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SOLVENT COMPOSITION EFFECTS ON THE RETENTION CHARACTERISTICS OF AROMATIC HYDROXYL COMPOUNDS WITH SILICA AND POLAR MOBILE PHASES AND INTERPRETATION BY THE SNYDER MODEL

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(First received January 25th, 1989; revised manuscript received March 9th, 1989)

SUMMARY

Equations from the Snyder chromatographic model were used to interpret the retention data of aromatic hydroxyl compounds on a high-performance silica column. A number of *n*-heptane–2-propanol and *n*-heptane–ethyl acetate mobile phases were used to obtain retention data. It was found that plots of log capacity factor (k') *vs.* solvent strength and log k' *vs.* log mole fraction of the strong solvent showed good linearity for all solutes studied. The slopes of log k' *vs.* solvent strength were used to show that the solutes localized or were hydrogen bonding on the stationary phase. Also, the slopes from various plots were used to obtain experimental molecular areas (A_s) of the solutes. The A_s values were compared a number of ways and the results showed that it was important to consider both the localization of the solutes and the polar mobile phases. In addition, the theoretical chromatographic models correlated better with the data from the ethyl acetate binary mobile phases compared to the data from the 2-propanol binary mobile phases.

INTRODUCTION

High-performance liquid chromatography is a very useful tool in the separation of complex organic mixtures. While reversed-phase systems (non-polar bonded-phase columns and polar mobile phases) are more commonly used, normal-phase systems are also important. Normal-phase systems frequently offer better resolution of isomers and functional classes than reversed-phase systems[1]. A particular advantage of normal-phase systems is in separating coal-derived liquids, since the coal liquids are often insoluble in the water-based solvents frequently used in reversed-phase liquid chromatography[2].

Mobile phase composition in normal-phase liquid chromatography plays a vital role in selectivity and retention of solutes[1,3,4]. A number of authors have developed models to explain solvent, solute, and stationary phase interactions in normal-phase liquid chromatography[5–18]. If a model can describe the interactions in a given chromatographic system, the model can be used to predict retention of solutes at

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different mobile phase compositions. This information would be useful in obtaining a mobile phase composition that would give the best separation.

Snyder and co-workers[10,11] have developed an adsorption model based on the displacement of solvent molecules by solute molecules from sites on the stationary phase. A similar model has been proposed by Soczewinski and co-workers[5–9] for adsorption chromatography.

These models have been used to predict retention in normal-phase chromatographic systems. Hurtubise *et al.*[19] concluded that the Snyder–Soczewinski model described the retention for alkyl phenols on an aminopropyl bonded-phase column. Hussain *et al.*[20] found that the Snyder–Soczewinski model basically described the retention for alkyl phenols on silica and cyanopropyl bonded-phase columns for certain mobile phase compositions. Ruckmick and Hurtubise[21] concluded that the Snyder model could be used to describe the behavior of polar solutes on a silica stationary phase. Ruckmick and Hurtubise[22] used the Snyder model to describe solvent composition effects for nitrogen heterocycles and hydroxyl aromatics on a nitrophenyl stationary phase. Scolla and Hurtubise[23] were able to use the Snyder model to relate the retention of aromatic nitrogen compounds on an aminopropyl bonded-phase column. Snyder[24] used his model to predict the retention characteristics of diastereomers on a silica column. Snyder and Schunk[25] used the displacement model to describe the behavior of solutes on an amino bonded-phase column. The Soczewinski model was used by Hara *et al.*[26] to interpret the retention results of steroids on a silica stationary phase. Using the displacement model of Snyder, Hammers *et al.*[27] concluded that an amino bonded-phase column behaved like partially deactivated silica gel. Wieser *et al.*[28], using the Snyder model, concluded that a cyanopropyl bonded-phase column acts like deactivated silica gel. Smith and Cooper[29] applied Snyder's model to an amino, cyano, and diol bonded-phase columns to explain the behavior of solutes in several different solvents.

The model by Scott[12] and Scott and Kucera[13–15] views the retention mechanism to be a sorption process emphasizing solute-mobile phase interactions. The models of Snyder, Soczewinski, and Scott have been reviewed by Snyder and co-workers[10,25,30].

Hennion *et al.*[16] proposed a retention model for normal-phase aminopropyl bonded silica. The Hennion model contains elements of the Snyder, Soczewinski, and Scott models. These models were reviewed and summarized by Snyder and Schunk[25].

Chang and co-workers[17,18] have postulated hydrogen bonding as well as other types of interactions to explain the retention of phenolic and amine type compounds on an amino bonded-phase column.

Most of the earlier investigations that have considered theoretical adsorption models for normal-phase liquid chromatography have used low-molecular-weight standards and relatively weak mobile phases. In this study, we investigated a silica column with various polar compositions of *n*-heptane–2-propanol and *n*-heptane–ethyl acetate mobile phases. In addition, a set of aromatic hydroxyl standards were used that had specific structural features.

EXPERIMENTAL

High-performance liquid chromatograph

The liquid chromatograph consisted of mainly Waters Assoc. (Milford, MA,

U.S.A.) equipment. A Model 510 pump and a UK6 injector were used along with a Model 450 variable-wavelength detector set at 280 nm. Also a Linear 1200 strip chart recorder was employed.

Column

The column used was a 30 cm × 3.9 mm I.D. prepacked μPorasil column obtained from Waters Assoc. The μPorasil column consisted of 10-μm porous silica. The column was kept at a constant temperature with a Haake Model FE water circulator set at 25°C.

Reagents

J. T. Baker HPLC-grade *n*-heptane, 2-propanol, and ethyl acetate were obtained from VWR Scientific (Denver, CO, U.S.A.). These solvents were prefiltered through MAGNA Nylon 66 membrane filters of 0.47 μm which were obtained from Fisher Scientific (Denver, CO, U.S.A.).

Chromatographic systems

All chromatographic systems investigated were normal-phase systems using a silica column and various *n*-heptane–2-propanol or *n*-heptane–ethyl acetate mobile phase compositions at a flow-rate of 1.5 ml/min. The *n*-heptane–2-propanol mobile phases consisted of the following compositions: 99.8:0.2, 99.5:0.5, 99:1, 98:2, 97:3, 96:4, and 95:5 (v/v). The *n*-heptane–ethyl acetate mobile phases consisted of the following compositions: 98:2, 96:4, 94:6, 92:8, 90:10, 88:12, 86:14, 84:16, 82:18, and 80:20 (v/v).

Standard compounds

Standard compounds were selected based on their similar structure and size but differing polarity, acidities, and hydrogen bonding abilities. The standards were obtained from commercially available sources. Solutions of 1 mg/ml of the standards were prepared in the mobile phase composition that was used to elute the solute. The retention volumes of the standards were determined from duplicate injections of 5 μl of the standard solutions. The capacity factors (k') were calculated using the equation $k' = (V_R - V_M)/V_M$, where V_R (ml) is the measured retention volume and V_M (ml) is the column void volume. The column void volume was determined for each mobile phase composition by injecting 0.5 μl toluene and measuring its retention volume.

RESULTS AND DISCUSSION

Theoretical considerations

The model developed by Snyder[11] involves the following equation

$$X_m + nS_a \rightleftarrows X_a + nS_m \tag{1}$$

where the subscripts m and a refer to the mobile phase and adsorbed phase, respectively. The adsorption of a solute molecule X results in the displacement of n solvent molecules S. Using the Snyder model the following equation can be derived

$$\log k' = \log(V_A W/V_M) + \alpha'(S^0 - \varepsilon^0 A_s) \tag{2}$$

where k' is the capacity factor, V_A is the adsorbent surface volume, W (g) is the weight of adsorbent in the column, V_M is the void volume of the column, α' is the adsorbent activity, S^0 is the solute adsorption energy, ε^0 is the solvent strength parameter, and A_s is the normalized molecular area of the solute.

If plots of log k' *vs.* S^0 for standard polycyclic aromatic hydrocarbons are made using heptane as the eluent, α' and log $(V_A W/V_M)$ are the slope and intercept, respectively, from eqn. 2. The solvent strengths of binary eluents can be calculated if α' is known according to Snyder and Schunk[25] and Snyder[31] using

$$\varepsilon_{AB} = \varepsilon_A + \log[N_B 10^{\alpha' n_b}(\varepsilon_A - \varepsilon_B) + 1 - N_B]/\alpha' n_b \quad (3)$$

where ε_{AB} is the solvent strength of the binary eluent, ε_A and ε_B are the solvent strengths of the pure weak and strong solvent respectively, N_B is the mole fraction of solvent B, and n_b is the relative molecular area of a molecule of solvent B. Snyder[4] and Snyder and Schunk[25] have concluded that ε_B can vary with N_B due to restricted-access delocalization of the strong solvent on silica.

According to Snyder[30], the variation of retention of a solute with two mobile phases can be described by the following equation

$$\log(k'_2/k'_1) = \alpha' A_s(\varepsilon_1 - \varepsilon_2) \quad (4)$$

where k'_1 is the capacity factor of the solute in mobile phase 1 that has a solvent strength of ε_1, and k'_2 is the capacity factor for the solute in the second mobile phase of solvent strength ε_2. If *n*-heptane is used as mobile phase 1, the solvent strength (ε_1) is zero. If plots of log k'_2 *vs.* ε_2 are made, the slope is equal to $\alpha' A_s$ and the intercept is log k'_1. From the slopes of the log k'_2 *vs.* ε_2 plots, experimental A_s values can be determined if α' is known. Snyder and Glajch[32] have stated that for high-performance chromatographic silica α' can be considered as a constant equal to 0.57. Experimental A_s values are expected to be larger than calculated A_s values if solutes are polar and can localize and/or hydrogen bond with the adsorbent. These factors cause site-competition delocalization of the solute to occur on silica stationary phases[11,25,32].

With the Snyder[30] approach and very polar binary mobile phases the following equation can be derived

$$\log k' = \log k'_0 - (A_s/n_b) \log X_s \quad (5)$$

where A_s is the molecular area of the solute, n_b is the strong solvent molecular area, k' is the capacity factor of the solute eluted in the binary mobile phase, k'_0 is the capacity factor of the solute eluted in the pure strong solvent, and X_s is the mole fraction of the strong solvent in the binary mobile phase. For plots of log k' *vs.* log X_s, Snyder[24,30] has indicated that the slope (A_s/n_b) for monofunctional solutes is equal to the ratio of the number of solute molecules to polar solvent molecules which are displaced from the adsorbent surface. Snyder[24,30] has concluded that the experimental A_s values for a solute molecule can be a function of the solute's configuration on the adsorbent.

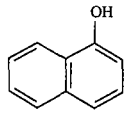
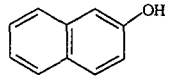
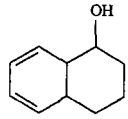
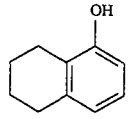
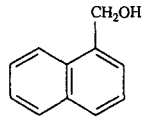
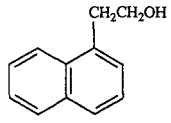
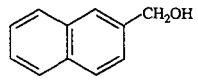
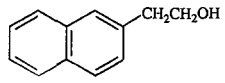
In this work, the A_s values were calculated using the following equation developed by Snyder[33] for unsubstituted aromatic hydrocarbons

$$A_s = 6 + 0.80(h - 6) + 0.25(c - h) \quad (6)$$

where h is the number of aromatic hydrogens in the solute and c is the number of aromatic carbons in the solute. The A_s values increase by 7.6 units and 8.5 units for aromatic –OH groups and aliphatic –OH groups, respectively[33].

TABLE I

COMPOUNDS STUDIED ON SILICA

No.	*Compound*	*Structure*	pK_a
1	1-Naphthol		9.3[a]
2	2-Naphthol		9.6[a]
3	1,2,3,4-Tetrahydro-1-naphthol		15.7[b]
4	5,6,7,8-Tetrahydro-1-naphthol		10.3[a]
5	1-Naphthalenemethanol		15.3[b]
6	1-Naphthaleneethanol		15.2[b]
7	2-Naphthalenemethanol		15.3[b]
8	2-Naphthaleneethanol		15.2[b]

[a] From ref. 34.

[b] Calculated from information in ref. 35.

Plots of log k' vs. solvent strength

The hydroxyl standards studied in this work appear in Table I along with their pK_a values[34,35]. The solvent strength values used in this study were determined from solvent strength data published by Snyder and Glajch[32]. Snyder and Glajch[32] listed solvent strength values for *n*-hexane–2-propanol and *n*-hexane–ethyl acetate mobile phases. Since *n*-heptane and *n*-hexane both have a solvent strength of zero on silica and both solvents give essentially the same selectivity, the solvent strength of a given mole fraction of strong solvent should be the same whether in *n*-hexane or *n*-heptane. For this work, solvent strengths were determined by graphing solvent strength values[32] *vs.* mole fraction of the strong solvent and then by knowing the mole fraction of the strong solvent used in this study the solvent strength values were obtained from the graphs. Table II gives a listing of various mobile phase compositions of *n*-heptane–2-propanol, log *k'* for the solutes, and solvent strength values used in this work. Similar information can be found in Table III for *n*-heptane–ethyl acetate mobile phases.

Using eqn. 4, plots of log *k'* *vs.* solvent strength for the solutes listed in Table I yielded linear correlation coefficients ranging from −0.994 to −1.00 for the *n*-heptane–2-propanol mobile phases, and the correlation coefficients were all −1.00 for *n*-heptane–ethyl acetate mobile phases. The results for *n*-heptane–2-propanol mobile phases were based on five compositions since results for the 96:4 and 95:5 mobile phases showed deviation from the log *k'* *vs.* solvent strength line. This is possibly due to the small *k'* values, ranging from 0.34 to 1.56, obtained at these compositions or to errors in determining the solvent strength in these solvent mixtures. The slope, intercept, and correlation coefficient values obtained are shown in Table IV. The linearity of the plots is shown by the high correlation coefficient values obtained. The lowest correlation coefficient was −0.994 for 1-naphthaleneethanol in the

TABLE II

LOG *k'* VALUES FOR THE MODEL COMPOUNDS ON SILICA WITH *n*-HEPTANE–2-PROPANOL MOBILE PHASES

Compound No.	*Mole fraction 2-propanol*						
	0.004	*0.010*	*0.019*	*0.038*	*0.056*	*0.074*	*0.092*
	n-Heptane-2-propanol (v/v)						
	99.8:0.2	*99.5:0.5*	*99:1*	*98:2*	*97:3*	*96:4*	*95:5*
1	1.224	0.760	0.387	0.039	−0.14	−0.28	−0.36
2	0.982	0.571	0.248	−0.07	−0.23	−0.36	−0.47
3	1.137	0.743	0.456	0.185	0.000	−0.11	−0.21
4	0.762	0.389	0.097	−0.18	−0.33	−0.47	−0.55
5	–	0.974	0.681	0.398	0.228	0.097	0.013
6	–	1.054	0.750	0.453	0.281	0.152	0.061
7	–	1.082	0.776	0.480	0.301	0.176	0.076
8	–	1.067	0.774	0.486	0.314	0.193	0.097
log X_s	−2.398	−2.000	−1.721	−1.420	−1.252	−1.131	−1.036
ε_{AB}	0.270	0.291	0.308	0.323	0.338	0.348	0.360

TABLE III

LOG k' VALUES FOR THE MODEL COMPOUNDS ON SILICA WITH *n*-HEPTANE–ETHYL ACETATE MOBILE PHASES

Compound No.	*Mole fraction ethyl acetate*									
	0.030	*0.059*	*0.087*	*0.115*	*0.143*	*0.170*	*0.196*	*0.222*	*0.248*	*0.273*
	n-Heptane–ethyl acetate (v/v)									
	98:2	*96:4*	*94:6*	*92:8*	*90:10*	*88:12*	*86:14*	*84:16*	*82:18*	*80:20*
1	1.096	0.721	0.498	0.350	0.220	0.107	0.025	−0.06	−0.14	−0.21
2	0.833	0.496	0.286	0.152	0.017	−0.06	−0.16	−0.24	−0.28	−0.39
3	1.367	1.046	0.855	0.721	0.582	0.473	0.393	0.307	0.233	0.187
4	0.733	0.382	0.167	−0.03	−0.10	−0.23	−0.28	−0.41	−0.48	−0.57
5	–	1.278	1.081	0.938	0.802	0.682	0.606	0.514	0.449	0.367
6	–	1.461	1.272	1.128	0.990	0.878	0.797	0.708	0.621	0.557
7	–	1.390	1.195	1.048	0.912	0.800	0.716	0.624	0.536	0.479
8	–	1.480	1.290	1.150	1.017	0.900	0.827	0.739	0.655	0.593
$\log X_s$	−1.523	−1.229	−1.060	−0.939	−0.845	−0.770	−0.708	−0.654	−0.605	−0.564
ε_{AB}	0.205	0.233	0.262	0.278	0.293	0.307	0.317	0.323	0.330	0.337

TABLE IV

SLOPE, INTERCEPT AND CORRELATION COEFFICIENT VALUES FOR LOG k' *vs.* SOLVENT STRENGTH FOR (a) 2-PROPANOL AND (b) ETHYL ACETATE BINARY MOBILE PHASES ON SILICA

Compound No.	*Slope*	*Intercept*	*Correlation coefficient*
(a) 1	−18.28	5.89	−1.00
2	−20.65	6.77	−1.00
3	−16.92	5.68	−1.00
4	−16.43	5.17	−1.00
5	−15.42	5.44	−1.00
6	−15.89	5.65	−0.994
7	−15.99	5.71	−1.00
8	−15.44	5.53	−1.00
(b) 1	−8.69	2.57	−1.00
2	−9.38	2.97	−1.00
3	−8.70	3.13	−1.00
4	−9.30	2.60	−1.00
5	−8.80	3.37	−1.00
6	−8.80	3.55	−1.00
7	−8.89	3.50	−1.00
8	−8.62	3.53	−1.00

n-heptane–2-propanol system. However, this is still a very good correlation. Fig. 1 shows a plot of log k' *vs.* solvent strength of 2-propanol for 1,2,3,4-tetrahydro-1-naphthol with *n*-heptane–2-propanol mobile phases. Fig. 2 shows similar results for the *n*-heptane–ethyl acetate mobile phases using 1,2,3,4-tetrahydro-1-naphthol.

Plots of log k' vs. log mole fraction of the strong solvent

The standard solutes investigated herein appear in Table I. For polar mobile phases eqn. 5 shows that plots of log k' *vs.* log mole fraction of the strong solvent should be linear[30]. Table V lists the slope, intercept, and correlation coefficient values for the results of log k' *vs.* log mole fraction of the strong solvent. The high values of the correlation coefficient indicates that the plots are very linear. Figs. 3 and 4 are plots of log k' *vs.* log mole fraction of the strong solvent for 1,2,3,4-tetrahydro-1-naphthol in *n*-heptane–2-propanol and *n*-heptane–ethyl acetate mobile phases, respectively. Unlike the results discussed for Table IV, the results for *n*-heptane–2-propanol mobile phases in Fig. 3 remained linear when plotted over the entire mobile phase composition range.

Theoretical interpretations

According to eqn. 4 the slope values in Table IV are equal to $\alpha' A_s$, and according to eqn. 5 the slope values in Table V are equal to A_s/n_b. The interpretation of the results for the slopes are given in the following two sections. Snyder and Glajch[32] have stated that α' for high-performance chromatographic silica is equal to 0.57. The n_b values for 2-propanol and ethyl acetate on silica have been determined by Snyder[11] to be equal to

TABLE V

SLOPE, INTERCEPT AND CORRELATION COEFFICIENT VALUES FOR LOG k' *vs.* LOG MOLE FRACTION OF THE STRONG SOLVENT FOR (a) 2-PROPANOL AND (b) ETHYL ACETATE BINARY MOBILE PHASES ON SILICA

Compound No.	*Slope*	*Intercept*	*Correlation coefficient*
(a) 1	−1.06	−1.57	−1.00
2	−1.18	−1.61	−1.00
3	−0.99	−1.23	−1.00
4	−0.97	−1.55	−1.00
5	−1.00	−1.02	−1.00
6	−1.03	−1.01	−1.00
7	−1.04	−1.00	−1.00
8	−1.00	−0.94	−1.00
(b) 1	−1.25	−1.05	−1.00
2	−1.35	−0.94	−1.00
3	−1.25	−0.49	−1.00
4	−1.34	−1.27	−1.00
5	−1.37	−0.38	−1.00
6	−1.37	−0.19	−1.00
7	−1.39	−0.28	−1.00
8	−1.35	−0.14	−1.00

4.4 and 5.2, respectively. Using this information, the slopes in Tables IV and V, and eqn. 6, the experimental and calculated A_s values can be determined. Table VI contains a list of calculated A_s, experimental A_s, and ΔA_s(calculated A_s − experimental A_s) from the log k' *vs.* solvent strength data for both *n*-heptane–2-propanol and *n*-heptane–ethyl acetate mobile phases. Table VII has similar information but from log k' *vs.* log mole fraction of the strong solvent data.

Log k' vs. solvent strength. Hydrogen bonding and localization of the solutes to the stationary phase is expected due to the polar nature of the functional groups. If localization or hydrogen bonding occurs, experimental A_s values should be larger than the calculated A_s values[3,4,24]. In all cases, this can be seen in Table VI with the *n*-heptane–2-propanol mobile phases. In Table VI for *n*-heptane–2-propanol, the largest absolute ΔA_s values occurred for compounds with the lowest pK_a values (see Table I). This would seem to follow since the smaller the pK_a value the more readily the compound would donate a proton and therefore more strongly localize or take part in hydrogen bonding with the stationary phase. Compound 4 has a pK_a of about 10.3 and the absolute ΔA_s value is smaller than for compounds 1 or 2 but larger than for compounds 5, 6, 7, and 8 which have pK_a values of around 15.2. Compounds 5, 6, 7, and 8 have the smallest absolute ΔA_s values suggesting they are not as strongly localized and are not as polar as the other compounds. The results for compound 3 do not correlate well, when comparing it's pK_a value to it's ΔA_s value. The pK_a values only give a very rough indication of the factors involved in obtaining experimental A_s values larger than calculated A_s values. Nevertheless, it is instructive to correlate the pK_a values with experimental A_s values because the pK_a values give a qualitative measure of the ability of the model compounds to donate protons.

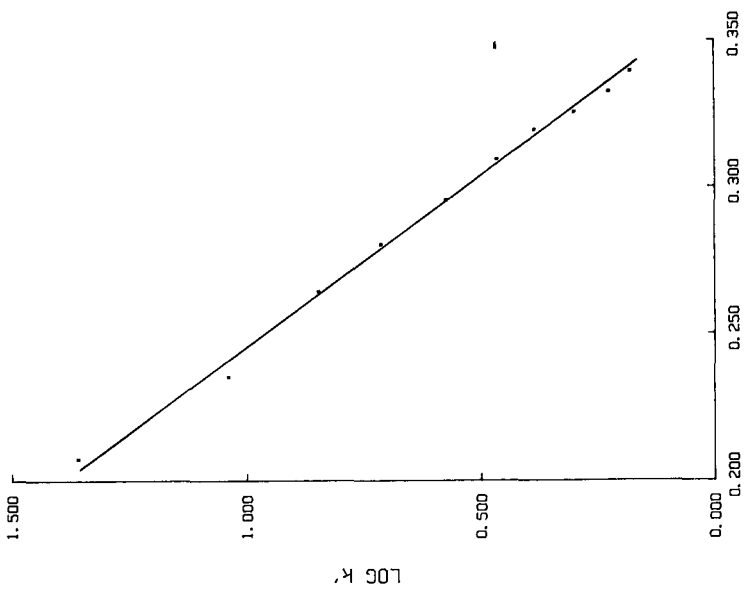


Fig. 1. Plot of log k' *vs.* solvent strength (ε) for 1,2,3,4-tetrahydro-1-naphthol using *n*-heptane–2-propanol mobile phases.

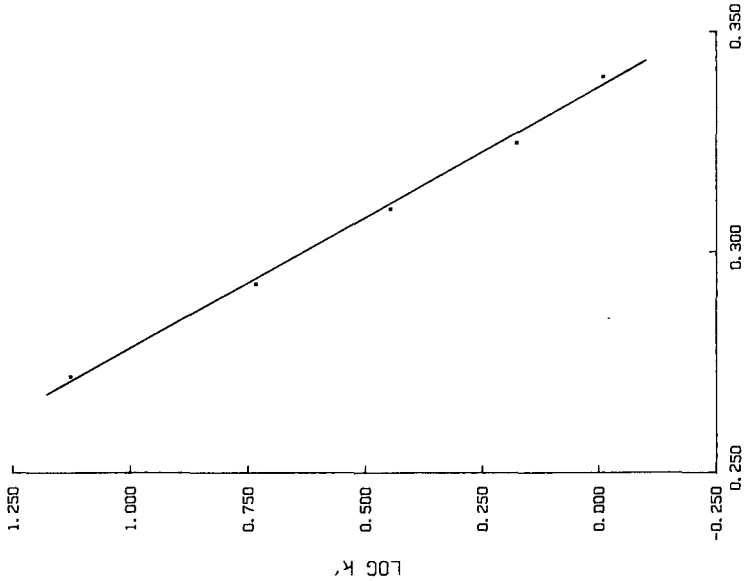


Fig. 2. Plot of log k' *vs.* solvent strength (ε) for 1,2,3,4-tetrahydro-1-naphthol using *n*-heptane–ethyl acetate mobile phases.

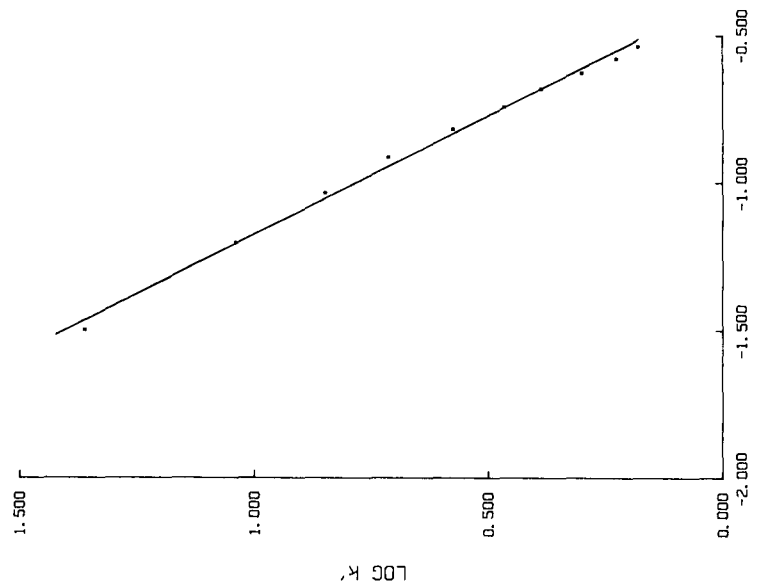


Fig. 3. Plot of log k' *vs.* log mole fraction 2-propanol for 1,2,3,4-tetrahydro-1-naphthol using *n*-heptane–2-propanol mobile phases.

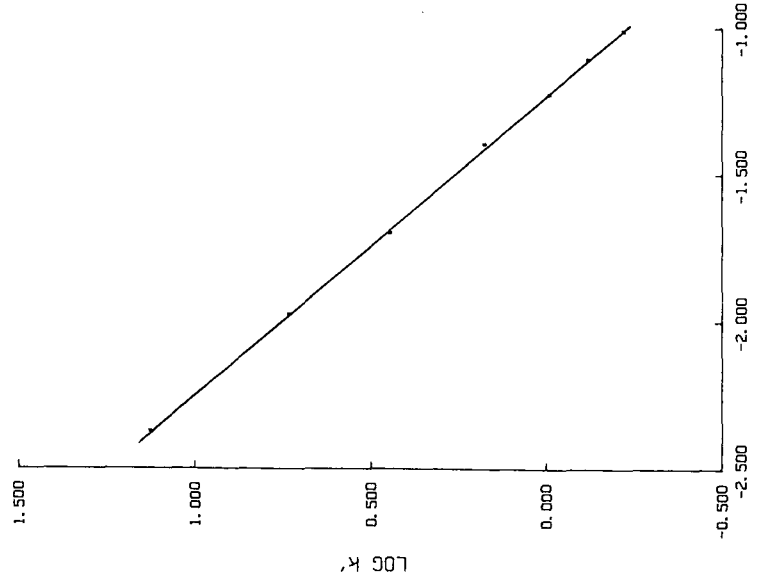


Fig. 4. Plot of log k' *vs.* log mole fraction ethyl acetate for 1,2,3,4-tetrahydro-1-naphthol using *n*-heptane–ethyl acetate mobile phases.

TABLE VI

CALCULATED AND EXPERIMENTAL A_s VALUES USING LOG k' *vs.* SOLVENT STRENGTH FOR (a) 2-PROPANOL AND (b) ETHYL ACETATE BINARY MOBILE PHASES ON SILICA

Compound No.	$A_s(calc)$	$A_s(exp)$	ΔA_s
(a) 1	15.7	32.1	−16.4
2	15.7	36.2	−20.5
3	18.1	29.7	−11.6
4	17.2	28.8	−11.6
5	17.5	27.0	−9.5
6	18.4	27.9	−9.5
7	17.5	28.1	−10.6
8	18.4	27.1	−8.7
(b) 1	15.7	15.3	0.4
2	15.7	16.5	−0.8
3	18.1	15.3	2.8
4	17.2	16.3	0.9
5	17.5	15.4	2.1
6	18.4	15.4	3.0
7	17.5	15.6	1.9
8	18.4	15.1	3.3

TABLE VII

RELATIVE MOLECULAR AREAS OF THE SOLUTES FROM LOG k' *vs.* LOG MOLE FRACTION OF THE STRONG SOLVENT FOR (a) 2-PROPANOL AND (b) ETHYL ACETATE BINARY MOBILE PHASES ON SILICA

Compound No.	$A_s(calc)$	$A_s(exp)$	ΔA_s	$A_s(exp)^a$	$\Delta A_s{}^a$
(a) 1	15.7	4.68	11.0	23.8	−8.1
2	15.7	5.17	10.5	26.5	−10.8
3	18.1	4.34	13.8	22.3	−4.2
4	17.2	4.25	13.0	21.8	−4.6
5	17.5	4.38	13.1	22.5	−5.0
6	18.4	4.52	13.9	23.2	−4.8
7	17.5	4.56	12.9	23.4	−5.9
8	18.4	4.40	14.0	22.5	−4.1
(b) 1	15.7	6.50	9.20	17.1	−1.4
2	15.7	7.02	8.68	18.5	−2.8
3	18.1	6.49	11.6	17.1	1.0
4	17.2	6.96	10.2	18.4	−1.2
5	17.5	7.13	10.4	18.7	−1.2
6	18.4	7.13	11.3	18.8	−0.4
7	17.5	7.21	10.3	19.0	−1.5
8	18.4	6.99	11.4	18.5	−0.1

[a] These A_s(exp) and ΔA_s values are based on the corrected n_b values which account for localization.

