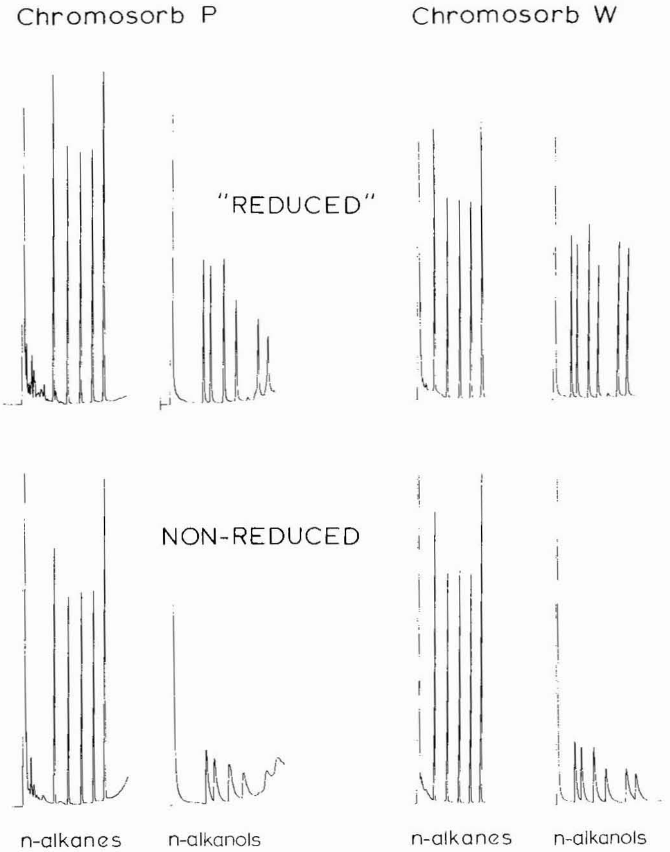

Fig. 1. Temperature-programmed chromatography of *n*-alkanes (C_{12}, C_{14}, C_{16}, C_{18}, C_{20}) and *n*-alkanols (C_7, C_8, C_{10}, C_{12}, C_{16}, C_{18}) on bonded phases from Carbowax 20M, on hydrogen-treated and non-hydrogen-treated Chromosorbs P and W.

whether it can be prolonged by, say, doping the carrier gas with minute amounts of hydrogen. It would also be interesting to see if phases of the hydrogen-treated variety behaved differently toward easily decomposed solutes; or if hydrogen treatment also brings about changes in those highly cleaned diatomaceous supports that have little iron left on their surface. Such studies could prove lengthy, however, and that puts them beyond our present designs.

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Note

Analysis of carbohydrates using the mass detector

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The analysis of carbohydrates using high-performance liquid chromatography (HPLC) is now well established. Most applications employ amino-bonded phases[1] but alternative techniques, such as ion-exchange partition[2] and the use of amine modifiers in the mobile phase[3], have been used with some success. The separation of complex mixtures of carbohydrates, ranging from simple monosaccharides to oligosaccharides and dextrins, can be readily achieved and in general experimental problems are those associated with detection rather than with the chromatographic separation. The most commonly used detector for carbohydrate analysis is the differential refractive index (RI) detector[4]. However, although RI detectors have a wide linear range they are not very sensitive and require elaborate temperature control to obtain stable base-lines. In addition it is impractical to carry out gradient elution, which is necessary for the analysis of mixtures containing a wide range of sugar types. Ultraviolet absorption at 200 nm[5], or even below, can be used but appears to provide little advantage over RI detection, although limited gradients can be run if solvents of sufficient purity are available. Liquid chromatography offers considerable advantages over gas chromatography for carbohydrate analysis but these advantages will only be realised with suitable detectors. The mass detector is a sensitive instrument which can be used for the detection of non-volatile solutes. The solvent is evaporated after nebulisation in a heated column producing finely divided solute particles which pass through a light-beam. Light scattered from the particles is detected by a photomultiplier placed at 120° to the light beam. The signal is amplified and recorded on a conventional chart recorder. A light trap is mounted directly opposite the light source to prevent internal reflection of non-scattered light, as shown in Fig. 1. The theoretical basis of the light-scattering process is elegantly described in a paper by Charlesworth[6].

This paper describes briefly the characteristics of the mass detector and its application to some carbohydrate analyses in foods.

EXPERIMENTAL

The instrument used in this study was a pre-production Applied Chromatography Systems (Luton, Great Britain) mass detector in conjunction with an Applied Chromatography Systems Model 750 gradient chromatograph. Injection was carried out via a Rheodyne injection valve, Model 7120 (20-μl loop). Peak areas were measured using a Pye DP88 integrator.

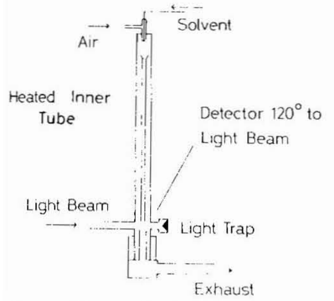

Fig. 1. Schematic diagram of mass detector.

Chromatographic columns (25 cm × 5 mm) of Spherisorb-5-NH_2 were either packed in our own laboratory or obtained from Hichrom (Woodley, Great Britain). Acetonitrile was of HPLC grade obtained from Rathburn (Walkerburn, Great Britain). All the chromatograms in this paper were produced using aqueous acetonitrile and the above columns usually with a flow-rate of 2 ml min^{-1}. Sugar standards were purchased from BDH (Poole, Great Britain).

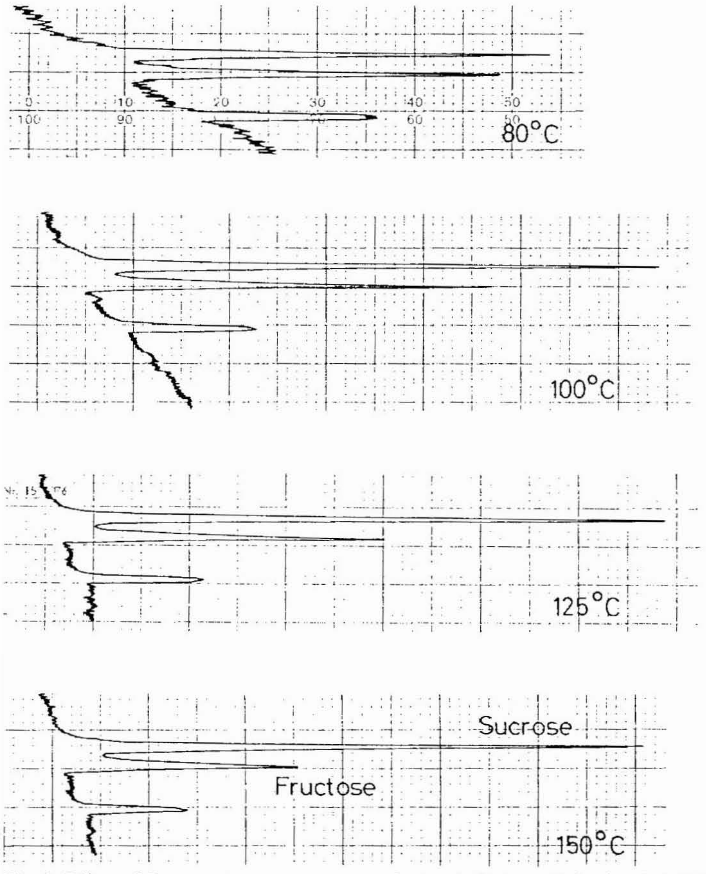

Fig. 2. Effect of detector temperature on noise level. Column Spherisorb-5-NH_2. Solvent water–acetonitrile (30:70), 2 ml min^{-1}. Detector ×4. Fructose and sucrose 20 μg.

Food samples, wheat germ, soyabeans and lupinseeds were prepared for analysis as described by Macrae and Zand-Moghaddam[7].

RESULTS AND DISCUSSION

The detector will only function efficiently if all the chromatographic solvent is removed by evaporation prior to detection. Thus, as expected, the noise level was found to be dependent on both the evaporation temperature and the amount of water in the solvent, in this case aqueous acetonitrile. Figs. 2 and 3 show this effect and also that the detector response is temperature dependent. In the case of gradient elution, it is therefore necessary to adjust the evaporation temperature such that the noise level is acceptable at the highest water composition. In sugar analyses with amino columns this may mean compositions up to 50% (v/v) water.

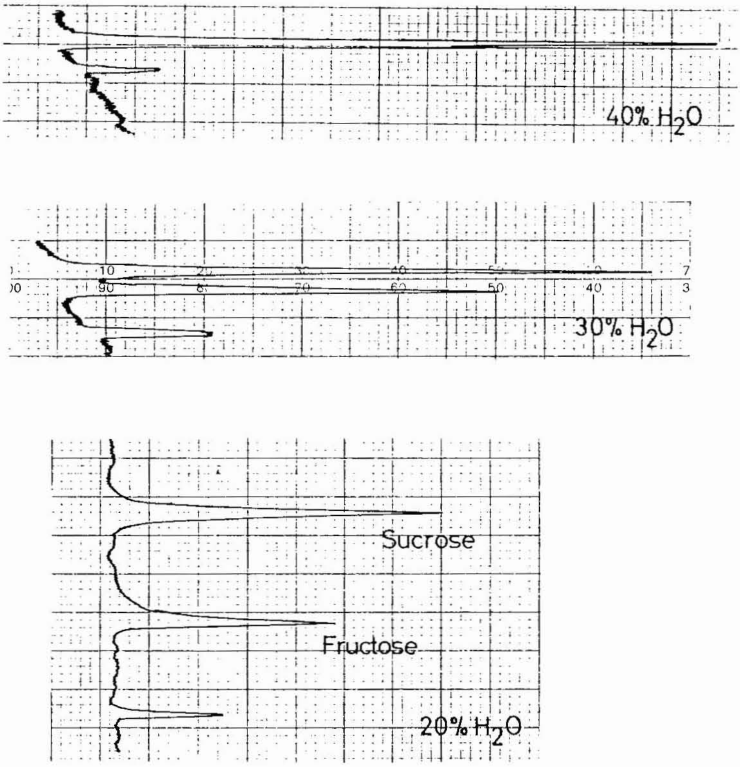

Fig. 3. Effect of solvent composition on noise level. Column Spherisorb-5-NH_2. Flow-rate 2 ml min^{-1}. Detector ×4, 100°C. Fructose and sucrose 20 μg.

The temperature dependence of the detector was confirmed with a complex mixture of monosaccharides as shown in Fig. 4. The pentoses, and the ketohexose, fructose, all show a marked decrease in response at elevated temperatures. In general, monosaccharides such as these would be analysed with solvents of low water content (15–25%, v/v) and thus there would be no need to operate at high temperatures to

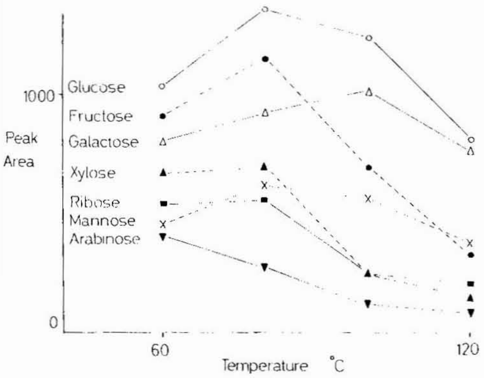

Fig. 4. Detector response variation with temperature. Column Spherisorb-5-NH_2. Solvent water–acetonitrile (11.5:88.5), 2 ml min^{-1}. All sugars 100 μg.

obtain a good baseline. Thus acceptable noise levels and high sensitivity can be achieved simultaneously.

The linearity of the detector was investigated with a glucose syrup of known composition. For glucose and maltose the response appeared to be linear over the range 10–200 μg on column, for which correlation data are shown in Fig. 5, with a clear negative deviation above this level. The sensitivity of the detector depends on the operating conditions, both in terms of signal output and signal-to-noise ratio, but 2–3 μg on column will produce a readily quantifiable peak as shown for fructose, glucose, sucrose and maltose in Fig. 6.

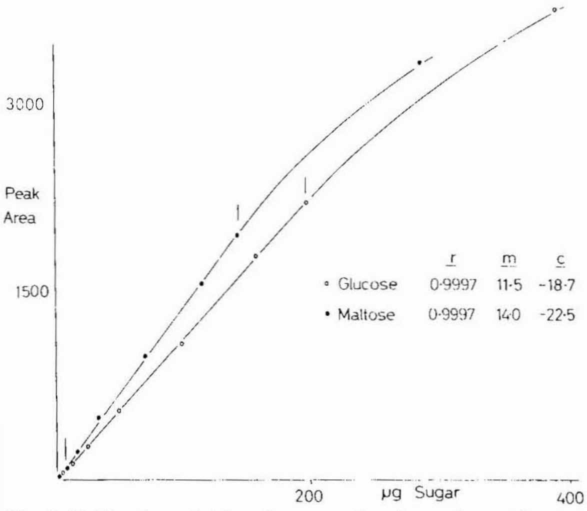

Fig. 5. Calibration plot for glucose and maltose, from glucose syrup. Column Spherisorb-5-NH_2. Solvent water–acetonitrile (35:65), 2 ml min^{-1}. Detector ×2, 120°C.

The ability to carry out gradient analyses allows the separation of complex mixtures of sugars including those that would normally require more than one chromatographic run. In the chromatogram shown in Fig. 7 mannose, glucose and galactose are adequately separated while maltose and sucrose are also eluted within a

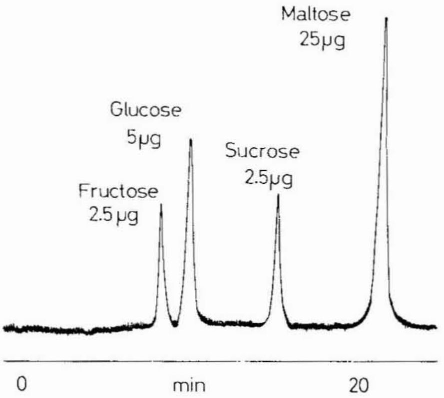

Fig. 6. Chromatogram of sugar standards. Column Spherisorb-5-NH_2. Solvent water–acetonitrile (20:80), 1.5 ml min^{-1}. Detector ×4.

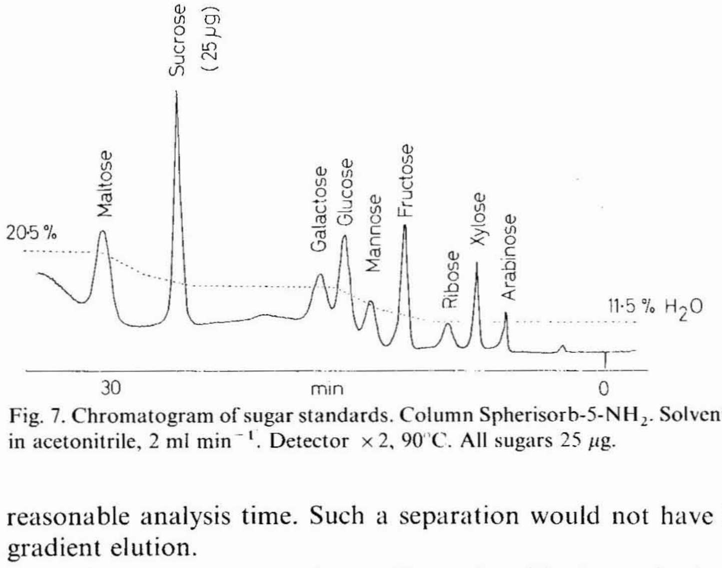

Fig. 7. Chromatogram of sugar standards. Column Spherisorb-5-NH_2. Solvent gradient 11.5–20.5% water in acetonitrile, 2 ml min^{-1}. Detector ×2, 90°C. All sugars 25 µg.

reasonable analysis time. Such a separation would not have been possible without gradient elution.

Glucose syrups can be readily analysed by isocratic elution. However, the use of gradient elution considerably reduces the analysis time and results in very little peak broadening even with dextrins up to degree of polymerization (DP) 12 (Fig. 8). This factor, coupled with the greater sensitivity of the mass detector, provides an elegant method for determining the small amounts of higher dextrins in syrups and malted products.

Under certain processing conditions monosaccharides can be formed in wheat germ and even low levels can be important in terms of the final product quality. In a chromatogram of a wheat germ extract shown in Fig. 9, which was produced isocratically, very small amounts could have been readily detected. In this case traces of unidentified disaccharides were also detected, which were not observed when a similar analysis was carried out with RI detection.

The oligosaccharide composition of lupin seeds and soyabeans has been re-

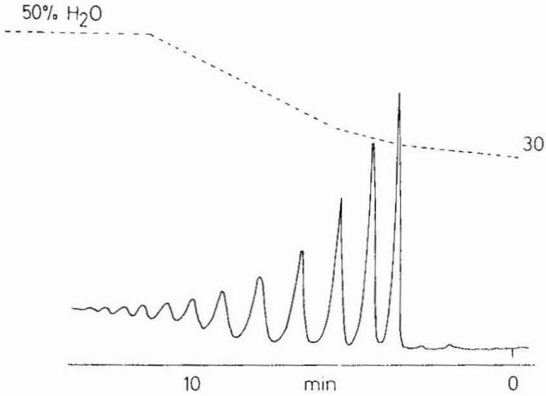

Fig. 8. Chromatogram of glucose syrup. Column Spherisorb-5-NH_2. Solvent gradient 30–50% water in acetonitrile, 2 ml min^{-1}. Detector × 1, 125°C.

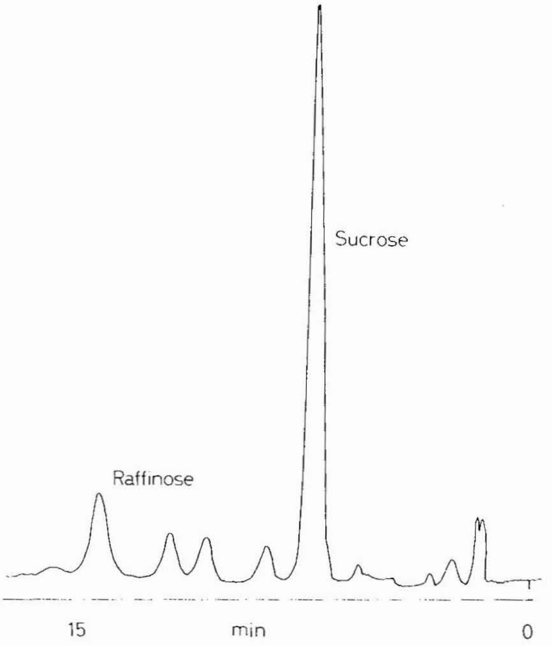

Fig. 9. Chromatogram of wheat germ extract. Column Spherisorb-5-NH_2. Solvent water–acetonitrile (30:70), 2 ml min^{-1}. Detector ×4, 100°C.

ported by Macrae and Zand-Moghaddam[7] using RI detection. These analyses were repeated using the mass detector and significantly improved chromatograms were obtained as shown in Figs. 10 and 11. Here again the ability to use gradient elution allowed improved separation of the monosaccharides, whilst verbascose (a pentasaccharide) was still eluted within a reasonable time. In the soyabean extract the small amount of verbascose present resulted in a small but readily quantifiable peak. Quantification was not possible with RI detection. The presence of dissacharides other than sucrose was also more clearly shown.

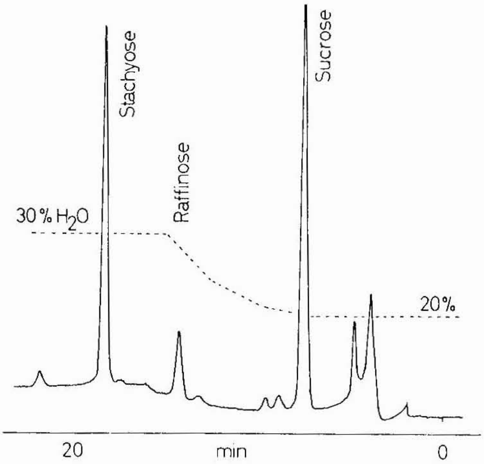

Fig. 10. Chromatogram of soyabean extract. Column Spherisorb-5-NH_2. Solvent gradient 20–30% water in acetonitrile, 2 ml min^{-1}. Detector × 1, 125°C.

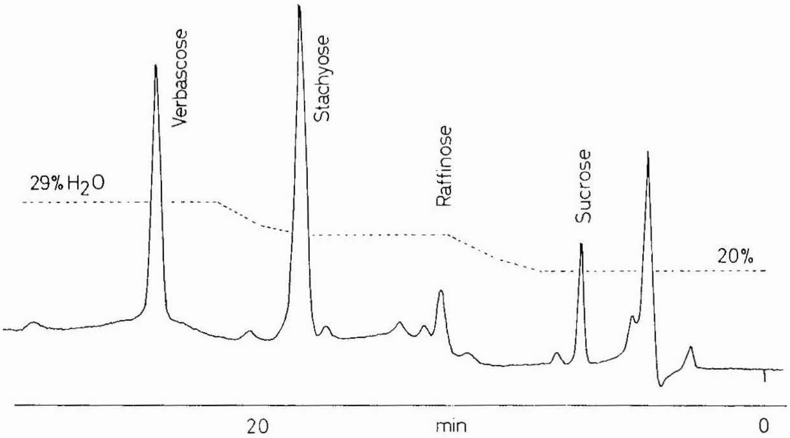

Fig. 11. Chromatogram of lupinseed extract. Column Spherisorb-5-NH_2. Solvent gradient 20–29% water in acetonitrile, 2 ml min^{-1}. Detector × 2, 125°C.

In this preliminary study the mass detector proved to have three major advantages over RI detection, namely increased sensitivity (in the order of a factor of ten), improved stability and the ability to run gradient elution. Thus, in spite of its more limited linear range, it will provide a much improved alternative to the well established use of RI detection.

ACKNOWLEDGEMENTS

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Note

Further calibration data for the gas chromatography of benzyl esters

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Benzyl esters have been found useful for gas chromatography (GC)[1–3] and GC–mass spectrometry (MS)[4–7] of carboxylic acids, especially short-chain and other low-molecular-weight species. Various data on GC retention times[1,2] of benzyl esters and MS studies[2,6,7] have been published; this note presents retention time data for further benzyl esters prepared during a mass spectral study. A range of chemical types has been included, with some of biological interest.

EXPERIMENTAL

Preparation of samples and esterification

Acids or salts were obtained from various commercial sources and used without further purification. Benzyl chloroformate was purchased as the ester. Tetrahydrofuran 2-carboxylic acid was prepared by platinum oxidation[8] of tetrahydrofurfuryl alcohol. Benzyl esters were prepared by esterification using diazotoluene (phenyldiazomethane) prepared from N-benzyl-N-nitroso-toluene-4-sulphonamide[9] as previously described[10]; excess solvent was removed under vacuum and the diazotoluene mixed with dry dimethylformamide to give an approximately 25% (v/v) solution. Acid salts were converted to the free acid using an ion-exchange resin[10] and dried or lyophilised. Esters were prepared by the addition of a slight excess of diazotoluene solution to the acid (1 to 2 mg) and on completion of the reaction the solution was diluted with ethyl acetate to give a final concentration of approximately 4 mg/ml.

Gas chromatography

Retention times were measured on a column packed with 3% OV-1 on Diatomite CQ, 100–120 mesh; column dimensions and other parameters were as previously described[1] except that column temperatures were as given in Table I. Identities of the peaks were confirmed by GC–MS[4] at GC column temperatures 30° higher than those in Table I.

RESULTS AND DISCUSSION

The results are summarised in Table I, which is divided into three temperature groups: (a) 140°, reference benzyl 2-bromopropionate, (b) 200°, reference benzyl

TABLE I

RETENTION DATA FOR BENZYL ESTERS OF CARBOXYLIC ACIDS

Column, 1.52 m × 4 mm I.D. glass, 3% OV-1 on Diatomite CQ, 100–120 mesh. Temperatures (°C) as indicated.

Parent acid	*Mol.wt. of benzyl ester*	*Relative retention*★
Group 1 —140° column, 200° injector		
Chloroformic	170	0.16
Pivalic (2,2-dimethylpropanoic)	192	0.51
Bromoacetic	229	0.65
2-Bromopropanoic	243	1.00[a]
Cyclohexylacetic	232	1.17
Tetrahydrofuran 2-carboxylic	206	1.18
Cyclopentylcarboxylic	204	1.21
Furan 2-carboxylic	202	1.46
3-Methoxybenzoic	242	1.84
trans-Cinnamic	238	1.97
Group 2 —200° column, 250° injector		
5-Bromofuran 2-carboxylic	281	0.33
2,6-Dimethylbenzoic	240	0.40
6-Bromohexanoic	285	0.43
Indole 2-carboxylic	251	0.92
1- and 2-naphthoic	262	1.00[b]
3,4,5-Trimethoxybenzoic	286	1.16
2-Naphthaleneacetic	276	1.27
2,3-Dihydroxymaleic	296	1.30
3-Hydroxy-2-naphthoic	278	1.53
Anthracene 9-carboxylic	312	1.85
Benzilic (2-Hydroxy-2,2-diphenylpropanoic)	318	1.95
Phenylcinnamic	314	2.03
Group 3 — 250° column, 300° injector		
11-Bromoundecanoic	359	0.46
Palmitic	346	0.59
Oleic	372	0.93
Linoleic	370	0.93
Linolenic	368	0.94
Stearic	374	1.00[c]
Naphthalene 2,3-dicarboxylic	396	1.36
Decane 1,10-dicarboxylic	382	1.53

★ Retention times (min) of reference esters: [a]11.9, [b]13.1, [c]12.3.

naphthoate and (c) 250°, reference benzyl stearate. (Retention characteristics of benzyl stearate have been published[1,6] but the ester is included here as a suitable reference and for comparison with the C_{18} unsaturated acid esters).

With regard to the previous use[1,4,6,7] of diazotoluene in light petroleum (b.p. 30–40°C) or diethyl ether, some difficulty was experienced with the solubility of certain acids in the present series. Use of dimethylformamide as a solvent for both diazotoluene and sample was found to overcome this difficulty and showed that dimethylformamide was an excellent solvent for the esterification system.

As observed previously[1], most esters eluted in approximately molecular weight order, but with the notable exception of the bromine-containing acids. The series benzyl bromoacetate, benzyl 2-bromopropionate, benzyl 5-bromofuroate, benzyl 6-bromohexanoate and benzyl 11-bromoundecanoate all gave much shorter retention times, relative to comparable molecular weight acids, of betweeen one third and one half of the predicted value.

In one aspect of this study, we investigated the esterification of acids exhibiting varying degrees of steric hindrance about the carboxyl group as judged by standard esterification techniques. In common with other diazo-esterifying reagents, and although the reagent possesses the relatively bulky benzyl group, esterification with diazotoluene proceeded rapidly (within 5 min) and smoothly with acids such as 2,6-dimethylbenzoic, 3-hydroxy-2-naphthoic, anthracene 9-carboxylic, phenylcinnamic, benzilic, naphthalene 2,3-dicarboxylic and pivalic. Dicarboxylic acids also reacted smoothly; certain shorter-chain dicarboxylic acid benzyl esters have been chromatographed previously[1,7]. The positional isomers 1- and 2-naphthoic acid benzyl esters did not resolve.

Fatty acid benzyl esters of the C_{18} group were tested on OV-1 in comparison with EGSS-X phase[6]. In common with observations of methyl esters on non-polar phases[11], $C_{18:0}$ resolved, albeit poorly, from others in the group, but $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ essentially co-eluted. This reflects the known property of OV-1 in often giving incomplete resolution of levels of unsaturation; the benzyl esters did not enhance the resolution. The separation of the benzyl esters of the C_{18} group on 10% SILAR-10C was similar to that of the methyl esters on this phase. Various aspects, of the esterification reaction and applications, in the use of diazotoluene have already been discussed[1,2,6,7].

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Note

High-performance liquid chromatographic determination of vincamine

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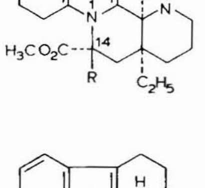
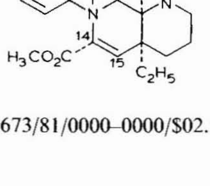
Vincamine (I) [eburnamenine-14-carboxylic acid, 14,15-dihydro-14-hydroxy-, methyl ester (3α, 14β, 16α)] is an efficient vasodilator for the treatment of the cerebral microcirculation system[1,2]. Pharmacokinetic studies of this product require a rapid, sensitive and specific assay. Methods for determining the vincamine content of biological fluids include radioisotope techniques[3] and gas chromatography–mass spectrometry[4,5]. However, these techniques are not suitable for routine analysis. More convenient and reliable methods for the determination of vincamine in blood by gas chromatography without a derivatization step[6] and by *in situ* spectrophotometry[7] have also been described. However, they are time consuming and lack sensitivity.

We report here a method for the measurement of vincamine using reversed-phase high-performance liquid chromatography (HPLC). Chromatography is performed at alkaline pH on bonded octadecylsilane with papaverine as an internal standard. The proposed method requires minimal sample preparation times and can determine as little as 5–10 ng of vincamine in 4 min. Moreover, the same system has been extended to the assay of vincamine either as a bulk drug or as a component of pharmaceutical preparations, even in the presence of the following impurities: 14-epivincamine (II) [eburnamenine-14-carboxylic acid, 14,15-dihydro-14-hydroxy-, methyl ester (3α, 14α, 16α)] and apovincamine (III) [eburnamenine-14-carboxylic acid, methyl ester (3α, 16α)].



I R = β –OH

II R = α –OH



III

EXPERIMENTAL

Materials

HPLC-grade methanol (LiChrosolv; Merck, Darmstadt, G.F.R.) was used. Water was distilled from an all-glass still and then passed through a 0.45-μm membrane filter (Millipore Type HA). Vincamine, 14-epivincamine and apovincamine are purchased from Ricerchimica (Milan, Italy) and papaverine and ammonium carbonate from Sigma (St. Louis, MO, U.S.A.).

Solutions

Standard solutions were prepared by dissolving vincamine and papaverine (0.02%, w/v), 14-epivincamine and apovincamine (0.004%, w/v) in methanol. Both liquid and solid dosage forms were weighed accurately and diluted with methanol to the desired volume (C_I = 0.02%, w/v).

To urine (diluted 1:10 with water) and plasma samples (0.5 ml) in a glass tube (10 ml) were added 4 μg of papaverine, 4–20 μg of vincamine and 200 μl of 1 *M* sodium hydroxide solution. The mixture was adjusted to 2 ml with distilled water to give diluted urine and plasma samples for assay by HPLC. Alternatively, diluted urine and plasma samples were extracted three times with 5 ml of diethyl ether by vortex mixing, followed by centrifugation. The ethereal phases were transferred by Pasteur pipette into a small tube, dried over anhydrous sodium sulphate and evaporated to dryness at 30°C under a stream of dry nitrogen. The residue was dissolved in 200 μl of methanol and vortexed for 20 sec, then 10 μl of this solution were injected into the liquid chromatograph.

Chromatographic conditions

The chromatographic system consisted of a Model 6000 A solvent delivery system, a Model U6K universal injector, a Model 440 ultraviolet detector and a Model 730 data module (Waters Assoc., Milford, MA, U.S.A.). Ultraviolet absorption was measured at 254 nm. Samples were chromatographed at room temperature over a 300 × 3.9 mm I.D. stainless-steel column packed with μBondapak C_{18} (10 μm) (Waters Assoc.). To extend the column life a pre-column packed with Bondapak C_{18}/Corasil was inserted between the pump and the injector. The mobile phase was methanol–0.01 *M* ammonium carbonate (75:25, v/v) at a flow-rate of 2 ml/min. The pH of the mobile phase was about 7.8–7.9; retention times were constant to ±2% over a 30-day period.

RESULTS AND DISCUSSION

Papaverine, 14-epivincamine, vincamine and apovincamine had capacity factors of 1.3, 2, 3.7 and 7.2, respectively (Fig. 1).

Calibration graphs of peak-height ratio (vincamine to papaverine) *versus* vincamine concentration in the range 0.2 to 0.8 μg/ml were constructed from give replicate injections (Fig. 2).

The determination of vincamine in methanol extracts of bulk drugs or pharmaceutical preparations and in diluted urine and plasma and in ether extracts thereof was studied (Fig. 3).

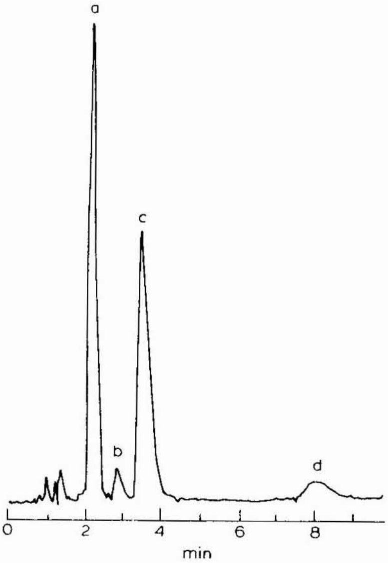


Fig. 1. High-performance liquid chromatogram of (a) papaverine, (b) 14-epivincamine, (c) vincamine and (d) apovincamine.