The experimental A_s values from the ethyl acetate binary mobile phases in Table VI, in most cases, are relatively smaller and much closer to the calculated A_s values compared to similar data from the 2-propanol binary mobile phases. The compounds with the smallest pK_a values (compounds 1, 2, and 4) have A_s experimental values relatively close to their calculated A_s values. For the remaining compounds, with the larger pK_a values, the experimental A_s values are relatively smaller compared to the calculated A_s values. The primary reason the experimental A_s values with the 2-propanol binary mobile phases are larger than the corresponding experimental A_s values with *n*-heptane–ethyl acetate mobile phases is due to the polarity of the 2-propanol binary mobile phases. The *n*-heptane–2-propanol mobile phases can localize and hydrogen bond to a greater extent than the *n*-heptane–ethyl acetate mobile phases[3,32]. Also, eqn. 4 recognizes the localization of the solute and solvent. However, eqn. 4 does not take into consideration the "interaction" of these two effects. Snyder *et al.*[3] have considered this and for the case of localizing solutes and solvents the term A_1 must be added to eqn. 4. The term A_1 corrects eqn. 4 for the "interaction" of solute and solvent localization, and its effect on k'. The term A_1 would become larger for increasing localization of the solute and solvent. The larger A_s(exp) values in Table VI for the 2-propanol binary mobile phases compared to the ethyl acetate binary mobile phases are due to 2-propanol localizing more strongly than ethyl acetate and the "interaction" effect being greater for 2-propanol compared to ethyl acetate.

Log k' vs. log mole fraction of the strong solvent. The method used to obtain experimental A_s values, as in Table VII, has been used in discussing solute configuration on the adsorbent[24,30]. A calculated A_s value assumes flatwise adsorption[33]. Snyder[30] has stated that experimental A_s values will be smaller than calculated if vertical adsorption occurs on the stationary phase. Vertical adsorption is often favored for strong solvents and silica[30]. Since the experimental A_s values in Table VII are considerably smaller than the calculated values, vertical adsorption is more likely than flatwise adsorption. The experimental A_s values in Table VII are smaller for the compounds in *n*-heptane–2-propanol mobile phases compared to the corresponding compounds in *n*-heptane–ethyl acetate mobile phases. This would seem to follow since 2-propanol is a stronger solvent than ethyl acetate.

However, another way to interpret the data is to consider the n_b values for the polar solvents. In using the n_b values to calculate the experimental A_s values, it was assumed that the solvents did not localize[32]. However, the solvents do localize and thus the n_b values would be larger than those used here due to localization. Snyder and Glajch[36] have commented on this earlier. Also, in the derivation of eqn. 5, it is assumed that solvent strength does not change with increasing percentage of strong solvent[11]. However, it has been shown that solvent strength does vary with percentage of strong solvent[32], and eqn. 5 may not be applicable to the data in this work. However, eqn. 5, or a slightly modified form of the equation, is widely used in chromatography; thus it is appropriate to comment on the use of eqn. 5 in the interpretation of chromatographic data. Snyder and Glajch[32] have reported localization functions for 2-propanol and ethyl acetate of 18.1 and 8.5, respectively. The appropriate localization function was added to the n_b values for 2-propanol and ethyl acetate to obtain corrected n_b values of 22.5 and 13.7, respectively. If these corrected n_b values are used to calculate the experimental A_s values, the new experimental A_s values for the *n*-heptane–2-propanol mobile phases are generally closer to the calculated A_s values compared to the

experimental A_s values calculated with uncorrected n_b values (see Table VII). Also, the experimental A_s values for the *n*-heptane–ethyl acetate mobile phases are much closer to the calculated A_s values in Table VII compared to the *n*-heptane–2-propanol mobile phases. Thus, by correcting the n_b values for the polar mobile phases, a better correlation is obtained between A_s(calc) and A_s(exp) for both 2-propanol and ethyl acetate binary mobile phases. The ΔA_s values for the 2-propanol binary mobile phases are still somewhat high. This is most likely due to the polar nature of 2-propanol.

Ideally, the A_s(exp) values in Table VI and Table VII should be very close to one another for a given compound. By comparing A_s(exp) values in Table VI for the ethyl acetate binary mobile phases with the corresponding A_s(exp) values in Table VII based on the larger n_b values, it can be seen that the A_s values are roughly the same for a given compound. By making the same comparison for the 2-propanol binary mobile phases in Tables VI and VII, the A_s(exp) values are considerably larger in Table VI compared to the A_s(exp) values in Table VII. However, the larger differences observed for the A_s(exp) values for the 2-propanol binary mobile phases is most likely related to the very polar nature of 2-propanol. In addition, eqn. 4 is considered to be more broadly based and the theoretical treatment of eqn. 4 has been discussed much more extensively in the literature than eqn. 5[11]. Thus, the data analysis using eqn. 4 is considered to be more valid. More work would have to be done to explain the differences observed in the A_s(exp) values for both binary mobile phases.

CONCLUSIONS

Linear relationships obtained for log k' *vs.* solvent strength and log k' *vs.* log mole fraction of the strong solvent showed that these relationships would be useful for predicting the retention characteristics of aromatic hydroxyl compounds. The interpretation of the retention data with equations from the Snyder model showed the importance of considering localization effects for both polar solutes and polar solvents. Comparison of A_s values for the aromatic hydroxyl compounds from graphs of log k' *vs.* solvent strength and log k' *vs.* log mole fraction of the strong solvent showed that the A_s values were roughly the same for ethyl acetate binary mobile phases. However, much larger differences in A_s values were obtained for the A_s values from the graphs with 2-propanol binary mobile phases. It was concluded that eqn. 4 was more valid in the interpretation of the retention data than eqn. 5.

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DETERMINATION OF 17-OXOSTEROID SULPHATES IN SERUM BY ION-PAIR EXTRACTION, PRELABELLING WITH DANSYLHYDRAZINE AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A new high-performance liquid chromatographic (HPLC) method with fluorescence detection is described for the direct determination of four serum 17-oxosteroid sulphates. Each serum sample was deproteinated with methanol, the methanol was evaporated and 17-oxosteroid sulphates in the residue were extracted with benzene as ion pairs in the presence of tetrapentylammonium ion. The ion pairs were labelled with dansylhydrazine and the hydrazones were separated by HPLC on a Capcell-Pak C_8 (silicone polymer-coated silica gel modified with octyl groups) reversed-phase column using methanol–0.5% (w/v) sodium acetate–50% (v/v) acetic acid (57:42:1, v/v/v) as the mobile phase. The eluent was monitored with a fluorometric detector at an excitation wavelength of 330 nm and an emission wavelength of 540 nm.

INTRODUCTION

The measurement of sulphates of 17-oxosteroids (17OS) in serum is of clinical significance for clarifying the situation of adrenal androgen. Sulphates of 17OS in serum include dehydroepiandrosterone (DHEA), androsterone (Ad), epiandrosterone (EA) and etiocholanolone (Ec) sulphates. Among these four steroids, DHEA sulphate is secreted abundantly from the adrenal. A low level of secretion is shown in adrenal cortex insufficiency, whereas a high level of secretion is indicated in cases of adrenal hyperplasia such as Cushing's syndrome. In contrast, cases of adenoma show a marked reduction in secretion, and this feature can be utilized in the differential diagnosis of adrenal lesions[1]. Thus, DHEA sulphate is the only adrenal androgen generally measured, and the clinical significance of the other 17OS sulphates has not yet been clarified.

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Measurement methods using gas chromatography (GC)[2–4] and GC combined with mass spectrometry[5–7] were reported. However, as the procedure necessary for such measurements is too complex, at present determinations are generally conducted by radioimmunoassay[8–11], but this method has a problem of cross-reaction.

For highly sensitive high-performance liquid chromatography (HPLC) with fluorescence detection, two methods have been reported[14,15]. In one, 17OS sulphates in serum are solvolysed after removal of free 17OS present in the serum[14]. In the other, serum is applied on to a Sep-Pak C_{18} cartridge[15].

DHEA sulphate and Ad sulphate have been separated by HPLC with fluorescence detection using dansylhydrazine as a prelabelling reagent, following these two pretreatment methods, but two other 17OS sulfates, EA and Ec sulphates, were not separated.

Independently, we have developed a method that involves the formation of 17OS sulphate ion pairs with tetrapentylammonium ion (TPA), extraction of the ion-paired 17OS sulphates with benzene, labelling of the ion pairs with dansylhydrazine, resulting in dansylhydrazones, and separate measurement by HPLC on a Capcell-Pak C_8 column followed by spectrofluorimetric detection. This method does not require hydrolysis. Compared with the usual methods of GC and radioimmunoassay, this new method requires a shorter measurement time and is highly accurate and reproducible. Furthermore it is able to measure DHEA, Ad, EA and Ec sulphates at the same time.

EXPERIMENTAL

Apparatus

The high-performance liquid chromatograph (Model 655A-11), equipped with a controller (Model L-5000LC), spectrofluorimeter (Model F-1000), recorder (Model D-2000 chromato-integrator) and column oven (Model 655A-52) were from Hitachi (Tokyo, Japan). A 250 mm × 4.6 mm I.D. stainless-steel column packed with Capcell-Pak C_8 (silicone polymer-coated silica gel modified with octyl groups; particle size 5 μm) was purchased from Shiseido (Tokyo, Japan).

Materials

DHEA, EA, Ec and Ad sulphates were obtained from Sigma (St. Louis, MO, U.S.A.) and tetrapentylammonium bromide from Aldrich (Milwaukee, WI, U.S.A.). All other reagents and solvents were of analytical-reagent grade or HPLC grade and were purchased from commercial sources.

Reagent solutions

TPA solution. A 0.1 *M* TPA solution was prepared by dissolving TPA bromide in water.

Dansylhydrazine solution. A 0.2% (w/v) solution was prepared by dissolving 20 mg of dansylhydrazine in 10 ml of acetonitrile; the solution was stored in a refrigerator until use.

Borate buffer. A 0.4 *M* buffer solution (pH 9.0) was prepared.

Standard solution. A 1-mg amount each of DHEA, EA, Ec and Ad sulphates were dissolved separately in 10 ml of water (concentration 100 mg/l) and an equal

volume of each was combined (four volumes), then 16 volumes of water were added (resulting in a concentration of each steroid of 5 mg/l), and the solution was stored in a refrigerator. Working standard solutions (0.05–5 mg/l of each steroid), used to determine calibration graphs, were prepared immediately before use by further dilution with water.

Mobile phase. A methanol–0.5% (w/v) sodium acetate–50% (v/v) acetic acid (57:42:1, v/v/v) mixture was prepared.

Procedure

Deproteination of serum sample. A 0.6-ml volume of serum was mixed with 3 ml of methanol in a glass test-tube, ultrasonicated for 15 min and centrifuged for 10 min at 1100 *g*. Then, 2.4 ml of the supernatant (0.4 ml as serum) were transferred into a glass test-tube and evaporated to dryness under reduced pressure.

Extraction of 17OS sulphates. The above residue was dissolved in 1.0 ml of water, then 0.6 ml of borate buffer was added and the mixture was shaken with 4 ml of benzene for 30 s. The mixture was centrifuged for 5 min at 700 *g* and the benzene layer was removed. Free 17OS was extracted in the benzene layer. 17OS can be measured by the method of Kawasaki *et al.*[14]. To the aqueous layer, 0.4 ml of TPA solution was added and mixed well. The mixture was shaken with 4 ml of benzene for 30 s, and centrifuged for 10 min at 700 *g*. A 2-ml volume of the benzene solution was transferred into a glass-stoppered test-tube and evaporated to dryness under reduced pressure (ion pairs of 17OS sulphates with TPA).

Labelling reaction. Labelling was done by adding 200 μl of dansylhydrazine solution and 50 μl of acetic acid to the extracted ion pairs of 17OS sulphates with TPA. The mixture was evaporated under reduced pressure at 50°C and allowed to stand for 20 min. The labelled ion pairs were dissolved in 100 μl of acetonitrile and an aliquot (5 μl for the calibration graph and normal values, 1 μl for high values, and 10 μl for low values) was then chromatographed.

Chromatographic conditions. The separation of 17OS sulphates was carried out on a Capcell-Pak C_8 column at 30°C with a mobile phase flow-rate of 1 ml/min. The effluent was monitored with a spectrofluorimeter at an excitation wavelength of 330 nm and an emission wavelength of 540 nm.

The above procedure was also used for working standard solutions.

Evaluation of serum deproteination

Preparation of sample. For evaluation of the methanol method and the Sep-Pak C_{18} cartridge method by the recovery test, serum–17OS sulphate solutions and serum–water solutions (9:1, v/v) were prepared. 17OS sulphate solutions were prepared by dissolving 1 mg each of the 17OS sulphates in 10 ml of water.

Methanol method[16,17]. A 1.0-ml serum sample was mixed with 5 ml of methanol and ultrasonicated for 15 min, before being centrifuged for 10 min at 1100 *g*. Then 1.0 ml (for direct dansylation) or 2.0 ml (for dansylation of ion pairs of 17OS sulphates with TPA) of the supernatant were evaporated. The 17OS sulphates were either directly dansylated, or extracted as ion pairs and dansylated by the extraction and dansylation procedure used for the present method.

Sep-Pak C_{18} cartridge[15]. The cartridge was activated with 5 ml of methanol and washed with 20 ml of water before use. A 0.6-ml volume of serum was diluted to

3.0 ml with 0.025 *M* phosphate buffer (pH 7.0) and applied to a Sep-Pak C_{18} cartridge, which was then washed with 4 ml of water. The 17OS sulphates adsorbed by the cartridge were eluted with 3 ml of methanol. Then 1 ml (for direct dansylation) or 2 ml (for dansylation of ion pairs) of the effluent were evaporated under reduced pressure.

Procedure for determinating 17OS sulphates by solvolysis

Solvolysis of 17OS sulphates[12,13]. The sample was acidified to pH 1.0 with 50% sulphuric acid, adjusted to a final salt concentration of 20% with sodium chloride and extracted with 4 ml of ethyl acetate. The organic phase was dried with 0.1 g of anhydrous sodium sulphate. The subsequent filtrate was kept at 30°C for 20 h, washed with 2 ml of 10% (w/v) potassium hydroxide and then twice with 2 ml of water. The washed solvent was dried with sodium sulphate and evaporated under reduced pressure (17OS from 17OS sulphates).

Labelling reaction of free-type 17OS. The 17OS in each residue was labelled by the dansylhydrazine method of Kawasaki *et al.*[14].

HPLC conditions. 17OS was separated by using a Zorbax SIL column (250 mm × 4.6 mm I.D.) at 25°C using the organic layer separated from the mixture of dichloromethane, ethanol and water (400:7:7, v/v/v). The effluent from the column was monitored with the spectrofluorimetric detector at an excitation wavelength of 350 nm and an emission wavelength of 505 nm[14].

RESULTS AND DISCUSSION

Optimization of assay conditions

Serum deproteination methods. Experiments were performed to find an adequate deproteination method for the determination of 17OS sulphates in serum.

Serum was deproteinated with either methanol or a Sep-Pak C_{18} cartridge. The 17OS sulphates in the deproteinated samples were directly dansylated either without using ion-pair extraction or after ion-pair extraction. The recoveries (± S.D., $n = 5$) of DHEA, EA, Ec and Ad sulphates obtained by deproteination with methanol, ion-pair extraction and dansylation were 96.8 ± 2.5%, 101.6 ± 3.3%, 101.1 ± 3.2% and 97.5 ± 2.8%, respectively, these values being better than those obtained with other methods. The recoveries (± S.D., $n = 5$) of 17OS sulphates obtained by deproteination with methanol and dansylation were 59.5 ± 2.3 to 78.7 ± 5.7% those obtained by treatment with the Sep-Pak C_{18} cartridge, ion-pair extraction and dansylation were 44.1 ± 3.3 to 52.2 ± 10.8% and those obtained by treatment with the Sep-Pak C_{18} Cartridge and dansylation were 32.1 ± 3.3 to 52.2 ± 10.8%.

Optimum pH extraction of 17OS sulphates. To determine the optimum pH for extraction of 17OS sulphates, we adjusted the pH of the mixture for five volumes of sample (4 mg/l of each DHEA sulphate) and one volume of TPA solution (pH 6, 7, 8, 9, 10, 11 or 11.5) with phosphate buffer (pH 6, 7 and 8) and borate buffer (pH 9, 10, 11 and 11.5) at a concentration of 0.2 *M*. For extraction with benzene, twice the volume of aqueous solution was used. The extraction (± S.D., $n = 5$) of DHEA sulphates in the pH range 6–11.5 was 98.8 ± 1.5%.

TPA extraction of 17OS sulphates. To determine the concentration of TPA at which extraction of 17OS sulphates with benzene would be maximized, 17OS sul-

phates were extracted with five different concentrations of TPA solutions. Maximum extraction was obtained at a molar ratio of TPA to DHEA sulphate of 50:1. When the molar ratios of TPA to DHEA sulphate were 10:1, 25:1, 50:1, 75:1 and 100:1, the extraction (± S.D., $n = 5$) was 78.0 ± 4.0, 91.5 ± 2.7, 101.1 ± 2.0, 99.6 ± 2.3 and 100.3 ± 2.5%, respectively.

Labelling. Dansylhydrazine has been used as a labelling agent for the chromatographic determination of free 17OS and 17OS sulphates. The dansylation has been conducted in the presence of a low concentration of strong acid in an organic solvent, including (1) concentrated hydrochloric acid in ethanol (final concentration 0.325%, v/v)[18]; (2) trichloroacetic acid in benzene (final concentration 0.33%, v/v[14], or 0.4%, v/v[15]) or (3) trichloroacetic acid in a mixture of benzene and ethanol (final concentration 0.33%, w/v)[19]. We tested various concentrations for dansylating ion pairs of the 17OS sulphates with TPA to determine directly the individual serum 17OS sulphates by HPLC. We found that acetic acid was as effective for dansylation as a low concentration of trichloroacetic acid. The optimum final concentrations of acetic acid and trichloroacetic acid were 20% (v/v) and 0.25% (w/v), respectively.

In order to select an adequate solvent for dansylation and for injection of the dansylated 17OS sulphates into the HPLC apparatus, the sample was dansylated by using ethanol, acetonitrile, 1,2-dichloroethane or benzene as the reaction solvent. The 17OS sulphate values obtained with benzene as reaction solvent were lower than those with acetonitrile and ethanol, and the values obtained with 1,2-dichloromethane were lower than those obtained with benzene. The relations of DHEA, EA, Ec and Ad sulphate values obtained with benzene or 1,2-dichloroethane to those obtained with acetonitrile were 0.20, 0.21, 0.23 and 0.23 (with benzene) and 0.11, 0.11, 0.12 and 0.11 (with 1,2-dichloroethane), respectively.

There was no difference between 17OS sulphate concentrations obtained with acetonitrile and those obtained with ethanol. However, with ethanol, some non-steroidal peaks were present on the chromatograms, and these peaks increased with time at room temperature. These peaks were not observed with acetonitrile (Fig. 1). We found that evaporation of the solvent in the reaction solution accelerated the labelling.

To bring the dansylation to an end after evaporation of the solvent in the reaction solution at the temperatures selected for the acceleration of the reaction, the optimum times for maintaining the various temperatures were determined, and were found to be 25 min at 40°C, 15–20 min at 50°C and 10 min at 60°C.

Mobile phase. In order to separate the four 17OS sulphates, the proportions of methanol, sodium acetate and acetic acid in the mobile phase on the Capcell-Park C_8 column were varied. The separation of 17OS sulphates was improved by decreasing the methanol concentration, but the capacity factor (k') increased and the retention time was prolonged. However the k' values decreased with increase in column temperature. The k' value of 17OS sulphates was slightly decreased with a reduction in the pH of the mobile phase (Fig. 2). The optimum mobile phase obtained from this experiment was methanol–5% (w/v) sodium acetate–50% (v/v) acetic acid (57:42:1, v/v/v).

Fluorescence spectrum. To obtain the fluorescence spectrum of DHEA sulphate extracted with benzene as ion pairs with TPA and then dansylated, the fractions of dansylated ion pairs separated by HPLC were collected, and monitored with a spec-

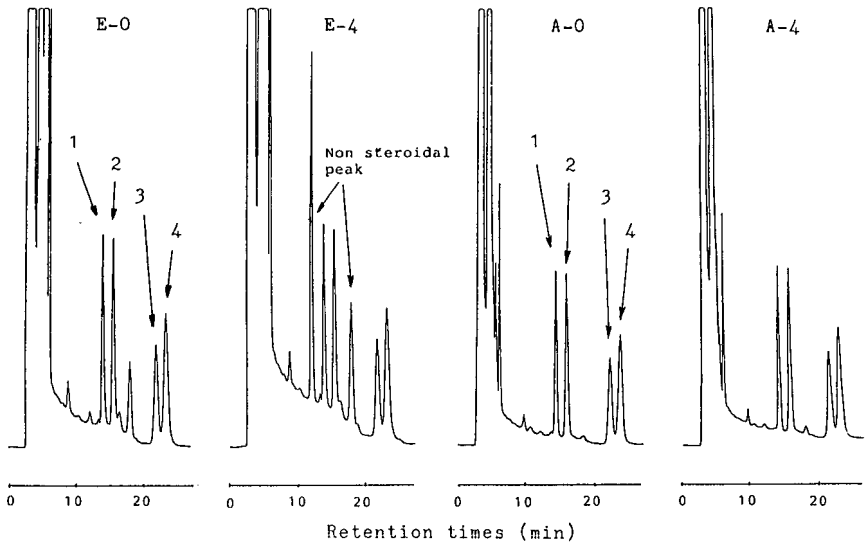

Fig. 1. Chromatograms obtained using ethanol (E-0, E-4) or acetonitrile (A-0, A-4) as solvents, for dansylated steroids E-0 and A-0 were obtained immediately after dansylation and E-4 and A-4 were obtained after E-0 and A-0 had remained for several hours at room temperature. Peaks: 1 = DHEA sulphate, 2 = EA sulphate; 3 = Ec sulphate; 4 = Ad sulphate.

trofluorimeter (Hitachi Model 4010). Dansylated ion pairs of DHEA sulphates with TPA had an extraction maximum at 332 nm and an emission maximum at 538 nm in the mobile phase.

Stability. The fluorescence of the dansylhydrazones obtained by dansylation of ion pairs of the 17OS sulphates with TPA following evaporation of the solvent was stable for 30 days, and the fluorescence of the dansylated sample in the solvent dissolved in acetonitrile was stable for 1 week (both when stored in a refrigerator).

Calibration graphs. Calibration graphs were prepared by using 0.05–5 mg/l working standard solutions. The equations for the calibration graphs were $y = 3.9x$ for DHEA sulphate, $y = 3.4x$ for EA sulphate, $y = 3.8x$ for Ec sulphate and $y = 3.8x$ for Ad sulphate (y = amount of steroid, ng/injection volume; x = peak area, mV min). Coefficients of variation (C.V.) of the values obtained with the working standard solutions containing 0.05, 0.1, 0.5, 1.0 and 5.0 mg/l each of the 17OS sulphates were 4.3–6.1, 2.7–3.2, 2.2–2.8, 1.5–2.6 and 1.7–2.5% ($n = 5$), respectively.

Analytical Recovery. The serum sample recovery was determined by adding 0.1 ml of solution containing either 5 or 10 mg of all four 17OS sulphates at a ratio of 1 l of water to 0.9 ml of serum sample (final concentration of each, 500 and 1000 μg/l). The recoveries obtained on addition of the solution of 5 or 10 mg of 17OS sulphates were in the ranges 95.8–104.0% ($n = 5$) and 96.3–102.2% ($n = 10$), respectively, as shown in Table I.

Within- and between-run precision. The within-day C.V. values, determined from ten repeated measurements of 17OS sulphates in serum and in serum with 5 or 10 mg of added 17OS sulphates as in the recoverey test, were in the range 2.56–7.40% and 2.78-3.34% (Table I). The between-day C.V. values, determined once a day on

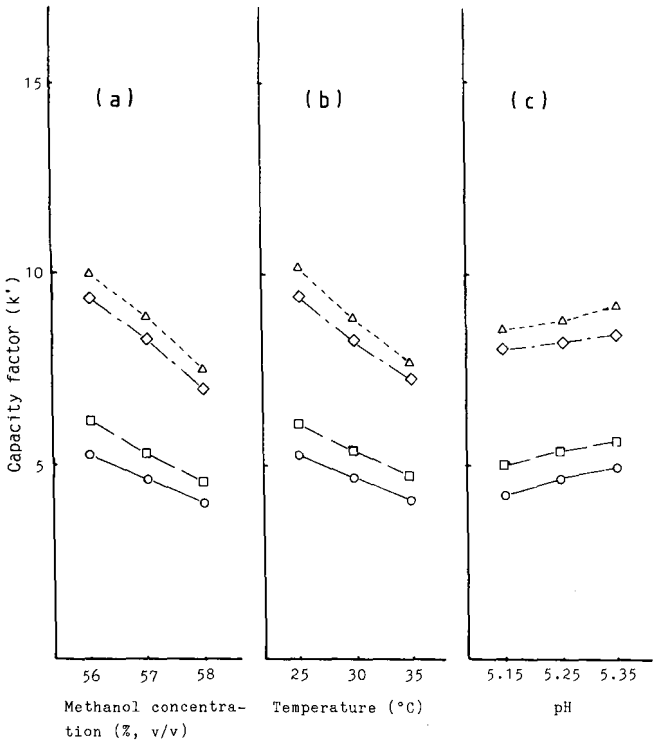

Fig. 2. Effect of methanol concentration and pH in mobile phase and column temperature. (a) The methanol concentration in the mobile phase [methanol–0.5% (w/v) sodium acetate–50% (v/v) acetic acid (57:42:1, v/v/v)] was changed from 56 to 58% at a column temperature of 30°C. (b) The column temperature was changed from 25 to 35°C using the proposed mobile phase. (c) The pH was changed from 5.15 to 5.35 by variation of the sodium acetate concentration in the mobile phase at a column temperature of 30°C. (○) DHEA sulphate; (□) EA sulphate; (◇) Ec sulphate; (△) Ad sulphate.

ten successive days from measurement of 17OS sulphates in the serum sample with added 17OS sulphates as in the recovery test, were in the range 4.11–5.98%.

Comparison with results obtained by deconjugation of 17OS sulphates in serum. The 17OS sulphate concentrations obtained with the present method (converted into free-type values) were compared with those obtained from solvolysis. Good correlations were observed for 17OS sulphates correlation coefficients of DHEA, EA, Ec and Ad were 0.927, 0.905, 0.929 and 0.948, respectively (n = 20)].

Results using samples from both patients and healthy males

Typical chromatograms from serum samples. Typical chromatograms of 17OS sulphates in serum from a patient with Cushing's syndrome and from a healthy male are shown in Fig. 3.

Concentration of 17OS sulphates in serum. The determination of 17OS sulphates in serum is generally only performed for DHEA sulphate by radioimmunoassay. However, radioimmunoassay of steroids has the problem of steroid cross-reactivity. The direct determination of 17OS sulphates in serum by HPLC shows excellent speci-

TABLE I

ANALYTICAL RECOVERY OF 17OS SULPHATES FROM AND PRECISION OF ASSAY

Sulphate	*Amount added (μg/l)*	*Final amount (μg/l)*	*Recovery (%)*	*n*	*C.V. (%)*
DHEA	0	2014		5	2.89
	500	2493	95.8	5	3.75
	0	1263		10	2.56
	1000	2226	96.3	10	3.13
EA	0	174		5	5.16
	500	694	104.0	5	4.04
	0	76		10	7.40
	1000	1094	102.2	10	2.86
Ec	0	227		5	5.32
	500	733	101.2	5	3.87
	0	110		10	7.07
	1000	1095	98.5	10	3.34
Ad	0	669		5	3.60
	500	1154	97.0	5	3.62
	0	475		10	3.16
	1000	1461	98.6	10	2.78

ficity, but so far has been reported only for DHEA and Ad sulphates by Kawasaki *et al.*[15]. We applied our method for the direct determination of DHEA, Ad and two other 17OS sulphates to serum from 50 healthy men and a patient with Cushing's syndrome.

Table II shows that the levels of DHEA and Ec sulphates in the serum of the patient were higher than those in healthy men. Yamaji and Ibrayashi[1] reported the levels of DHEA sulphate for healthy men and women to be 1650 ± 471 μg/l (21–30-year-old men, n = 25) and 1090 ± 298 μg/l (21–30-year-old women, n = 20), Seki-

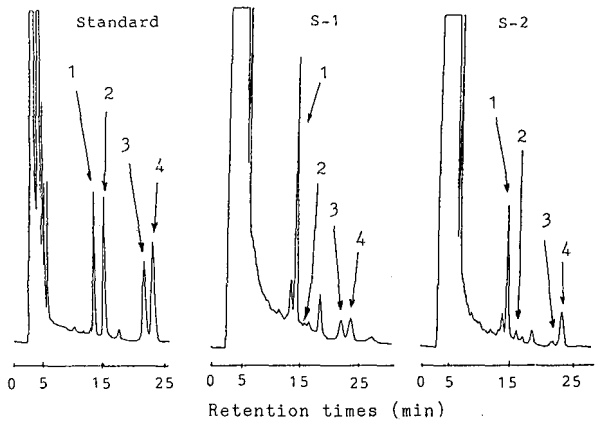

Fig. 3. Chromatograms of Serum 17OS sulphates. Standard: 2 mg/l of each steroid. S-1 = serum from 29-year-old man with Cushing's syndrome. S-2 = serum from a 25-year-old healthy man. Mobile phase: methanol–0.5% (w/v) sodium acetate–50% (v/v) (57:42:1, v/v/v).

TABLE II

17OS SULPHATE CONCENTRATIONS IN SERUM

Sulphate	*Concentration (μg/l) 29-year-old man with Cushing's syndrome*	*Healthy 31–55-year-old men*
DHEA	2595	1156 (± 572)
EA	162	97 (± 80)
Ec	298	91 (± 39)
Ad	273	454 (± 287)

hara *et al.*[20] 1780 ± 630 μg/l (20–29-year-old men, n = 15) and 980 ± 410 μg/l (20–29-year-old women, n = 14) and Kokubo *et al.*[11] 1290 ± 590 μg/l (20–50-year-old men, n = 10) and 830 ± 620 μg/l (20–50-year-old women, n = 5). The value obtained by the present method was 1156 ± 574 μg/l (31–55-year-old men, n = 50). Any difference between the previously reported values, determined by radioimmunoassay, and the values obtained by the present method are likely to have been due to steroid cross-reactivity (Kawasaki *et al.*[15] reported a method for the separation of DHEA and Ad sulphates by HPLC, but no values were given).

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Note

Evaluation of the moving belt as an interface for the high-performance liquid chromatographic–mass spectrometric analysis of the flavonoid aglycones

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The moving belt[1] is a transport interface that makes possible the direct coupling of high-performance liquid chromatography (HPLC) and mass spectrometry (MS), obtaining true electron impact (EI) and chemical ionization (CI) mass spectra with free selection of reagent gases[2–4].

Although EI and/or CI mass spectra alone can hardly establish completely the structure of a given flavonoid aglycone, they can always determine the molecular weight of these compounds and almost always the distribution of substituents between the A- and B-rings (see the structure insert in Fig. 1)[5–11]. The mass spectra of the flavonoids are also quite characteristic and can differentiate many isomers: compare, for instance, the spectra of quercetin and morin in Table I. Further, detailed EI mass spectrometric studies are available of the flavonoids that permit assignement of complicated substitution patterns[12–14]. Thus, with the combination of HPLC/UV–visible techniques[15], HPLC–EI-MS and HPLC–CI-MS analysis would be enough for the complete identification of many flavonoid aglycones, if a system for such HPLC–MS analysis was available.

Previously, we have studied some phenolic compounds by HPLC–MS using the moving belt as interface[16–22]. Here we report a study of the suitability of the belt for the HPLC–MS analysis of representative compounds of four flavonoid aglycone classes: flavones, flavonols, flavanones and flavanonols, covering an extensive range of polarities.