The results suggest that for urine samples ether extraction is not necessary, and direct analysis of diluted urine (1:10) is preferred. The mean recovery in the specified concentration ranges was 99.8 ± 1.5% (n = 12) and the precision over a 30-day period was ±3.5%, using different urine samples. For plasma samples, ether extraction is recommended in order to prevent contamination of the column by plasma proteins. Ether extraction, although time consuming, results in a more accurate pro-

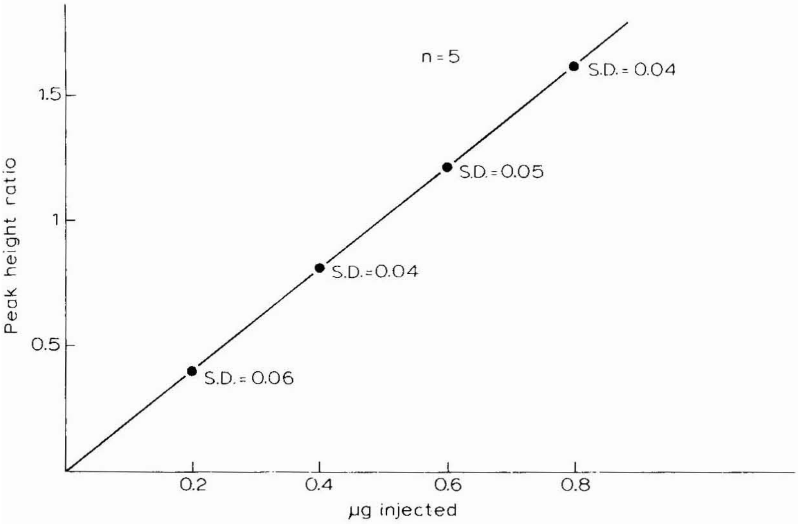


Fig. 2. Calibration graph of peak-height ratio (vincamine to papaverine) *versus* amount of vincamine injected.

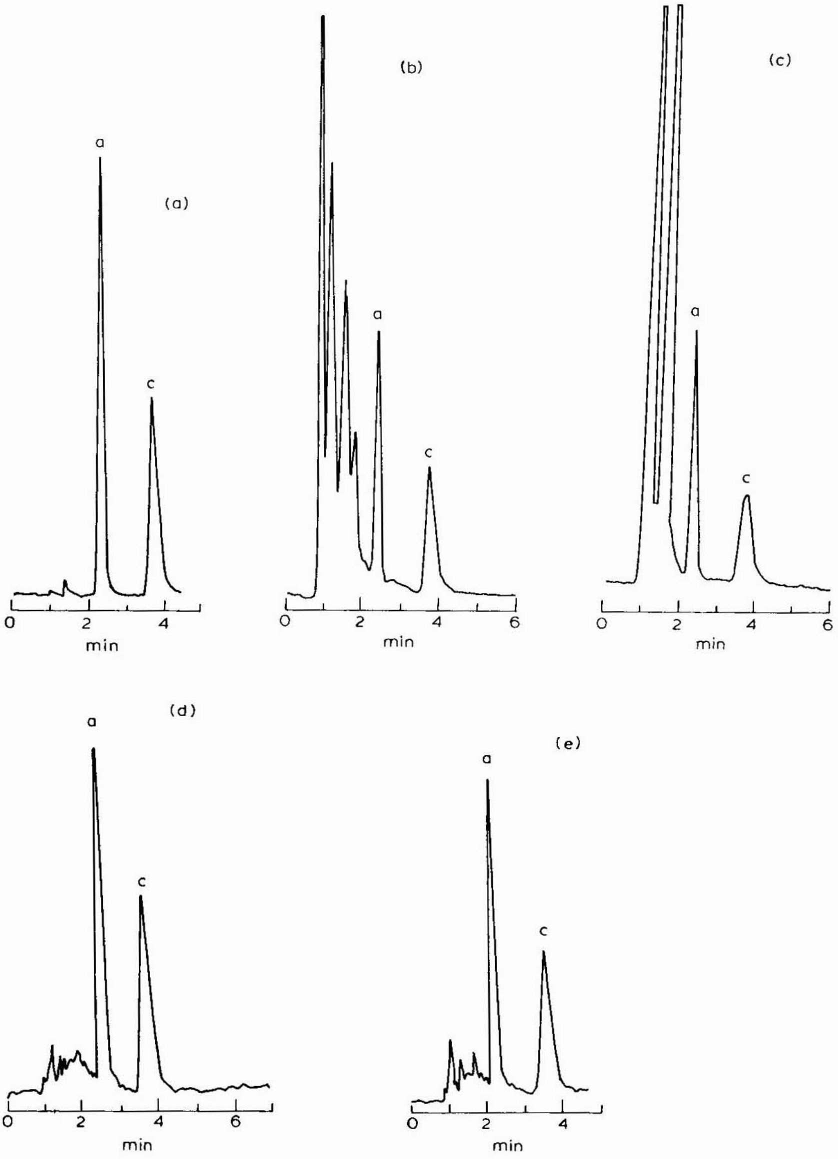


Fig. 3. High-performance liquid chromatogram of internal standard and vincamine in drugs and urine and plasma samples. Eluent: methanol–0.01 *M* ammonium carbonate (75:25, v/v), flow-rate 2 ml/min. Column: 300 × 3.9 mm, μBondapak C_{18}. UV detection at 254 nm. (a) Drugs; (b) diluted urine; (c) diluted plasma; (d) ether extract of diluted urine; (e) ether extract of diluted plasma.

cedure, with a mean extraction recovery of $100.5 \pm 1.3\%$ ($n = 12$) and a precision of $\pm 2.9\%$.

The HPLC assay described is simple, rapid and provides a sensitivity of 5–10 ng of vincamine in plasma and urine. No interfering peaks were noted in plasma or urine samples from different sources. Moreover, the urine assay allows the quantitation of total vincamine in urine, as the conjugated drug is easily hydrolysed at alkaline pH.

In conclusion, the proposed method is accurate, sensitive and offers a useful routine alternative to gas chromatography or *in situ* spectrophotometry.

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* *Editor's Note:* See also the paper by C. Dubruc, H. Caqueret and G. Bianchetti, *J. Chromatogr.*, 204 (1981) 335–339.

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Note

High-performance liquid chromatographic analysis of pyrrolizidine (*Senecio*) alkaloids using a reversed-phase styrene–divinylbenzene resin column

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(Received February 9th, 1981)

Pyrrolizidine alkaloids (PAs) have attracted considerable attention due to their hepatotoxic effects[1] and occurrence in a large number of plant species[2]. PAs have caused significant losses of livestock[3] and have been implicated in human poisoning[4]. *Senecio jacobaea* (tansy ragwort) and *S. vulgaris* (common groundsel) are two common PA-containing plants occurring in the Pacific Northwest. Tansy ragwort is responsible for serious livestock losses in that area[5] and contamination of milk[6] and honey[7] by its alkaloids has been reported.

We have previously used[8] a C_8 reversed-phase high-performance liquid chromatographic (HPLC) system for the analysis of *Senecio* PAs. This method, as well as one using other reversed-phase columns[9,10], has certain drawbacks, however, such as shortened column life due to the relatively high pH of the buffers used, significant peak tailing with resulting limitations in resolution, limited sensitivity with gradient analysis because of solvent (methanol or tetrahydrofuran) absorption and pump seal wear from buffer salts. We report here a method using a reversed-phase styrene–divinylbenzene resin HPLC column that overcomes most of these problems and which may be ideally suited for use in quantitative analysis of the PAs.

MATERIALS AND METHODS

S. vulgaris plants and *S. jacobaea* flower tops were collected in the vicinity of Corvallis, OR, U.S.A., during May and July of 1979, respectively. Inorganic chemicals used were reagent grade (Mallinckrodt, St. Louis, MO, U.S.A.) and extraction solvents were glass distilled in our laboratory. The PAs jacobine, jacoline, senecionine and seneciphylline were isolated from recrystallized *S. jacobaea* extracts by preparative reversed-phase HPLC. The recovered PAs were recrystallized and their purity verified by analytical HPLC, melting point and gas chromatographic–mass spectrometric analysis.

Extracts were prepared from each plant species in the same manner, following the method used by Culvenor and co-workers[2,11] and others. The plant material was extracted with methanol in a Soxhlet apparatus for 16–24 h, the extract filtered and the solvent removed at reduced pressure. The residue was taken up in a mixture of *n*-

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hexane and 0.2 *N* sulfuric acid. The phases were separated and then the aqueous layer was extracted three times with *n*-hexane and four times with dichloromethane. The alkaloid N-oxides were reduced by adding zinc dust to the acidic aqueous solution and the mixture was stirred for 1 h. It was then filtered and the filtrate was extracted four times with dichloromethane. The pH of the aqueous phase was then adjusted to 10.5 with concentrated NH_4OH. The solution was then extracted three times with dichloromethane. The combined organic fractions were dried over anhydrous Na_2SO_4. After filtration, the solvent was removed at reduced pressure. The extracts were dried over NaOH *in vacuo*.

A PRP-1 reversed-phase resin column (15 cm × 4.1 mm) (Hamilton, Reno, NV, U.S.A.) was used for the separation of the PAs. HPLC was performed on an Altex Model 322 chromatograph equipped with a Schoeffel SF-770 detector set at 220 nm. The chromatograms were recorded with a Spectra-Physics 4100 integrator.

The mobile phase consisted of acetonitrile (HPLC grade; J. T. Baker, Phillipsburg, NJ, U.S.A.) and 0.1 *M* NH_4OH made up using water purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Loss of NH_3 from this solution via volatilization did not cause any appreciable variation in results if the NH_4OH solution was used within two days of its preparation. *S. jacobaea* alkaloids were separated with a 20-min linear gradient of 10% to 30% acetonitrile. Separation of *S. vulgaris* alkaloids was achieved isocratically with 25% acetonitrile. A flow-rate of 1 ml/min was used in both cases.

Samples were dissolved in methanol–water (1:1) for injection. Peaks were collected and the solvents removed under vacuum. Direct probe electron impact mass spectra were obtained on a Varian CH-7 spectrometer coupled with a Systems Industries 150 computer.

RESULTS

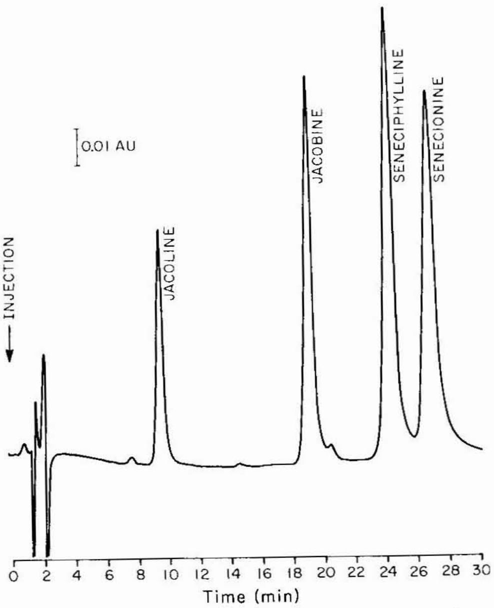
Fig. 1 shows the separation of a mixture of pure jacoline, jacobine, seneciphylline and senecionine chromatographed with a 10 to 30% acetonitrile–NH_4OH gradient. The peaks are essentially symmetrical and retention times correspond to those of peaks in the chromatogram of a *S. jacobaea* extract (Fig. 2). Mass spectrometry of collected fractions from the crude *S. jacobaea* extract yielded the following assignments by comparison with published PA spectra and fragmentation patterns[2,7,10,12]: peak 3 = jacoline, peak 8 = jacozine, peak 9 = jacobine, peak 10 = jaconine, peak 11 = seneciphylline and peak 12 = senecionine.

Peaks 1, 2, 4 and 7 do not appear to be PAs. Peaks 5 and 6 gave spectra containing fragments characteristic of *Senecio* PAs but these compounds have not yet been identified.

Fig. 3 shows a chromatogram of a *S. vulgaris* extract obtained with the isocratic system. As with the gradient system, the peak shape is good. The major peaks were identified by comparison of the mass spectra with those available in the literature[2,7,12]: peak 1 = retrorsine, peak 2 = seneciphylline, and peak 3 = senecionine.

Fig. 4 shows detector response curves for jacoline, jacobine, senecionine and seneciphylline. Response (in area units) is linear over a 40-fold range of PA injected.

Three 1.00-g samples of dried *S. jacobaea* flowers were extracted and analyzed for jacoline, jacobine, seneciphylline and senecionine content using the 10–30% ace-



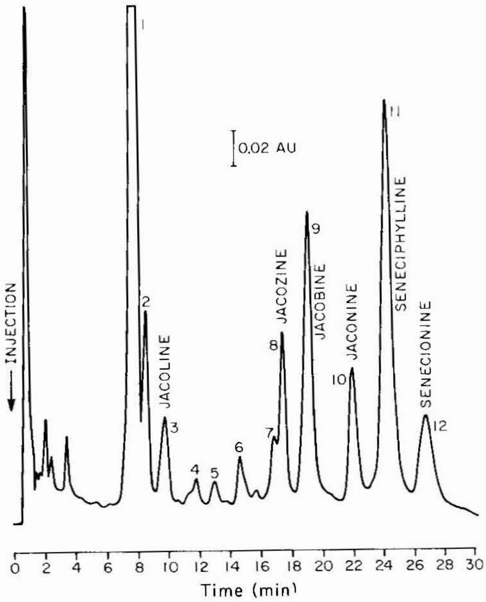


Fig. 1. Separation of a mixture of pure PAs. Solvent: 1 ml/min acetonitrile (10% to 30% in 20 min)–0.1 *M* NH_4OH.

Fig. 2. Separation of a *S. jacobaea* extract. Conditions as in Fig. 1.

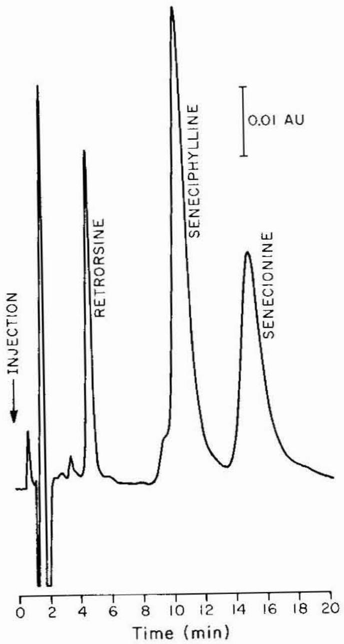


Fig. 3. Separation of a *S. vulgaris* extract. Solvent: isocratic, 1 ml/min, acetonitrile–0.1 *M* NH_4OH (25:75).

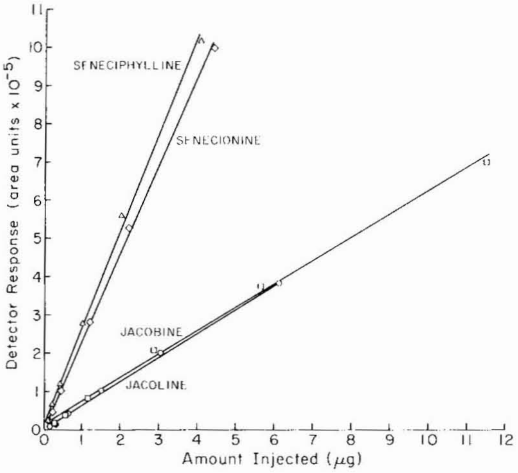


Fig. 4. Detector response *vs.* amount injected. Conditions as in Fig. 1. Each data point represents a single determination.

tonitrile gradient system. Average values for these PAs in µg/g dry weight (± standard deviation) were, respectively, 155 (± 17), 1019 (± 35), 405 (± 20) and 148 (± 12). Total PA concentrations in dried *S. jacobaea* flowers have been previously reported to be 0.15–0.30%[7] and 0.2%[13].

DISCUSSION

A styrene–divinylbenzene resin column allows use of high pH mobile phases that would destroy silica-based columns. One result is good peak symmetry for basic compounds without the use of ion-pairing reagents. The acetonitrile–NH_4OH system offers several advantages. Preparative work is simplified since the mobile phase solvents can be directly evaporated under vacuum, thus avoiding the extractions necessary when using phosphate buffers. The low absorbance of HPLC-grade acetonitrile at 220 nm allows gradient analysis of PAs and high sensitivity without prohibitive baseline shifts. The problems associated with the use of solid buffer salts (including increased pump seal wear) are avoided with this system.