EXPERIMENTAL

A VG Analytical moving belt (which provides sample flash evaporation directly into the ion source) interfaced to a VG Analytical double focusing 7070E mass spectrometer, operated at low resolution (R_s = 1000) and equipped with an EI–CI ion source has been used.

A polyimide (Kapton®) belt at 1.6 cm/s was used. Conditions: infrared solvent heater, 150°C (indicated); sample evaporator temperature, 200°C (indicated); ion

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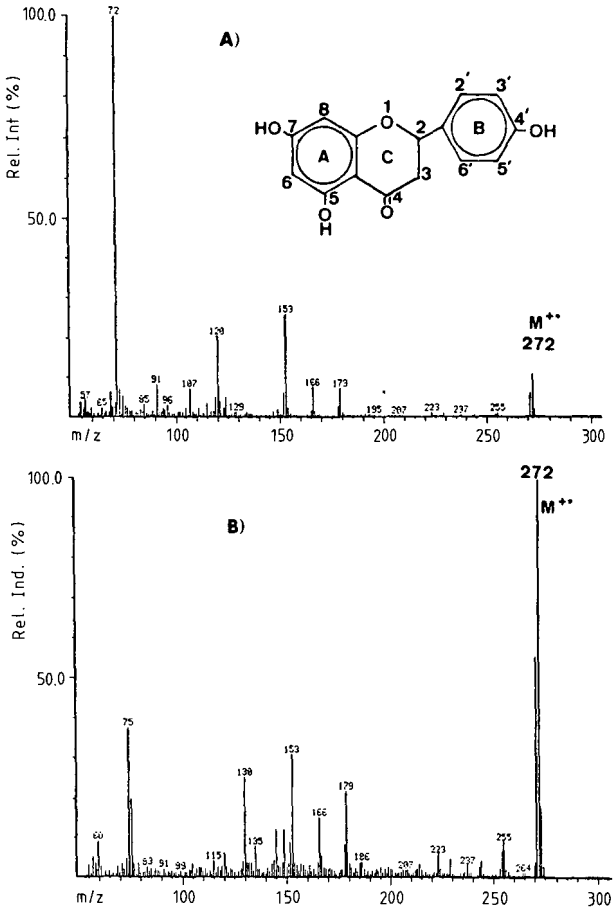

Fig. 1. EI mass spectra of naringenin obtained using the moving belt as inlet system: (A) and (B), 2 and 4 μg of the compound spotted on the belt, respectively.

source temperature, 200–210°C in EI and CI mode; ion source pressures, (a) $2 \cdot 10^{-6}$ Torr in EI mode that increased to $6 \cdot 10^{-6}$–$8 \cdot 10^{-6}$ Torr when working with LC on line and (b) $4 \cdot 10^{-5}$ Torr in CI mode (methane as reagent gas); electron energy and emission current, 70 eV and 200 μA, respectively. The scan rates and mass ranges were (a) 2 s per decade and 35 to 500 a.m.u. for the spotting experiments in the EI mode; and (b) 3 s per decade and 90 to 500 a.m.u. for the HPLC–MS analysis, detection limit and spotting studies in the CI mode.

The chromatographic system consisted of Waters 6000 A pumps, an M660 solvent programmer and an M441 UV absorbance detector (254 nm). The column was a Hypersil ODS 2 (10 cm × 4.6 mm I.D.) with a 3-μm particle diameter.

The solvent used was methanol–5% aqueous acetic acid (60:40, v/v) at a flow-rate of 0.9 ml/min directly fed on the belt with an electrically heated spray deposition device.

Flavonoid standards were obtained from commercial suppliers (Fluka, Madaus, Sarsyntex) or as described in refs. 23 and 24.

TABLE I

EI AND METHANE CI MASS SPECTRA OF SELECTED FLAVONOID AGLYCONES OBTAINED SPOTTING THE COMPOUNDS IN METHANOLIC SOLUTION DIRECTLY ONTO THE MOVING BELT INTERFACE

Only structurally significative peaks are quoted (see analytical conditions in the Experimental section).

Compound and substitution pattern	*Molecular weight*		*m/z (% relative intensity)*
Flavones			
Flavone	222	EI	222(100), 221(34), 194(30), 165(7), 120(42), 97(4), 92(7)
		CI	251(8), 224(15), 223(100), 222(6)
Chrysin 5,7-$(OH)_2$	254	EI	254(100), 253(16), 226(31), 152(49), 124(36), 105(7), 102(11), 96(15)
		CI	283(12), 256(21), 255(100), 254(26), 237(6), 153(18)
Apigenin 5,7,4′-$(OH)_3$	270	EI	270(100), 269(12), 242(15), 124(23), 121(31)
		CI	299(12), 272(22), 271(100), 270(19)
Acacetin 5,7-$(OH)_2$-4′-OCH_3	284	EI	284(100), 283(10), 256(4), 241(10), 152(8), 132(21), 124(5)
		CI	313(5), 286(19), 285(100), 284(13), 257(4)
Luteolin 5,7,3′,4′-$(OH)_4$	286	EI	286(100), 285(9), 258(17), 229(9), 153(67), 137(6), 134(21), 124(18), 96(9)
Xanthomicrol 5,4′-$(OH)_2$-6,7,8-$(OCH_3)_3$	344	EI	344(61), 343(3), 329(100), 314(6), 301(4), 211(24), 183(16), 118(9)
		CI	373(36), 346(72), 345(100), 344(82), 343(9), 329(33), 315(5), 314(4)
Cirsimaritin 5,4′-$(OH)_2$-6,7-$(OCH_3)_2$	314	EI	314(98), 313(23), 299(100), 285(25), 271(33), 181(29)
		CI	343(8), 316(21), 315(100), 314(17), 299(6), 285(9), 271(6), 265(12), 255(21), 205(12)
Diosmetin 5,7,3′-$(OH)_3$-4′-OCH_3	300	EI	300(100), 299(2), 284(9), 271(5), 257(18), 229(12), 153(12), 148(6), 133(8), 105(3)
		CI	329(9), 302(15), 301(100), 300(19), 285(7), 283(5), 153(4)
Flavonols			
Kaempferol 3,5,7,4′-$(OH)_4$	286	EI	286(100), 285(25), 258(9), 229(7), 213(6), 153(5), 143(4), 121(10)
		CI	315(4), 288(19), 287(100), 286(20)
Quercetin 3,5,7,3′,4′-$(OH)_5$	302	EI	302(100), 301(24), 273(13), 257(9), 245(9), 229(11), 153(29), 137(43), 109(22)
		CI	331(13), 304(23), 303(100), 302(32)
Morin 3,5,7,2′,4′-$(OH)_5$	302	EI	302(99), 301(7), 286(54), 285(100), 153(32), 137(16)
		CI	304(18), 303(100), 302(11)
Flavanones			
Naringenin 5,7,4′-$(OH)_3$	272	EI	272(100), 271(56), 254(7), 244(4), 179(22), 166(15), 153(31), 120(6)
		CI	301(6), 274(20), 273(100), 272(16), 265(7), 255(7), 179(31), 153(35), 147(7)

TABLE I (*continued*)

Compound and substitution pattern	*Molecular weight*		*m/z (% relative intensity)*
Eriodictyol 5,7,3′,4′-$(OH)_4$	288	EI	288(10), 287(5), 179(21), 166(31), 153(100), 136(76), 123(46), 110(18)
Flavononols			
Taxifolin 3,5,7,3′,4′-$(OH)_5$	304	EI	304(18), 286(5), 275(31), 165(17), 153(79), 152(31), 137(30), 123(45)
		CI	306(15), 305(100), 304(4), 289(17), 287(33), 275(15), 259(19), 195(9), 181(9), 153(14)

RESULTS AND DISCUSSION

Polarity and possibility of handling the flavonoid aglycones by the belt

At the moment, no HPLC–MS system or interface is of universal application[3,4] In order to determine which types of flavonoids can be investigated by the VG moving belt, initial studies were conducted with a series of flavonoids (Table I), by spotting solutions of the samples (1–2 μl of a 1-mg/ml solution of each compound in methanol) onto the belt under a variety of source and sample evaporator temperature settings. Flavone, xanthomicrol, cirsimaritin and other apolar flavonoids provided very good EI and CI mass spectra from the moving belt interface and temperature control was not very critical. On the other hand, the more polar and involatile compounds, as luteolin and taxifolin, for instance, were very sensitive with respect to the temperature settings. 200°C for the sample evaporator and 200–210°C for the ion source were the most convenient for most of the flavonoids studied as well in the EI as in the CI mode. However, the belt was unsuitable for the mass spectral analysis of high-polarity and high-molecular-weight compounds as the flavonolignans silybin, silydianin and silychristin. There was not a recognizable molecular ion for these flavonoids using the belt as inlet system.

With regard to the spectral quality, there are two features worthy of consideration. (i) The background contribution to the spectra (even after background subtraction) with the more involatile samples is intense and it often gives an intense peak at *m/z* 149 due to belt plasticizers. This is specially inconvenient for the EI-MS analysis of the flavonoids because of its proximity to the diagnostic peaks at *m/z* 152 and/or *m/z* 153 of many 5,7-dihydroxyflavonoids and a peak at *m/z* 177 that interferes with the diagnostic peak at *m/z* 179, $[M - (B\text{-ring})]^+$, of the 5,7-dihydroxyflavanones. (ii) With some polar compounds, there is also a notable diminution of the relative intensities of the highest mass ions (molecular ion included) compared with the corresponding direct-probe inlet EI mass spectra. This, added to the relatively high background levels in the spectra of these compounds, leads to useful mass spectra but only of medium quality.

Thus, in the given conditions, the moving belt studied is a good EI-as well as CI-MS inlet for the flavonoids of low to medium polarity examined, but limited with respect to high polar and involatile compounds.

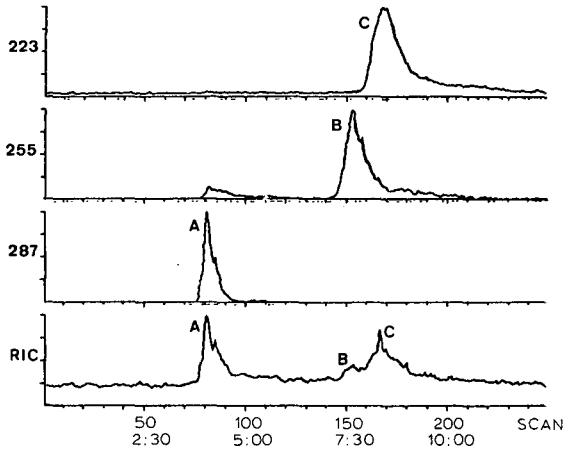

Fig. 2. Methane CI HPLC–MS computer reconstructed total-ion current trace (RIC) and extracted mass chromatograms ($[M + H]^+$ ions) obtained for a mixture of 10 μg of kaempferol (mol.wt. 286; peak A), 3 μg of chrysin (mol.wt. 254; peak B) and 3 μg of flavone (mol.wt. 222; peak C) injected on column (see chromatographic and MS conditions in the Experimental section).

Detection limits and HPLC–MS on line of the flavonoid aglycones

The mass spectra obtained using the moving belt are dependent on the quantity of sample spotted on the surface[21,25]. In our case, spotting 2 or 4 μg of naringenin on the belt has a dramatic effect on the relative intensity of the molecular ion, as it is shown in Fig. 1. Because of this, it is worthy to study the practical detection limits of the flavonoid aglycones using this interface at full scan, both spotting the samples directly on the belt and in true HPLC–MC conditions. The minimum amounts of

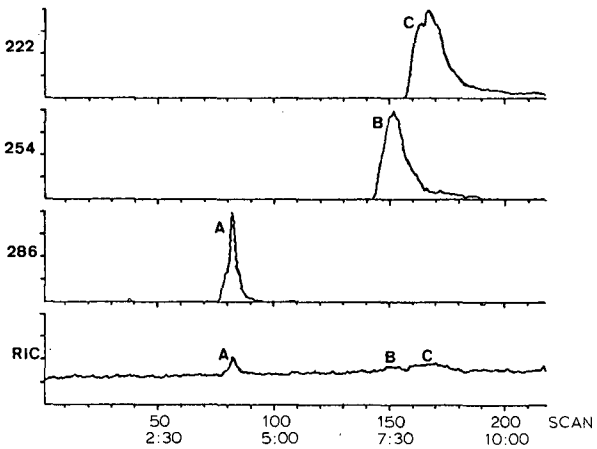

Fig. 3. HPLC–EI-MS computer reconstructed total-ion current trace (RIC) and extracted mass chromatograms ($M^{+\cdot}$ ions) obtained for a mixture of 10 μg of kaempferol (peak A), 3 μg of chrysin (peak B) and 3 μg of flavone (peak C) injected on column (see chromatographic and MS conditions in the Experimental section).

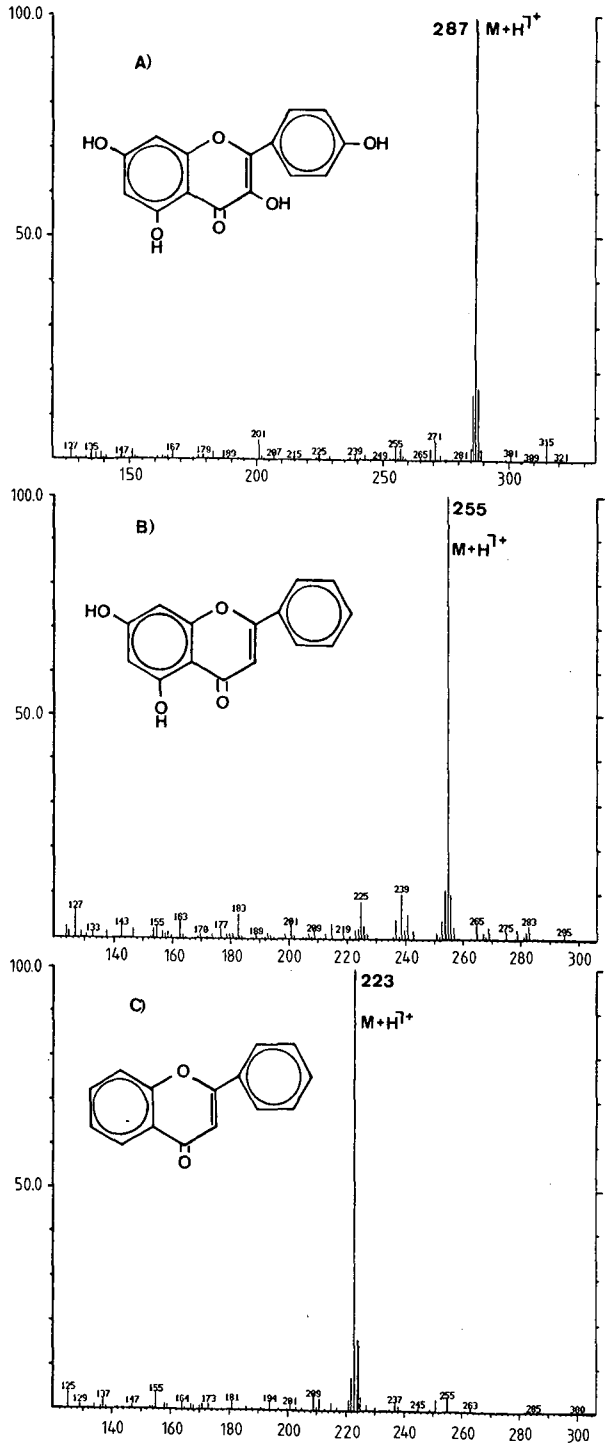

Fig. 4. (A), (B) and (C), methane CI mass spectra of peaks A, B and C, respectively, in the HPLC–MS chromatogram shown in Fig. 2.

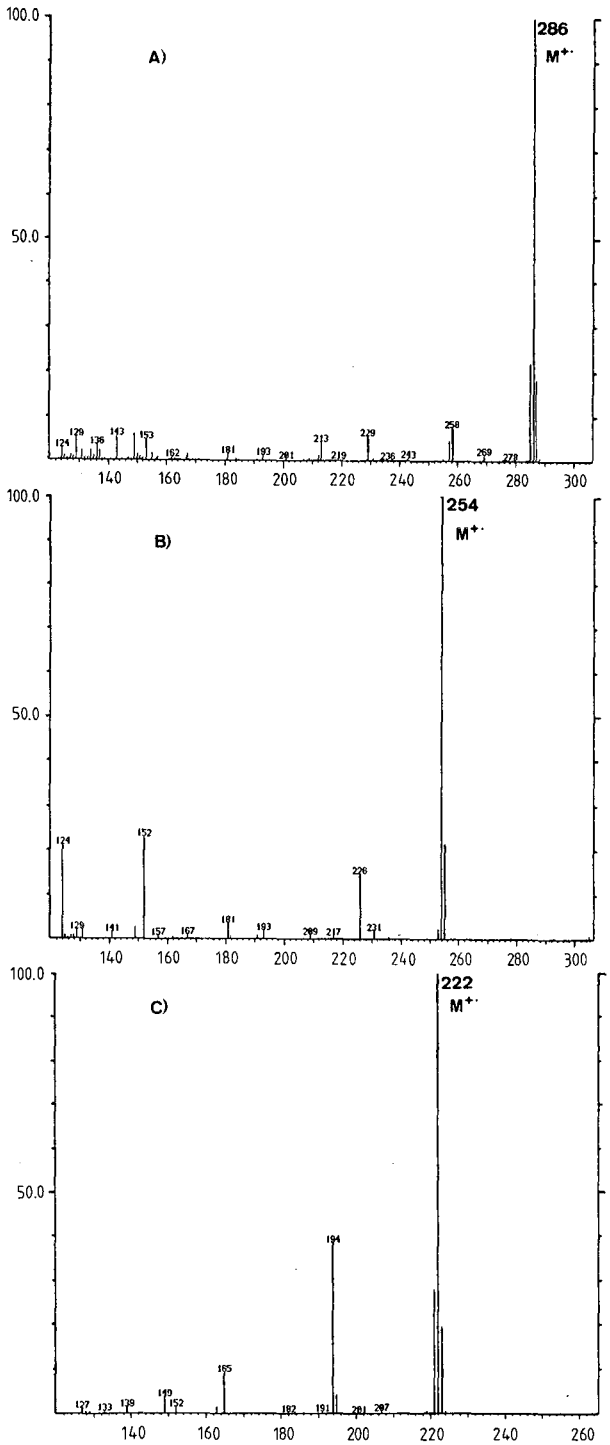

Fig. 5. (A), (B) and (C), EI mass spectra of peaks A, B and C, respectively, in the HPLC–MS chromatogram in Fig. 3.

sample effectively spotted or injected on the column that give an interpretable mass spectrum and comparable to the direct inlet spectrum of the same compound are as follows: flavone, 0.05 μg spotting and 0.1 μg injecting; kaempferol, 0.5 μg spotting and 4.0 μg injecting. For this purpose, we have chosen a flavonoid of low polarity (flavone) and one of medium-high-polarity (kaempferol) in order to cover a reasonable polarity range. Obviously, because of the column dilution effect, when working on line, the limits are higher than when spotting directly onto the belt. Thus, for those compounds which tend to be adsorbed on the belt surface, the amount of sample needed for the detection and structural analysis of the flavonoids injected on column is quite high.

With respect to the HPLC–MS flavonoid mixture analysis using the VG moving belt, as in many HPLC–MS mixture analysis, with this and other systems, the computer reconstructed total ion current traces are poor, even scanning 90 a.m.u. upwards to avoid solvent interferences (Figs. 2 and 3). But, for low molecular weight and/or low polarity flavonoids, the $[M + H]^+$ ion (CI mode) or $M^{+\cdot}$ ion (EI mode) mass chromatograms are acceptable and the spectra of every peak, even for those not well resolved, provide very valuable structural information, as it is shown in Figs. 2–5.

Thus, whereas the detection of single flavonoids and simple mixtures of low-polarity compounds injected on column is possible with the HPLC–MS system used, it is unsuitable for the analysis of complex mixtures including high-polarity and involatile flavonoids as the flavonolignans, for instance.

Perhaps newer belt interface designs[21,26] can overcome some of these drawbacks. On the other hand, given the results obtained with fast atom bombardment (FAB) MS studies of the flavonoid aglycones[27], HPLC–FAB-MS using the moving belt[26,28] could be an useful system for the HPLC–MS analysis of these compounds.

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Note

Determination of oxine-copper in orange by gas chromatography

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Oxine-copper [bis(quinolin-8-olato)copper(I)] is commonly used as a fungicide for controlling crop diseases. Several analytical methods have been reported for its determination. Bromometric titration[1] and a spectrophorometric method[2] have been developed for the determination of 8-hydroxyquinoline which is a decomposition product of oxine-copper. Oxine-copper has also been determined by coupling with *p*-nitrobenzenediazonium fluoroborate after being distilled with steam[3]. However, there is a limitation to these colorimetric methods in either the sensitivity or selectivity when a trace amount of oxine-copper is to be determined. In addition, the clean-up procedure is still not satisfactory. Some samples cause foams during steam distillation. For such samples, the steam distillation is not pertinent. For residue analysis of oxine-copper in crops, recently a derivative spectrophotometric method[4] and an high-performance liquid chromatographic (HPLC) method[5] using a fluorimetric detector were applied. In the latter method, 8-hydroxyquinoline was determined as an aluminium chelate. Although these are selective methods suitable for the determination of oxine-copper, there still remain some shortcomings in respect of the sensitivity and clean-up procedure.

In this paper, we describe a simple and effective clean-up procedure in which an Extrelut column is used instead of steam distillation, and a sensitive gas chromatographic (GC) determination of oxine-copper after derivatization with pentafluorobenzyl bromide (PFB-Br).

EXPERIMENTAL

Materials

Oxine-copper and 8-hydroxyquinoline were obtained from Kanesho Chemical (Tokyo, Japan) and Wako Pure Chem. (Tokyo, Japan), respectively. Aluminium nitrate and PFB-Br were obtained from Kanto Chem. (Tokyo, Japan) and Aldrich (Milwaukee, WI, U.S.A.), respectively. Extrelut-20 columns were from E. Merck (Darmstadt, F.R.G.). Oranges used in this study were obtained from The Agricultural Center of Mie Prefecture.

The identification of the PFB derivative of oxine-copper was verified by gas chromatography–mass spectrometry (GC–MS) (JEOL JMS-DX 300 GC-MS equipped with a JMA-DA 5000 GC–MS data system).

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Instrumentation

Gas chromatography was performed using an Hewlett-Packard 5890-A instrument equipped with a nitrogen–phosphorus flame ionization detector. A column (4 ft. × 3 mm I.D.) packed with 1% PEG-HT on Chromosorb W AW DMCS (80–100 mesh) was used. The column, injection port and detector temperatures were maintained at 220, 250 and 300°C, respectively. Helium at 30 ml/min was used as the carrier gas. The hydrogen and air flow-rates were 3 and 100 ml/min, respectively.

HPLC was carried out on a Tri Rotar SP chromatograph (Japan Spectroscopic, Tokyo, Japan) connected to a Hitachi 650-10S fluorescence spectrophotometer with excitation at 380 nm and emission at 520 nm. The Pyrex column (500 mm × 3 mm I.D.) was packed with Hitachi gel No. 3030. The mobile phase consisted of 1% aluminium nitrate in methanol, and the flow-rate was 1.2 ml/min. All operations were carried out an ambient temperature.

Sample extraction

A 5-ml volume of 1 *M* hydrochloric acid and 150 ml of acetone were added to 50 g of homogenized orange (flesh). The mixture was shaken vigorously for 30 min at room temperature, then filtered *in vacuo*. After the residue was washed with 50 ml of acetone, the pooled filtrates were concentrated to approximately 5 ml. To the concentrated solution, 70 ml of distilled water were added. The mixture was acidified to pH 1 by adding 1 *M* hydrochloric acid and washed with 50 ml of *n*-hexane. The aqueous phase was concentrated to approximately 5 ml, and adjusted to pH 13 by adding 10% sodium hydroxide. To this mixture, 1 ml of methanol and 10 ml of distilled water were added. After being stirred, the solution was charged on an Extrelut-20 column for approximately 20 min. Oxine-copper was eluted from the column with 100 ml of dichloromethane. The effluent was evaporated and the residue obtained was dissolved in 18 ml of acetone.

Pentafluorobenzylation

To an 18-ml volume of the extract containing the oxine-copper, 2 ml of 1% PFB-Br in acetone solution and 1 ml of 50% potassium carbonate solution were added. The mixture was heated at 100°C for 3 h, then allowed to cool. Distilled water (30 ml) was added and extracted twice with 10 ml of benzene. The pooled organic layers were dried over anhydrous sodium sulphate, then evaporated *in vacuo*. The residue obtained was dissolved in an appropriate volume of acetone and a 2-μl aliquot of the solution was injected into the gas chromatograph.

Determination

The oxine-copper was determined by comparing peak heights of samples to a calibration graph prepared by plotting the peak height *versus* the amounts of oxine-copper injected.

RESULTS AND DISCUSSION

Effect of pH on elution of oxine-copper from an Extrelut column

An Extrelut column has effectively been used for the clean-up of water-soluble compounds. It was therefore applied as a simple clean-up method for oxine-copper.

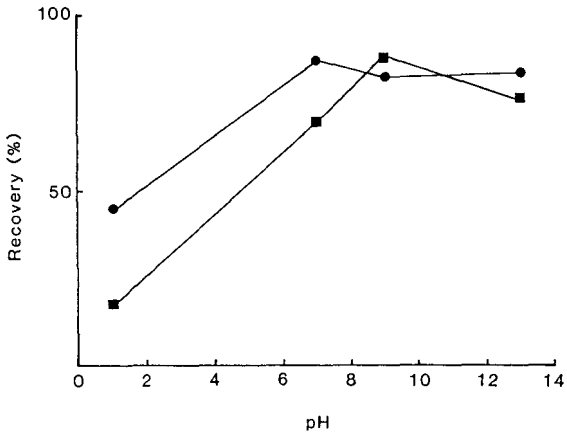

Fig. 1. Effect of pH on the elution of oxine-copper (●) and 8-hydroxyquinoline (■) from an Extrelut column. Values are duplicate determinations. Amounts of oxine-copper and 8-hydroxyquinoline: 100 μg. These data were determined by HPLC.

An 100-μg amount of oxine-copper or 8-hydroxyquinoline in acetone was adjusted to pH 1, 7, 9 or 13 with 1% sodium hydroxide or 1 *M* hydrochloric acid, and then applied to an Extrelut column and eluted with dichloromethane. The recoveries were directly determined by HPLC. As shown in Fig. 1, the recoveries from an Extrelut column were maximal in the ranges pH 7–13 and 9–13 for oxine-copper and 8-hydroxyquinoline, respectively. Then, the method was applied to the recovery test of oxine-copper from orange. The results are listed in Table I and compared with those obtained by the steam distillation method. The recoveries at pH 13 obtained by the Extrelut method were slightly better than those of the steam distillation method.

Thus, the method using an Extrelut column is effective for clean-up of oxine-copper.

TABLE I

RECOVERY (%) OF OXINE-COPPER AND 8-HYDROXYQUINOLINE FROM ORANGE

Values are the means of duplicate experiments. The sample solution was adjusted to pH 13 or 9 with 10% NaOH prior to being charged on an Extrelut column.

Compound	*Added (ppm)*	*HPLC*[a]			*GC*[a]
		Extrelut		*Steam distillation*	*Extrelut pH 13*
		pH 13	*pH 9*		
Oxine-copper	0.2	83.0	81.8	83.0	84.0
	0.4				90.4
	0.5	97.5	89.7	80.5	87.8
	2.0	88.3	88.5	88.2	92.4
8-Hydroxy-quinoline	2.0	98.6	95.1		

[a] Method of detection.

Effect of reaction temperature, reaction time and amount of potassium carbonate on the PFB derivatization of oxine-copper

In general, phenols react with PFB-Br to form PFB derivatives. 8-Hydroxyquinoline which is a decomposition product from oxine-copper also gave a PFB derivative with PFB-Br. Consequently, an examination of the hydrolysis in alkaline media and of the derivatization with PFB-Br of oxine-copper was simultaneously carried out.

The effects of the reaction temperature and reaction time on the PFB derivatization of oxine-copper were examined. A 100-μg amount of oxine-copper was dissolved in 18 ml of acetone and 20 mg of potassium carbonate followed by 2 ml of 1% PFB-Br in acetone were added. The mixture was heated for 1, 3 and 5 h at 70 or 100°C. The PFB derivative of 8-hydroxyquinoline heated at 100°C for 3 h was used as a control compound for the determination of the PFB derivative of oxine-copper. Table II shows that the derivatization of oxine-copper reached equilibrium after heating at 100°C for 3 h. However, the yield was only 1.8%. It can be assumed that the amount of potassium carbonate was not sufficient for the reaction.

To examine the effect of the amount of potassium carbonate, several amounts of potassium carbonate were added to the acetone solution containing 100 μg of oxine-copper and the mixture was heated at 100°C for 3 h. The results are listed in Tables II and III. They indicate that the preparation of the PFB derivative of oxine-copper was strongly affected by the concentration of potassium carbonate. Table II shows that the yield was less than 26% in the presence of 20, 50, 100, 200 and 300 mg of potassium carbonate. So, the derivatization was conducted in 30, 50 or 70% potassium carbonate solutions. As summarized in Table III, the best results can be obtained by adding 1 ml of 50 or 70% potassium carbonate solution. If the hydrolysis and the PFB derivatization of oxine-copper occur quantitatively, the relative yield was 82.5% {[(2 · PFB-hydroxyquinoline/oxine-copper)/(PFB-hydroxyquinoline/8-hy-

TABLE II

EFFECTS OF THE AMOUNT OF POTASSIUM CARBONATE, REACTION TEMPERATURE AND REACTION TIME ON THE PFB DERIVATIZATION OF OXINE-COPPER

Values are the means of duplicate experiments. Reaction conditions: oxine-copper, 100 μg; 8-hydroxyquinoline, 100 μg; temperature, 100°C; time, 3 h; 1% PFB-Br in acetone, 2 ml.

Amount of K_2CO_3 (mg)	*Temperature (°C)*	*Time (h)*	*Relative yield[a] (%) mean ± S.D.*
20	70	1	0.7
		3	0.9
		5	1.5
20	100	1	1.4
		3	1.8
		5	1.8
50	100	3	3.1
100	100	3	8.1
200	100	3	26.3
300	100	3	17.0

[a] Ratio of peak heights: (oxine-copper/8-hydroxyquinoline) · 100.

TABLE III

EFFECT OF POTASSIUM CARBONATE ON THE PFB DERIVATIZATION OF OXINE-COPPER

Values are the means of triplicate experiments. Reaction conditions: oxine-copper, 100 μg; 8-hydroxyquinoline, 100 μg; temperature, 100°C; time, 3 h; 1% PFB-Br in acetone, 2 ml.

Percentage of K_2KO_3	*Volume of K_2CO_3 (ml)*	*Relative yield[a] (%) mean ± S.D.*
30	0.2	7.6 ± 1.0
	0.5	34.5 ± 4.6
	1.0	90.4 ± 9.0
50	0.5	26.0 ± 4.3
	1.0	116.4 ± 3.6
	1.5	99.1 ± 19.2
	2.0	89.8 ± 4.8
70	0.5	23.1 ± 2.9
	1.0	116.1 ± 12.8

[a] Ratio of peak heights: (oxine-copper/8-hydroxyquinoline) · 100.

droxyquinoline)] · 100 = (650/351.9)/(325/145.15) · 100} because 1 mol of oxinecopper theoretically produces 2 mol of 8-hydroxyquinoline. However, in this study, the yield was 116%. This is considered to be due to the low yield of 8-hydroxyquinoline used as a control compound.