The method presented here will be useful for analysis of small samples of *S. jacobaea* and *S. vulgaris*. It should prove useful for analysis of other PA-containing extracts as well.

ACKNOWLEDGEMENTS

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* *Editor's Note:* See also H. J. Huizing and Th. M. Malingré, *J. Chromatogr.*, 176 (1979) 274.

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Note

High-performance liquid chromatographic determination of grandaxin-(a 2,3-benzodiazepine) and its trace impurities

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Grandaxin [1-(3,4-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-5H-2,3-benzodiazepine], an anxiolytic agent marketed by Egyt (Budapest, Hungary) is synthesized as shown in Fig. 1[1]. For the simultaneous determination of grandaxin and its trace impurities (compounds III and IV), high-performance liquid chromatography (HPLC) proved to be superior to either thin-layer or gas chromatography in terms of efficiency and loadability.

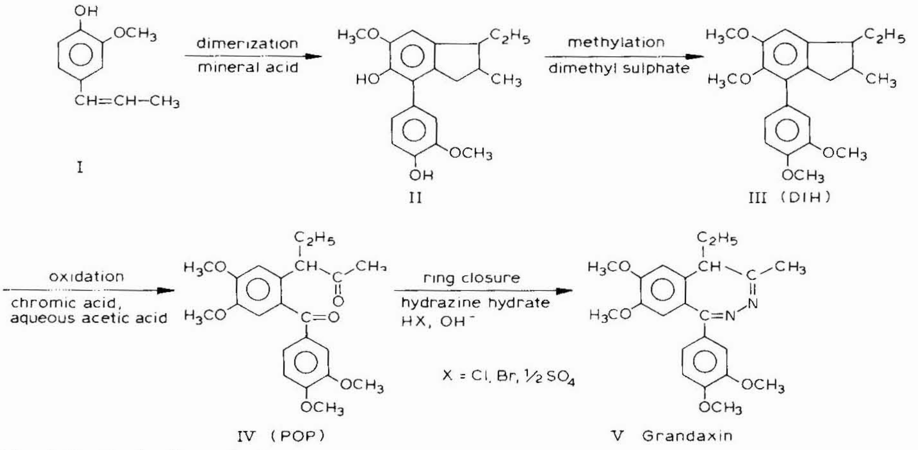

Fig. 1. Synthesis of grandaxin.

This paper reports normal- and reversed-phase HPLC methods and the sensitivity that was achieved for the trace components by optimization of column parameters. The method has also been used for the examination of grandaxin-containing tablets.

EXPERIMENTAL

Chromatographic separations were performed on a Varian 8500 liquid chromatograph equipped with a Variscan variable-wavelength UV-visible detector (231

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nm) and a Model A-25 strip-chart recorder. Two of the HPLC columns and eluents tested are specified in the legends of Figs. 2 and 3, the latter being the system of choice for the determinations. Spherisorb S10 W (10 μm) and LiChrosorb RP-8 (5 μm) packing materials were supplied by Phase Separations (Queensferry, Great Britain) and Merck (Darmstadt, G.F.R.), respectively. The columns were slurry-packed in our laboratory by an ascending technique[2]. Dioxane (Chrom AR grade) was purchased from Mallinckrodt (St. Louis, MO, U.S.A.) and acetonitrile, *n*-hexane (Uvasol) and methanol (Selectipur) were obtained from Merck. Grandaxin and tablets of grandaxin were prepared by Egyt. Other chemicals were of the highest grade available.

When tablets of grandaxin were examined, the extraction procedure was as follows. Five tablets (each consisting of 50 mg of grandaxin, 92 mg of lactose, 20.5 mg of amylum solani, 10 mg of microcrystalline cellulose, 3.5 mg of gelatine, 2 mg of stearine and 2 mg of talcum) were weighed and powdered. A portion of the powder (equivalent to 25 mg of grandaxin) and 3.3 ml of solvent [dioxane–acetonitrile–methanol (25:5:3)], the polar part of the eluent, were placed in a stoppered test-tube. The mixture was kept in a 50°C water-bath in an ultrasonic apparatus (Heat Systems Ultrasonics, Plainview, NY, U.S.A.) for 20 min, then cooled and 6.7 ml of *n*-hexane were added. The mixture was shaken and allowed to stand for a few minutes, then 40 μl of the clear supernatant liquid were injected (via a stop-flow septumless injection system) for the determination of the trace components and 2–5 μl when only the grandaxin content was to be determined. The efficiency of the extraction procedure was over 99%, so no internal standard was used.

When examining crystalline grandaxin, the substance was dissolved in the eluent to give a 1 mg/ml solution, of which 5 μl was injected.

Calculations were based on calibration graphs prepared with the corresponding reference standards (peak height *versus* amount injected).

RESULTS AND DISCUSSION

The detection wavelength was chosen with a view to obtaining the highest sensitivity for III, the component with the weakest UV absorption and the smallest concentration (see DIH in Fig. 1 and Table I). When optimizing the column parameters from the point of view of the lowest possible detection limits, we relied mainly on the work of Karger *et al.*[3].

As the trace compounds (III and IV) exhibit a significant difference in polarity (see Figs. 1 and 2), optimization for both is impossible in reversed-phase systems

TABLE I

UV ABSORPTION DATA FOR GRANDAXIN AND ITS POTENTIAL IMPURITIES

UV absorption data	*III (DIH) (diisohomogenol)*		*IV (POP) (primary oxidation product)*			*V (grandaxin)*		
$\lambda_{\max.}^{\text{ethanol}}$ (nm)	231	280	234	279	312	238	273	311
$A_{1\ \text{cm}}^{1\ \%}$	436	209	654	322	340	686	292	450

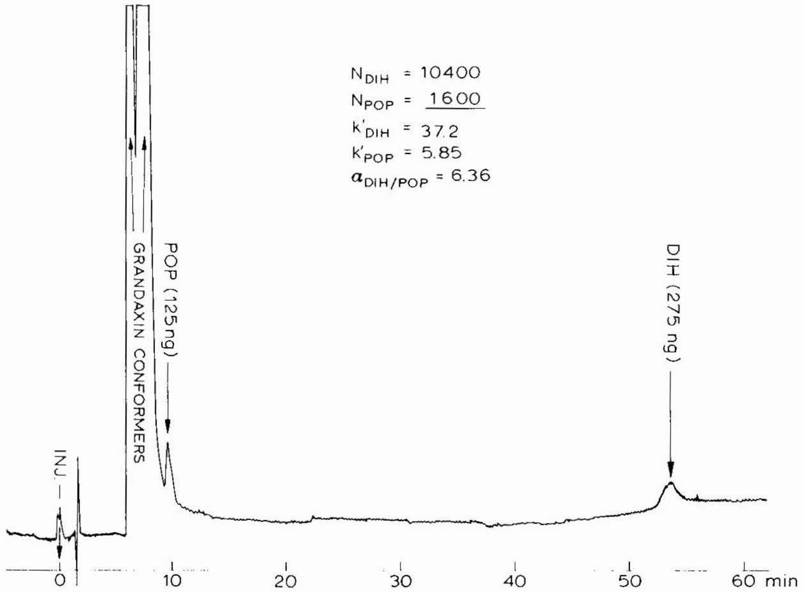

Fig. 2. Separation of grandaxin and its intermediates by reversed-phase HPLC. Sample, grandaxin containing DIH and POP as impurities; column, LiChrosorb RP-8, 200 × 4.6 mm I.D.; eluent, acetonitrile–1% (w/v) ammonium carbonate water (40:10:50); flow-rate, 50 ml/h; chart speed, 30 cm/h; temperature, ambient.

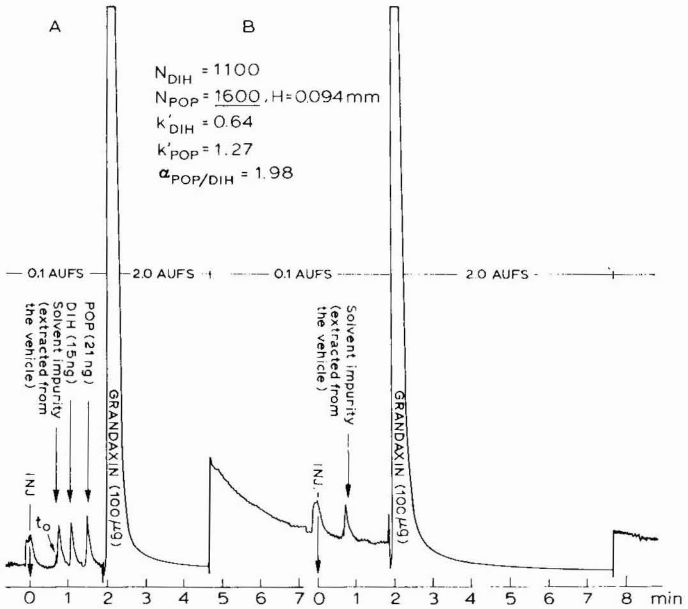

Fig. 3. Separation of grandaxin and its intermediates by normal-phase HPLC. Samples, tablet extract containing DIH and POP as impurities (A) and tablet extract with no DIH and POP (B); column, Spherisorb S10 W, 150 × 3 mm I.D.; eluent, *n*-hexane–dioxane–acetonitrile methanol 25% (w/v) ammonia (67:25:5:3:0.02); flow-rate, 50 ml/h; chart speed, 100 cm/h; temperature, ambient.

($\alpha_{DIH/POP}$ = 6.3–8.0), and there are also problems in normal-phase systems ($\alpha_{POP/DIH}$ = 2–4).

In a satisfactorily optimized normal-phase system (see Fig. 3) capacity factors (k') of III and IV were in the optimal range (0.5–2.0), but the resolution (R_s) was still about twice as high as the optimal value (2.8 instead of 1.5). To achieve optimal resolution with selectivity factor $\alpha = 2$ and $k'_2 = 1.27$, a column with only 460 plates would be needed. With the theoretical plate height (H) in Fig. 3, this would mean an optimal column length of 4.3 cm, and a column length of about 0.5 cm or below, if efficient columns ($H \leqslant 0.01$ mm) are used. Although such short columns cause little dilution, they make high demands on minimizing the extra-column band broadening effects (dead volumes) and on the dynamic characteristics of the detection and recording units. For instance, the reduction of the column length to the theoretical optimum (4.3 cm in our case) resulted in a poorer than expected resolution, owing to the increased role of the dead volume in the lines between the injector and the column head (the adverse effect of this dead volume is demonstrated by the fact that, even with longer columns, substantially lower plate numbers were obtained for early peaks than for highly retained ones; see Figs. 2 and 3).

Thus, as control of α by gradient elution is rarely possible in sensitive trace analysis, it is reasonable to accept that combined optimization with component pairs having an α value greater than 2 is technically a greater problem than it is with components of fairly similar nature (*e.g.*, when α = 1.08–1.10).

In the system presented in Fig. 3, detection limits of DIH and POP were 2.5 and 3.0 ng, respectively. The detection limit for grandaxin, which may be of use in metabolism studies, was 2.5 ng at 238 nm. The precision was ±1.5% for grandaxin and ±3.5% for the trace components.

Grandaxin is eluted as double peaks in reversed-phase systems (see Fig. 2) and also in some normal-phase systems (with plate numbers higher than 3000). The explanation lies in the fact that, in solution, the molecule exists as a mixture of two boat conformers[4]. The ratio of conformers is a function of time, temperature and solvent.

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Note

Determination of psilocybin in *Psilocybe semilanceata* using high-performance liquid chromatography on a silica column

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During the last few years an increasing number of young Norwegians have used *Psilocybe semilanceata* (Fr. *ex* Secr.) Kummer as a narcotic. The first report on misuse of this mushroom was made in autumn 1977, and knowledge of its hallucinogenic properties has gradually become public through reports in the popular media. The ingestion of *P. semilanceata* has in some cases resulted in the need for treatment in hospital[1,2].

Many species of the genus *Psilocybe* are found in Norway[3], but only *P. semilanceata* is regarded as hallucinogenic. It occurs on grassy sites in most parts of the country from the middle of August to the middle of October. *P. semilanceata* is known to contain indole alkaloids, of which psilocybin is considered to be the main constituent. In order to carry out detailed studies of the potency of Norwegian *P. semilanceata* a quantitative method was required for the assay of psilocybin.

Several methods have previously been used for analysis of the hallucinogenic components of *Psilocybe* mushrooms. Both paper chromatography[4,5] and thin-layer chromatography[6,7] have been employed in conjunction with colorimetric reagents as well as with UV spectroscopy. Gas chromatography and gas chromatography–mass spectrometry[8] have been applied for the analysis of psilocin and psilocybin.

Recently two high-performance liquid chromatographic (HPLC) methods have been published[9,10]. White[9] separated the three compounds psilocin, psilocybin and baeocystin on a silica column, and Perkal *et al.*[10] described the quantitation of psilocin and psilocybin by ion-exchange chromatography. We have developed a HPLC method based on a silica column which provides a simple, rapid and accurate quantitation of the psilocybin content of Norwegian *P. semilanceata.*

EXPERIMENTAL

Chemicals

Psilocin and psilocybin were supplied by Sandoz (Basel, Switzerland). Analytical-grade methanol was obtained from E. Merck (Darmstadt, G.F.R.).

Apparatus

A reciprocating pump (Glenco Scientific, Houston, TX, U.S.A.) was used to

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deliver solvent at 1 ml min^{-1}, and a Spectra-Physics UV detector (Model 225) was used to monitor the eluent at 254 nm. A Kontron® spectrofluorometer SFM23 was used for the fluorimetric detection. Samples were introduced through a Rheodyne (Model 7120) rotary valve injector which was equipped with an external 10-μl sample loop. The column was a 25 cm × 4.6 mm I.D. stainless-steel tube, slurry-packed with small-particle silica (6-μm Partisil 5, Whatman, Maidstone, Great Britain). Separation of the components was achieved with methanol–water–1 *N* ammonium nitrate solution (220:70:10). The 1 *N* ammonium nitrate solution was buffered to pH 9.6 with 2 *N* ammonia. A VG Micromass 7070F mass spectrometer was used for the mass spectrometric investigation.

Extraction procedure

The mushrooms were dried in an oven at 50°C overnight. One accurately weighed mushroom was ground to a powder in a mortar and transferred to a glass-stoppered centrifuge-tube. A 3-ml volume of 10% 1 *N* ammonium nitrate in methanol was added and the tube was rotated for 30 min using a rotary mixer (Cenco Instruments). After centrifugation the supernatant was transferred to a 5-ml volumetric flask, and the extraction was repeated with 2 ml of the extraction solution. The two extracts were combined and diluted in 10% 1 *N* ammonium nitrate in methanol to 5.00 ml.

Quantitation

Quantitative analyses of psilocybin were based on peak height measurements. UV detection was used for routine analyses of mushroom extracts. A calibration graph was constructed for the concentration range 0.005–0.2 mg/ml. The relative standard deviation was calculated after eight assays of each of the three solutions at 0.01, 0.05 and 0.2 mg/ml. Fluorescence detection was used as a comparative method to check for interferences. A standard curve was constructed for the concentration range 0.05–0.2 mg/ml.

The calibration graphs of y (the peak height of psilocybin) against x (the concentration of psilocybin in mg/ml) were calculated according to the method of least squares.

RESULTS AND DISCUSSION

Extraction procedure

For routine HPLC analyses of the psilocybin content in dried mushrooms, a rapid and efficient extraction procedure was necessary. Methanol with 10% 1 *N* ammonium nitrate was employed as extraction solution. The mushroom extracts were stable for several weeks and had a similar composition to the mobile phase. During the drying procedure, the mushrooms lost 92% in weight.

A one-step extraction procedure for Australian *Psilocybe* species has recently been reported[10]. Homogenization for 2 min in 3 ml methanol was considered to be optimal for extraction of the active components. But Australian *P. subaeruginosa* has a low psilocybin content (0.01–0.2%) compared to the Norwegian *P. semilanceata* which we have found to be much more potent[11]. A one-step extraction procedure with 5 ml of the extraction solution was investigated, but only 91% of the psilocybin content was extracted. The yield of psilocybin was not increased by performing the extraction at 60°C. It therefore seemed clear that extraction of Norwegian *P. semilanceata* required more than one step.

Fig. 1 shows the percentage yield of psilocybin after successive extractions with 3 ml, 2 ml, 1 ml and 1 ml of 10% 1 *N* ammonium nitrate in methanol. The proposed two-step extraction procedure guarantees that at least 98% of the total psilocybin is extracted, which is considered to be satisfactory. All the detectable mushroom components seemed to be extracted to similar extents.

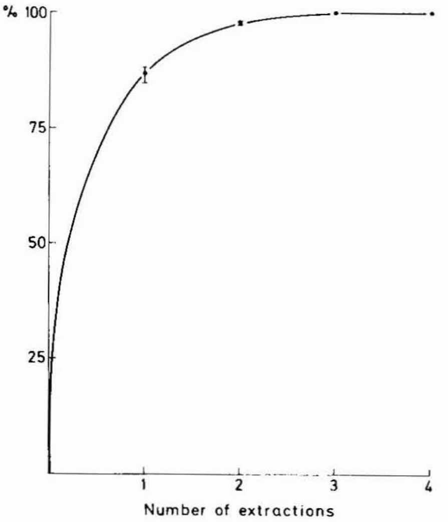

Fig. 1. Percentage yield of psilocybin (the mean value from three different mushrooms) after successive extractions with 3 ml, 2 ml, 1 ml and 1 ml of 10% 1 *N* ammonium nitrate in methanol.

Chromatography

The Australian[10] and English[9] HPLC examinations of *Psilocybe* mushrooms resulted in chromatograms having two and three peaks, respectively. The above conditions gave at least four distinct peaks, and the total analysis time was about 13 min. Typical chromatograms of Norwegian *P. semilanceata* are shown in Fig. 2. Peaks 1, 2 and 3 have not yet been identified. Baeocystin and norbaeocystin (the monomethyl and demethyl analogues of psilocybin) have previously been isolated from *P. baeocystis* grown in submerged culture[12]. The presence in English *P. semilanceata* of a compound provisionally identified as baeocystin has been reported[9]. It is possible that one or both of these substances are present. Further investigation of the chemical constituents of Norwegian *P. semilanceata* will be carried out.

From Fig. 2 it is seen that psilocin is eluted just after psilocybin. This is in contrast to the results obtained by a similar chromatographic system with Partisil 5 as stationary phase and methanol–water–1 *N* ammonium nitrate solution (240:50:10), buffered to pH 9.7 with ammonia (sp.gr. 0.88), as mobile phase[9]. Under these conditions psilocin was eluted much earlier. Because of this large variation in the retention of psilocin, the identity of our psilocin standard was verified by mass spectral analysis.

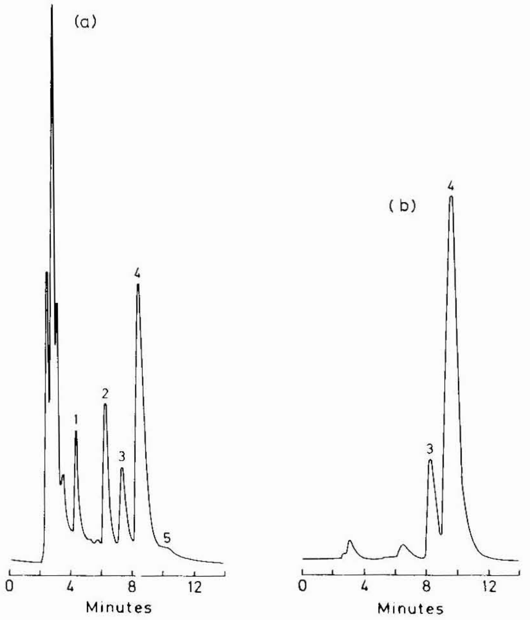

Fig. 2. Liquid chromatograms of Norwegian *P. semilanceata* extract. Detection by UV absorption at 254 nm (a) or by fluorescence at 335 nm (excitation at 267 nm) (b). Peaks: 1, 2, 3 = unknown; 4 = psilocybin; 5 = psilocin.

Separation of the components was also studied on ODS columns and the more polar Spherisorb Phenyl column, but the very polar and water soluble psilocybin did not seem to be sufficiently retarded.

Quantitative analysis

By using UV detection, a calibration graph was obtained which could be described by the equation $y = 54.73x - 0.02$ with a correlation coefficient of 0.9999. The relative standard deviations were 4.7%, 2.4% and 2.5% respectively for 0.01, 0.05 and 0.2 mg/ml solutions of psilocybin.

To check the results obtained by UV detection, a fluorescence spectrometer was used to monitor the eluent. Psilocybin has been found to fluoresce strongly at 335 nm with excitation at 267 nm[10], and the fluorescence detector was set at these wavelengths. Excellent linearity was obtained in the psilocybin fluorescence response. The standard curve could be described by the equation $y = 98.61x - 0.14$ with a correlation coefficient of 0.9993.

Mushroom extracts

Quantitative data for the psilocybin content in nine different mushrooms are given in Table I. The results obtained by UV absorption and fluorescence detection are well correlated. The fluorescence method is very specific for psilocybin, and the excellent agreement indicates that interferences can be neglected.

All the extracts analyzed contained various amounts of psilocybin and at least

TABLE I

QUANTITATIVE HPLC DATA FOR PSILOCYBIN IN DRIED MUSHROOM SAMPLES

Sample No.	*Psilocybin content (%, w/w)*	
	UV detector	*Fluorescence detector*
1	0.73	0.75
2	1.00	1.01
3	0.82	0.83
4	0.65	0.64
5	0.72	0.75
6	0.70	0.72
7	0.58	0.57
8	0.55	0.58
9	0.77	0.77

three unidentified components. Only traces of psilocin could be detected in some mushrooms.

The method described has been used in an extensive study of the potency of Norwegian *P. semilanceata*. The mushrooms were found to contain 0.2–2.0% psilocybin[11]. Norwegian *P. semilanceata* must therefore be regarded as a potent narcotic.

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Note

Separation and detection of synthetic food colors by ion-pair high-performance liquid chromatography

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In recent years concern has arisen over the extensive use of synthetic coloring matter in foods. In order to prevent indiscriminate use, regulations have been developed by many countries limiting the types, purity, uses and quantities of food colors permitted in foods. As a result the need has arisen to continuously monitor foods for permitted and non-permitted food colors particularly in imported foods. In the past, methodology for both qualitative and quantitative determinations have incorporated techniques such as paper and thin-layer chromatography[1–5], titration[6,7], electrophoresis[8] and spectrophotometry[9,10].

In recent years, high-performance liquid chromatography (HPLC) has been shown to have much potential for synthetic food color analysis in terms of qualitative identification, quantitation and speed of analysis[11–17]. Although most of these used ion-exchange with gradient elution or were employed to determine impurities in perhaps a single color, they clearly illustrated the power of HPLC in the area of food colors. Reversed-phase ion-pair chromatography has also been found to be particularly useful for the separation and detection of a large number of food colors[18,19]. This technique is particularly convenient because it makes use of a reversed-phase column which, when not employed for ion-pair chromatography, can be used for usual reversed-phase analyses. We report here the use of an ion-pairing chromatographic technique for the separation and quantitation of twelve primary food colors or subsidiaries with application to the analysis of several food colors in grape beverages.

EXPERIMENTAL

Reagents

Stock solutions of the colors (except Erythrosine) were prepared at concentrations of 0.01–0.02% in water containing 0.005 *M* tetra-*n*-butylammonium phosphate (TBAP).

Erythrosine was dissolved in methanol–water (1:1) containing 0.005 *M* TBAP in order to prevent precipitation of the ion pair.

Preparation of TBAP was done by adding 52 ml of 1.11 *M* KH_2PO_4 to 25 ml of 1.54 *M* tetra-*n*-butylammonium hydroxide solution which produces a solution 0.5 *M* TBAP, pH 7.2. The solution was prepared in a brown bottle and stored in the dark

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when not in use. This was used for adjustment of all standard and sample solutions, and the mobile phases.

The colors used in the study are given in Table I. All organic solvents were distilled-in-glass grade materials. Water was distilled and deionized.

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) Model 6000A pump, a Rheodyne 7125 syringe loading injection port with 20-μl loop, and a Waters Assoc. Model 450 variable-wavelength detector were employed. Separations were carried out on a Merck (Darmstadt, G.F.R.) Hibar II LiChrosorb RP-18, 10 μm (4.6 mm × 25 cm) column at ambient temperature. The mobile phases (degassed) usually consisted of methanol–water at ratios (v/v) of either (45:55) or (60:40) each containing 0.005 *M* TBAP. All mobile phases and samples were filtered through a 0.45-μm Millipore filter before use with the HPLC apparatus. At the end of each day the chromatographic system was rinsed thoroughly with 100 ml water followed by about 25 ml of methanol. This rinsing was important to protect the chromatographic system.

The Sep-Pak™ (Waters) cartridges (C_{18}) were prepared for use according to manufacturer's directions. Preparation of the beverage samples was as follows. A 100-ml volume of grape soda was made to 0.005 *M* TBAP, then 20 ml of this was passed through a Sep-Pak cartridge. The color remained on the column. The cartridge was washed with *ca.* 20 ml water then the colors eluted with *ca.* 4 ml methanol–water (1:1). The eluent was collected and evaporated to 0.5 ml, filtered through a 0.45-μm Millipore filter before HPLC analysis. The same procedure was used for a grape drink sample except only 2.0 ml of the drink was loaded onto the Sep-Pak cartridge. The synthetic colors remained on the column while most natural color was unretained as evidenced by the colored eluate which was discarded. Also, Benzyl Violet 4B required an additional 2 ml of 100% methanol for elution from the cartridge.

When carrying out the analysis of a series of food colors with the (45:55) mobile phase system, the detector wavelength and flow-rate were changed as indicated in Fig. 1 in order to best determine the compounds. The compromise wavelengths were 610 nm for the blue and green colors, and 480 nm for the reds, oranges and yellows.

RESULTS AND DISCUSSION

Table I summarizes the retention times for the various food colors examined. While six different combinations of mobile phases were evaluated, the two most useful ones were methanol–water (45:55) or (60:40), the first being the choice for most colors except Ponceau SX, Fast Red E and Benzyl Violet 4B which were analysed with latter mobile phase, and erythrosine which was analysed with methanol–water (70:30). Skyark, a subsidiary of Sunset Yellow FCF could not be completely resolved from Indigotine. However, since these two absorb at different wavelengths (Skyark, 480 nm; Indigotine, 610 nm), they could be identified by appropriate wavelength selection. Initially it was attempted to separate as many of the food colors as possible in a single run without recourse to gradient elution. Fig. 1 shows a chromatographic analysis of eight food colors using the (45:55) mobile phase. The minor peaks of

TABLE I

RETENTION TIMES OF COLOURS TESTED IN VARIOUS MOBILE PHASES

Mobile phase: methanol–water containing 0.005 *M* TBAP. Flow-rate, 1.0 ml/min. t_0 = 2.7 min.

Methanol–water ratio	*Retention times (min)*											
	Indigotine	*Tartrazine*	*Amaranth*	*Sunset yellow FCF*	*Allura Red AC*	*Fast Green FCF*	*Brilliant Blue FCF*	*Erythrosine*	*Ponceau SX*	*Fast Red E*	*Skyark*	*Benzyl Violet 4B*
40:60	8.7	18	25	32	65	> 70*	> 70*	–**	–	–	–	–
45:55	5.4	7.3	9.0	11	17	33	40	–	–	56	5.7	–
50:50	4.4	5.4	7.5	9.0	–	24	30	> 40	–	–	–	–
55:45	4.0	4.7	5.0	5.9	7.5	9.0	9.8	–	> 50	21	4.7	–
60:40	–	–	3.6	–	–	–	–	44	14.8	6.9	–	32*
70:30	–	–	2.7	–	–	–	–	9.9	4.8	–	–	–

* For Fast Green FCF, Brilliant Blue FCF and Benzyl Violet 4B, the major peak is given.

** Dashes indicate that no analyses were carried out.

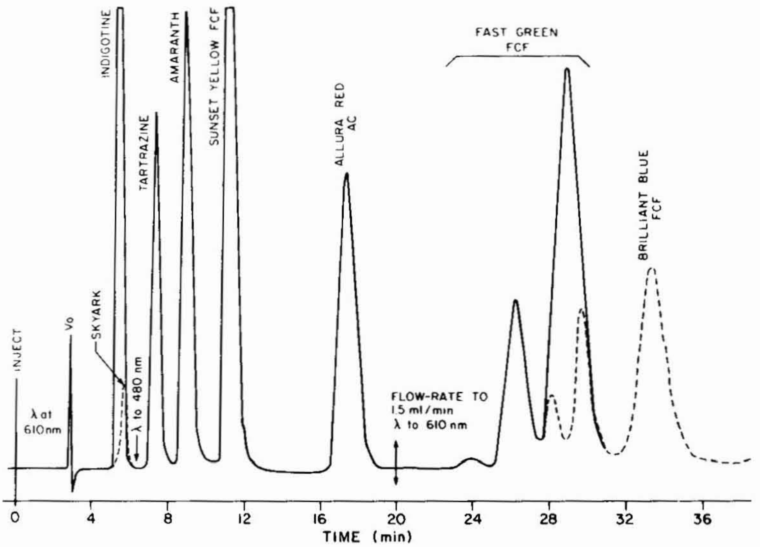

Fig. 1. Chromatogram of eight colors. Conditions as described in the text. Mobile phase methanol–water (45:55) containing 0.005 *M* TBAP; flow-rate 1.0 ml/min up to 20.0 min, then changed to 1.5 ml/min. Wavelength changes were made as indicated. Skyark and Brilliant Blue FCF peaks are in dashed lines.

Brilliant Blue FCF elute in the region of the major peak of Fast Green FCF. In order to monitor all food colors it was necessary to make detector wavelength changes during the run as indicated in Fig. 1. The flow-rate was increased at 20 min to 1.5 ml/min to speed up the elution of Fast Green FCF and Brilliant Blue FCF. Fig. 2

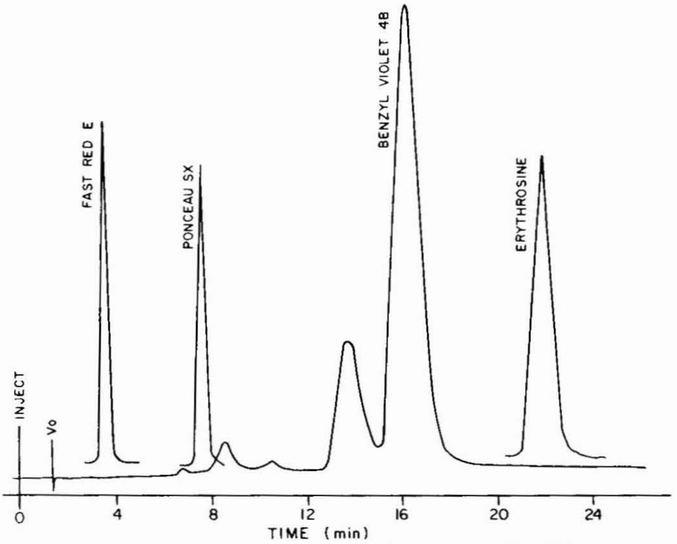

Fig. 2. Composite chromatogram of four food colors. Conditions as described in the next. Mobile phase, methanol–water (60:40) containing 0.005 *M* TBAP; flow-rate 2.0 ml/min throughout. Fast Red E, Ponceau SX and Erythrosine detected at 500 nm; Benzyl Violet 4B detected at 545 nm.

shows typical results for four other colors studied employing the (60:40) mobile phase. All four are easily separated from each other, however, for Erythrosine analysis we found that a methanol–water (70:30) mobile phase was superior in terms of analysis time and peak shape.

Application of the technique to beverage analysis is shown in Fig. 3 for a commercial grape soda drink. The results indicate the presence of both the permitted food colors, Amaranth and Brilliant Blue FCF. Results from a grape drink sample spiked with a mixture of Tartrazine, Allura Red AC, Brilliant Blue FCT and Benzyl Violet 4B showed no interferences from coextractives or natural food colors while all color peaks were quantitatively recovered when spiked in the concentration range of 4–44 μg/g.

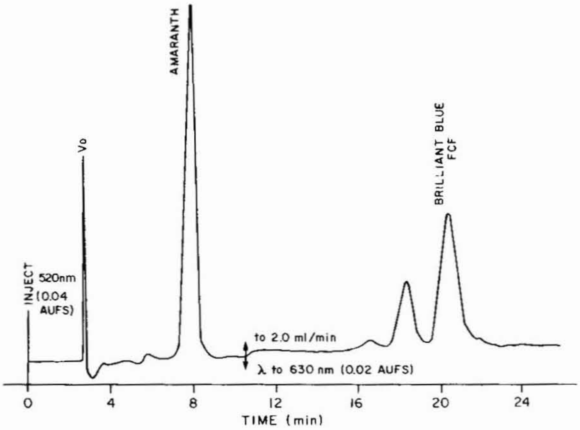

Fig. 3. Analysis of a grape soda beverage. Conditions as described in the text. Mobile phase methanol–water (50:50) containing 0.005 *M* TBAP. Wavelength and sensitivity settings (absorbance units full scale, AUFS) were as indicated.