From these results, the reaction conditions for the PFB derivatization of oxine-copper in alkaline media were selected as follows: reaction temperature, 100°C; reaction time, 3 h; volume of 50% potassium carbonate, 1 ml.

Mass spectrum of the PFB derivative of oxine-copper

The electron impact (EI) mass spectra of the PFB derivatives of oxine-copper and 8-hydroxyquinoline were identical, as shown in Fig. 2. The molecular ion appeared at *m/z* 325 and major fragment ions were found at *m/z* 181 $[C_6F_5CH_2]^+$, 158 $[M-C_6F_5]^+$, 144 $[M-C_6F_5CH_2]^+$, 129 $[C_6F_3]^+$ and 116 $[144-CO]^+$.

Limit of detection

Under the given GC conditions, the minimum detectable amount of oxine-copper was 0.25 ng. Therefore, the limit of detection was 0.005 ppm when a 2-μg portion of 2 ml of the final acetone extract obtained from 50 g of orange was injected into the gas chromatograph.

This value was significantly better than those (> 0.02 ppm) from the Extrelut and steam distillation methods.

Results from orange

The recovery experiments for the PFB derivatization of oxine-copper were carried out at fortification levels of 0.2, 0.4, 0.5 and 2 ppm. Untreated orange was treated with standard acetone solution of oxine-copper prior to the addition of the extraction solvent. The results are shown in Fig. 3 and Table I. The chromatogram shows no interfering peak from the sample, and oxine-copper was quantitatively recovered.

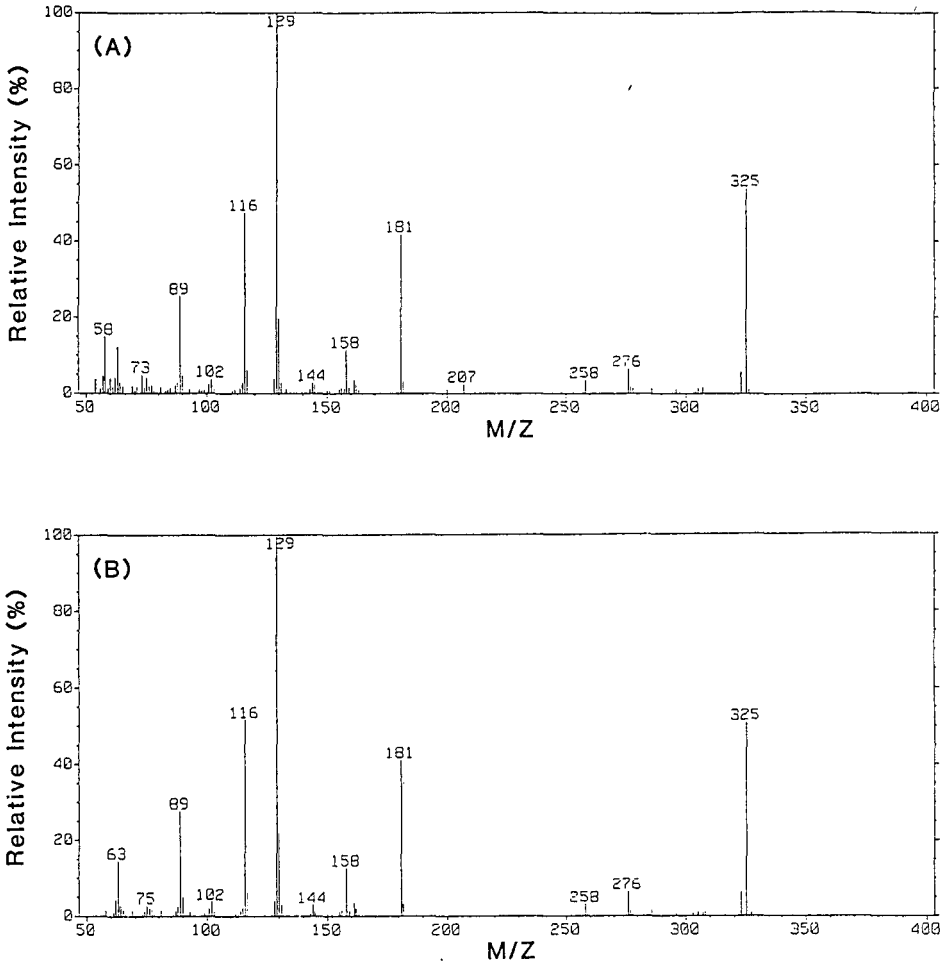

Fig. 2. Mass spectra of the PFB derivatives of oxine-copper (A) and 8-hydroxyquinoline (B).

TABLE IV

RESIDUES (ppm) OF OXINE-COPPER IN ORANGE FLESH (FIELD TRIALS)

The values in parentheses are mean ± S.D. Active content of formulation: 80%. Concentrate in spray: 600 l per 10 acre. Type of formulation: wettable powder.

No. of applications	*Days after last application*	*HPLC*[a]		*GLC*[a]
		Extrelut	*Steam distillation*	*Extrelut*
5	30	0.27, 0.25, 0.25 (0.26 ± 0.01)	0.26, 0.25, 0.28 (0.26 ± 0.02)	0.27, 0.23, 0.29 (0.26 ± 0.03)

[a] Method of detection.

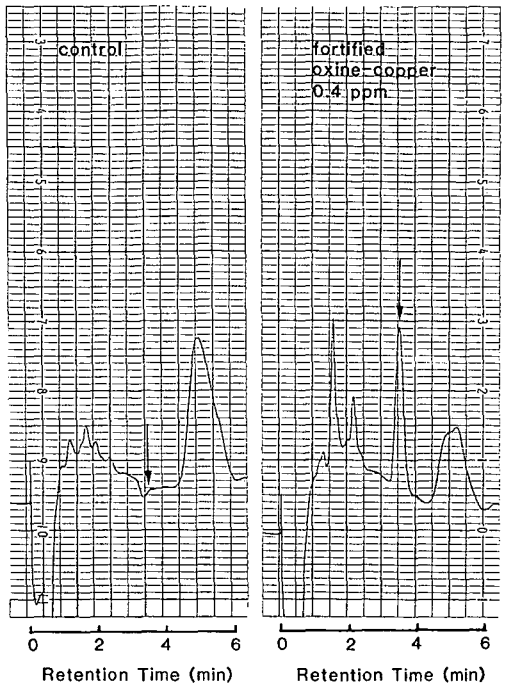

Fig. 3. Gas chromatograms of the recovery and control of oxine-copper from orange. Arrows indicate the retention times of the PFB derivative of oxine-copper. Conditions: final acetone solution, 2 ml (control); 4 ml (recovery). Injection volume: 2 μl. Other conditions was given in the text.

These results indicate that the method is eligible for determination of trace amounts of oxine-copper.

The results obtained for actual samples are summarized in Table IV. The mean residue in oranges sprayed five times with 600 l per 10 acre was 0.27 ppm at 30 days after the last application, in accord with the analytical data obtained by steam distillation. Therefore, the clean-up with an Extrelut-20 column and the derivatization with PFB-Br will be very suitable for the determination of oxine-copper residues.

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Note

On-line system for supercritical fluid extraction and capillary gas chromatography with electron-capture detection

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Supercritical fluids have been succesfully used for industrial extractions for many years[1] because of their strong solvating power, low viscosity and high solute diffusivities, yielding good mass transfer during extraction. More recently, analytical chemists have studied the potential of supercritical fluid extraction (SFE) as an alternative to time-consuming classical methods such as Soxhlet extraction and steam distillation. Off-line SFE of different adsorbents spiked with polycyclic aromatic hydrocarbons and other pollutants was described by Raymer and co-workers[2,3] and Wright *et al.*[4] Sugiyama and Saito[5] described a simple (off-line) micro-scale SFE system and its application to gas chromatography–mass spectrometry of lemon peel oil. McNally and Wheeler[6] studied the efficiency of SFE from complex matrices via retention characteristics in packed supercritical fluid chromatography (SFC). Engelhardt and Gross[7] combined the benefits of SFE with packed SFC in an on-line system.

In environmental trace analysis, one often deals with components that have a relatively good thermal stability (*e.g.*, polycyclic and polychlorinated aromatic hydrocarbons) and can be easily determined by capillary gas chromatography (GC). The gaseous effluent that is obtained in SFE after decompression is in principle compatible with GC. An on-line SFE–GC system allows the entire extract, rather than an aliquot, to be concentrated and analysed. As the final aim of our present project (the short-term sampling of air) usually yields only a few picograms of relevant analytes, improvement of the detection limit of the method is essential. Introduction of the entire extract into a chromatographic system, via on-line SFE–GC, might achieve this.

Hawthorne and co. workers[8–10] designed an on-line SFE–GC interface consisting of a linear fused-silica restrictor which is inserted in the on-column injector of a capillary gas chromatograph. However, the restrictor becomes fragile after a few extractions and is therefore replaced after each extraction. Wright *et al.*[11] described a

[a] Author deceased.

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similar method. Another interface for on-line SFE–GC was reported by Levy *et al.*[12], who proposed decompression via a stainless-steel restrictor which is inserted, through a septum, into a hot split–splitless GC injector.

Both interfaces can be succesfully used for the extraction and determination of particular components in (environmental) solids, adsorbents and other materials at the ppm level. However, both approaches suffer from several disadvantages. Stainless-steel restrictors are not inert and may interfere with trace-level analysis. In addition, the introduction of the entire gaseous effluent, *e.g.*, carbon dioxide or dinitrogen oxide, into the capillary GC column restricts the flow of SFE and consequently increases the extraction time and/or the allowable inner diameter of the GC column. Also, the stability of, *e.g.*, an electron-capture detector, might be negatively influenced; in fact, this detector cannot be used with dinitrogen oxide. Finally, the frequent replacement of fused-silica restrictors is disadvantagous for routine analysis. Therefore, the aim of this study was the design of an on-line SFE–GC system that can be utilized for the analysis of environmental samples at the picogram level, without the drawbacks mentioned above.

EXPERIMENTAL

Apparatus

The on-line SFE–GC system is shown in Fig. 1 and consists of (1) a cylinder with carbon dioxide (Rommenhöller, Rotterdam, The Netherlands) having a dip tube; (2) a stainless-steel frit (2 μm); (3) a Carlo Erba (Milan, Italy) Phoenix 20 syringe pump, equipped with a control unit for pressure programming and (4) a coolant supply set at 5°C; (5) a thermostated water-bath, set at 42°C; (6) a heat exchanger; (7) the extraction vessel, which was made from an empty 5.0 cm × 2.0 mm I.D. high-performance liquid chromatographic column; (8) a Rheodyne (Cotati, CA, U.S.A.) Model 7335 stainless-steel 0.5-μm filter; (9) a Valco (Houston, TX, U.S.A.) Model C6W switching valve; (10) a Whitey (Highland Heights, OH, U.S.A.) Model SS-43S4 shut-off valve; (11) an LDC/Milton Roy (Ivyland, PA, U.S.A.) Critical Ex-

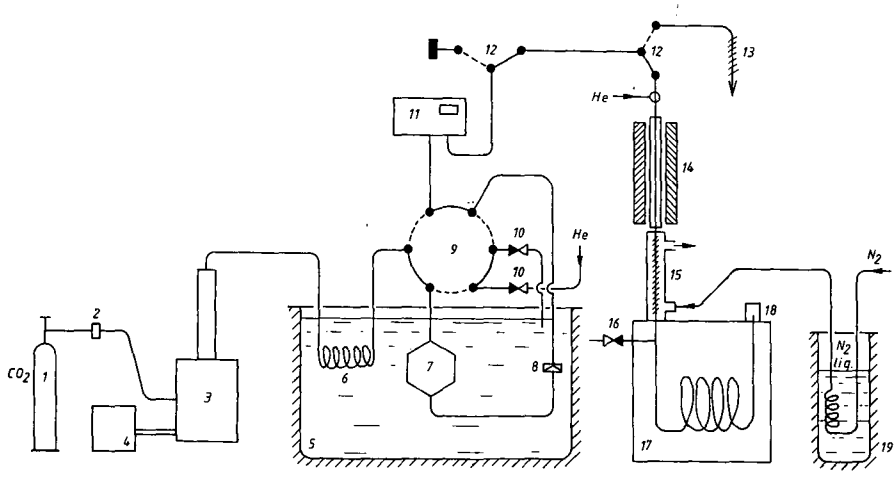

Fig. 1. Design of the on-line SFE–GC system. For explanation, see text.

traction Monitor, set at 210 nm; (12) a Valco Model C3W switching valve; (13) a laboratory-made electrically heated linear fused-silica restrictor with a collection tube for off-line sampling; (14 and 15) a thermodesorption/cold trap injection system[13] which contains a 20 cm × 25 μm I.D. fused-silica restrictor (Chrompack, Middelburg, The Netherlands); (16) a vent; (17) a Carlo Erba Model 5300 Mega high-resolution gas chromatograph, equipped with (18) an electron-capture detector and a 60 m × 0.22 mm I.D. DB-1 column (J&W Scientific, Folsom, CA, U.S.A.), with helium at a pressure of 220 kPa as carrier gas; and (19) a Dewar vessel filled with liquid nitrogen.

Chemicals

Stock solutions and dilutions of hexachlorobenzene (HCB), PCB 101, PCB 153 and PCB 180 were prepared in Nanograde acetone (Promochem, Wesel, F.R.G.).

Procedure

The extraction vessel is filled with 100 mg of Tenax GC (Chrompack) and cleaned for 16 h with a flow of purified helium in an oven at 250°C. The Tenax is spiked via injection of 10 μl of an acetone solution containing 1500 pg of HCB and 300 pg each of PCB 101, PCB 153 and PCB 180. Then the extraction vessel is closed and mounted in the SFE–GC system. Starting with the situation as shown in Fig. 1, the two valves 10 are opened and valve 9 is switched, so that the contents of the extraction vessel can be dried on-line with purified helium at 120 ml/min. After 5 min, valves 10 and 9 are switched to their original positions. Next, valve 12 is switched to the capped position and the extraction vessel is pressurized to 20 MPa at a water-bath temperature of 42°C (these conditions have been reported[2,3] to provide adequate extraction of PCBs from Tenax using supercritical carbon dioxide). Then the vent 16 is opened and the capillary cold trap, 15 is cooled to 5 ± 2°C, while the restrictor is heated to 300°C in the oven (14). When these conditions have been reached, valve 12 is switched to its original position and the extraction proceeds. The extracted components are deposited in the cold-trap (15), while the gaseous carbon dioxide leaves the system via the vent. The extraction is stopped, by switching valve 12 to the capped position, when 11.5 ml of carbon dioxide, measured at the pump, have passed through the sample. After a 1-min delay, the vent is closed, the cold-trap flash-heated to 300°C and the released components are transferred to the capillary GC column. The extraction requires less than 35 min. The extraction vessel can be decompressed during the GC separation by switching valve 9 and opening valve 10. The next extraction vessel can then be mounted in the SFE–GC system.

RESULTS AND DISCUSSION

Design of the on-line SFE–GC system

The proposed SFE–GC system is basically an elution system, and does not suffer from the drawbacks of a recycling system[14], in which the extract may be contaminated by, or lost in, the recycle pump and where usually only a fraction of the extract is transferred to the chromatographic system. Another important feature is the restrictor, which is shown in Fig. 2. The fused-silica restrictor is inserted in an 18 cm × 0.6 mm I.D. glass tube which fits in a conventional thermodesorption/cold-trap

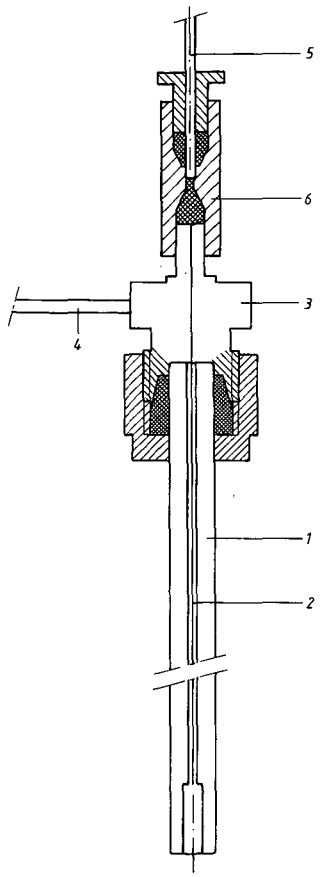

Fig. 2. Design of the restrictor. 1 = Glass tube (18 cm × 0.6 mm I.D.); 2 = 20 cm × 25 μm I.D. fused-silica capillary; 3 = 1/4-in. T-piece; 4 = helium carrier line; 5 = supercritical fluid; 6 = 1/16–1/32-in. reducing union.

injector. The restrictor is protected in this way and is re-usable; in practice, we have used the same restrictor for several weeks. In addition, the desorption oven is used as a restrictor oven and prevents condensation or precipitation of the extracted components during decompression in the restrictor.

The co-axial addition of helium carrier gas to the restrictor effluent allows pressure-controlled operation and prevents backflushing of supercritical carbon dioxide into the helium carrier line. The use of a vent is very practical, because now the carbon dioxide flow-rate is not restricted by the inner diameter of the capillary GC column, and no backflushing of gaseous carbon dioxide into the helium carrier line will occur.

Cryogenic operation of the entire gas chromatograph is unnecessary. Refocusing of the analytes occurs in the cold-trap of the injection system, which can be cooled to −50°C if required.

The on-line SFE–GC system is equipped with an UV absorbance monitor and a

second heated restrictor for detection and (off-line) collection of components which are present in relatively high concentrations. The system also allows on-line density-programmed SFE–GC. The influence of the addition of modifiers to the supercritical carbon dioxide on the refocusing efficiency in the cold-trap has not yet been investigated.

Purity of supercritical carbon dioxide

One of the most serious problems associated with on-line SFE–GC is the purity of the supercritical fluid, *e.g.*, carbon dioxide. In SFC, impurities will cause a background signal which may interfere with trace analyses when programmed-density SFC is applied. In on-line SFE–GC, the impurities in the supercritical fluid are, as a rule, preconcentrated in the cold-trap and subsequently injected into the capillary gas chromatograph. This results in a high background, which seriously limits trace analysis. We have compared carbon dioxide obtained from several manufactures. Unfortunately, none of these turned out to be really suitable for trace-level analyses. Even research-grade purity (99.999%) carbon dioxide still contains a few ppm of water and "total hydrocarbons". The water content limits the temperature range of the cold-trap to values above 0°C, and the hydrocarbons often show typical oil patterns in a flame ionization detector; occasionally the detector flame was even extinguished (!). Both phenomena will have an impact on the recovery: too high a temperature of the cold-trap causes breakthrough of the more volatile solutes. Oil residues will create a film in the cold-trap which may act as a stationary phase, thereby reducing the desorption efficiency and introducing a memory effect. So far, the best results were obtained with "food-grade" carbon dioxide obtained from Rommenhöller.

Application to the determination of polychlorinated aromatic hydrocarbons

The GC–electron capture detection (ECD) system was calibrated by direct injection of the sample into a plug of quartz-wool that had been inserted into a glass thermodesorption tube. After evaporation of the solvent, the tube was placed in the thermodesorption/cold-trap injector and analysed under similar time and temperature conditions as for SFE–GC. The spiked 100-mg Tenax samples were analysed as described under Experimental. The recovery was calculated relative to the results obtained in calibration experiments. The reproducibility was determined by performing three experiments on different days. The memory effect was determined by a second SFE–GC run on each sample. The results are given in Table I.

TABLE I

RESULTS OF THE ANALYSIS OF SPIKED TENAX SAMPLES

Component	*Amount added*		*Recovery (%)*	*Reproducibility (%)*	*Memory (%)*
	pg	*ppb*[a]			
HCB	1500	15.0	52	12	2
PCB-101	300	3.0	58	12	7
PCB-153	300	3.0	59	10	9
PCB-180	300	3.0	63	9	13

[a] The American billion (10^9) is meant throughout.

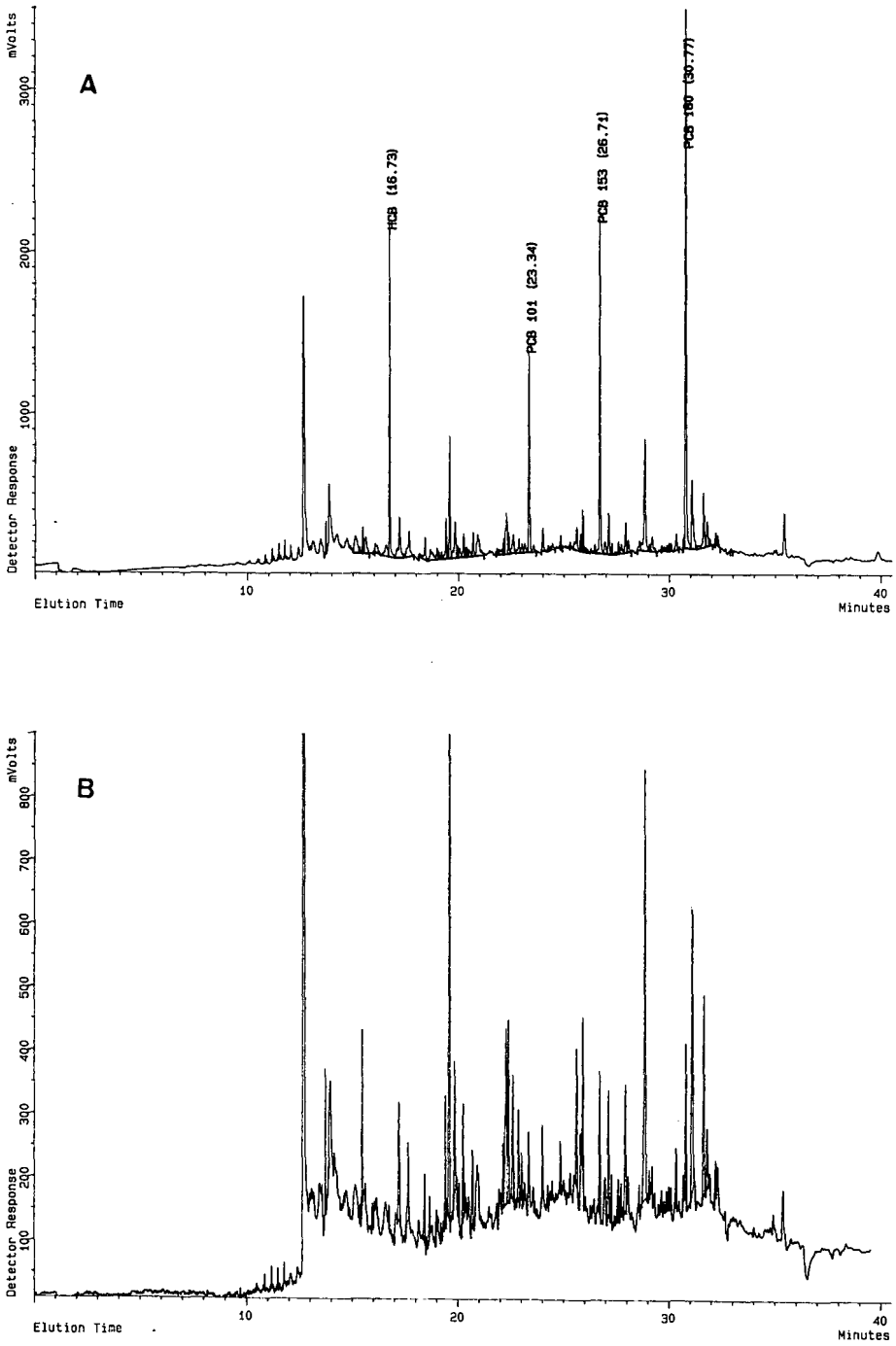

Fig. 3. Chromatograms of (A) Tenax, spiked with 1500 pg of HCB and 300 pg of the PCB 101, 153 and 180, and extracted using 11.5 ml of supercritical carbon dioxide; and (B) 11.5 ml of supercritical carbon dioxide (note the difference in the detector response scale).

The recoveries are of the order of 50–65%. A second extraction with a further 11.5 ml or a single extraction with double the volume of supercritical carbon dioxide increases the recovery by only 2–13%, as indicated by the memory effect. An explanation for the incomplete recovery might be the reduced sensitivity of ECD for the components of interest during co-elution with the oil residues. Note that the oil contamination was absent when the GC–ECD system was calibrated, resulting in a calibration error and an apparent loss of components. In addition, the most volatile component, HCB, may be partly lost by breakthrough in the cold-trap (it should be noted that the flow-rate through the cold-trap is increased during SFE compared with the calibration of the GC–ECD system); moreover, fogging may occur in the cold-trap, which can cause a partial loss of the components.

The memory effect is not caused by incomplete desorption from the cold-trap, as was indicated by a second thermodesorption between the first and second extractions. Obviously, incomplete extraction and/or retention in the system are responsible for the memory effect.

Despite the incomplete recoveries, the system was found to be fairly stable, as indicated by the reproducibility of 9–12%. With the present procedure, the attainable detection limit is about 30 pg (0.3 ppb) for the individual PCBs. A typical chromatogram is shown in Fig. 3A. The spiked components are seen to be superimposed on the impurities present in the supercritical fluid itself, as demonstrated by comparison with Fig. 3B.

CONCLUSIONS

The on-line SFE–GC system shows several advantages over the systems described in the literature. The system is robust and compatible with existing thermodesorption/cold-trap equipment, it does not restrict the choice of GC columns or detectors and has potential for environmental trace analyses, as was demonstrated by the analysis of Tenax spiked with polychlorinated aromatic hydrocarbons at the picogram level. The recovery of these analytes is satisfactory.

The detection limit for the individual PCBs is 30 pg, which appears to be a significant improvement over results such as reported in, *e.g.*, refs. 9 and 15. For a further decrease in the detection limit to 1–5 pg (which is no problem for ordinary capillary GC–ECD and will be sufficient for our air-sampling project), the availability of carbon dioxide of higher purity is required. Should it become available, then the recovery of more volatile components will also increase, because the temperature of the cold-trap can be lowered.

Efficient purification of carbon dioxide, further optimization of the recovery of the analytes and application of the on-line SFE–GC system for the analysis of air samples are currently being investigated.

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Note

Polyoxyalkyleneglycols immobilized on Sepharose 6B for the sequential extraction of three enzymes from a crude extract of *Pseudomonas testosteroni*

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Mild hydrophobic interaction chromatography is attracting increasing interest, for instance in the purification of enzymes acting on hydrophobic substrates, such as steroids or lipids. This technique exploits the moderately hydrophobic character of certain polymers, essentially various polyethers so far, to obtain high purification ratios together with the recovery of specific activities which generally are better than those obtained by traditional hydrophobic interaction chromatography (HIC)[1–8].

In previous papers[6,7] we reported the purification of $\Delta_{5\rightarrow4}$ 3-oxosteroid isomerase from *Pseudomonas testosteroni* by chromatography on stationary phases obtained by the immobilization of polyethylene glycol (PEG) on Sepharose 6B by means of spacer arms totally devoid of hydrophobic moieties strong enough to play a role in the separation process and therefore to lead to erroneous interpretations.

In this paper, we report preliminary results concerning the ability of this technique to meet a more complicated challenge, *i.e.*, to obtain good purification ratios and high recoveries for three closely related enzymes, simply by stepwise elution and starting from the same crude extract.

EXPERIMENTAL

Sepharose 6B was obtained from Pharmacia (Uppsala, Sweden). Polyethylene glycol monomethyl ether ($\bar{M}_w \approx 750$) and N,N'-carbonyldiimidazole were supplied by Aldrich (F.R.G.). Pluronic L 64 was purchased from Serva (Heidelberg, F.R.G.). The crude extract of *Pseudomonas testosteroni* was prepared as described previously[6]. The three enzymes tested were $\Delta_{5\rightarrow4}$ 3-oxosteroid isomerase (E.C. 5.3.3.1), 3α-hydroxysteroid dehydrogenase (α-HSD) (E.C. 1.1.1.50 and 3β,17β-hydroxysteroid dehydrogenase (β-HSD) (E.C. 1.1.1.51). Their enzymatic activities were assayed with Δ_5-androstene-3,17-dione, androsterone and testosterone, respectively, according to previously published procedures[9,10]. Protein concentrations were measured according to the Amido Black technique[11]. The term potassium phosphate implies a mixture of K_2HPO_4 and KH_2PO_4 in the ratio 306.9:168.6 (w/w).

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RESULTS

Stationary phases were prepared according to a procedure described previously[7] by immobilization of two different polyethers on to Sepharose 6B, after preliminary activation of this matrix with N,N′-carbonyldiimidazole. The two polyethers concerned are polyethylene glycol monomethyl ether, $CH_3O(CH_2CH_2O)_nH$ ($\bar{M}_w \approx 750$), and Pluronic L 64, a copolymer of polyethylene glycol (PEG) and polypropylene glycol (PPG), $HO(CH_2CH_2O)_{n/2}[CH_2CH(CH_3)O]_p(CH_2CH_2O)_{n/2}H$, with $p = 30$ and $n = 27$ ($\bar{M}_w \approx 3000$), These two polyethers differ essentially in their hydrophilic–lipophilic balance (HLB), their lipophilic character increasing with their content of PPG moieties.

As hydrophobic interactions are highly dependent on ionic strength, the strategy of mild hydrophobic interaction chromatography implies a careful selection of this parameter during both the adsorption and desorption steps. A high ionic strength (yet as low as possible in order to minimize the retention of slightly hydrophobic contaminants) is required for the adsorption step, whereas a low ionic strength (yet as high as possible in order to minimize the release of strongly interacting contaminants) is necessary during the desorption.

Fig. 1A shows the elution profile obtained with Sepharose–PEG, starting with 20% potassium phosphate in the mobile phase. The three enzymes are separated from unretarded contaminants. However, the desorption step results in a poor purification of α-HSD and no separation between β-HSD and isomerase. If the experiment is

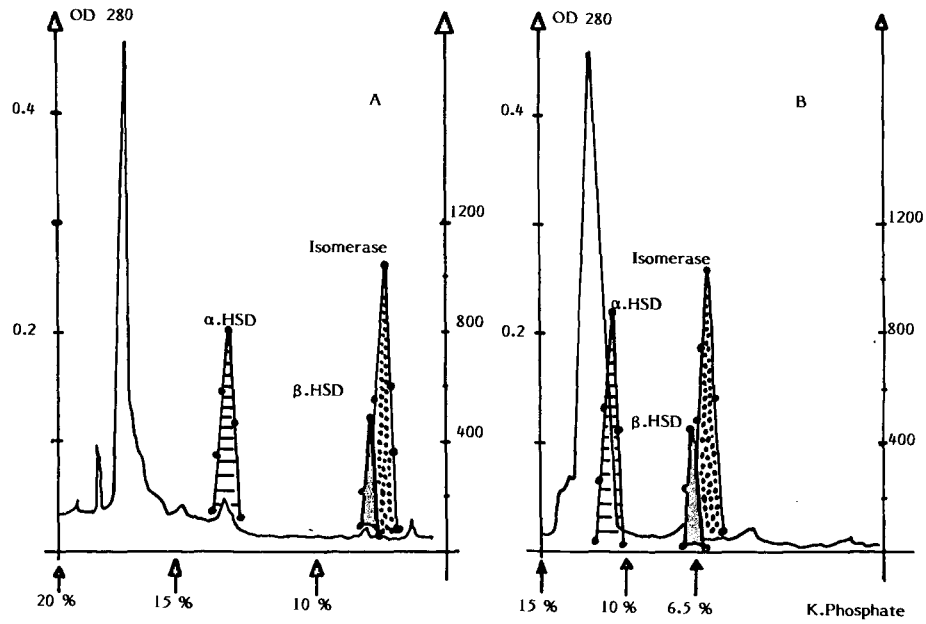

Fig. 1. Stepwise elution of the *Pseudomonas testosteroni* crude extract (0.4 ml) with various concentrations of potassium phosphate, pH 7.0 (ionic strength changes are indicated by arrows). Stationary phase: Sepharose–polyethyleneglycol monomethyl ether. Column, 38 cm × 1 cm I.D.; flow-rate, 26 ml/h; fractions taken every 5 min; room temperature. The enzymatic activities are indicated in arbitrary units.