CONCLUSION

The described HPLC method is capable of separating and quantitating twelve major food colors or subsidiary dyes. Application to grape beverages indicates the potential of the method for rapid screening of samples for non-permitted colors as well as for quantitating permitted food colors by comparison to known standards to ensure that they do not exceed the legal limit. The method is faster and less tedious than the thin-layer chromatographic–spectrophotometric techniques currently in use.

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Note

Partial purification of microsomal proteolipid(s) from *Neurospora crassa* by high-performance liquid chromatography on silica gel

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Certain membranes, in particular myelin and mitochondria, contain intrinsic proteins of extreme hydrophobicity. Those hydrophobic proteins which are soluble in chloroform–methanol (2:1) have been operationally defined as proteolipids[1]. Although the amino acid composition of different proteolipids differs, a striking feature common to these proteins is their high content of hydrophobic and neutral amino acid residues[2]. In addition to hydrophobic interaction with complex lipids, at least one proteolipid, the myelin proteolipid, contains covalently bound fatty acids[3]. Proteolipids have been purified by chloroform–methanol extraction, followed by dialysis in chloroform–methanol to remove non-covalently bound lipid; however, proteolipids of molecular weight less than 12,000 are lost from the dialysis rentate by this procedure[3]. Thus, dialysis is inappropriate for purification of low-molecular-weight proteolipids. Solubilization and chromatography in sodium dodecyl sulfate (SDS) have also been used to purify hydrophobic subunits of enzyme complexes such as cytochrome oxidase[4] or cytochrome *b*[5]; however, the associated SDS is difficult to remove and can interfere with subsequent analysis. Improved fractionation procedures would facilitate the work in this field.

The microsomal fraction of *Neurospora crassa* contains a proteolipid(s) of approximately 6500 daltons as measured by SDS–urea gels[6]. The synthesis of the proteolipid is blocked by the mitochondrial protein synthesis inhibitor chloramphenicol, but not by the cytoplasmic protein synthesis inhibitor cycloheximide, suggesting mitochondrial synthesis of the microsomal proteolipid. Only a small fraction of cellular protein is synthesized in the presence of cycloheximide. The microsomal proteolipid can be specifically radioactively labeled under these conditions and initial solubilization of this labeled proteolipid can be achieved by extraction with chloroform–methanol. The extract contains microsomal lipids such as phosphatidyl choline,

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ergosterol, fatty acids and triglycerides plus a number of proteolipids[6]. The large amount of lipid in the extract and the low molecular weight of the proteolipid are major factors complicating further purification of the proteolipid. We report here the use of high-performance liquid chromatographic (HPLC) silica gel columns to obtain an approximately 174-fold purification of this chloramphenicol-sensitive, cycloheximide-insensitive proteolipid with respect to protein specific activity, with the concomitant removal of the major contaminating lipids. The methods described here may be applicable to the purification of other proteolipids.

METHODS

Radioactive labeling and extraction of N. crassa proteolipids

N. crassa was cultured, labeled and fractionated as described previously[6]. Briefly, cells were grown for 18 h at 30°C. Cultures were exposed to cycloheximide for 5 min and subsequently labeled for 20 min with [^{3}H]phenylalanine. The cells were ground twice in a mortar with sand in 0.25 *M* sucrose, 50 m*M* Tris, 1 m*M* EDTA pH 7.5, containing 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF) to inhibit protease activity. The microsomal fraction was isolated by density gradient centrifugation at the 0.6/1.3 *M* sucrose interface of discontinuous sucrose gradients. A crude proteolipid preparation, containing proteolipids and lipids was prepared by extraction of the microsomes with chloroform–methanol (2:1). For large-scale preparations of the proteolipid, approximately 20 mg labeled microsomal protein was mixed with 700 mg unlabeled microsomal protein and the proteolipid fraction was extracted with chloroform–methanol.

Chromatographic separations

The proteolipid preparation was analyzed for lipids and proteolipids by thin-layer chromatography (TLC) on silica gel HR (Merck, Darmstadt, G.F.R.)[6]. The sample was separated by successive development in chloroform–methanol–glacial acetic acid–water (65:25:8:4) and then light petroleum (b.p. 35–60°C)–diethyl ether–glacial acetic acid (75:25:1). Lipids were visualized with iodine vapor; protein was visualized by ninhydrin reactivity. Bands were scraped from the plate into counting vials and radioactivity was determined by counting in toluene-liquifluor (New England Nuclear, Boston, MA, U.S.A.).

Analysis by paper chromatography utilized a tank saturated with *tert.*-amyl alcohol–methyl ethyl ketone–water (3:1:1). The chloroform–methanol-soluble sample was applied to Whatman paper and developed for 12 h. The paper was analyzed for protein and for radioactivity.

Separation of the crude proteolipid preparation was attempted by application of the fraction to Sephadex LH-20 or LH-60 columns (60 × 2 cm) equilibrated in chloroform–methanol (2:1). The columns were eluted at 9 ml/h for 24 h. Fractions were collected and analyzed for absorbance at 280 nm and for radioactivity.

Preparative HPLC separation was performed on the crude proteolipid preparation. Silica gel columns were packed with 5-μm LiChrosorb (Altex Labs., Berkeley, CA, U.S.A.). The HPLC instrument used was a ISCO Model 384 (Omaha, NE, U.S.A.). A 1-ml volume of crude proteolipid extract was injected onto a preparative silica column (25 × 1 cm) equilibrated with benzene–ethanol (95:5). A gradient was applied for 20 min which raised the final percentage of ethanol to 66%.

Other procedures

Protein was determined by the method of Lowry *et al.*[7] as adapted by Lees and Paxman[8], using bovine serum albumin as the standard.

Amino acids were determined after acid hydrolysis of the partially purified proteolipid. Protein was hydrolyzed in 6 *N* hydrochloric acid at 110°C for 72 h. Amino acids were separated on silica gel plates (EM Labs.) with ethanol–water (7:3) and visualized with ninhydrin.

RESULTS AND DISCUSSION

A crude microsomal proteolipid fraction was prepared by extraction of [^{3}H]phenylalanine-labeled microsomes with chloroform–methanol. Approximately 1.9% of the total microsomal protein was solubilized by chloroform–methanol. The crude proteolipid fraction was initially separated by chromatography on a Sephadex LH-20 column (Fig. 1). Two major radioactive peaks were obtained and further analyzed using a Sephadex LH-60 column. The radioactive fractions I and II migrated as single peaks and were separated from non-radioactive proteins on the LH-60 column. Analysis of peaks I and II after chromatography on LH-60 was attempted by paper chromatography (Fig. 2). The two fractions did not appear to be significantly different, nor particularly pure. Both fractions still contained non-radioactive ninhydrin-positive spots and most of the radioactive material remained at the origin. It is possible that they could represent a single radioactive protein which migrates as multiple peaks in LH-20 and LH-60 depending on association with lipids or on its state of aggregation. As a consequence of their chemical nature, the chromatographic behavior of proteolipids is somewhat unusual. The myelin proteolipid, for example, which on the basis of several criteria is considered to consist of a single protein[2], produces multiple peaks upon silica gel chromatography in chloroform–methanol[9]. These peaks have identical amino acid compositions and certain of them can be interconverted, suggesting that these multiple peaks may contain the same protein

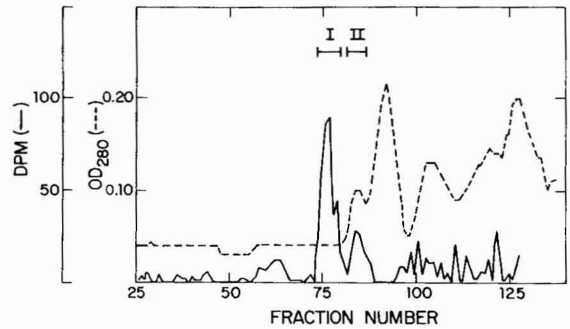


Fig. 1. Fractionation of chloroform–methanol extract of *N. crassa* microsomes on Sephadex LH-20 column. Microsomes were prepared from cells radioactively labeled with [^{3}H]phenylalanine in the presence of cycloheximide. 2.75 mg microsomal protein (0.6 ml) were extracted in 15 ml chloroform–methanol (2:1). After centrifugation to remove unextracted material, the supernatant was concentrated under nitrogen to approximately 2 ml and applied to a Sephadex LH-20 column equilibrated with chloroform–methanol (2:1). The column was eluted at 9 ml/h. Fractions (1 ml) were collected and analyzed for protein by absorbance at 280 nm and for radioactivity.

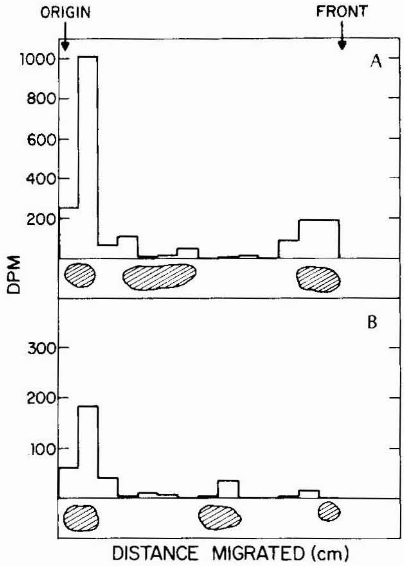


Fig. 2. Analysis of Sephadex LH-20–LH-60 column fractions I and II by paper chromatography. Sephadex LH-20 fractions I and II were re-chromatographed on a Sephadex LH-60 column and then concentrated under nitrogen, applied to Whatman paper and chromatographed. Crosshatched regions represent ninhydrin-positive spots. A, LH-20 fraction I; B, LH-20 fraction II.

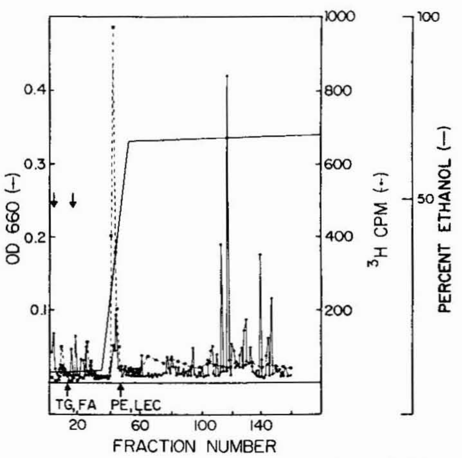
which interacts with the silica gel differently depending on associated lipids or its state of aggregation.

In the present study, the usefulness of chromatography on LH-20 or LH-60 columns was limited to an analytical assessment of the microsomal proteolipid. Although some purification of the radioactive proteolipid was possible by chromatography on LH-20 and LH-60 columns, in general, the procedures were unsatisfactory for purification of the microsomal proteolipids. The radioactive proteolipid was not completely separated from the non-radioactive material and the recovery of material from the columns was quite poor as a consequence of non-specific adsorption of radioactive material to the columns. Once the proteolipid fraction was taken to dryness for further separation by TLC or paper chromatography, re-extraction of the protein was virtually impossible. Furthermore, the TLC or paper chromatographic systems tried by us resulted in poor resolution of the proteolipid. Thus, these chromatographic methods were inadequate for purification of the proteolipid.

A different approach to separation of the crude proteolipid preparation was attempted by analytical and preparative HPLC utilizing silica gel columns. A number of solvents were used for proteolipid fractionation in the HPLC system. Solvent systems employing chloroform–methanol or chloroform–methanol–acetic acid in various proportions did not resolve the proteolipid significantly on silica gel columns nor did use of a stronger acid, such as hydrochloric acid, with the chloroform–methanol. The most successful solvent system consisted of an initial solvent of benzene–ethanol (95:5) and a gradient of ethanol to a final concentration of 66%. The major radioactive fraction eluted as a sharp peak at benzene–ethanol (*ca.* 42:58) (Fig. 3). Direct counting of the silica gel from the columns indicated less than 1% of the proteolipid radioactivity was retained in the columns.

The radioactive fraction from the silica gel column was analyzed by TLC which indicated that this HPLC system freed the proteolipid extract of ergosterol, triglycerides and free fatty acids, although the proteolipid fraction was still contaminated with some phospholipid (Fig. 4). The mobility of the partially purified proteolipid in this TLC system was similar to, although broader than that of the major radioactive component of the crude extract[6]. After acid hydrolysis of the proteolipid, amino acids were analyzed by TLC in an amino acid separating system. No free amino acids were detectable prior to hydrolysis. On the basis of protein specific activity, the partially purified proteolipid was purified 2.5-fold from the crude proteolipid preparation and 174-fold from the original microsomal fraction (Table I). Furthermore, 85% of the contaminating lipids in the chloroform–methanol extract were also removed by this HPLC procedure, leaving only phospholipid as the major contaminant. Since more than 90% of the chloroform–methanol extract was lipid, the actual purification of the proteolipid was significantly higher than 174-fold.

The use of silica gel chromatography for fractionation of various lipid classes is well established. Silica gel columns containing a finely grained silica gel (5 μm) have been used to fractionate growth-promoting polypeptides which have substantial



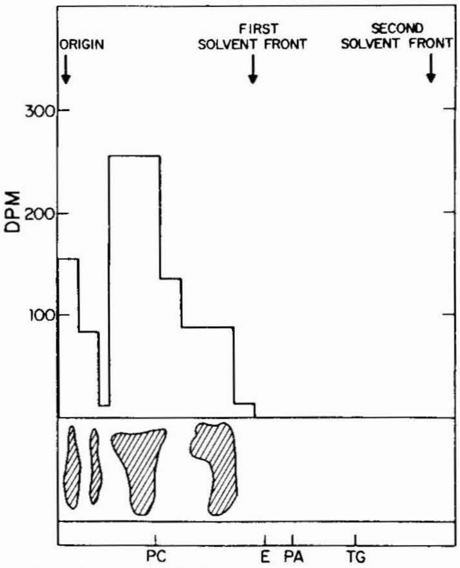


Fig. 3. Silica gel HPLC fractionation of chloroform–methanol extract of *N. crassa* microsomes. Two aliquots of 350 μg protein each were injected onto the column. The two arrows indicate times of sample injection onto the column. The column was originally equilibrated with benzene–ethanol (95:5) and was eluted with a 20-min gradient of 5–66% ethanol. Solid line represents the gradient. Fractions were analyzed for protein (circles), radioactivity (squares) and lipid content. Lipid standards: phosphatidyl ethanolamine (PE), phosphatidyl choline (LEC), triglycerides (TG) and fatty acids (FA).

Fig. 4. TLC analysis of microsomal proteolipid after partial purification by silica gel HPLC. After chromatography on silica gel HPLC, the proteolipid preparation was concentrated under nitrogen, and applied to silica gel HR thin layer plates (Merck). Plates were successively developed in chloroform–methanol–glacial acetic acid–water (65:25:8:4) and then light petroleum (b.p. 35–60°C)–diethyl ether–glacial acetic acid (75:25:1). Crosshatched area represents ninhydrin-positive spots. Plates were scraped and analyzed for radioactivity. Sample lipids and lipid standards were visualized with iodine. Standards were phosphatidyl choline (PC), ergosterol (E), palmitic acid (PA) and triolein (TG).

TABLE I

PURIFICATION OF CHLORAMPHENICOL-SENSITIVE, CYCLOHEXIMIDE-INSENSITIVE PROTEOLIPID FROM *N. CRASSA*, BY HPLC ON SILICA GEL

The purification described here is relative to protein only. The increase in purity is significantly higher relative to total weight, since lipids constitute more than 90% (w/w) of the chloroform–methanol microsomal extract and more than 85% of these lipids were removed by HPLC.

Proteolipid preparation	*Protein (mg)*	*Protein (cpm/mg)*
Microsomal fraction	720	–
Chloroform–methanol extract from microsomes	13.4	930
Radioactive peak from silica gel HPLC	4.2	2330
Increase in purity	174-fold relative to the microsomal fraction	2.5-fold relative to the chloroform–methanol extract from microsomes

hydrophobic character[10]. More recently, various types of silica gel columns and especially reversed-phase silica gel columns have been used to fractionate a variety of polypeptides and proteins, such as interferon and mitochondrial proteolipids which had previously been resistant to isolation[11–14]. The separation described in this paper increased the protein specific activity of the labeled proteolipid by approximately 174-fold. In addition, a major purification of the chloroform-methanol extract was obtained by removal of lipid contaminants. The results described in this paper may be applicable to a variety of other types of biological proteins of extreme hydrophobic character such as serum lipoproteins or the hydrophobic subunits of enzymes such as cytochrome oxidase or cytochrome *b*.

ACKNOWLEDGEMENTS

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Note

Isolation of human urine urokinase by column chromatography on sawdust and some properties of the enzyme obtained

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(Received January 20th, 1981)

The plasminogen activation factor in human urine was discovered by Williams[1] and designated as urokinase (E.C. 3.4.99.26) by Sobel *et al.*[2]. The enzyme has been applied to the therapy of thrombosis, and several papers have been published on its isolation and purification[3–9].

Recently we noticed that urokinase lost its activity in the presence of lignin, but that the addition of sodium chloride to this mixture restored the activity. Sawdust is, in a sense, small pieces of naturally occurring cellulose–lignin complex. The use of sawdust for the isolation and purification of urokinase from urine was studied and was found to be effective.

This paper deals with the conditions for the isolation and purification of urine urokinase using a column of sawdust and some properties of the enzyme obtained.

MATERIALS AND METHODS

Human urine was freshly collected from about a dozen normal male adults and a small amount of *n*-butanol was added as an antiseptic.

Acetylglycyl-L-lysine methyl ester (AGLMe) as the substrate was purchased from the Foundation for Promotion of Protein Research (Osaka, Japan). Human fibrinogen and thrombin were obtained from Green Cross Corp. (Osaka, Japan). "Uronase", 6000 I.U. (International Units) per vial, as the standard urokinase for plasminogen activation activity was obtained from Mochida Pharmaceutial Co. (Tokyo, Japan). Myoglobin, chymotrypsinogen A, ovalbumin and bovine serum albumin as the standard for the determination of molecular weight by gel chromatography were obtained from Serva (Heidelberg, G.F.R.). The protein standard kit for the determination of molecular weight by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was obtained from Boehringer (Mannheim, G.F.R.).

The sawdust, derived mainly from North American cedar, was sifted to screen pieces of sizes between 32 and 100 mesh, and was suspended in water under reduced pressure to remove air. It was then suspended in about 40 volumes of 2 *N* sodium hydroxide solution with stirring at 40°C until no further brown colour was produced

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(*ca.* 24 h). The sawdust was washed thoroughly with water, then suspended in about 40 volumes of 2 *N* hydrochloric acid with stirring at 40°C for 3–4 h. The acid-treated sawdust was washed thoroughly with water and air dried at 40°C. The washed sawdust was packed in a column after suspension in water. One gram of sawdust on a dry basis gave a packed volume of 6–7 ml.

The ester hydrolytic activity of urokinase was determined by the method of White and Barlow[10], but using AGLMe as the substrate instead of acetyl-L-lysine methyl ester. One unit of esterase activity was defined as the amount of the enzyme that liberated 1 μmole of methanol per minute at 37°C.

Plasminogen activation activity was assayed according to the method of Nishizaki and Kawamura[11] using a fibrin plate, and the activity was expressed in International Units.

Protein concentration was determined spectrophotometrically with a Hitachi Model 124 spectrophotometer assuming that the $A^{1\%}_{280\,\mathrm{nm}}$ value of urokinase was 13.6[5]. The specific activity was tentatively expressed by dividing the urokinase activity (I.U./ml) by the protein concentration (mg/ml) of the enzyme solution.

The gel chromatography for the determination of molecular weight was carried out on a Sephadex G-75 column, using 0.02 *M* phosphate buffer (pH 7.4) containing 0.15 *M* sodium chloride and 2 m*M* EDTA as the developing solvent.

SDS (0.1%) disc electrophoresis in 7.5% polyacrylamide gel was performed according to the method of Weber and Osborn[12] to check the purity and determine the molecular weight, except that no 2-mercaptoethanol was added as it would have caused complete inactivation of the enzyme. The gel after electrophoresis was stained with 0.1% Coomassie Brilliant Blue and destained with 7% acetic acid.

RESULTS

Human urine, freshly collected and diluted with an equal volume of water, was applied to a sawdust column equilibrated with 0.01 *M* Tris–hydrochloric acid buffer (pH 7.2) containing 0.15 *M* sodium chloride, and the column was washed with the same buffer until no further urine colour was observed in the effluent. The urokinase adsorbed on the column was eluted with 0.02 *M* borate buffer containing 1.0 *M* sodium chloride (pH 10.0). As shown in Fig. 1, most of the activity of urokinase in the fresh urine was adsorbed on the sawdust column, and the activity adsorbed was eluted with a yield of 94%. A small proportion (15–25%, depending on the urine employed) of the enzyme activity of urine appeared in the effluent. The specific activity of the eluted enzyme was 5100 I.U./mg protein, an 18,500-fold increase compared with the original urine. The sawdust column used was reactivated by washing successively with water, 0.2 *N* hydrochloric acid, water, 0.2 *N* sodium hydroxide solution and water.

The active fraction obtained by the first chromatography was dialysed against 0.01 *M* phosphate buffer (pH 7.4) containing 0.15 *M* sodium chloride and 2 m*M* EDTA at 4°C, and applied to the sawdust column equilibrated with the same buffer as used for dialysis. After washing the column with the same buffer, the enzyme adsorbed was eluted with 0.02 *M* borate buffer containing 1.0 *M* sodium chloride and 2 m*M* EDTA (pH 10.0) at 4°C. The urokinase recovery was 85% and the specific activity was 23,000 I.U./mg protein. The urokinase recovered was dialysed and sub-

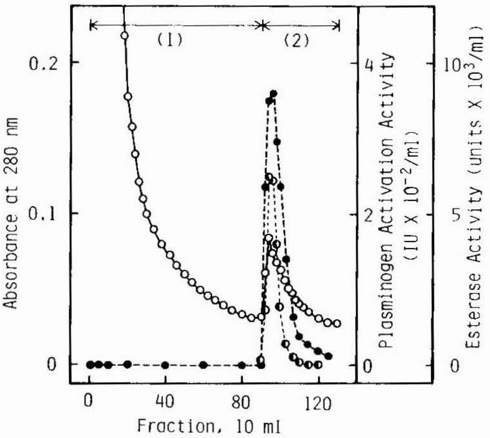

Fig. 1. Elution pattern of urokinase from human urine by chromatography on a sawdust column. Column, 2.0 × 7.5 cm; twice diluted urine, 7900 ml ($A_{280\ nm}$ = 22.1), plasminogen activation activity, 4.5 I.U./ml; flow-rate, 300 ml/h; temperature, 4°C. (1) 0.01 *M* Tris–HCl buffer containing 0.15 *M* NaCl (pH 7.2); (2) 0.02 *M* borate buffer containing 1.0 *M* NaCl (pH 10.0). ○, Absorbance at 280 nm; ●, plasminogen activation activity; ◐, esterase activity.

jected to a third chromatographic step on the same sawdust column as described above. The specific activity increased to 60,000 I.U./mg protein, and the recovery of activity in this step was 85%. The elution patterns of urokinase in this third chromatography are shown in Fig. 2.

As shown in Figs. 1 and 2, the plasminogen activation and esterase activities were eluted in parallel from the sawdust column and their ratio was about 50,000:1 in all of the active fractions.

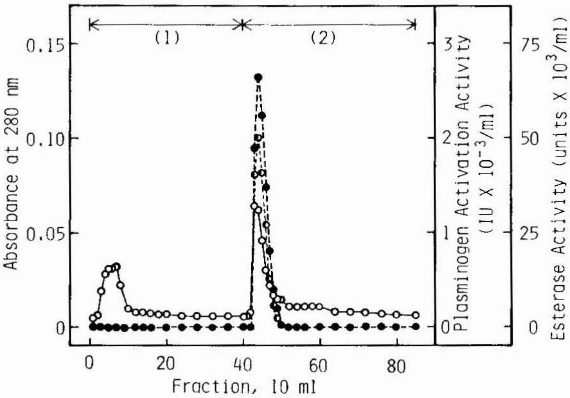

Fig. 2. Elution pattern of urokinase in third chromatographic step on sawdust column. Column as in Fig. 1; enzyme applied, 50 ml ($A_{280\ nm}$ = 0.121), plasminogen activation activity, 2435 I.U./ml, esterase activity, $49.6 \cdot 10^{-3}$ U./ml; flow-rate, 300 ml/h; temperature, 4°C. (1) 0.01 *M* phosphate buffer containing 0.15 *M* NaCl and 2 m*M* EDTA (pH 7.4); (2) 0.02 *M* borate buffer containing 1.0 *M* NaCl and 2 m*M* EDTA (pH 10.0). ○, Absorbance at 280 nm; ●, plasminogen activation activity; ◐, esterase activity.

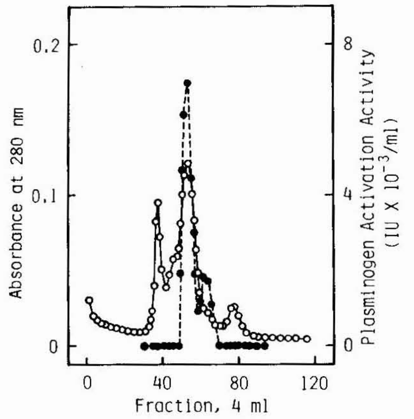

Fig. 3. Gel chromatography of the urokinase on Sephadex G-75. Column, 2.25 × 126 cm; 0.02 *M* phosphate buffer containing 0.15 *M* NaCl and 2 m*M* EDTA, (pH 7.4); enzyme applied, 9.0 ml ($A_{280\,nm}$ = 1.000), plasminogen activation activity, 31,000 I.U./ml; flow-rate, 17 ml/h; temperature, 4°C. ○, Absorbance at 280 nm; ●, plasminogen activation activity.

The protein components of the urokinase preparation obtained by the sawdust column chromatography were examined. The urokinase preparations obtained by performing several series of purifications by triple chromatography were collected and concentrated by ultrafiltration (Model UHP-43, ultrafilter UK-10; Toyo Kagaku Sangyo Co., Osaka, Japan). The specific activity of the enzyme decreased to 70% on ultrafiltration. The concentrate was subjected to gel chromatography using a column of Sephadex G-75. As shown in Fig. 3, protein appeared, separating into peaks for three fractions. The plasminogen activation activity was eluted together with the middle protein peak. However, a small peak of enzyme activity appeared behind the major activity. The molecular weights of the major and minor enzyme active fractions were estimated to be $5.4 \cdot 10^4$ and $3.6 \cdot 10^4$, respectively (Fig. 4).

The concentrated preparation described above was also examined by SDS polyacrylamide gel electrophoresis, as shown in Fig. 5. The protein of the preparation was separated into five components, with molecular weights estimated to be $9.2 \cdot 10^4$

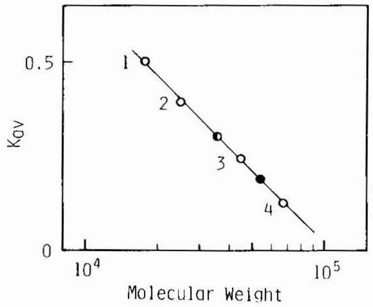

Fig. 4. Determination of molecular weight of urokinase by gel chromatography on Sephadex G-75. Molecular weights of the reference proteins: 1, myoglobin, $1.78 \cdot 10^4$; 2, chymotrypsinogen A, $2.5 \cdot 10^4$; 3, ovalbumin, $4.5 \cdot 10^4$; 4, bovine serum albumin, $6.7 \cdot 10^4$. ●, Urokinase of the major active fraction; ◐, urokinase of the minor fraction. K_{av}: partition coefficient.

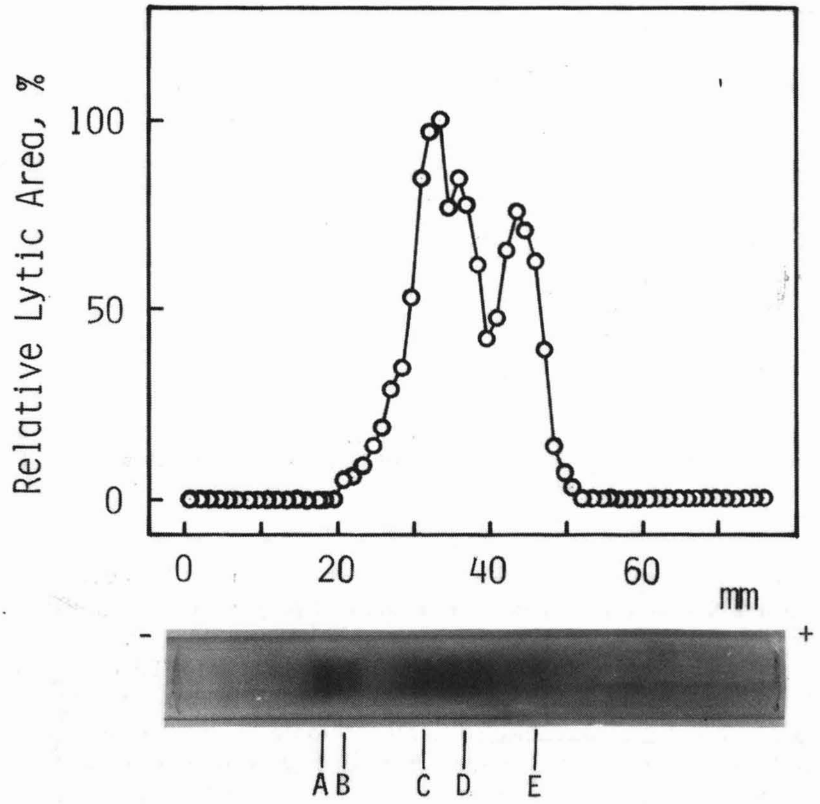

Fig. 5. SDS polyacrylamide gel electrophoresis of urokinase and distribution of urokinase activity on the gel after electrophoresis. The sample was applied after incubation with SDS at 37°C for 24 h but without addition of 2-mercaptoethanol. Molecular weights of the components: A, $9.2 \cdot 10^4$; B, $8.2 \cdot 10^4$; C, $5.4 \cdot 10^4$; D, $4.7 \cdot 10^4$; E, $3.6 \cdot 10^4$. The gels (1.25 mm thick) were placed directly on a fibrin plate and their lytic areas were measured.

(A), $8.2 \cdot 10^4$ (B), $5.4 \cdot 10^4$ (C), $4.7 \cdot 10^4$ (D) and $3.6 \cdot 10^4$ (E). The unstained gel column that had been run at the same time was sectioned into 1.25-mm thick portions, and the sections were placed on a fibrin plate for analysis of the plasminogen activation activity. As shown in Fig. 5, components C, D and E showed plasminogen activation activity, although the method used demonstrated only the presence of enzyme activity and was not effective in establishing the exact relative activities between the components.

DISCUSSION

The adsorption of urokinase by sawdust seems to be specific, although the adsorption capacity is not as prominent as with various other ion exchangers. The purification of human pancreatic juice elastase II, the isoelectric point of which is 8.8[13], and hen egg white lysozyme has also been performed by chromatography using a sawdust column. It is probable that sawdust adsorbs basic proteins by an ion-exchange reaction. In fact, sawdust activated by washing with dilute hydrochloric acid and sodium hydroxide solution showed a cation-exchange capacity of about 0.14 mequiv./g. On the one hand, urokinse was found to be markedly inhibited by soluble lignin, which suggested that the enzyme binds to lignin in sawdust.

Two kinds of urokinase are known at present, with molecular weights of about $5.4 \cdot 10^4$ (ref. 5, 9, 14) and $3.2 \cdot 10^4$ (ref. 5, 8, 9, 15, 16). The high-molecular-weight urokinase may change into the low-molecular-weight variety under certain conditions. In an experiment carried out independently, the urokinase of molecular weight $3.2 \cdot 10^4$ was passed through the sawdust column under the conditions described above. The activity ratio of plasminogen activation to esterase of the low-molecular-weight urokinase was half that of the high-molecular-weight urokinase. In this study, the purified enzyme showed a constant activity ratio of esterase and plasminogen activation, indicating that both enzyme activities were attributed to the high-molecular-weight urokinase, although a small amount of the low-molecular-weight enzyme was present. This enzyme may have appeared during the repeated purification by the sawdust adsorption method.

This study also demonstrated that in addition to the above two enzymes, a new urokinase of intermediate molecular weight appeared when examined by SDS polyacrylamide gel electrophoresis. This urokinase may be an artificial product. It is possible that human urokinase exists with several molecular weights with different activities of esterase and plasminogen activation; further study is necessary in order to clarify this aspect.