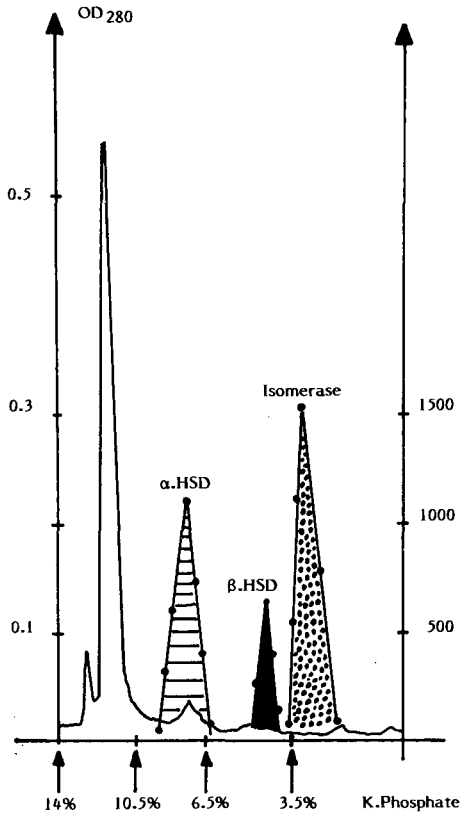

Fig. 2. Stepwise elution of the *Pseudomonas testosteroni* crude extract. Stationary phase: Sepharose–Pluronic L64. Other conditions as in Fig. 1.

carried out with a slightly more hydrophobic stationary phase, *i.e.*, Sepharose–Pluronic, the minimum ionic strength permitting the adsorption of the three enzymes without retardation of the contaminants is weaker (14% potassium phosphate), as expected (Fig. 2). Unlike the Sepharose–PEG, the desorption step on Sepharose–Pluronic leads to total separation of the three enzymes from one another, with good recoveries of enzymatic activities (αHSD 60%; β-HSD 40%; isomerase 65%) and almost quantitative removal of the contaminants in each fraction (Table I). Under

TABLE I

CONTAMINANTS IN THE FRACTIONS CONTAINING THE THREE DIFFERENT ENZYMES

Results are % of the total proteins in the crude extract.

Stationary phase	*Enzyme*		
	α-HSD	*β-HSD*	*Isomerase*
Sepharose–PEG	5.7	2.8	0.5
Sepharose–Pluronic	1.5	2.0	Not detectable

these ionic strength conditions, the experiment carried out on Sepharose-PEG leads to no retention at all of α-HSD and complete overlapping of the peaks corresponding to β-HSD and isomerase (Fig. 1B).

These results indicate that slight modifications of the hydrophobic character of the stationary phase and the ionic strength conditions during chromatography can result in different separation characteristics. Stationary phases synthesized by immobilization of more hydrophobic polyethers, such as polypropylene glycol (PPG) or polytetramethylene glycol (PTMG), are currently under investigation. They should bring additional potential to this field of mild hydrophobic interaction chromatography.

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Note

Activation of thin adsorbent layers by an anhydrous organic solvent

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Earlier, by using adsorption thin-layer chromatography (TLC), we have shown that the qualitative composition of polar lipids from soy bean seeds is highly complex[1]. Therefore, to determine it completely an highly efficient and selective TLC procedure is necessary.

To achieve maximum efficiency and selectivity while solving this separation problem, the activity of the adsorbent employed must be optimal[2,3]. However, when the relative humidity (r.h.) of ambient air is above a certain level, almost all of the conventional TLC techniques do not ensure the attainment of this goal (see below). Therefore, we have adapted a procedure for the activation of a thin layer[4]. According to this procedure, after applying lipids on the plate, physically absorbed water is removed from its surface by treating the adsorbent with anhydrous diethyl ether; until now, to the best of our knowledge, organic solvents with various water contents have been used for the conditioning of silica gel activity only in column chromatography[5,6].

EXPERIMENTAL

Diethyl ether (reagent grade) was dried and distilled[7]. Methanol (reagent grade) and chloroform (pharmaceutical grade) were purified only by distillation. Aqueous solutions of NH_3 (26% w/v, commercial grade) and phosphomolybdic acid (analytical grade) were used without further purification. Silufol TLC plates (Kavalier, Votice, Czechoslovakia) were predeveloped with chloroform–methanol (3:1, v/v). The r.h. was determined using a BM-2 hygrometer.

The mixture of seed lipids (20 μg)[1] was applied on the plate not activated by heating. The various TLC conditions are shown in Table I. Those used for the control experiments (variants A and B) were the same as described previously (continuous flow TLC in the chamber described by Van den Eijnden[8]), whereas a specially designed TLC apparatus was used in variant C (Fig. 1). After assembling the apparatus (fumehood!), 3 ml of diethyl ether were introduced into chamber 1 through the tube 8. The ether together with water dissolved therein migrated through the adsorbent layer and a sheet of paper, then passed out of chamber 1 and evaporated. There was no desorption of polar lipids from the starting point on the plate. Just after the disappearance of ether from the thin layer surface and chamber bottom, *i.e.*, after about 20

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TABLE I

CONDITIONS FOR TLC SEPARATION OF POLAR LIPIDS[a]

Variant	*r.h. (%)*	*Type of TLC chamber*	*Chloroform–methanol–ammonia mobile phase (v/v/v)*
A	40	Continuous flow	26:7:0.7
B	70	Continuous flow	32:7:0.7
C	70	See Fig. 1	32:7:0.7

[a] See text for A, B, C and r.h.

min, 20 ml of mobile phase were introduced into the chamber in the same way (Table I) and the plate was developed for 2 h. Vaskovsky and Dragendorff colour reagents as well as ninhydrin and phosphomolybdic acid were applied to visualize and identify the lipids[9]. They were also identified by using TLC standards and by comparing found R_F values with those obtained by other workers[1].

RESULTS AND DISCUSSION

The results of TLC of the lipid mixture are presented in Fig. 2. At r.h. = 40% (A), sufficiently high selectivity was achieved, but at r.h. = 70% (B) there was no separation of phosphatidylcholines from phosphatidylethanolamines although in the latter case the mobile phase was less polar. Finally, the variant C (r.h. = 70%), due to the activation of the layer by the removal of water, was quite similar to variant A as regards the selectivity of lipid fractionation. The same results were obtained over the range of r.h. 50–90%.

Thus, the increase in r.h. substantially diminished the selectivity of separation although in other cases the opposite was observed[3]. Apparently, in our work this was due to the decrease in the activity of the adsorbent layer, which in turn resulted from

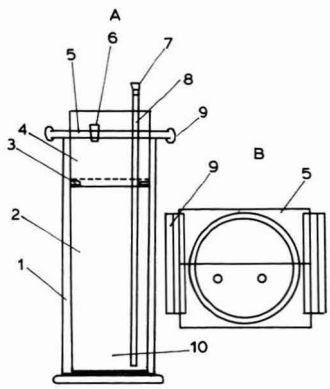

Fig. 1. Apparatus for adsorbent activation and TLC analysis. (A) Front view, (B) top view. 1 = Chamber, 180 mm × 60 mm; 2 = TLC plate, 150 mm × 54 mm; 3 = metal clamps; 4 = filter-paper; 5 = chamber cover consisting of two glass plates ground to the upper edge of the chamber and to each other (all ground surfaces are lubricated with glycerol); 6 and 7 = rubber stoppers; 8 = PTFE tube; 9 = spring clamps; 10 = starting zone of TLC plate.

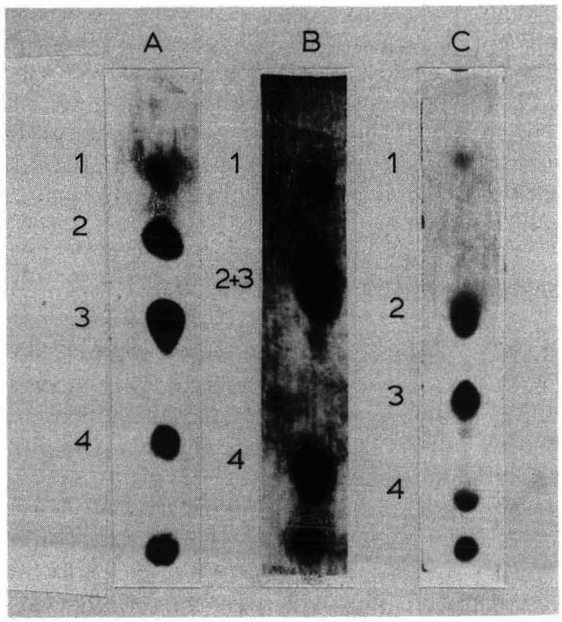

Fig. 2. Chromatograms of a mixture of polar lipids. 1 = N-acylphosphatidylethanolamine; 2 = phosphatidylethanolamine; 3 = phosphatidylcholine; 4 = phosphatidylinositol. See Table I for TLC separation conditions for the variants A, B and C.

the absorption of ambient moisture by this layer[2]. The extent of the latter process is known to rise sharply at r.h $\geqslant$55%: thus, at r.h. = 45.6 and 74.5%, moisture makes up 13.3 and 38.8% respectively of the layer weight[10], and at r.h. $\geqslant$60% an equilibrium between the water content of the adsorbent and of the surrounding atmosphere was reached within the first few minutes of their contact[2,10]. Thus, the extent of moisture absorption by the adsorbent layer depends on the r.h. of the ambient air. At the same time, it is inversely proportional to the initial humidity of the layer[3].

In our experiments another factor responsible for the decrease in selectivity with increasing r.h. is the extent of mobile phase demixing under these conditions[11,12]. This phenomenon consists in the migration of one or more secondary mobile phase fronts on the plate in addition to the major one. In our experiments it brought about a significant distortion of the normal shape of chromatographic zones and a drastic change in their usual mobility which resulted in a partial or even complete loss of separation selectivity of polar lipids. Most investigators also consider demixing to be harmful for TLC separation[2,3,12].

Demixing is usually observed when the mobile phase consists of solvents which differ considerably in their polarities, the more hydrophobic and more polar components being concentrated above and below the secondary front, respectively. In our experiments, demixing appeared only at higher r.h.; however, under other separation conditions it was observed even at low ambient humidity[3]. Therefore, the possibility of its appearance should be taken into account in each TLC separation, including cases where the migration of the secondary front on the plate is not obvious[11].

Within a certain r.h. range, demixing can be prevented by preliminary saturation of the layer with the mobile phase vapour[3,12]. This, however, considerably

increases the duration of the analysis, and therefore a special technique has been developed making it possible to perform the TLC analysis after the appearance of demixing. To this end, continuous flow TLC in a sandwich chamber is used[3,13]; the sample is applied through the special holes in the cover below the secondary solvent front[12]. TLC after the demixing had appeared is again subject to considerable difficulties. First, the chamber required is not produced commercially. Moreover, the attainment of equilibrium between the mobile and stationary phases requires much time. Finally, because the sample is applied directly into the continuous flow of solvent the chromatographic zones become very diffuse[6].

Demixing was observed in almost all our experiments at high r.h. (variant B), but treatment of the layer by diethyl ether completely prevented it.

To assess the activation technique proposed here one must compare it with other similar methods. The major approach employed to this end in TLC comprises heating the plates at 110–115°C for 3–30 min[3,14–16]. In order to prevent subsequent moisture absorption from ambient air, the plate thus treated is immediately covered with a dry glass sheet; the sample to be analyzed can be applied only after this point[2,16]. However, when transferring the plate to the TLC chamber and in the course of subsequent separation (during 30 min or more) the thin layer is permanently in contact with the environment which has practically the same humidity as the laboratory air. Therefore, the layer loses its adsorptive capacity very rapidly[3].

To activate the layer without using an high temperature, the plate with the applied sample was placed in a TLC chamber which, in addition to the mobile phase, contained a vessel with a moisture-absorbing medium (sulphuric acid solution). The plate was kept in the chamber for a long time, up to 12 h, and then brought into contact with the mobile phase while maintaining the chamber hermetically sealed[10,17]. A significant drawback of this approach is the considerable duration of the experiment, which involves also a long-term contact of the sample with atmospheric oxygen. Moreover, the layer absorbs sulphuric acid vapours, which may adversely affect the results of TLC analysis[3]. On the basis of this principle, the twin trough chamber and the Vario-K S chamber (Camag, Birmensdorf, Switzerland) were constructed[3]; both of these chambers share with the original technique the drawbacks mentioned above.

It is possible that high activity of the layer will also be preserved by performing TLC analysis in a closed desk-top cabinet (similar to the "glove-in chamber" used in tracer experiments[6,18]) with a constantly maintained low humidity. However, on a practical scale such an approach would be subject to considerable technical difficulties[19].

As is seen from the preceding two paragraphs, the techniques of adsorbent activation employed presently in TLC are not very efficient. Therefore, we turned our attention to the corresponding methods used in column chromatography. These methods are also usually based on preliminary heating[20]. At the same time, adsorbent dehydration may also be achieved at room temperatures. Thus, the percolation of 2,2-dimethoxypropane (DMP) through a column in the presence of acetic acid or some other acidic catalyst brings about complete disappearance of adsorbed water: $H_2O + CH_3C(OCH_3)_2CH_3 \rightarrow CH_3COCH_3 + 2CH_3OH$[21]. However, DMP cannot be used in planar chromatography including TLC since under these conditions the whole active surface of the adsorbent saturated with DMP would be in contact with

moist air. Besides, the traces of DMP which remained on the plate may promote decomposition of the sample, *e.g.*, the conversion of acyl lipids into fatty acid methyl esters[22].

According to another approach (that used in our work) a non-polar organic solvent with limited hydrophilicity is percolated through a column[4]. In TLC experiments we used diethyl ether as such a solvent because it does not elute polar lipids from the silica gel surface. Ether can dissolve up to 1.2% (w/w) of water[23] and is characterized by high volatility and relatively low chemical activity. Finally, it is readily available.

CONCLUSION

In conclusion it must be stressed that in many geographical regions the laboratory r.h. remains high for up to 5 months or more every year. Consequently, during this prolonged period of time the execution of TLC experiments will be more or less complicated. The technique proposed here may contribute to overcoming these difficulties, because it makes it possible to prepare active TLC plates at any r.h. Such plates are suitable for the separation of both polar lipids and other organic compounds of the same or lower lipophilicity. It is true that this technique cannot be used to analyse ether-soluble compounds such as neutral lipids. However, according to our experience, the separation of these compounds is less dependent on air humidity than the separation of more hydrophilic substances because mixtures of solvents sharply differing in their polarities (see above) are not usually employed for this purpose.

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CHROM. 21 540

Note

Determination of optical purity by high-performance liquid chromatography on chiral stationary phases: pantothenic acid and related compounds

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In the last few years, numerous chiral stationary phases (CSPs) have been developed for optical resolution by high-performance liquid chromatography (HPLC). The CSPs include many types of columns[1], for instance, Pirkle[2], ligand-exchange[3], protein-conjugated[4,5], etc.

D-Pantothenic acid is a precursor of the biologically important coenzyme A. It is well known that D-(*R*)-pantothenic acid and related compounds are biologically active, but the L-isomers are inactive. A few chromatographic methods have been presented for the chiral separation of pantothenic acid and related substances by gas chromatography[6–9]. In these cases, it is necessary to prepare the chiral reagents or chiral stationary phases.

This paper describes two enantiospecific HPLC methods for the separation of pantothenic acid, panthenol and pantolactone enantiomers: (1) after conversion into pantoic acid or direct resolution on a ligand-exchange CSP, and (2) derivatization with the 3,5-dinitrobenzoyl reagents, followed by separation on chiral acrylic polymer CSP or "brush type"[1] CSPs.

EXPERIMENTAL

Chemicals and reagents

DL-Calcium pantothenate and DL-panthenol of reagent grade were obtained from Sigma (St. Louis, MO, U.S.A.), D-, L-calcium pantothenate, D-, L-pantolactone, D-panthenol of pharmaceutical grade from Daiichi Seiyaku (Tokyo, Japan), 3,5-dinitrobenzoyl chloride (DNBC) of reagent grade from Nakarai Chemical (Kyoto, Japan) and 3,5-dinitrophenyl isocyanate (DNPI) from Sumitomo Chemical (Osaka, Japan). All other reagents and solvents were of reagent grade.

High-performance liquid chromatography

An Hitachi Model L-6200 high-performance liquid chromatograph equipped with a spectrophotometric detector was operated at 254 nm.

Chromatographic separation of underivatized calcium pantothenate enantiomer and the related compound enantiomers, which were converted into pan-

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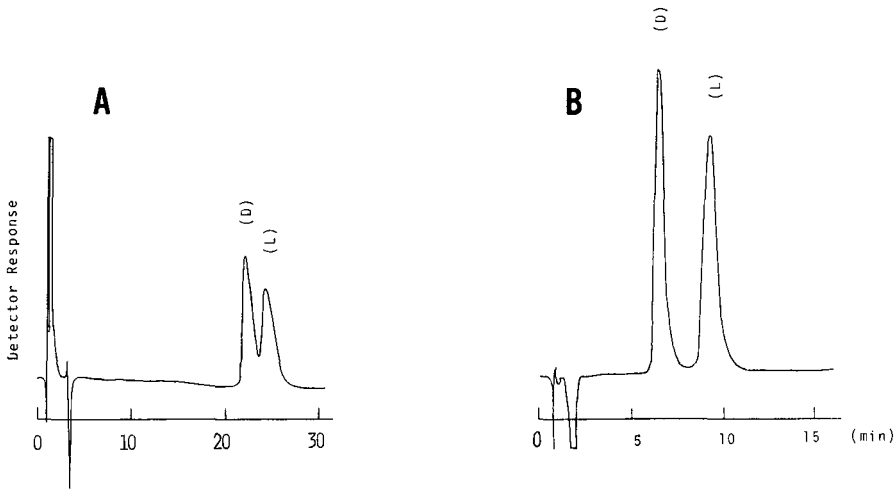


Fig. 1. Chromatograms of racemic calcium pantothenate (A) and racemic pantoic acid converted from panthenol (B). Chromatographic conditions: column; MCI gel CRS 10W 50 mm × 4.6 mm I.D. Mobile phases: 2 m*M* $CuSO_4$ (A), 2 m*M* $CuSO_4$ containing 10% acetonitrile (B). Flow-rates: 0.6 (A), 0.8 ml/min (B), Column temperatures: 25 (A), 35°C (B). Sample amount: *ca.* 50 μg as the racemic mixture.

toic acid, was achieved on MCI gel CRS 10W (50 mm × 4.6 mm I.D., Mitsubishi Kasei Kogyo, Tokyo, Japan), particle size 3 μm. Fig. 1 shows typical chromatograms of these enantiomers.

Chromatographic separation of the 3,5-dinitrobenzoyl (DNB) and 3,5-dinitrophenyl (DNP) derivatives was achieved on a YMC-packed A-KO3 column (250 mm × 4.6 mm I.D., Yamamura Chemical, Kyoto, Japan) which comprises a conjugated D-naphthylethylamine polymer on a silica gel surface, Sumipax OA-4000 (250 mm × 4.6 mm I.D.; Sumitomo Chemical, Osaka, Japan) and Enantio P1 (250 mm × 4.6 mm I.D.; TOSOH, Tokyo, Japan) which are brush-type columns.

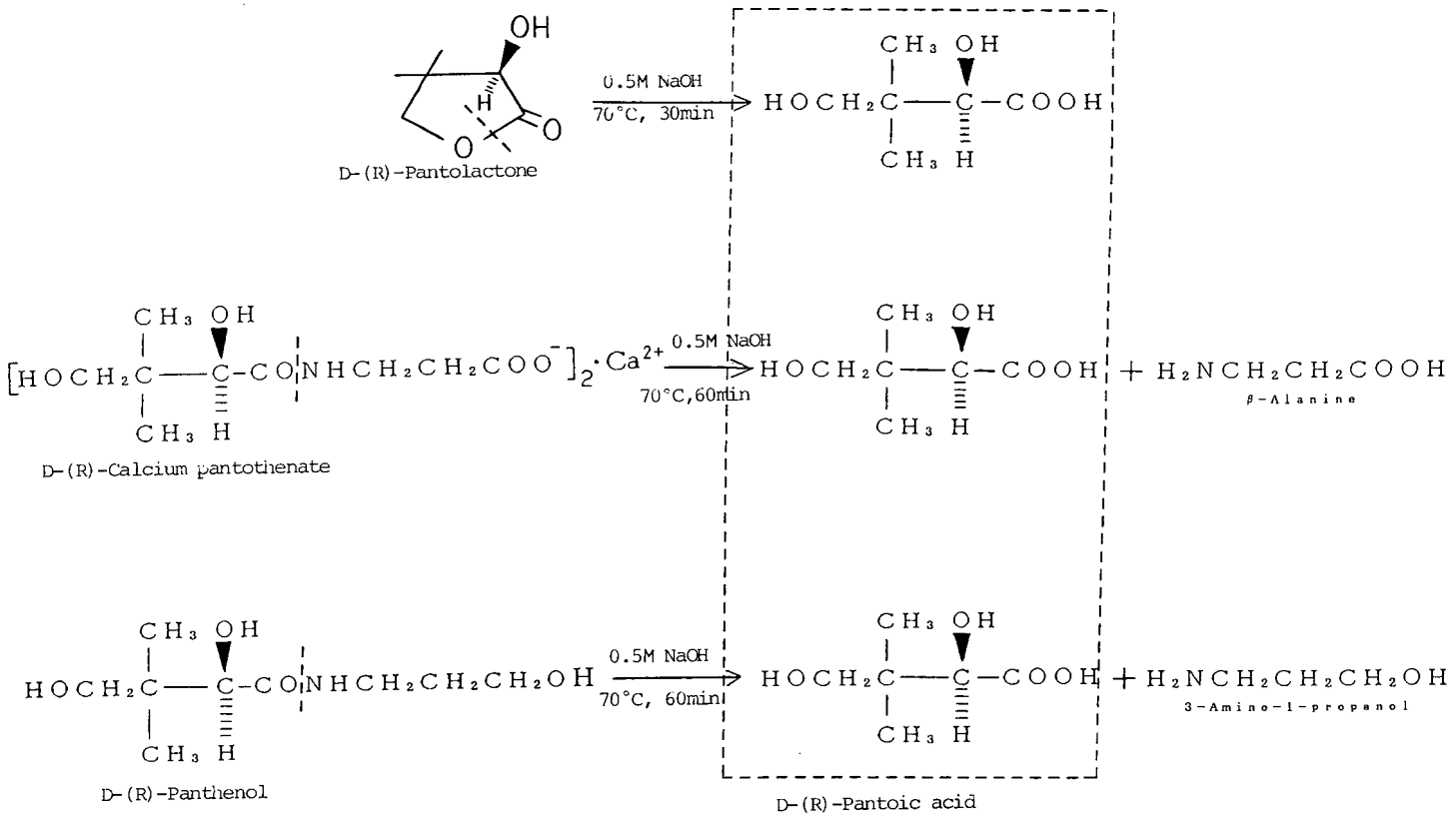
Conversion into pantoic acid (Scheme 1)

D-, L-Pantolactone, D-, L-calcium pantothenate and D-, L-panthenol (10–20 mg) were incubated in 0.5 *M* NaOH (2 ml) solution in vials at 70°C for 30–60 min (see Scheme 1) in a water-bath. After the conversion into pantoic acid, this solution was injected into the chromatograph with a ligand-exchange CSP.

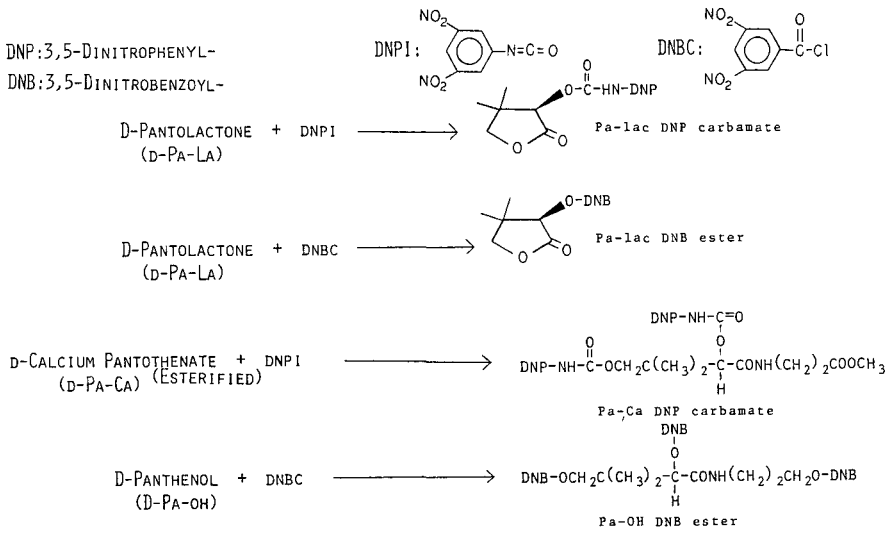
Derivatization with DNPI and DNBC (Scheme 2)

Samples (10 mg) of D-, L- and DL-pantothenic acid were esterified with 5 ml of 1.5 *M* hydrochloric acid in dry methanol at 50°C for 30 min in a screw-cap vial. After removal of the methanol under reduced pressure, 2 ml of dry toluene, 40–60 mg of DNPI and 50 μl of pyridine were added, and the reaction mixture was kept at 60°C for 30 min[10]. After removal of the excess of reagents, the sample was dissolved in 5 ml of chloroform, washed with 5 ml of 1 *M* HCl and water, dried with sodium sulphate (anhydrous) and used for HPLC investigation.

Samples (10 mg) of D-, L-panthenol or D-, L-pantolactone were converted into 3,5-dinitrophenyl carbamates in the same way as pantothenic acid, but without esterification.



Scheme 1. Conversion of pantothenic acid and related compounds into pantoic acid.



Scheme 2. Derivatization of pantothenic acid and related compounds with DNPI and DNBC.

Samples (10 mg) of D-, L-pantolactone were dissolved in 2 ml of tetrahydrofuran, and 40–60 mg of DNBC and 50 μl of pyridine were added. The mixture was heated at 60°C for 60 min[11]. After removal of the excess of reagent, the samples were dissolved in 5 ml of chloroform, washed with 5 ml of 1 *M* HCl, 5% $NaHCO_3$ and water, dried with sodium sulphate and used for HPLC investigation.

RESULTS AND DISCUSSION

MCI gel CRS 10W is a chiral stationary phase with ligand-exchange properties. Partial separation of the calcium pantothenate enantiomers was achieved directly with 2 m*M* copper sulphate solution. The complete resolution of pantoic acid enantiomers was obtained with 2 m*M* copper sulphate solution containing 10% acetonitrile. The peak area ratios of D-, L-pantoic acid are indications of the optical purity of the compounds. This method is applicable to the determination of the optical purity of D-pantolactone and D-calcium pantothenate. A good correlation between the theoretical value (D/D + L %) and the observed value was obtained for both pantolactone and calcium pantothenate (pantolactone: $y = 0.931x + 0.643$, $r = 0.992$; calcium pantothenate: $y = 0.971x + 0.801$, $r = 0.998$). In this application, it was confirmed that the racemization of D- and L-isomers did not occur in alkaline solution.

On the other hand, Pirkle *et al.*[12,13] and Ôi *et al.*[10,14] have reported the separation of many enantiomers as their 3,5-dinitrobenzoyl esters or their 3,5-dinitrophenyl carbamates. We, therefore, attempted to separate the enantiomers of pantothenic acid and related compounds as their 3,5-dinitrobenzoyl or 3,5-dinitrophenyl derivatives. The chromatographic results of these experiments are summarized in Table I and Fig. 2.

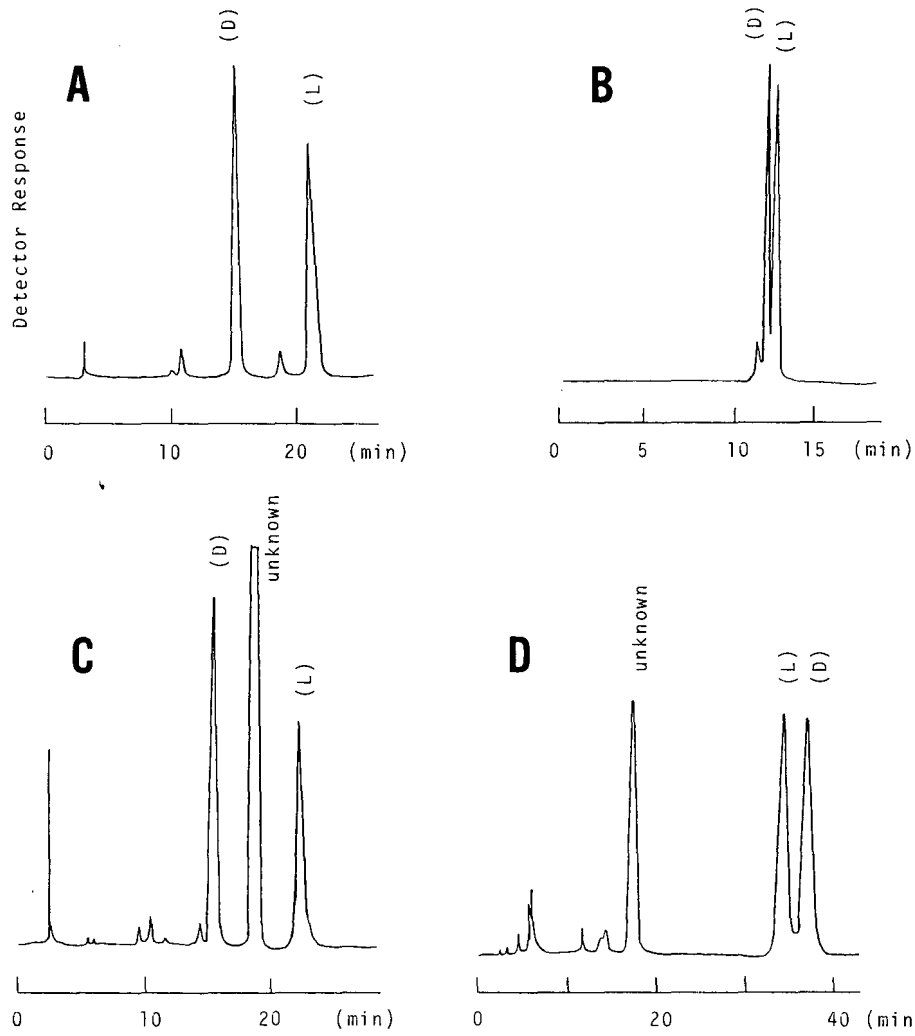


Fig. 2. Typical chromatograms of enantiomeric 3,5-dinitrophenyl (-benzoyl) carbamates (esters) on CSP (YMC-KO3): (A) Pa-lac DNP carbamate; (B) Pa-lac DNB ester; (C) Pa-Ca DNP carbamate; (D) Pa-OH DNB ester. Chromatographic conditions as in table I.

Retention volumes of D-isomer derivatives were smaller than those of L-isomer derivatives except in the case of panthenol. As a result, it was found that the 3,5-dinitrophenyl carbamate derivatives were better separated than the 3,5-dinitrobenzoyl ester derivatives. It can be assumed that the 3,5-dinitrobenzoyl esters lack the –NH– function for hydrogen bonding interaction with the CSPs which is important for chiral recognition.

In conclusion, the methods described here are expected to be useful for determination of the optical purity of pantothenic acid and related compounds by HPLC.

TABLE I

ENANTIOMER SEPARATION OF CALCIUM PANTOTHENATE (Pa-Ca), PANTOTHENYL ALCOHOL (Pa-OH) AND PANTOYL LACTONE (Pa-lac) AS 3,5-DINITROPHENYL (-BENZOYL) DERIVATIVES WITH CHIRAL STATIONARY PHASES (CSPs)

Racemate	*CSP I*			*CSP II*			*CSP III*		
	α^a	k'^b	*Mobile phase*[c]	α^a	k'^b	*Mobile phase*[c]	α^a	k'^b	*Mobile phase*[c]
Pa-Pc DNP carbamate	1.52	4.90	A	1.55	1.46	C	1.37	2.89	C
Pa-lac DNP carbamate	1.51	4.79	A	1.54	1.45	C	1.37	2.67	C
Pa-lac DNB ester	1.09	0.88	A	1.00	0.67	D	1.00	0.67	C
Pa-OH DNB ester	1.08	12.05	B	1.00	10.58	D	1.00	3.18	C

[a] The separation factor of the enantiomers, α, is the ratio of the capacity factors of the enantiomers.

[b] k' is the capacity factor for the first enantiomer eluted (D-isomer except in the case of Pa-OH).

[c] Mobile phases: hexane–dichloromethane–ethanol, 70:30:8 (A), 70:30:10 (B), 70:30:5 (C), 70:30:1 (D). CSP: YMC A-KO3 (I), Sumipax OA-4000 (II), TSKgel Enantio PI (III). Flow-rates of 0.8 ml/min were typically used at 35°C.

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Note

Analysis of natural and modified amino acids and hexosamines by reversed-phase high-performance liquid chromatography[a]

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Because of its speed and sensitivity, amino acid analysis by high-performance liquid chromatography (HPLC) of the phenylthiocarbamyl (PTC) derivatives of amino acids has become a widely used procedure. Based on methodology developed by Koop *et al.*[1], the method has been popularized as the "Pico-Tag" system by Waters Instruments[2] in which pretested reversed-phase HPLC columns are recommended. Similar resolution of amino acids can also be obtained using other high-efficiency reversed-phase supports[2–7]; it appears, however, that each support requires different chromatographic conditions to achieve adequate resolution and that these conditions must be adjusted for each individual column[4]. This process can be time consuming since there are many different variables that affect resolution including column temperature, flow-rate, composition of solvents and gradient shape. Systematic studies indicating how each of these variables affect the retention times of individual amino acids, such as that carried out by Ebert[5] on the effect of triethylamine concentration, are helpful in minimizing the amount of time required for adjusting chromatographic conditions since the effects of modifications can be predicted.

Amino acid analysis is frequently used to analyze compounds other than the standard amino acids present in simple proteins. For example, glycoproteins contain glucosamine and galactosamine which can be derivatized with phenylisothiocyanate (PITC) and which may interfere with quantification of other amino acids since they elute very close to Ser and Gly. Cheng[8] has published a procedure for analyzing the PTC derivatives of the hexosamines and hexosaminitols but this method uses a mixed bed ion-exchange step to separate amino acids from sugars and it is not clear whether the early eluting amino acids can be resolved from the hexosamines in this procedure. Other compounds of interest include rare amino acids and amino acid derivatives generated by chemical procedures employed in protein chemistry. Retention times for several of the less common amino acids have been determined by O'Hare *et al.*[4] but it is not clear whether these were resolved from the standard set of 17 amino acids. Tarr[9] has also indicated elution positions for some of these derivatives.

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This study investigates the effect of buffer pH on the retention times of the PTC derivatives of 26 amino acids and derivatives as well as galactosamine and glucosamine. For general use, the optimum pH was found to be 5.5, and at this pH hexosamines and amino acids can be resolved within 18 min. The data presented can also be used to predict optimal conditions for separating closely eluting peaks and as a guide for manipulating conditions to obtain optimal resolution with new columns.

EXPERIMENTAL

Materials

Spherisorb ODS-2 columns (150 × 4.6 mm I.D., 3 μm in particle size) were purchased from Alltech. An amino acid standard mix containing 2.5 μmol/ml of each of the 17 amino acids and 1.25 μmol/ml L-cystine was purchased from Beckman. Other amino acids and hexosamines were either from Sigma or from Fluka. HPLC-grade triethylamine (TEA), phosphoric acid and acetonitrile were purchased from Fisher Scientific. Sodium acetate (HPLC grade) was from Merck. Constant boiling hydrochloric acid and phenylisothiocyanate were from Pierce. Sheep submaxillary mucin was prepared as described[10]. Tubes for amino acid analysis were washed with chromic acid before use.

Methods

Hydrolysis. Samples of sheep submaxillary mucin (100 μg) and lysozyme (100 μg) were hydrolyzed for 20 h at 110°C in 1.0 ml of constant boiling, 6*M* hydrochloric acid[11,12]. Lower ratios of acid to glycoprotein causes destruction of both amino acids and hexosamines[12]. For hexosamine analysis, the glycoprotein was hydrolysed in 4 *M* hydrochloric acid for 4 h at 100°C[13]. α-Aminobutyric acid (25 nmol) was added as an internal standard. After completion of hydrolysis the samples were cooled and then immediately dried in a vacuum drier from Savant Instruments since storage of samples in hydrochloric acid caused losses of some of the amino acids. The last traces of hydrochloric acid were removed by adding 200 μl of water and redrying. This process was repeated at least two times. Samples containing particulate material were filtered through 0.22-μm filters (Gelman). Losses of amino acids occuring during hydrolysis were corrected either by hydrolyzing for 4, 8, 12, 16 and 24 h and extrapolating to zero time or by hydrolyzing the standard amino acid mix and then calculating molar response ratios for the amino acids in the hydrolyzed standard. Samples were hydrolyzed and processed in duplicate.

Derivatization with PITC. The derivatization procedure employed was that of Bidlingmeyer *et al.*[2]. To the dried sample was added 10 μl of ethanol–water–TEA (2:2:1); the sample was then dried under a stream of nitrogen. Derivatizing reagent, 20 μl ethanol–water–TEA–PITC (7:1:1:1), was added and the sample incubated at room temperature for 30 min in the dark. Each sample was then dried thoroughly under nitrogen, immediately redissolved in 250 μl sample diluent buffer (see below) and aliquots analyzed by HPLC. Solubilized samples could be stored at −20°C for up to 24 h whereas dried samples could be stored for at least one week. When samples were solubilized and left at room temperature, losses of aspartic acid and generation of artifactual peaks were observed. Sample diluent buffer was prepared by dissolving 6.32 g of dibasic sodium phosphate in 100 ml of 25% aqueous acetonitrile, adjusting

the pH to 7.0, and filtering through 0.22-μm Millipore filters. Alternatively, a 2:1 mixture of solvents A and B (see below) was used as the sample diluent buffer.

Chromatography. The instruments employed were a Varian Model 5000 liquid chromatograph, a Varian Model 2050 variable-wavelength detector, and a Shimadzu C-R3A integrator. The flow-rate was 1.4 ml/min and the column temperature was maintained at 39°C using a circulating water bath and column jacket.

The gradient consisted of a linear gradient from 6% solvent B to 25% B for the first 5.5 min, isocratic elution at 25% B for 1.5 min, a second linear gradient reaching 30% B at 8 min, a linear gradient to 35% B at 10 min, and another linear gradient reaching 51% B at 20 min. This was followed by a washing cycle consisting of a linear gradient to 100% B at 21 min followed by 4 min at 100% B. The column was reequilibrated to initial conditions by eluting with 6% B for 5–7 min prior to injecting the next sample. In most experiments, solvent A contained 0.14 *M* sodium acetate and 0.08% TEA with the pH adjusted to 5.5 with phosphoric acid. In experiments testing the effect of pH on retention times, the pH was adjusted to various values ranging from 3.5 to 7.5 using phosphoric acid. Solvent B consisted of 60% aqueous acetonitrile.

The effect of pH on retention times was studied by coinjecting PTC amino acids from the standard amino acid mixture together with one of the following mixtures: (1) cysteic acid, carboxymethylcysteine, aminoethylcysteine, glucosamine and galactosamine, (2) homoserine, methionine sulfone, methionine sulfoxide and hydroxyproline, or (3) norvaline, norleucine and α-aminobutyric acid.

RESULTS AND DISCUSSION

The separation of the standard 17 PTC amino acids as well as the PTC derivatives of galactosamine and glucosamine on a Spherisorb 3-μm ODS-2 column using a pH 5.5 buffer is shown in Fig. 1. The resolution of the amino acids is similar to that achieved with other systems that have been described[2–7]. The effect of decreasing the concentration of sodium acetate from 0.14 to 0.01 *M* is shown in Fig. 2. Under these conditions, the retention times are decreased, Lys elutes as a broad peak, and the relative retention times of both His and Arg are increased, causing the coelution of Ala and Arg. The shape of the gradient is critical to the ability of the system to resolve closely eluting amino acids; shallow gradients give better resolution at the expense of increased analysis time. The gradient used in these studies was chosen to maximize resolution of the hexosamines from the other early peaks without inordinately increasing total analysis time. These conditions were found to be the best compromise between resolution and speed. Other parameters such as TEA concentration, which has been studied by Ebert[5], and flow-rate were not investigated in detail.

The compounds that must be resolved in this system include neutral species, zwitterions and species containing one or two negatively charged groups. Thus, it seemed logical to investigate the effect of buffer pH on resolution, particularly since the practical pH range for HPLC on silica supports is in the vicinity of the pK_a values for carboxyl groups. The effect of pH on retention times for the 28 compounds investigated is shown in Fig. 3. Almost all of these compounds demonstrate increased retention times with decreased pH; this presumably reflects the partial titration of their free carboxyl groups. The greater sensitivity to pH shown by aspartic acid,

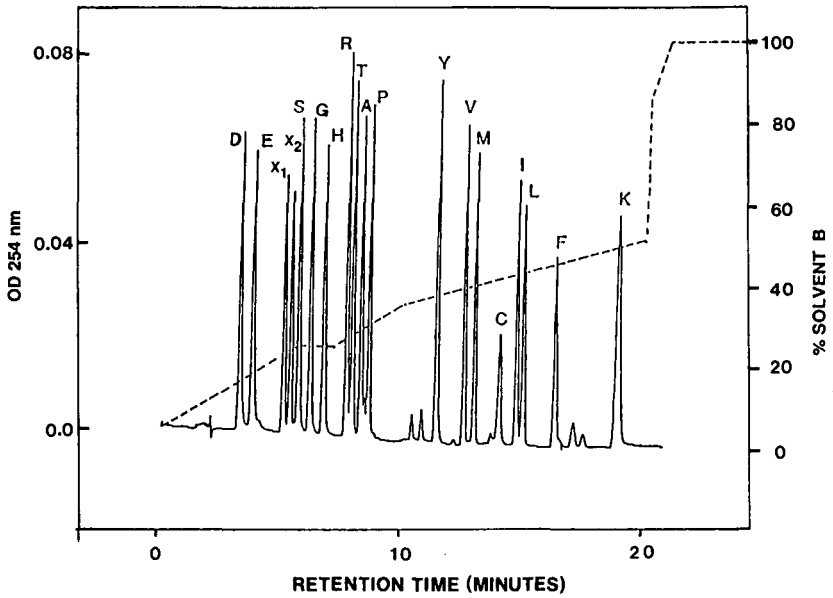

Fig. 1. Separation of PTC amino acids from PTC hexosamines at pH 5.5. Glucosamine and galactosamine were added to a standard mixture of amino acids. The mixture was derivatized with phenylisothiocyanate and separated by reversed-phase HPLC as described in *Methods*. Amino acids are identified by the single letter code; galactosamine is indicated by (X_1) and glucosamine by (X_2).

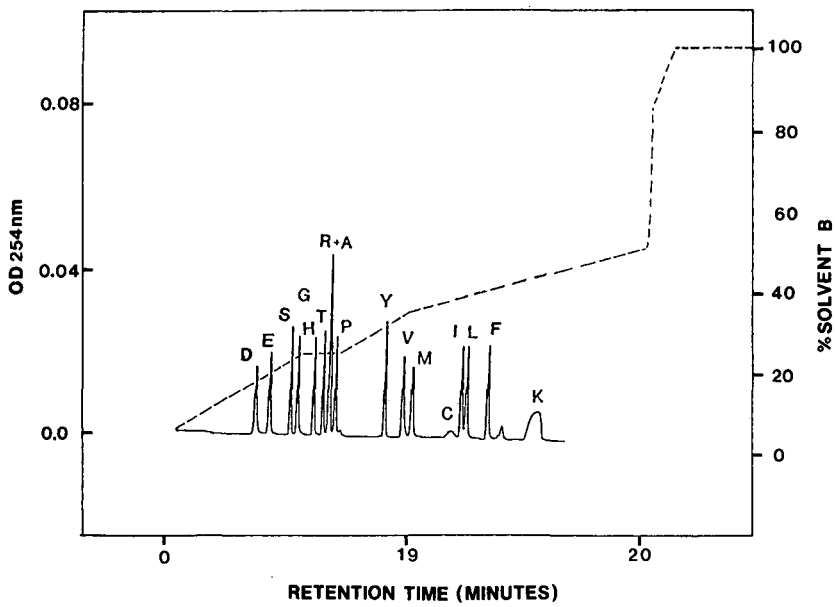

Fig. 2. Effect of the ionic strength of buffer A on separation of PTC amino acids. Conditions used were the same as in Fig. 1 except that buffer A contained 0.01 *M* rather than 0.14 *M* sodium acetate.

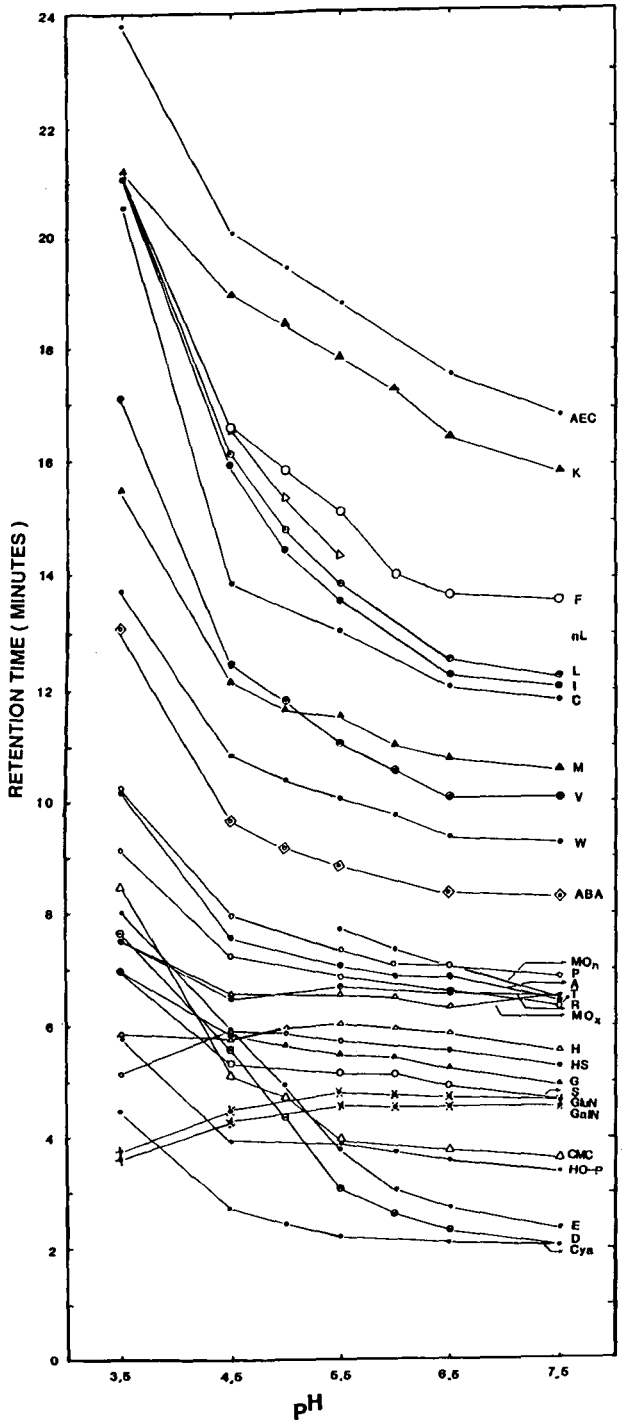

Fig. 3. The effect of pH on the retention times of amino acids and hexosamines. Mixtures of PTC amino acids and hexosamines were separated by HPLC using the conditions described in *Methods* except that buffer A was adjusted to different pH values ranging from 3.5 to 7.5. Amino acids are identified by the one letter code. Other compounds tested included the following: galactosamine, GalN; glucosamine, GlcN; homoserine, H-S; norleucine, nL; norvaline, nV; α-aminobutyric acid, ABA; aminoethyl cysteine, AEC; cysteic acid, Cya; carboxymethyl cysteine, CMC; hydroxyproline, HO-P; methionine sulfoxide, MO_x; methionine sulfone, MO_n.

carboxymethylcysteine and glutamic acid is consistent with the fact that they contain two carboxyl groups while the lack of increased sensitivity of cysteic acid to pH is consistent with the much lower pK_a value of sulfonates. The retention times of the PTC derivatives of the hexosamines, which are uncharged, are not greatly affected by pH and in fact decrease at lower pH values.

It is clear from Fig. 3 that α-aminobutyric acid represents a good choice for an internal standard since it is well separated from the other peaks at all pH values. Norleucine elutes in a crowded region of the chromatogram while norvaline coelutes with methionine at pH 5.5.

Amino acid analysis has frequently been used to quantify other compounds containing free amino groups in addition to the standard amino acids commonly found in proteins. These include compounds that occur naturally in specific proteins such as galactosamine and glucosamine in glycoproteins, hydroxyproline in collagen and methionine sulfoxide and sulfone which are formed from methionine under oxidizing conditions. Modified amino acids such as homoserine and aminoethyl cysteine are generated by standard procedures used in protein chemistry while the cysteine content of proteins is normally measured by making derivatives of cysteine such as carboxymethylcysteine or cysteic acid. Separation of these compounds in the chromatographic system is necessary both for their determination and also to prevent them from interfering with the analysis of the standard amino acids. An examination of Fig. 3 demonstrates that all peaks are resolved at pH 5.5 with the exception of hydroxyproline which coelutes with carboxymethylcysteine. These can, however, be resolved at pH 6.5. Similarly, phenylthiourea (from the reaction of PITC with ammo-

TABLE I

AMINO ACID ANALYSIS OF SHEEP SUBMAXILLARY MUCIN AND LYSOZYME

Values are given in terms of mol per thousand mol of amino acids. The literature data for lysozyme were taken from ref. 14 and the data for sheep submaxillary mucin from ref. 15.

Amino acid	*Sheep submaxillary mucin*		*Lysozyme*	
	Observed	*Literature*	*Observed*	*Literature*
Asx	20	22	185	183
Glx	46	63	39	43
N-Acetylgalactosamine	334	312	0	0
Ser	198	179	92	87
Gly	212	182	106	104
His	2	2	8	8
Arg	33	37	95	96
Thr	142	142	61	61
Ala	132	135	98	104
Pro	104	100	18	18
Tyr	4	2	32	26
Val	46	65	45	53
Met	0	0	11	18
Ile	9	16	51	51
Leu	31	35	75	76
Phe	16	16	26	26
Lys	6	7	59	60

nia) elutes as a very broad peak in the vicinity of the Ala and Pro peaks (data not shown) and may interfere with their quantification using this system. This peak can be suppressed by repeating the ethanol–water–TEA additions and drying steps prior to derivatization. Alternatively, other gradients are capable of separating phenylthiourea from the PTC amino acids[9].

The application of this methodology to the amino acid analysis of lysozyme and sheep submaxillary mucin is shown in Table I. Measured values for amino acid composition match literature values[14,15] reasonably well. Furthermore, the yield of amino acids recovered after hydrolysis of lysozyme accounted for 95.3% of the dry weight of the sample as calculated from the amount of internal standard added. Similarly, 94.1% of the dry weight of sheep submaxillary mucin was recovered as amino acids (40.8%), N-acetylgalactosamine (25.7%) and sialic acid (33.5%) using the known ratio of sialic acid to N-acetylgalactosamine of 0.91[16] to calculate amounts of sialic acid.

A number of manufacturers produce highly efficient reversed-phase HPLC columns capable of separating PTC amino acids[2–7]. Conditions for achieving adequate resolution appear to be somewhat different for each brand of stationary phase; appropriate conditions vary somewhat with different columns from the same producer and may also change as a column ages. Achieving separation by trial and error is tedious; this work on relating the buffer pH to retention times and that of Ebert[5] correlating TEA concentrations to separation provide a rational framework for predicting appropriate changes in elution conditions required to gain the desired separations.

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Note

Chromatographic fractionation of proteins at high organic solvent modifier concentrations

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A skewed U-shaped (or bimodal) dependency exists between retention times and concentration of organic solvent during reversed-phase chromatography of some small organic compounds, peptides and proteins[1–5]. Recently, we described a reversed-phase high-performance liquid chromatography (RP-HPLC) procedure (inverse-gradient RP-HPLC), based upon this phenomenon, for recovering subnanomole amounts of protein from sodium dodecylsulfate polyacrylamide gel electroeluates in a form suitable for microsequence analysis[5–9]. Using small pore size (600–120 Å) reversed-phase packings with large surface areas (200–400 m^2/g) and high carbon content (7–15%) proteins can be retained at high concentrations of 1-propanol (90–100%). Under these conditions sodium dodecylsulfate and acrylamide gel-related contaminants are not retained and are washed through the column. Retained proteins can be recovered from the column by the addition of an ion-pairing agent (*e.g.* trifluoroacetic acid) into the mobile phase and elution with a gradient of decreasing 1-propanol concentration (*i.e.*, an "inverse-gradient"). Proteins recovered from gel electroeluates by this method are free of high concentrations of sodium dodecylsulfate and acrylamide-related artifacts. Such artifacts interfere with the Edman chemistry, HPLC-based phenythiohydantion-amino acid analysis and peptide mapping. Inverse-gradient RP-HPLC has been successfully employed to recover a wide variety of proteins, many of which are not amenable to conventional RP-HPLC, from sodium dodecylsulfate gel electroeluates in a form suitable for N-terminal sequence analysis in the 10–500 pmol range[5,8,10]. Recently, the utility of this method was extended to recover proteins from the detergent mixtures (*e.g.* 2% sodium dodecylsulfate–1% Triton X-100) used to elute electroblotted Coomassie blue-stained proteins from poly(vinylidine difluoride) membranes[7].

As a continuation of these studies, we present in this paper an evaluation of other commercially-available silica-based reversed-phase supports which exhibit U-shaped (or bimodal) behavior (*i.e.*, protein retention at high organic modifier concentrations). In addition we demonstrate that protein mixtures can be fractionated by inverse-gradient RP-HPLC.

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EXPERIMENTAL

The proteins employed in this study were purchased from Sigma (St. Louis, MO, U.S.A.) and were of the highest available commercial grade. Trifluoroacetic acid (99+% grade) was from Pierce (Rockford, IL, U.S.A.). Deionised water, obtained from a tandem Milli-RO and Milli-Q system (Millipore, MA, U.S.A.) was used for all buffers. HPLC-grade organic solvents were purchased from Mallinckrodt (Melbourne, Australia). The HPLC system used has been described elsewhere[5,11]. The following reversed-phase supports were used in this study (a) Brownlee VeloSep Octyl (C_8) or Octadecyl (C_{18}) cartridges (3 μm, 100 Å, 40 × 3.2 mm I.D.) obtained from Applied Biosystems (Foster City, CA, U.S.A.). (b) ODS-Hypersil (C_{18}) columns (5 μm, 120 Å, 100 × 2.1 mm I.D.; 3 μm, 120 Å, 60 × 4.6 mm I.D.; or cartridges (5 μm, 120 Å, 20 × 2.1 mm I.D.) were obtained from Hewlett-Packard (Waldbronn, F.R.G.). ODS-Hypersil (C_{18}) microbore columns (3 μm, 120 Å, 50 × 2.1 mm I.D. or 50 × 1.0 mm I.D.) were packed as previously described[5]. Brownlee RP-300 Octyl (C_8) cartridges 7 μm, 300 Å, 100 × 2.1 mm I.D.) were obtained from Applied Biosystems. (d) LiChrospher Diol (Merck, Darmstadt, F.R.G.) columns (5 μm, 75 × 4.6 mm I.D.) were packed as previously described[5].

RESULTS AND DISCUSSION

Details of the silica-based packings employed in this study are summarized in Table I. Of the supports examined, the small pore size (100-120 Å) large surface area (170–200 m^2/g) supports (*e.g.* ODS-Hypersil and Brownlee C_8 VeloSep) exhibited comparable efficiences for a number of proteins chromatographed in the inverse-gradient elution mode (Fig. 1). These supports are commonly utilized for the RP-HPLC of low-molecular-weight compounds (*e.g.* peptides)[6,8,9,12]. Interestingly, the large pore size (300 Å) support used in this study (Brownlee RP-300) was not considered useful in the inverse-gradient mode since proteins were recovered in unacceptably large volumes (600–1500 μl) (Fig. 1).

Table II shows the retention times obtained for ten proteins of known primary structure on different columns operated in the classical reversed-phase or inverse-gradient reversed-phase elution mode. Unlike the chromatographic behavior of small peptides where it is well documented that a clear relationship exists between the

TABLE I

DATA FOR SPHERICAL POROUS SILICA SUPPORTS USED IN THIS STUDY

Obtained directly from the manufacturer. NA = Not available.

Support	*Carbon content (%)*	*Surface area (m^2/g)*	*Surface coverage ($\mu mol/m^2$)*	*Particle size (μm)*	*Pore volume (ml/g)*	*Designations*
ODS-Hypersil	9.5–10.0	170	2.06	3–5	0.7	C_{18}-120Å
Brownlee VeloSep C_8	7.4–8	200	2.2	3	0.8	C_8-100Å
Brownlee VeloSep C_8	12–13.2	200	1.9	3	0.8	C_{18}-100Å
Brownlee RP-300	7	80–110	8.7	7	0.5–0.6	C_8-300Å
Merck Diol	NA	250	NA	5	NA	Diol-100Å

TABLE II

RETENTION TIMES, t_R (min), FOR STANDARD PROTEINS ON DIFFERENT COLUMNS OPERATED IN EITHER REVERSED-PHASE OR INVERSE-GRADIENT REVERSED-PHASE ELUTION MODE

Columns: 1 = ODS-Hypersil, 20 × 2.1 mm I.D.; 2 = ODS-Hypersil, 100 × 2.1 mm I.D.; 3 = Brownlee RP-300, 30 × 2.1 mm I.D.; 4 = Brownlee C_8 VeloSep 40 × 3.2 mm I.D.; 5 = Brownlee C_{18} VeloSep, 40 × 3.2 mm I.D. Chromatographic conditions: reversed-phase, linear 50-min gradient from 0 to 100% B where solvent A = 0.1% (v/v) aqueous trifluoroacetic acid and solvent B = *n*-propanol–water (50:50) containing 0.1% (v/v) trifluoroacetic acid; inverse-gradient reversed-phase, linear 50-min gradient from 0 to 100% B where solvent A = 100% *n*-propanol and solvent B = *n*-propanol–water (50:50) containing 0.4% (v/v) trifluoroacetic acid (values in parenthesis were for solvent B = *n*-propanol–water (50:50) containing 0.1% (v/v) trifluoroacetic acid). Flow-rate: columns 1–3, 200 μl min^{-1}; columns 4 and 5, 400 μl min^{-1}. Detection, UV at 280 nm. Column temperature, 40°C. Sample load: 10 μg protein in 20 μl water. Data were obtained in duplicate with at least two independent sample preparations and averaged with resulting precision of 1–2%.ND = not determined.

Protein	M_r *(kDa)*	*HI*[a]	t_R *(min)*											
			Reversed-phase elution mode					*Inverse-gradient reversed-phase elution mode*						
			1	*2*	*3*	*4*	*5*	*1*	*2*	*3*	*4*		*5*	
Insulin	5.8	1180	21.67	26.46	20.96	20.62	19.91	12.57	8.14	6.29	7.35	(11.83)	5.67	(8.24)
Cytochrome *c*	11.6	1110	25.71	30.57	23.21	23.85	22.85	15.67	11.80	11.35	9.93	(16.73)	7.36	(11.26)
Ribonuclease B	13.5	870	20.29	25.37	19.24	19.08	17.89	15.32	11.47	11.56	9.62	(16.18)	7.38	(13.78)
α-Lactalbumin	14.1	1150	29.04	33.97	26.54	27.32	26.07	15.24	11.42	8.01	9.65	(15.78)	7.33	(10.95)
Lysozyme	14.7	970	26.59	31.84	24.13	24.70	23.52	15.43	11.45	10.78	9.71	(16.00)	7.33	(10.96)
Trypsin inhibitor	20	1040	32.98	38.77	28.54	30.35	28.68	15.86	12.17	8.53	10.12	(17.16)	7.51	(11.67)
Carbonic anhydrase	30	1060	35.78	41.25	30.70	32.63	30.47	17.51	13.68	11.56	11.06	(19.53)	8.17	(12.92)
Ovalbumin	45	1110	42.14	48.05	34.60	37.95	35.73	16.86	13.72	9.48	11.06	(19.62)	8.06	(12.93)
α-Amylase	52	1070	36.67	41.06	29.87	32.10	30.12	17.65	14.83	11.05	11.79	(21.14)	8.64	(13.82)
Transferrin	90	ND	33.04	38.58	26.63	28.73	26.59	18.10	14.79	14.80	11.77	(21.33)	8.42	(11.17)

[a] Average hydrophobicity, obtained from ref. 14.