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CHROM. 13,725

Note

Determination of 5′-nucleotidase by automated ion-exchange column chromatography

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A simple anion-exchange chromatographic separation of adenine nucleotides using G MP-1 resin has been described[1,2]. The resin has a strong affinity for adenosine phosphates but not for adenosine. Adenosine is eluted out with water at near void volume while AMP remains absorbed on the column.

This paper describes a simplified chromatographic procedure for automated quantitation of adenosine produced by 5′-nucleotidase reaction.

EXPERIMENTAL

Materials

AG MP-1 resin was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.); and AMP from Sigma (St. Louis, MO, U.S.A.).

Preparation of reaction mixture

A 0.5-g amount of the human cervix was homogenized in 2 ml of 0.25 *M* sucrose for 2 min at 900 rpm using a motor-driven tissue grinder. The homogenate was then centrifuged at 300 *g* for 10 min and 1 to 20 μl of the supernatant solution were used for the assay.

The reaction mixture in a total volume of 1 ml contained 50 m*M* glycylglycine (pH 7.4), 10 m*M* $MgCl_2$, 2 m*M* AMP and the tissue extract. The reaction was initiated by the addition of AMP at 37°C and terminated after 10 min by addition of 1 ml ice-cold 6% trichloroacetic acid. A blank for each sample was prepared by adding AMP after the enzyme was inactivated by the addition of trichloroacetic acid. The precipitate was removed by centrifugation and an aliquot was neutralized with 2 *M* Tris–base (5:1) for chromatography.

Automated column chromatography

In addition to the previous setup[2] for column chromatography, an automatic sample injector was attached to the column. Samples in a volume of 200 μl were injected at 10-min intervals while the column was eluted with water at a flow-rate of 2 ml/min. The effluent was monitored at 257 nm. The adenosine peaks appeared one after another, were recorded and then areas were integrated using an extinction

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coefficient of $15.4 \cdot 10^3$ l·mol^{-1}·cm^{-1}. After 10 injections the column was washed with 1 *M* hydrochloric acid for 5 min then with water for 10 min and the process was repeated.

RESULTS AND DISCUSSION

A 200-µl aliquot of a reaction blank containing 166 nmoles of AMP were injected at 10-min intervals. The adenosine peaks of ten injections were very constant. They gave an average value of 1.22 ± 0.22 (S.D.) nmoles. When 10 nmoles of adenosine were added to the above solution, the experimental error was minimized to less than 5%. Continuous injection of the blanks over 15 times tended to increase the value slightly. Therefore, alternate injection of a sample and its blank would avoid any experimental error.

The enzyme activity as reflected by the amount of adenosine produced was linearly proportional to the incubation time. The activity levelled off after one-half of AMP was dephosphorylated.

Fig. 1 showed the results of 5′-nucleotide activity *versus* the amount of enzyme present in the crude extract. It was linearly related up to 50 mU/ml and approximately 25% of AMP was consumed.

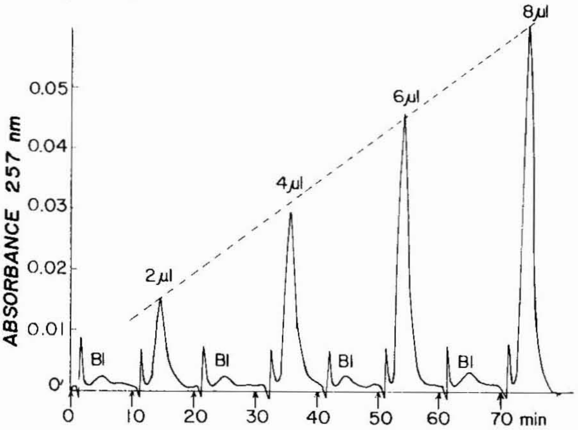

Fig. 1. Amount of adenosine produced *versus* the enzyme concentration. Four reaction mixtures containing different amounts of the enzyme extract (2–8 µl), and respective blanks (Bl) were injected to a column at the points marked by an arrow. The small sharp peaks appeared at the void volume and were from UV-absorbing substances such as unprecipitated proteins. The amount of adenosine and peaks of UV absorption at 257 nm were linearly proportional.

We estimated from the previous study[2] that the column bed (5 × 1.1 cm) has a total exchange capacity of 3 mmoles AMP. AMP was strongly bound to the resin and could not be eluted out with water. As far as the sample solution is neutralized to slightly basic, there is no leakage of AMP by repeating injection of 20 samples to a similar column. To determine the enzyme activity at lower limit, it is suggested to regenerate the column after every 10 injections.

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Note

Separation of isomeric thiohydantoins by thin-layer chromatography

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(Received January 29th, 1981)

During the synthesis of 2-thiohydantoins[1,2], 5,5-diaryl-, 3-aryl- and 5,5-diaryl-1,3-diaryl-2-thiohydantoins were obtained from benzils and various monoaryl- and *sym.*-diarylthioureas, respectively, and it was necessary to study the separation and identification of the final product by thin-layer chromatography (TLC). In recent years, this technique has also been applied successfully to the separation of isomeric compounds using suitable solvent systems[3–6]. The present investigation has shown that it is not only possible to separate thiohydantoins from corresponding thioureas but also to separate isomeric thiohydantoins from one another.

EXPERIMENTAL

A slurry was prepared by mixing 25 g of silica gel G (according to Stahl, Type 60; E. Merck, Darmstadt, G.F.R.) in 50 ml of distilled water. Ten well cleaned glass plates (20 × 10 cm) were coated with the adsorbent to a thickness of 250 μm using an adjustable Desaga Model S II (Stahl-type) applicator. When the layer had almost dried, the coated glass plates were activated by heating them in an air oven at 175°C for 45 min. The spotted plates were developed in air-tight glass chambers (25 × 12 × 25 cm) that had been previously saturated with the solvent vapour; the developing time was 58–80 min, depending on the solvent used. The operating temperature was 25–30°C and the relative humidity of the atmosphere was 60–70%. The developed chromatograms, after being dried, were sprayed with sodium azide and iodine–potassium iodide reagent followed by starch solution. The thiohydantoin compounds were detected as colourless spots on a bluish background.

RESULTS

A large number of solvent systems were tried, and those which gave reasonable differences in the R_F values of the isomers are listed in Table I. The separation of the isomeric compounds 5,5-di(*p*-chlorophenyl)-3-*o*-, *m*-, *p*-chlorophenyl-, 5,5-di(*p*-chlorophenyl)-1,3-di-*o*-, *m*-, *p*-chlorophenyl-2-thiohydantoins, 5,5-di(*p*-methoxyphenyl)-3-*o*-, *m*-, *p*-tolyl-, 5,5-di(*p*-methoxyphenyl)-1,3-di-*o*-, *m*-, *p*-tolyl-2-thiohydantoins and 5,5-distyril-3-*o*-, *m*-, *p*-nitrophenyl-, 5,5-distyril-1,3-di-*o*-, *m*-, *p*-nitrophenyl-2-thiohydantoins were studied.

An equimolar mixture of the isomeric compounds was chromatographed by

TABLE I

R_F VALUES OF ISOMERS IN DIFFERENT SOLVENT SYSTEMS

Solvent systems: 1:1 mixtures of light petroleum (b.p. 60–80°C) with (A) benzene, (B) methanol, (C) ethanol, (D) isopropanol and (E) *n*-butanol.

Compound	*R_F value*				
	A	*B*	*C*	*D*	*E*
5,5-Di(*p*-chlorophenyl)-3-*o*-chlorophenyl-2-thiohydantoin	0.03	0.52	0.58	0.54	0.55
5,5-Di(*p*-chlorophenyl)-3-*m*-chlorophenyl-2-thiohydantoin	0.15	0.62	0.67	0.63	0.66
5,5-Di(*p*-chlorophenyl)-3-*p*-chlorophenyl-2-thiohydantoin	0.31	0.83	0.85	0.82	0.84
5,5-Di(*p*-chlorophenyl)-1,3-di-*o*-chlorophenyl-2-thiohydantoin	0.08	0.58	0.59	0.54	0.53
5,5-Di(*p*-chlorophenyl)-1,3-di-*m*-chlorophenyl-2-thiohydantoin	0.21	0.67	0.68	0.62	0.61
5,5-Di(*p*-chlorophenyl)-1,3-di-*p*-chlorophenyl-2-thiohydantoin	0.39	0.78	0.77	0.74	0.73
5,5-Di(*p*-methoxyphenyl-3-*o*-tolyl-2-thiohydantoin	0.24	0.56	0.58	0.54	0.52
5,5-Di(*p*-methoxyphenyl)-3-*m*-tolyl-2-thiohydantoin	0.36	0.64	0.60	0.62	0.61
5,5-Di(*p*-methoxyphenyl)-3-*p*-tolyl-2-thiohydantoin	0.46	0.74	0.70	0.70	0.72
5,5-Di(*p*-methoxyphenyl)-1,3-di-*o*-tolyl-2-thiohydantoin	0.34	0.32	0.31	0.30	0.83
5,5-Di(*p*-methoxyphenyl)-1,3-di-*m*-tolyl-2-thiohydantoin	0.39	0.44	0.43	0.42	0.47
5,5-Di(*p*-methoxyphenyl)-1,3-di-*p*-tolyl-2-thiohydantoin	0.49	0.51	0.52	0.51	0.56
5,5-Distyril-3-*o*-nitrophenyl-2-thiohydantoin	0.41	0.45	0.43	0.42	0.41
5,5-Distyril-3-*m*-nitrophenyl-2-thiohydantoin	0.58	0.57	0.56	0.53	0.51
5,5-Distyril-3-*p*-nitrophenyl-2-thiohydantoin	0.67	0.64	0.62	0.61	0.60
5,5-Distyril-1,3-di-*o*-nitrophenyl-2-thiohydantoin	0.40	0.41	0.40	0.41	0.39
5,5-Distyril-1,3-di-*m*-nitrophenyl-2-thiohydantoin	0.51	0.56	0.54	0.54	0.52
5,5-Distyril-1,3-di-*p*-nitrophenyl-2-thiohydantoin	0.62	0.62	0.61	0.62	0.61
Time of development (min)	58	62	80	75	65

ascending one-dimensional TLC. Effective separations of all of the above compounds were possible with 1:1 binary solvent systems of light petroleum (b.p. 60–80°C) with benzene, methanol, ethanol, isopropanol and *n*-butanol, marked differences in the R_F values of the *o*-, *m*- and *p*-isomers being obtained.

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CHROM. 13,684

Book Review

Dictionary of chemical technology (in five languages: English, German, French, Polish, Russian), edited by D. Kryt, Elsevier, Amsterdam, Oxford, New York, 1980, XII + 601 pp., price Dfl. 195.00, US$ 95.00, ISBN 0-444-99788-1.

This dictionary was compiled by an outstanding team of Polish scientists, among them also several renowned chromatographers. It lists in alphabetical order 3805 English terms, each with a short definition followed by the German, French, Polish and Russian equivalents. As the book will be mainly used by Polish and Russian scientists, we have paid attention more to the Western languages.

Some of the German terms seem to be pure invention, for example, ion exchange capacity = Salzspaltkapazität and ion exclusion = Elektrolytvorlaufverfahren. They remind me of an English tourist who once bumped into me and said "Ich bin betrübt", *i.e.*, he translated literally "I am sorry".

Some French terms are equally wonderful, for example, R_M value = symbole R_M (why not "valeur de R_M"?) and R_f value = coefficient R_f (why not "valeur de R_f"?).

For some terms, such as No. 3112, "Sawhorse formula", the dictionary leaves all languages blank and gives only a Polish equivalent. One wonders why? I could not find "gel filtration" or "size exclusion chromatography" at all. Term No. 2465 is called "Openauer oxidation" in English and "Oppenauer-Oxydation" in German. Did the gentleman change his name when emigrating? Or is it a printer's error?

Like most dictionaries it irritates one by not listing the term one really needs but abounds in terms which are the same in most languages, such as "eluate", "isobar" and "isomer".

Lausanne (Switzerland) M. JANOVSKY

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Book Review

Size exclusion chromatography (GPC) (ACS Symposium Series 138), edited by T. Provder, American Chemical Society, Washington, DC, 1980, VIII + 312 pp., price US$ 30.75, ISBN 0-8412-0586-8.

The volume contains seventeen original papers which were presented at a symposium at the 178th National Meeting of the American Chemical Society, September 10–14, 1979. Almost all of the papers deal with synthetic polymers and oligomers and many of the authors work in industry. The only paper dealing with proteins is an evaluation of Spherogel TSK-SW-type gels and is well out of date.

Looking through these papers, one realizes that industrial chemists have already branched out into symbols and nomenclature of their own. For example, in the first paper on "Particles size analysis" one finds "Particle separation can be characterized by the separation factor, R_F, which is the ratio of the eluent to particle elution volumes..." Now which is it —the separation factor or something else? And why R_F?

The volume, although not typeset but reproduced from the typed manuscripts, is well produced and contains much material for the chemist engaged in the separation of synthetic industrial polymers. It offers little for workers in other fields.

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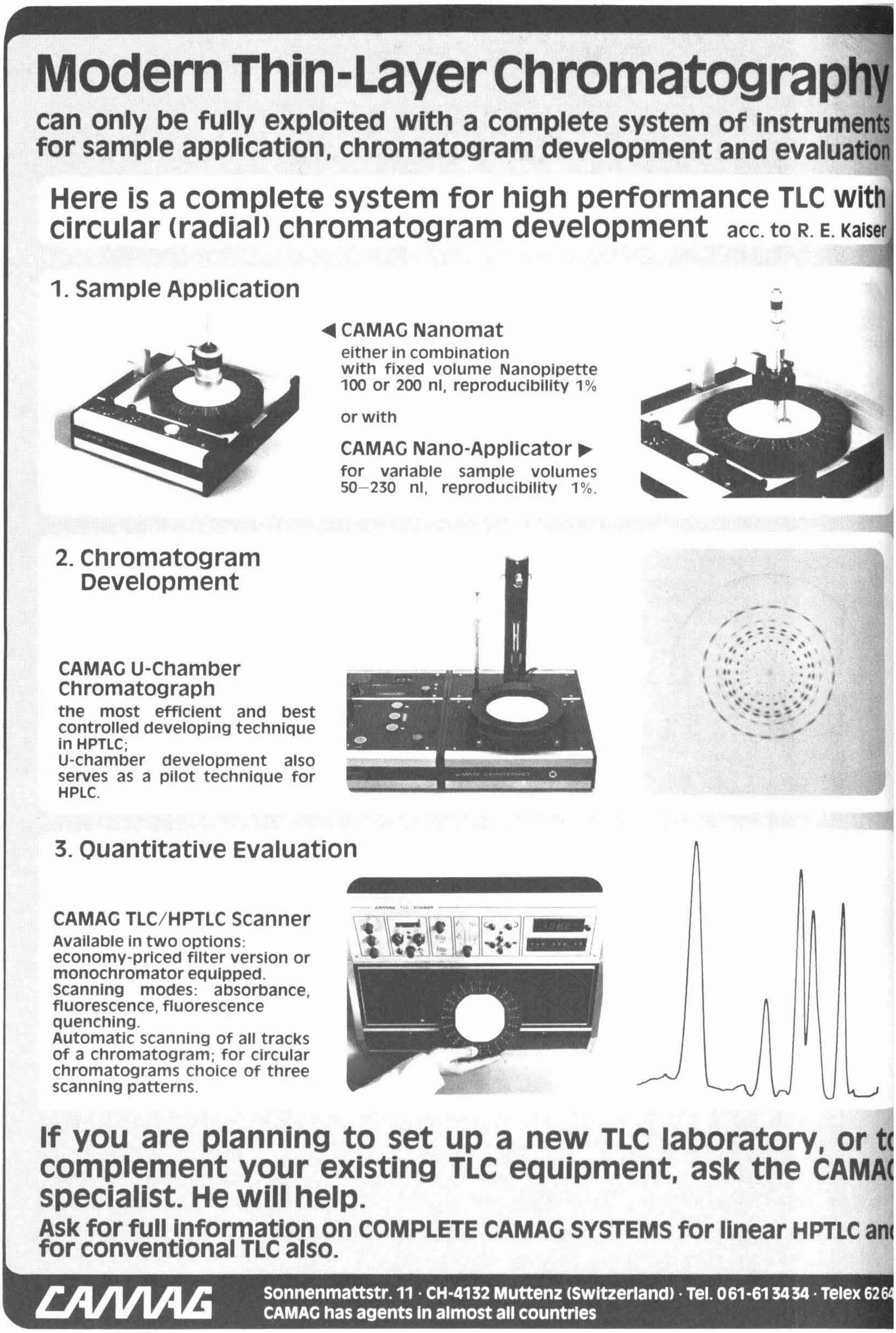

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