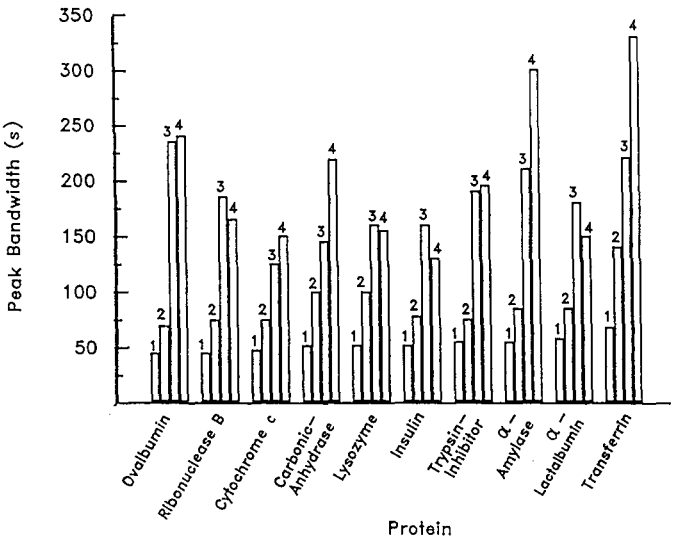

Fig. 1. Plot of the peakwidth (seconds) of eluted proteins from various columns operated in the inverse-gradient reversed-phase elution mode. Chromatographic conditions are given in Table II. Columns: (1) Brownlee VeloSep C_8 (40 × 3.2 mm I.D.); (2) ODS-Hypersil (100 × 2.1 mm I.D.); (3) Brownlee RP-300 (100 × 2.1 mm I.D.); Merck LiChrosorb Diol (75 × 4.6 mm I.D.). Proteins: ovalbumin; ribonuclease B; cytochrome *c*; carbonic anhydrase; lysozyme; insulin; trypsin inhibitor; α-amylase; α-lactalbumin transferrin.

polarity of a peptide and its retention order[13], an examination of the chromatographic behavior of the panel of proteins used in this study reveals no clear correlation between the calculated hydrophobicities[14] of these proteins (summarized in Table II) and their elutions times with 1-propanol.

The data presented in Table II clearly shows that the order of protein retention on the columns studied is largely independent of the chromatographic mode employed. Thus, for a particular support the protein retention order is essentially the same in both the conventional reversed-phase and inverse-gradient reversed-phase elution mode. However, upon close scrutinization of the retention data in Table II, some reversals in protein selectivity pattern are apparent. For example, the retention order of insulin and ribonuclease as well as ovalbumin and transferrin (Table II) are reversed in the two chromatographic modes. These findings are in accord with previously reported observations, that multiple retention processes may be involved in the binding of organic compounds[1], peptides and proteins[2,3] to silica-based reversed-phase supports. This change in selectivity pattern is suggestive of normal (or polar) phase chromatographic behavior in the inverse-gradient mode and is due, presumably, to residual silanol groups remaining on reversed-phase supports[1–5] (*i.e.*, a silanophilic mechanism[15]). This notion is supported by the work of Bij *et al.*[1] who demonstrate that the addition of *n*-butylamine to the eluent attenuated silanophilic interactions which resulted in regular retention behaviour for peptides. However, the observation by others[2,3,5] that other alkylamines (*e.g.*, triethylamine) do not dramatically influence this irregular U-shaped behaviour for peptides suggests that processes other than silanophilic interactions may be involved.

It is well known from the literature[16–19] that conformational transitions in

proteins can be induced by apolar compounds such as detergents and organic solvents. For instance it has been recently demonstrated (using circular dichroism, fluorescence and visible spectroscopy) that 1-propanol can induce a reversible conformational change in proteins to an apparently ordered helical form[20]. Consistent with this hypothesis is the recent report[17] that protein conformation can have a marked influence on protein retention behavior on reversed-phase supports. In these studies Benedek *et al.*[17] established that "native" and "denatured" forms of proteins can be clearly resolved on reversed-phase packings and that the kinetics of protein unfolding is a function of both the organic modifier employed and the incubation time that a protein spends on the bonded-phase surface prior to development of the column[17]. In the case of small peptides it has been demonstrated that their retention behavior on reversed-phase supports can be strikingly influenced if the peptide can be induced to form an amphipathic helix[21,22]. Hence, at high organic solvent concentrations protein structures may be disrupted to produce periodic but dispersed polar/apolar helical exteriors[23] which, in turn, may influence the interaction between protein and

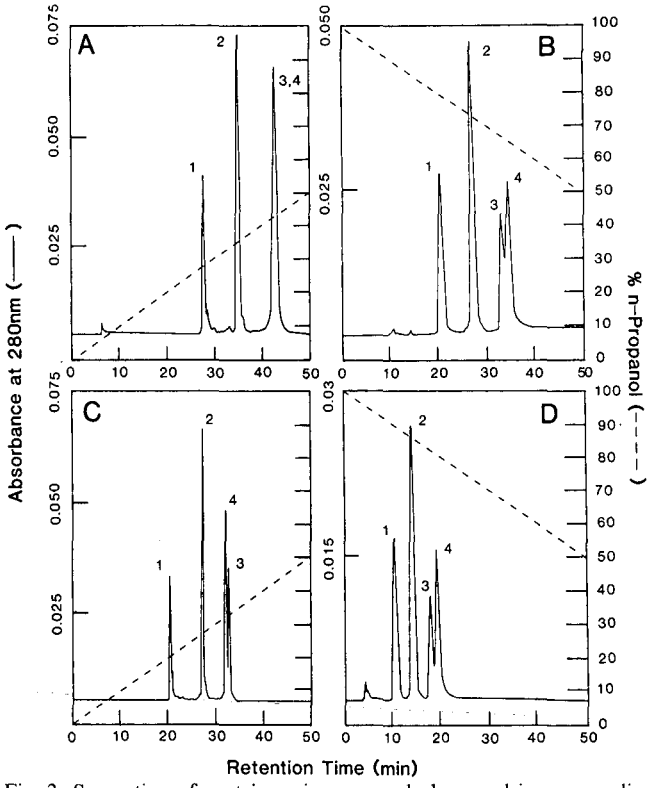

Fig. 2. Separation of proteins using reversed-phase and inverse-gradient reversed-phase elution modes. Chromatographic conditions: (A) ODS-Hypersil (100 × 2.1 mm I.D.); reversed-phase elution mode (linear 50-min gradient from 0 to 100% B, where solvent A = 0.1% (v/v) trifluoroacetic acid, solvent B = 50% (v/v) aqueous *n*-propanol containing 0.1% (v/v) trifluoroacetic acid). (B) ODS-Hypersil (100 × 2.1 mm I.D.). Inverse-gradient reversed-phase elution mode (linear 50-min gradient from 0 to 100% B, where solvent A = 100% *n*-propanol, solvent B = 50% aqueous *n*-propanol containing 0.1% (v/v) trifluoroacetic acid. (C) Brownlee C_8 VeloSep (40 × 3.2 mm I.D.). Reversed-phase elution mode (same as in Table II). (D) Brownlee C_8 VeloSep (40 × 3.2 mm I.D.). Inverse-gradient reversed-phase elution mode (same as in Table II). Flow-rate 400 μl min^{-1}. Column temperature, 40°C. Proteins: 1 = bovine insulin; 2 = α-lactalbumin; 3 = carbonic anhydrase; 4 = α-amylase. Sample load: 10 μg in 20 μl water.

chromatographic support. Thus, the selectivity changes may result from proteins assuming different conformations in the conventional reversed-phase and inverse-gradient reversed-phase elution modes; *i.e.*, different proteins conformations may be induced by the mobile phase interactions of the different chromatographic modes.

As described earlier[5], the ion-pairing agent trifluoroacetic acid modulates protein retention behavior in the "inverse-gradient" chromatographic mode (see also Table II) as well as chromatographic efficiencies and protein recoveries. For practical purposes, we routinely use 0.1–0.4% (v/v) trifluoroacetic acid in the second mobile phase in order to minimise peak bandwidth; under these conditions proteins were typically recovered in 100–300 μl using 2.1 or 3.2 mm I.D. columns[24].

The chromatographic efficiency of proteins in the inverse-gradient reversed-phase elution mode, approximately 90% of that achieved in the conventional reversed-phase elution mode, permits protein fractionations. Indeed, for the panel of proteins employed in this study α-amylase and carbonic anhydrase are better resolved in the inverse-gradient mode than the reversed-phase mode (Fig. 2). Thus the inverse-gradient RP-HPLC procedure described here offers the potential for resolving preparative amounts of proteins at high organic solvent concentrations within the confines of a chromatographic column.

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Note

Isolation and purification of a fibrinogenolysin from the venom of the saw-scaled viper (*Echis carinatus*) by high-performance liquid chromatography

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Traditionally, isolation of biologically active proteins from snake venoms has been achieved by open column ion-exchange chromatography. More recently, the venom of *Echis carinatus* (saw-tailed viper) has been fractionated by chromatographic and electrophoretic techniques, affinity chromatography and fast protein liquid chromatography[1–3]. Attempts at utilizing reversed-phase high-performance liquid chromatography (HPLC) have often resulted in denaturation of the venom proteins by the organic mobile phase with concomitant loss of biological activity[4,5]. Schaeffer *et al.*[6] have recently fractionated *E. carinatus* venom by gel permeation HPLC. Protein separations by means of hydrophobic interaction HPLC have been reported by Goheen and Englehorn[7]. This paper describes the successful fractionation of crude snake venoms by hydrophobic interaction HPLC, with retention of biological activity. A fibrinogenolysin, with its biological activity intact, was isolated from crude *E. carinatus* venom with use of a hydrophobic interaction column (TSK-Phenyl 5PW). After further purification by ion-exchange and reversed-phase HPLC, fourteen amino acid residues of its N-terminal sequence were determined.

EXPERIMENTAL

Materials

Lyophilised snake venoms were obtained from Sigma (St. Louis, MO, U.S.A.). "Centricon" micro-concentrators (10 000 M.W. cut off) were obtained from Amicon (Melbourne, Australia). Thrombin, topical-bovine origin (Parke-Davis & Co., De-

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troit, MI, U.S.A.) and human fibrinogen (Commonwealth Serum Laboratories, Melbourne, Australia) were used for assaying biological activity. CM-Trisacryl M gel (LKB) was purchased from Linbrook (Sydney, Australia).

HPLC instrumentation and conditions

Both Kortec and Waters Assoc. HPLC systems were used. The Kortec system consisted of a Rheodyne Model 7125 syringe-loading injector with a 200-μl sample loop, two single piston pumps (Model K45M), a Model K45 gradient controller and a Model K95 variable-wavelength detector set at 254 nm. A TSK-Phenyl 5PW column (LKB, 75 mm × 7.5 mm) was used for hydrophobic interaction separations, with a linear ammonium sulphate gradient over 30 min. The gradient was run at room temperature and started with 2 *M* ammonium sulphate and 0.1 *M* ammonium phosphate buffer at pH 7.0, and ended with the same buffer without the ammonium sulphate.

A TSK-DEAE 5PW column (LKB, 75 mm × 7.5 mm) was used for ion-exchange chromatography. A linear gradient, run at room temperature over 30 min, started with 0.1 *M* ammonium acetate at pH 8.0 and ended with 0.5 *M* ammonium acetate at pH 6.0.

The Waters Assoc. HPLC system consisted of a Model U6K syringe-loading injector and 200-μl sample loop, Model M600 and M45 pumps, Model 720 gradient controller and Model 441 UV detector with a fixed wavelength of 254nm. It was used with a μBondapak C_{18} column (Waters Assoc., 30 mm × 3.9 mm) for reversed-phase chromatography. A linear gradient, run at room temperature, over 30 min, started with 0.05% (v/v) trifluoroacetic acid (TFA) in water and ended with 0.05% (v/v) TFA in acetonitrile–water (60:40). A flow-rate of 1 ml/min was used for both HPLC systems. A CM-Trisacryl open column (12 cm × 1.5 cm) was packed at room temperature. An elution gradient, starting with 0.05 *M* ammonium acetate, pH 7.0, and finishing with final buffer of 0.05 *M* ammonium acetate, pH 7.0 containing 2 *M* sodium chloride, with a 25–30 ml/h flow-rate was used. Detection was carried out at 280 nm at 2 a.u.f.s. using a Pharmacia single path monitor, Model UV1. The amino acid sequence was determined with a gas-phase protein sequencer, Model 470A (Applied Biosystems, Foster City, CA, U.S.A.).

RESULTS AND DISCUSSION

A fibrinogenolytic protein was isolated from 250 mg of crude *E. carinatus* venom using a CM-Trisacryl open column (Fig. 1). Detection of fibrinogenolytic activity was by the thrombin time test[8] after incubation of fractions with fibrinogen (2 mg/ml) for 60 min at 37°C. Prolonged thrombin times were considered indicative of fibrinogenolytic activity. The active fractions (Fig. 1) were pooled and lyophilised prior to HPLC analysis.

Separations of crude *E. carinatus* venom were carried out by HPLC, using TSK-Phenyl 5PW, TSK-DEAE 5PW and μBondapak C_{18} columns. Injection of the fibrinogenolytically-active material from the open column chromatogram into each of these columns enabled the peak corresponding to active material to be identified by retention time (Fig. 2).

Fibrinogenolytic activity, although present in the crude venom, could not be

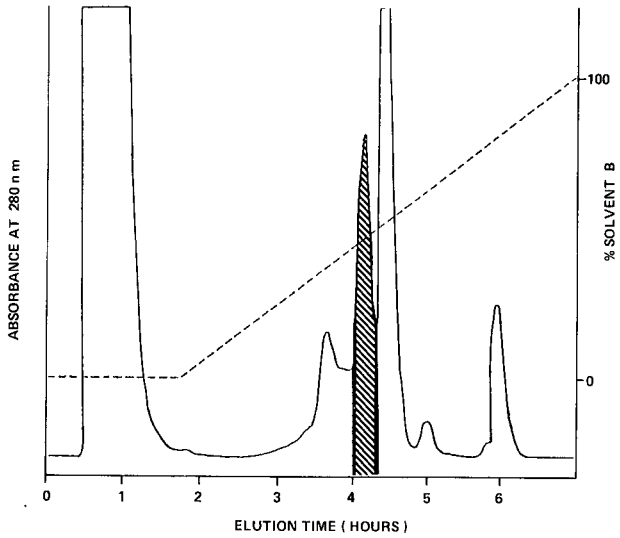

Fig. 1. Elution profile for crude *E. carinatus* venom. Crude venom (250 mg) was dissolved in 5 ml of starting buffer. After centrifugation, the supernatant was loaded on to a CM-Trisacryl open column (12 cm × 1.5 cm). Buffers and elution conditions are outlined in the Experimental section. Fractions of 10 ml were collected. The fraction showing the fibrinogenolytic activity is shaded. The gradient profile is indicated by the dashed line. a.u.f.s. = 2.

measured prior to isolation of the active component due to interference by coagulant enzymes. Activity was retained in the fractions collected from the TSK-Phenyl 5PW and TSK-DEAE 5PW columns. Material recovered from the reversed-phase μBondapak C_{18} column had lost all activity. Separations obtained by injection of crude venoms of four other species of snakes with these three columns are included for comparison (Fig. 3).

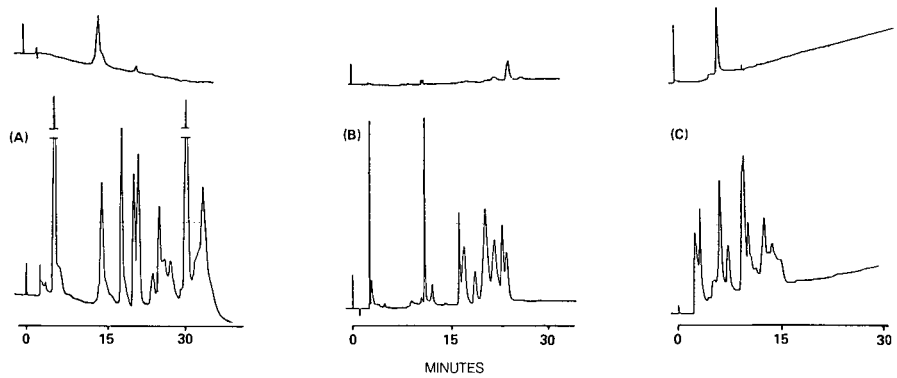

Fig. 2. Crude *E. carinatus* venom (1 mg) was dissolved in 200 μl of starting buffer. After centrifugation, the supernatant was injected into the HPLC chromatograph (lower profile). A volume of 200 μl of fibrinogenolytic material of the CM-Trisacryl fraction (Fig. 1) was injected directly into the HPLC chromatograph (upper profile). HPLC system: (A) TSK-Phenyl 5PW column, a.u.f.s. = 1; (B) TSK-DEAE 5PW column, a.u.f.s. = 0.125; (C) μ-Bondapak C_{18} column, a.u.f.s. = 0.5.

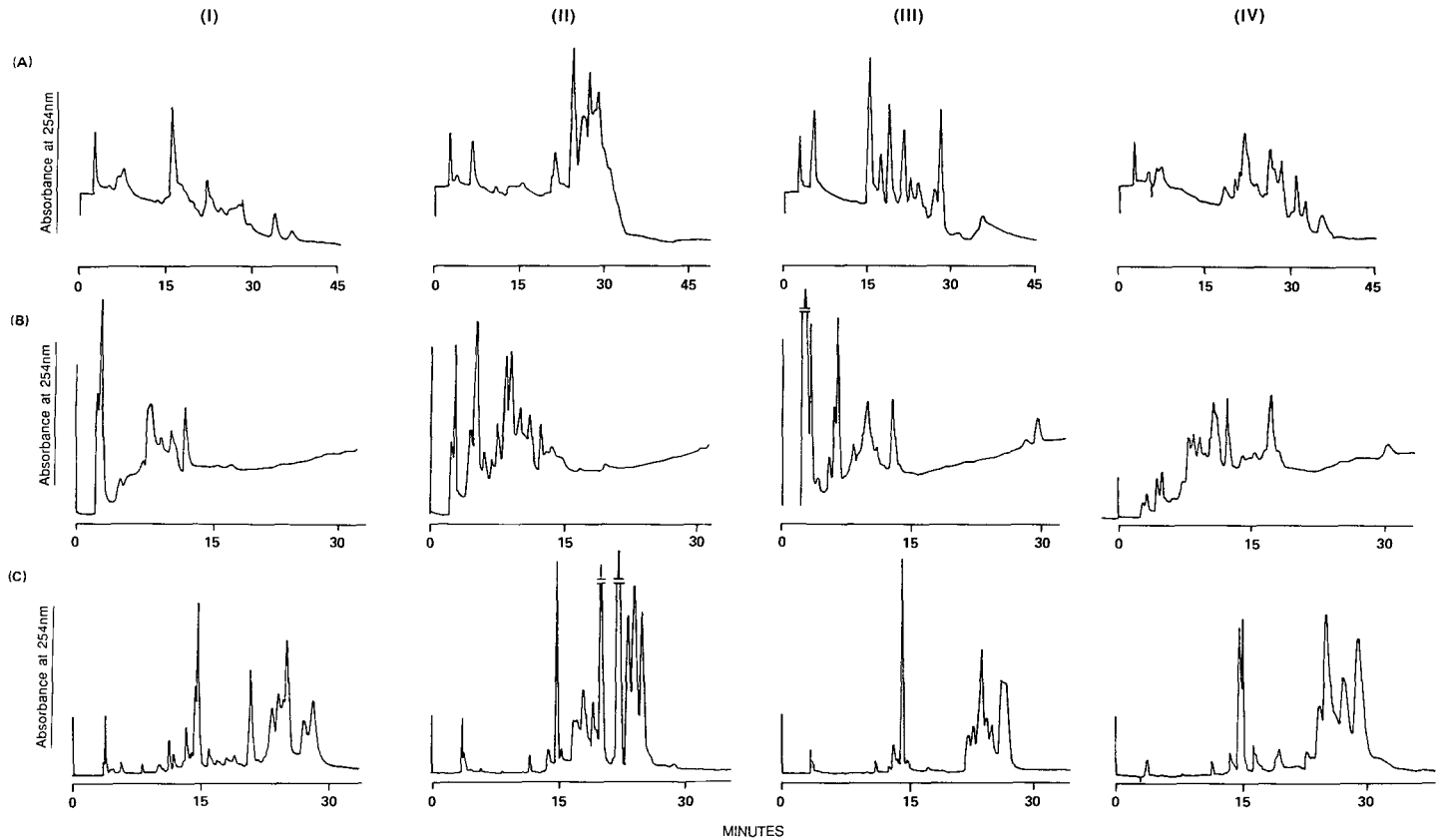

Fig. 3. HPLC separations of crude snake venoms. All crude venom was dissolved in starting buffer (1 mg/200 μl). After centrifugation, the supernatant was injected into the HPLC system. (I) *Bothrops atrox*, (II) *Crotalus scutulatus*, (III) *Agistrodon rhodostoma* and (IV) *Bothrops jaracaca* obtained with (A) TSK-Phenyl 5PW column, a.u.f.s. = 0.1; (B) TSK-DEAE 5PW column, a.u.f.s. = 0.1; (C) μBondapak C_{18} column; a.u.f.s. = 0.2.

TABLE I

SUPPLEMENTARY TABLE TO AMINO ACID SEQUENCE

Cycle	*Amino acid*	*Relative yield*[a] *(%)*
1	Gln	100
2	Arg	79
3	Phe	395
4	Asp	385
5	Pro	266
6	Arg	62
7	Tyr	480
8	Ile	95
9	Glu	371
10	Leu	340
11	Val	429
12	Val	523
13	Val	733
14	Ala	628

[a] Relative yield equals the yield of PTH-amino acid derivative produced in cycle *n* relative to yield in the first cycle, which was assigned the value of 100.

Subsequently, 30 mg of crude *E. carinatus* venom was dissolved in 200 μl 0.5 *M* ammonium acetate, pH 7.0, and injected into the TSK-Phenyl 5PW column. The peak exhibiting fibrinogenolytic activity was collected, desalted and concentrated with a "Centricon" microconcentrator. The concentrate was washed twice with the same ammonium acetate solution prior to lyophilisation. Approximately 1.5 mg (5.0%) of fibrinogenolysin was recovered from the venom by this procedure. This material was then subjected to further purification by HPLC with the μBondapak C_{18} column.

Preliminary investigations of the activity of the fibrinogenolysin by sodium dodecyl sulphate (SDS) electrophoresis[9] of the products of its reaction with human fibrinogen, reduced with mercaptoethanol and unreduced, indicated that fragmentation of the α-chain occurred initially, followed by that of the β-chain, whilst the gamma chain remained unaffected. The M_r of the fibrinogenolysin was estimated to be 20 000 by the SDS-polyacrylamide gel electrophoresis method[10]. Using gas phase Edman degradation[11], the sequence of its N-terminus was determined as: H_2N-Gln-Arg-Phe-Asp-Pro-Arg-Tyr-Ile-Glu-Leu-Val-Val-Val-Ala- (see Table I). The sequence traces showed no evidence of any inhomogeneity of the sample.

ACKNOWLEDGEMENTS

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Note

Application of autofocusing in the isolation of peroxidase

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Previously we have drawn attention to autofocusing as a method for isoelectric focusing without carrier ampholyte, working with the compound to be separated simply dissolved in distilled water. By autofocusing two pure enzymes have been prepared so far in our laboratory: uricase (E.C. 1.7.3.3) and α-amylase (E.C. 3.2.1.1)[1,2]. Here we report on the purification of horseradish peroxidase (E.C. 1.11.1.7), the first enzyme to be isolated by this method from plant material, present mainly in horseradish, sunflower and "giant" turnip-cabbage[3–8] from which it can be purified in three separation steps.

The necessary experimental backround and interpretation of results in enzyme autofocusing have been reported elsewhere[9–11].

EXPERIMENTAL

Horseradish was selected as a source of the enzyme peroxidase. After homogenization of 500 g of horseradish root in 2000 ml of 100 m*M* phosphate buffer (pH 7.0)[7] (3 × 5 min) and centrifugation at 1000 *g* for 15 min, the supernatant was dialysed against distilled water at 4°C for 24 h. The conductivity of raw peroxidase was adjusted to 360 μS cm^{-1} by addition of distilled water.

This solution was divided into two parts and each part was purified separately. The first part was subjected to ion-exchange chromatography on a CM-cellulose column (35 cm × 2 cm I.D.). The starting solution was a 10 m*M* sodium acetate buffer made 100 m*M* with respect to sodium chloride at pH 4.4. The second eluent was 100 m*M* sodium acetate with 1 *M* sodium chloride at pH 5.4. A linear gradient was applied at a flow-rate of 15 ml h^{-1}; 6-ml fractions were collected in which the peroxidase activity was determined[12]. The active fractions were pooled and subjected to ultrafiltration with disposable Centriflo membrane cones rated at MW 50 000 (for filtrate) and at MW 25 000 (for residue).

The second portion was subjected to autofocusing; 1 l of crude peroxidase solution with a conductivity of 360 μS cm^{-1} was focused in an autofocuser (Realizing Centre of Slovak Academy of Sciences, Košice, Czechoslovakia) at 4°C for 32 h in an

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electric field of strength varying from 250 to 1000 V d.c. until the current decreased to its minimum value. The autofocused medium was then divided into twenty equal fractions in which the pH, peroxidase activity and protein concentration were determined. The fractions containing peroxidase activity were pooled and loaded on to a 62 × 3 cm I.D. Spheron P-40 column equilibrated with 0.05 *M* phosphate buffer (pH 7.0)[8]. The column was operated at 4°C at a flow-rate of 100 ml h^{-1} and 15-ml fractions were collected by an automatic fraction collector (FCC 60, Laboratorní přístroje, Prague, Czechoslovakia). All fractions were tested for their protein content and peroxidase activity and the active fractions were pooled for final evaluation.

For peroxidase activity detection 9 m*M* pyrogallol and 4 m*M* hydrogen peroxide solution was freshly prepared in 4 ml of the peroxidase solution and incubated at 30°C for 5 min. The reaction was then stopped by adding 0.2 ml of 100 m*M* potassium cyanide to the reaction mixture. The yellow-brown colour at 380 nm was measured against a blank sample[12]. The protein concentration in individual fractions was determined by the Lowry method[13].

RESULTS AND DISCUSSION

Fig. 1 shows the purification of peroxidase by ion-exchange chromatography on CM-cellulose. The course of the purification process using ion-exchange chromatography is summarized in Table I. The active peroxidase fraction after ion-exchange chromatography contained as many as six surrounding protein fractions by polyacrylamide gel electrophoresis (PAGE) (data not shown) and still three fractions after the ultrafiltration step (for the effect of ultrafiltration in peroxidase purification see Table II).

Fig. 2 shows the results of autofocusing. The bulk of proteins focused within the pH range 2.4–3.1 while the fractions containing peroxidase activity occurred between

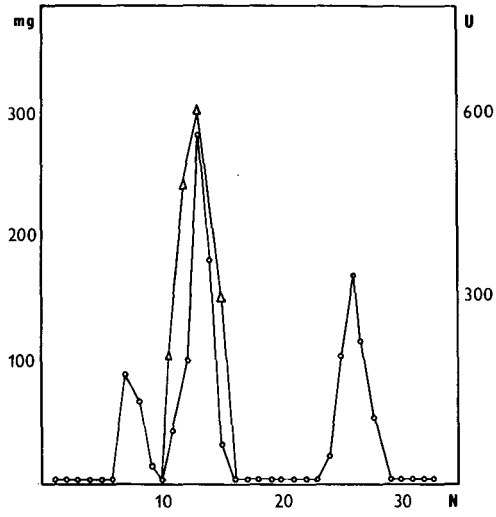

Fig. 1. Purification of peroxidase by ion-exchange chromatography. *N* = fraction number. ○, Protein concentration in mg per fraction; △, activity of peroxidase in U.

TABLE I

PURIFICATION OF PEROXIDASE BY ION-EXCHANGE CHROMATOGRAPHY AND ULTRAFILTRATION

Step	*Total protein (mg)*	*Peroxidase activity (U)*	*Specific activity (U/mg)*	*Purification*	*Recovery (%)*
Centrifugation	1320	1740	1.32	1.00	100
Ion-exchange chromatography	580	1500	2.59	1.96	86
Ultrafiltration (MW 50 000)	390	1176	3.02	2.29	68
Ultrafiltration (MW 20 000)	208	890	4.28	3.24	51

TABLE II

PURIFICATION OF PEROXIDASE BY ULTRAFILTRATION USING FILTERS LIMITING THE PROTEIN MOLECULES IN THE RANGE MW 25 000–50 000

Parameter	*MW 50 000*		*MW 25 000*	
	Residue	*Filtrate*	*Residue*	*Filtrate*
Total protein (mg)	75	390	208	166
Total activity (U)	–	1176	890	–

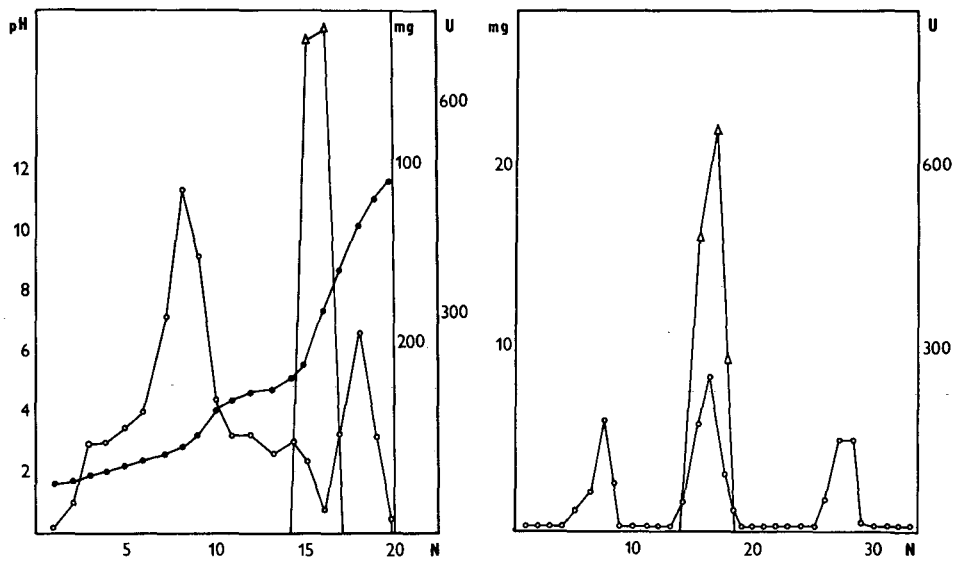

Fig. 2. Isolation of peroxidase by autofocusing. N = fraction number. ○, Protein concentration in mg per fraction; ●, pH gradient; △, activity of peroxidase in U.

Fig. 3. Purification of peroxidase after autofocusing by Spheron P 40 gel filtration. N = fraction number, ○, Protein concentration in mg per fraction; △, activity of peroxidase in U.

TABLE III
PURIFICATION OF PEROXIDASE BY AUTOFOCUSING AND GEL CHROMATOGRAPHY

Step	*Total protein (mg)*	*Peroxidase activity (U)*	*Specific activity (U/mg)*	*Purification*	*Recovery (%)*
Centrifugation	1650.00	1800	1.14	1.00	100
Autofocusing	25.50	1715	67.26	59.00	91
Spheron pool	17.20	1472	85.58	75.07	78

pH 5.45 and 7.12. Fig. 3 shows the purification of the focused peroxidase by subsequent gel chromatography. As can be seen in Table III, the specific activity of the enzyme increased 59-fold after autofocusing. At this stage four surrounding protein fractions were found by PAGE. After the gel chromatography step the specific activity increased 75-fold and the isolated enzyme was electrophoretically homogeneous (Fig. 4).

The purified enzyme was freeze-dried on addition of 25 μM glutathione. The enzyme activity of 85 U per mg protein remained unchanged for as long as 6 months.

The advantages of autofocusing over ion-exchange chromatography are obvious from the comparison of Tables I and III. While ion-exchange chromatography yields a 3.24-fold enrichment (compare also refs. 14–16), autofocusing followed by gel permeation chromatography offers a 75-fold enrichment with a very high recovery.

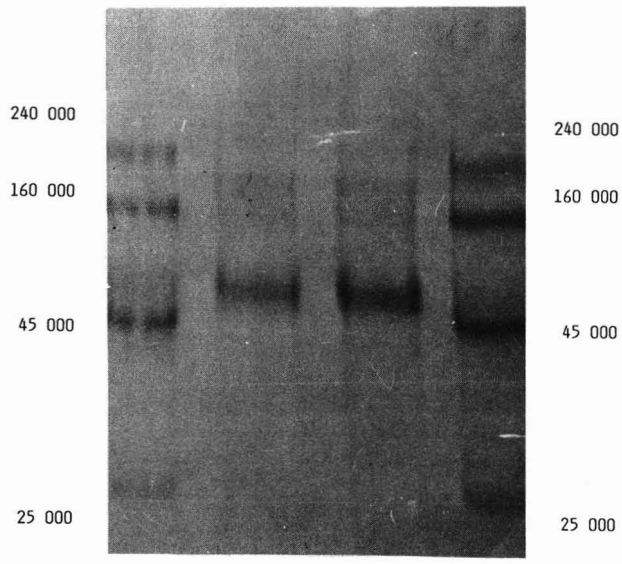

Fig. 4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of the peroxidase purified by autofocusing and gel chromatography. Lanes 1 and 4, molecular weight standards given in the margin; lanes 2 and 3, purified peroxidase.

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Note

An improved method for the separation and quantification of glutathione S-transferase subunits in rat tissue using high-performance liquid chromatography

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The glutathione S-transferases are a family of isoenzymes that play an essential role in the biotransformation of xenobiotics. They catalyse the conjugation of glutathione with numerous electrophilic agents, but also perform a number of other functions in cellular metabolism, *e.g.*, reduction of organic hydroperoxides, steroid isomerization and binding of non-substrate hydrophobic ligands such as bile acids, bilirubin and a number of drugs[1,2]. These enzymes have been found in numerous species and in many organs of mammals[2]. They exist as dimers and each dimer consists of two identical (homo-dimer) or two different (hetero-dimer) subunits with molecular weights in the region of 25 000 daltons. Each subunit has a characteristic enzymatic activity, which is additive in the different dimers[3]. Glutathione S-transferases are usually isolated from different sources with affinity chromatography, using epoxy-activated Sepharose to which S-hexylglutathione is linked. This method has to be used carefully, however, since some isoenzymes might not be bound. For example, rat glutathione S-transferase 5-5 does not bind to the S-hexylglutathione-Sepharose matrix[4]. Separation of the individual isoenzymes is usually performed with chromatofocusing, *e.g.*, on a Pharmacia fast protein liquid chromatography (FPLC) system[4]. Recently, Farrants *et al.*[5] described a method for the separation of glutathione S-transferase subunits using high-performance liquid chromatography (HPLC). This method appears very suitable for development into a system for the quantification of subunits in small amounts of sample or samples with low transferase activity. However, in the method described, the subunit peaks are rather broad and baseline resolution is poor, especially for the rat subunits 3, 4, 7 and 2. Chromatograms also suffer from a significant increase of the baseline during an analysis.

In this paper an improved method is presented for the separation and quantification of glutathione S-transferase subunits. Baseline separation of rat subunits 1, 2, 3, 4 and 7 is performed within 30 min.

EXPERIMENTAL

Chemicals and reagents

S-Hexylglutathione was obtained from Sigma (St. Louis, MO, U.S.A.). Epoxy-activated Sepharose 6B was obtained from Pharmacia/LKB (Uppsala, Sweden). Cen-

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tricon-10 microconcentrator tubes were from Amicon (Danvers, MA, U.S.A.). HPLC-grade acetonitrile was obtained from Promochem (Wesel, F.R.G.) and HPLC-grade trifluoroacetic acid (TFA) from Baker (Deventer, The Netherlands).

Isolation and purification of glutathione S-transferases

Cytosol of liver, kidney, lung and testis was prepared by homogenizing tissue with three volumes of 0.01 *M* Tris–HCl/0.14 *M* KCl pH 7.4 with a Potter-Elvehjem tissue homogenizer and centrifuging at 105 000 *g* for 75 min. Cytosol of the small intestinal mucosa was prepared by a modification of the method described by Borm *et al.*[6].

S-Hexylglutathione was linked to epoxy-activated Sepharose 6B as described by Mannervik and Guthenberg[7]. Rat glutathione S-transferases 1-1, 2-2, 3-3, 4-4 and 7-7 were isolated and purified from rat liver and kidney using S-hexylglutathione affinity chromatography and chromatofocusing on a Pharmacia FPLC system equipped with a Mono-P HR 5/20 column, as described previously[8]. The protein content of the purified isoenzymes was determined with the Lowry assay, using bovine serum albumin (BSA) as a standard. The identification and estimation of purity of the isoenzymes was performed with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing using the Pharmacia PhastSystem.

For the determination of glutathione S-transferase subunits in different tissues, 1 ml of cytosol was applied to a S-hexylglutathione affinity column (2.5 cm × 1 cm I.D.) equilibrated with 10–15 ml of 10 m*M* Tris–HCl/1 m*M* EDTA/0.2 m*M* dithiothreitol, pH 7.8. After rinsing the column with 15 ml of equilibration buffer containing 0.2 *M* NaCl, the glutathione S-transferases were eluted with 6 ml equilibration buffer containing 0.2 *M* NaCl and 2.5 m*M* S-hexylglutathione. The flow-rate was 0.5–0.7 ml/min. The recovery of transferase activity in the eluate ranged from 86 (small intestine) to 99% (liver). Enzyme activities were determined using 1-chloro-2,4-dinitrobenzene as a second substrate, according to Habig *et al.*[9].

High-performance liquid chromatography

A modular HPLC system was assembled with a 2150 HPLC pump (LKB), a 2152 LC controller (LKB), a 2156 solvent conditioner (LKB) and a 2140 Rapid Spectral Detector (LKB) operating at 214 nm. Peak areas were integrated with Nelson Analytical Model 2600 chromatography software. The separation of glutathione S-transferase subunits was performed with a Vydac 201 TP 5 (200 mm × 3 mm I.D.) chromatography column (Chromsep system, Chrompack, The Netherlands) and a gradient of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The gradient consisted of a linear gradient from 35 to 45% (v/v) solvent B in 18 min, followed by a linear gradient from 45 to 55% (v/v) solvent B in 5 min and isocratic elution at 55% solvent B for 7 min. The flow-rate was 0.6 ml/min. From the S-hexylglutathione eluates of liver and testis, 50 µl were directly injected on the HPLC column. Eluates of kidney and lung were concentrated ten times and the eluate of the small intestine twenty times, using Centricon-10 microconcentrator tubes, before injection of 50 µl.

RESULTS

Elution profiles

Fig. 1A shows a typical chromatogram of a standard mixture of rat glutathione S-transferases 1-1, 2-2, 3-3, 4-4 and 7-7 each in a concentration of 50 μg/ml. Under the denaturating conditions employed, the dimers dissociate and the individual subunits are separated. Subunits 3, 4, 7, 2 and 1 were eluted with retention times of 15.0, 16.5, 17.2, 19.3 and 24.5 min respectively (capacity factors, $k' = 13.1, 14.6, 15.3, 17.3$ and 21.8), as established by injecting the purified homodimers. Subunit 1 separates into two major peaks because of its microheterogeneity, as observed before[5,10]. In Fig. 1B–F, chromatograms of glutathione S-transferase subunits derived from rat liver, kidneys, testis, lung and small intestinal mucosa are presented. In liver, subunits

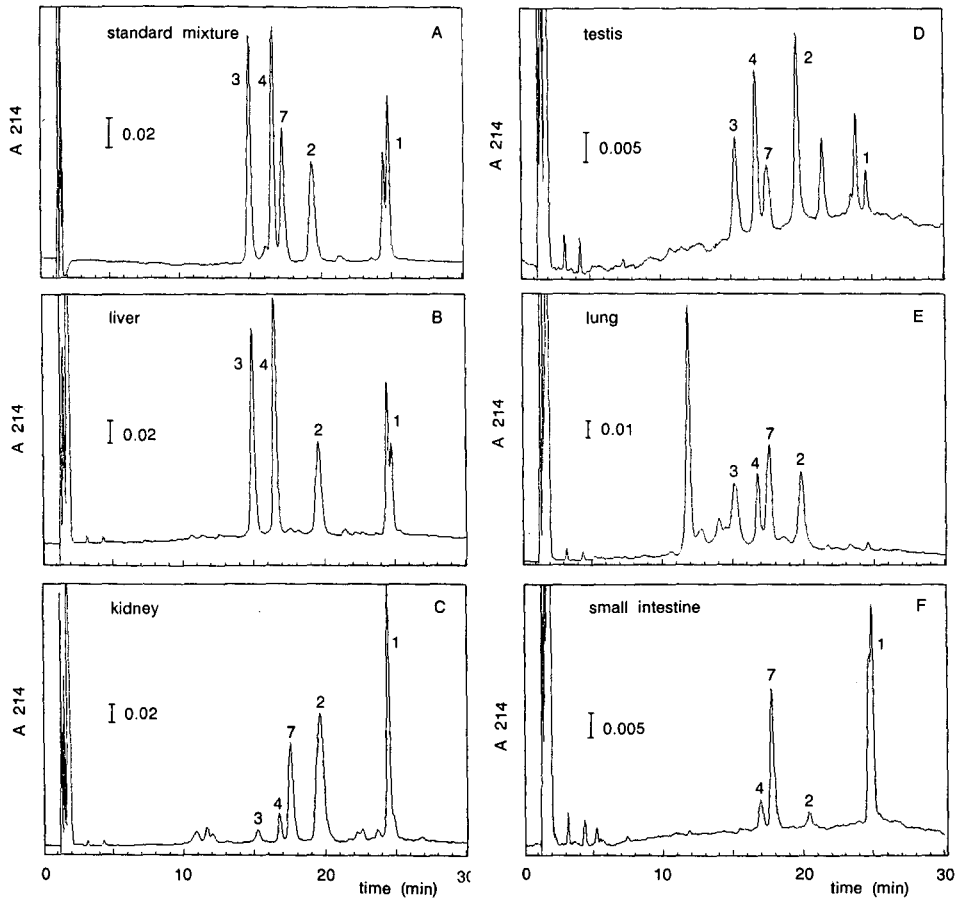

Fig. 1. Elution profile of a standard mixture of glutathione S-transferases 1-1, 2-2, 3-3, 4-4 and 7-7 in a concentration of 50 μg/ml (2.5 μg injected) (A), and elution profiles of S-hexylglutathione eluates of 2.1 mg liver (B), 2.1 mg testis (C), 16.7 mg kidney (D), 16.7 mg lung (E) and 0.53 mg small intestinal protein (F). The HPLC conditions are described in Experimental.

TABLE I

CONCENTRATION OF GLUTATHIONE S-TRANSFERASE SUBUNITS IN RAT LIVER, KIDNEY, TESTIS, LUNG AND SMALL INTESTINAL MUCOSA

Preparation of cytosol, isolation of glutathione S-transferases and HPLC separation and quantification of subunits were performed according to the procedures described in Experimental. The HPLC elution profiles of the different tissues are presented in Fig. 1B–F.

Organ	*Subunit concentration*				
	3	*4*	*7*	*2*	*1*
Liver[a]	1494	1661	–	1433	1245
Kidney[a]	26	33	217	414	239
Testis[a]	224	283	240	515	51
Lung[a]	85	65	148	126	–
Small intestine[b]	–	0.47	2.96	0.40	3.72

[a] Expressed in μg/g tissue.
[b] Expressed in μg/g protein.

3, 4, 2 and 1 are the only major components. In kidney, subunits 3 and 4 are present in minor amounts, while subunits 7, 2 and 1 are the major components. In liver, subunit 7 exists only in trace amounts[11].

In testis, a number of isoenzyme subunits exist. Subunits 3, 4, 7, 2 and 1 are clearly identified. Subunit 1 is present in minor amounts. Between subunits 2 and 1, at retention times of 21.5 and 24 min respectively, two peaks are present. The elution profile is in good agreement with that of seminiferous tubulus of rat testis as described by Farrants *et al.*[5], the peak at 21.5 min probably being due to subunit 6.

Fig. 1E shows the isoenzyme pattern of lung cytosol. Subunits 3, 4, 7 and 2 are major components. Subunit 1 seems to be almost absent. At 12 min, however, an unknown component is eluted, which is abundant in lung. It is different from the subunits 1, 2, 3, 4, 6 and 7. It cannot be identified with subunit 5, because the latter has no affinity for the S-hexylglutathione-Sepharose matrix[4]. Further investigation will be needed to identify this component. Fig. 1F presents the elution profile of the small intestinal mucosa. Subunits 7 and 1 are the major components, while subunit 4 and 2 exist only in minor amounts. Subunit 3 seems to be totally absent. The subunit concentration, calculated with the standard mixture of Fig. 1A, in the different organs is presented in Table I.

Linearity, precision and sensitivity of the assay

To estimate the relationship between the amount of isoenzyme injected and the integrated peak area, four different concentrations of a mixture containing isoenzymes 1-1, 2-2, 3-3 and 4-4 in the range from 15 to 90 μg/ml were injected on the HPLC column. The correlation coefficient varied between 0.997 and 0.999. The intercepts did not differ significantly from zero.

To determine the precision of the assay, the subunit concentration was determined in a pooled liver cytosol on different days, running the whole procedure. The interassay variation for the subunits 1, 2, 3 and 4 was 4, 4, 10 and 13%, respectively.

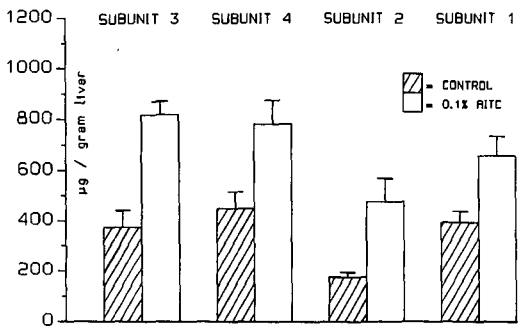

Fig. 2. Subunit composition in a rat control group (n = 5) and a group who received 0.1% allyl isothiocyanate (AITC) in the diet for 4 weeks (n = 5). Isolation of isoenzymes and separation of subunits were performed according to the procedures described in Experimental. Means and standard deviations are presented.

The method described is sensitive, detection of 50 ng (2 pmol) of the glutathione S-transferase subunits (absorption 0.002) being possible.

DISCUSSION

An improved HPLC method has been developed for the separation of glutathione S-transferase subunits. A Vydac TP reversed-phase column was used: the wide pores in TP silica make it ideal for the separation of large bio-molecules. Using the system outlined above, baseline separation between subunits 1, 2, 3, 4 and 7 can be performed within 30 min. The composition of the subunits was determined in rat liver, kidney, testis, lung and small intestinal mucosa, and was found to be in good agreement with the results obtained by Hayes and Mantle[11], who used immunoblotting for subunit identification. The method described is not only suitable for the quantification of subunits in different tissues, even in small samples or samples with low transferase activity, but can also be applied in experiments to estimate which subunits are induced by xenobiotics. For example, in Fig. 2 the subunit contents in liver are presented of a feeding study with rats, consisting of a control group and a group who received 0.1% of allyl isothiocyanate in the diet for 4 weeks, respectively. Subunits 2 and 3 were induced 169 and 119% by the allyl isothiocyanate, while subunits 1 and 4 were induced to much lower extents (67 and 74% respectively). Finally, in addition to other procedures, the method can be applied for the identification of isoenzymes and determining impurities during isolation and purification procedures.

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Note

Separation of optical isomers by capillary zone electrophoresis based on host–guest complexation with cyclodextrins

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Sympathomimetic drugs with substitution at either the α or β carbon atoms exhibit optical isomerism. When the drug is lavorotatory arising from substitution at the β carbon, it has greater peripherical activity, *e.g.*, L-epinephrine and L-norepinephrine are ten times more potent than their dextrorotary isomers[1].

Optical isomer resolution is an important and attractive field of research; sympathomimetic amine racemates are resolved by high-performance liquid chromatography (HPLC) and gas chromatography (GC) using optically active compounds in the stationary phase or by derivatization[2–5]. Recently, capillary zone electrophoresis (CZE) has been used to resolve dansyl-amino acid racemates using either cyclodextrins or the aspartame copper complex[6,7].

The optical isomer resolution of ephedrine and related compounds was studied by capillary isotachophoresis[8] but the resolution of ephedrine and norephedrine was not achieved. There appears to be no report of enantiomer resolutions of epinephrine, norepinephrine and isoproterenol with electrophoretic techniques. Being interested in optical resolution by electrophoretic techniques[9–11], we used CZE for the separation of ephedrine, norephedrine, epinephrine, norepinephrine and isoproterenol enantiomers. Cyclodextrins were added as chiral agents to the background electrolyte (BGE) and the influence of the shape of the cyclodextrin and its concentration on the effective mobility was studied. Complete resolution of the five racemates by using low pH and 18 m*M* heptakis(2,6-di-O-methyl-β-cyclodextrin) were obtained.

EXPERIMENTAL

Apparatus

The experiments were performed with a Bio-Rad HPE 100 apparatus (Richmond, CA, U.S.A.) equipped with an UV detector with a deuterium lamp (190–380 nm). The volumes of the electrode vessels were about 1 ml and 100 μl; for electrophoretic sampling the vessel with the lower volume was used. The apparatus was equipped with a power supply able to deliver up to 12 kV. Sampling and electrophoresis were controlled by a microprocessor.

Separations were performed in a Bio-Rad 148-3002 HPE capillary cartridge (20 cm × 0.025 mm, coated). The capillary was filled with the BGE by using an

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Hamilton microsyringe of 50 μl. Electropherograms were recorded with an LKB 2210 line recorder at a chart speed of 10 mm/min.

Chemicals

Tris(hydroxymethyl)aminomethane (Tris) and phosphoric acid were obtained from Carlo Erba (Milan, Italy), β-cyclodextrin (β-CD), ephedrine (E), norephedrine (nor-E), epinephrine (Ep), norepinephrine (nor-Ep), isoproterenol(i-P) and their enantiomers from Sigma (St. Louis, MO, U.S.A.). Heptakis(2,6-di-O-methyl-β-cyclodextrin) (di-OMe-β-CD) and heptakis(2,3,6-tri-O-methyl-β-cyclodextrin) (tri-OMe-β-CD) were kindly supplied by Dr. D. Sybilska, Institute of Physical Chemistry, Warsaw, Poland.

RESULTS AND DISCUSSION

The resolution of the sympathomimetic drugs is based on the host–guest complexation between the enantiomers and cyclodextrins. Cyclodextrins are neutral polymers with different units of D(+)-glucopyranose and the most commonly CD used in such separation techniques are α, β and γ with six, seven and eight glucose units respectively. Modified CDs, *e.g.*, di- and tri-O-methyl-β-CD are also available.

The enantioselectivity of the CDs arises from the chiral atom present in the glucose units and depends on the stability of the complexes formed with the compounds studied.

Snopek *et al.*[8] in their study by capillary isotachophoresis achieved good enantiomeric resolution of organic bases by using cyclodextrins at pH 5.4. At this pH no resolution of ephedrine and norephedrine was obtained.

We investigated the effect of β-CD, di-OMe-β-CD and tri-OMe-β-CD added to the BGE at low pH on the effective electrophoretic mobility of ephedrine, norephedrine, epinephrine, norepinephrine and isoproterenol.

As shown previously[12], in order to obtain highly efficient separations, electromigration dispersion must be minimized, *e.g.*, by using a co-ion in the BGE having similar effective mobility to that of the analyte. On the other hand the BGE should not adsorb at the wavelength used and should not form inclusion complexes with cyclodextrins. Tris, with a relatively low mobility, was selected as the cation in the BGE; phosphate was the anion at a pH of 2.4.

In order to obtain high separation efficiencies and short analysis time, the electrophoretic experiments were carried out by using a short capillary with a relatively small I.D. The dissipation of the heat generated during electrophoresis is greater when capillaries with small I.D.s are used[13,14].

The column length plays no rôle in separation efficiency but influences the migration time. The capillary used in the experiments was a commercially available coated one that allowed the electroosmotic flow to be minimized to the benefit of the resolution. The resolution of compounds with very similar mobilities can be achieved by balancing the electroomotic flow and effective mobility[15].

Fig. 1 shows the complete separation of the five racemic sympathomimetic drugs. The analysis was carried out in only 4 min. In this experiment it was possible to obtain a relatively high electric field (400 V/cm) by using a relatively low voltage (8 kV).

In the separation shown in Fig. 1 the amount injected was estimated by the formula proposed by Jorgenson and Lukacs[15]

$$Q = (u + u_{os})VACt_i/L \quad (1)$$

where Q is the quantity injected in mole, u the effective mobility, u_{os} the electroosmotic flow, V the injection voltage, A the cross-sectional area of the capillary, C the concentration of the sample, t_i the injection time and L the length of the capillary.

The quantity of epinephrine injected in the separation shown in Fig. 1 was found to be approximately $45 \cdot 10^{-15}$ mole and the volume injected was about 2 nl.

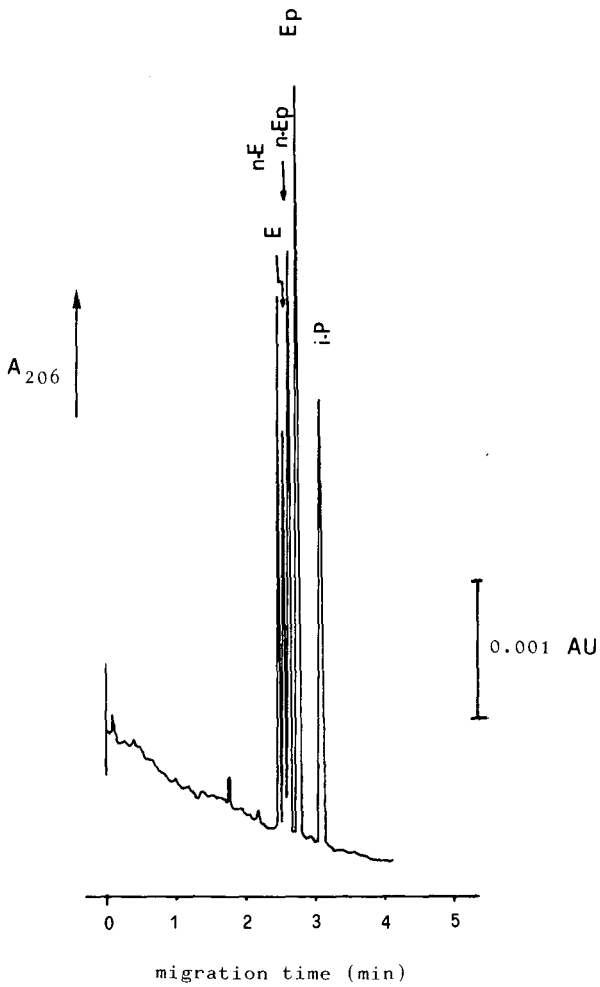

Fig. 1. Electropherogram of the separation of the racemic amines: n-E = norephedrine; E = ephedrine; n-Ep = norepinephrine; Ep = epinephrine and i-P = isoproterenol. BGE: 10 mM Tris-H_3PO_4, pH 2.4. Sampling: electrophoresis at 6 kV for 6 s. The mixture contained $2 \cdot 10^{-5}$ M for each racemic compound. Electrophoretic experiment: 8 kV; I = 6.8 μA.

Different amounts of β-CD were added to the BGE and the migration times of the compounds studied were measured. By increasing the concentration of β-CD the migration times of the five racemic compounds analyzed were reduced. This indicates that all the analytes form inclusion complexes with the CD used. Ephedrine and norephedrine showed the highest reduction in the effective mobility. Despite being a good complexing agent towards racemic compounds, β-CD was not able to resolve the sympathomimetic drugs into their enantiomers. Very poor resolution was obtained at a relatively high concentration of β-CD (20 m*M*) for ephedrine and isoproterenol.

Fig. 2A and B shows the effect of the concentration of heptakis(2,6-di-O-methyl-β-CD) on the migration time of E, nor-E, Ep, nor-Ep and i-P. From these results di-OMe-β-CD seems to be a very good enantioselective complexing agent towards all five compounds studied by CZE.

Complete enantiomer resolution was achieved for nor-E, Ep and i-P when 9 m*M* of di-OMe-β-CD was added to the BGE. To resolve completely the racemic E and nor-Ep, 18 m*M* of the chiral agent was used. In all cases the (+) isomer shows the lowest migration time and this indicates that the inclusion complexation is higher than that obtained with the (−) isomer.

From the data shown in Fig. 2 it is evident that E and nor-E fit closely the cavity of the di-OMe-β-CD and thus are more complexed than the catecholamines studied. In this case the shape of the guest (analytes) plays an important rôle in the complexation: E and nor-E possess the aromatic group without any substitution.

For optical resolution of the compounds studied, di-OMe-β-CD was found to be a very good resolving agent for all samples but the best discriminating effect was observed in the optical resolution of catecholamines.

Fig. 3a and b shows the resolution of racemic nor-E and E, nor-Ep, Ep and i-P respectively.

In order to explain the different complexation of the enantiomers and modified CD is necessary to consider that CDs contain five chiral atoms for each glucose[16]. Furthermore, the complex is influenced by the hydroxyl and O-methyl groups present on the rim of the cavity of the CD. The rim is relatively hydrophobic and can offer the possibility to form hydrogen bonds.

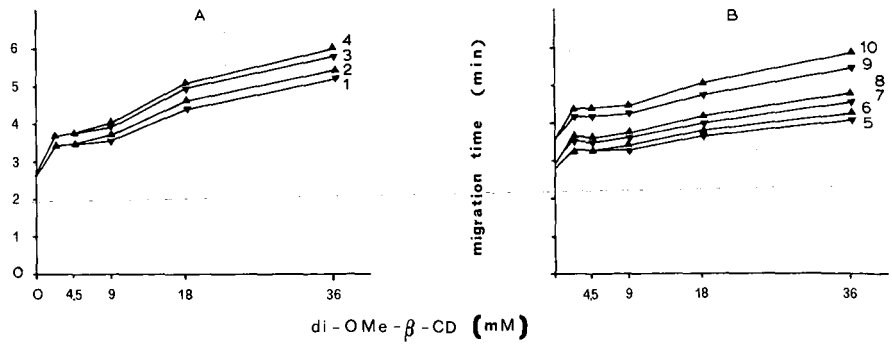

Fig. 2. Effect of the concentration of heptakis(2, 6-di-O-methyl-β-CD) in the BGE on the migration time of: (A) 1 = (−)norephedrine; 2 = (+)norephedrine; 3 = (−)ephedrine; 4 = (+)ephedrine; (B) 5 = (−)norepinephrine; 6 = (+)norepinephrine; 7 = (−)epinephrine; 8 = (+)epinephrine; 9 = (−)isoproterenol; 10 = (+)isoproterenol.

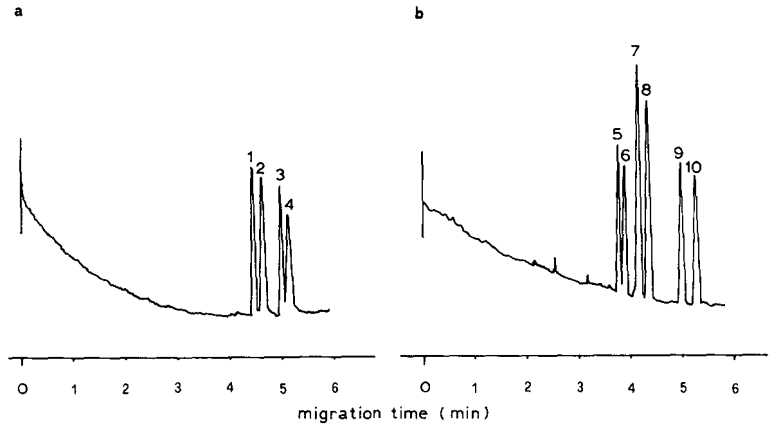

Fig. 3. Electropherograms of the optical isomer resolution of: (a) norephedrine and ephedrine; (b) norepinephrine, epinephrine and isoproterenol. BGE: 10 m*M* Tris-H_3PO_4, pH 2.4 and 18 m*M* of di-OMe-β-CD. Other conditions as in Figs. 1 and 2.

Experiments carried out by adding different amounts of tri-OMe-β-CD to the BGE showed retardation of all the five racemic compounds studied but a chiral resolution was not obtained.

CONCLUSIONS

Our experiments show that the resolution of racemic mixtures of sympathomimetic drugs can be obtained rapidly with CZE by adding to the BGE a chiral host–guest complexing agent. The resolution of the optical isomers studied depends on the type of cyclodextrin. The hydroxyl and the O-methyl groups on the rim of the cavity of CDs influence the resolution power; the presence of both groups in the entrance of the hydrophobic cavity improves the optical resolution. Very poor resolution is obtained when the CD possesses the same groups in the 2, 3 and 6 positions either OH (β-CD) or OCH_3 (tri-OMe-β-CD). The optical resolution is also influenced by the alkyl group bonded to the nitrogen atom of the sample. Isoproterenol with an isopropyl group is the best resolved racemic compounds. An increase in the amount of CD in the BGE improves the resolutions.

CZE is a very promising analytical technique for the optical isomer resolution of the compounds studied.

The drawbacks of other techniques such as HPLC where sophisticated stationary phases and/or the relatively high quantity of the chiral agent in the mobile phase do not exist in CZE. In fact the separations are generally performed in free solutions and the volume of the BGE in each experiment is relatively low. In this study only 2 ml of BGE was used for each run.

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Note

Simultaneous determination of stevioside, rebaudioside A and C and dulcoside A in foods by high-performance liquid chromatography

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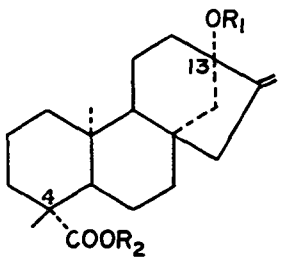
Some sweet-tasting compounds from the leaves of *Stevia rebaudiana* Bertoni, a plant native to Paraguay, have been reported[1,2]. These stevia sweeteners are similar in structure in that a steviol aglycone is connected at C-4 and C-13 to mono-, di- or trisaccharides consisting of glucose and/or rhamnose residues, as shown in Fig. 1. Stevioside has been shown to be the most effective sweetener, and in addition rebaudioside A and C and dulcoside A are also important. In Japan, food-grade stevia sweetener products have been used in a wide range of foods, so it is desirable to establish a simple, rapid and accurate method for the determination of these glycosides in various foods.

Various methods for the determination of stevioside and rebaudioside A after enzymatic or acidic hydrolysis of the stevia glycosides have been reported, including gas–liquid chromatography[3], thin-layer chromatography[4] and "thinchrography"[5], a combination of rod-type thin-layer chromatography and gas chromatography with flame ionization detection. However, these methods are non-specific for the determinaton of individual stevia sweeteners and are time consuming. Recently, several high-performance liquid chromatographic (HPLC) methods for the separation and determination of the stevia glycosides have been reported[6–12]. In particular, Ahmed and Dobberstein[9] and Makapugay *et al.*[11] developed excellent methods for the



	R_1	R_2
Stevioside	glc^{2}-glc	glc
Rebaudioside A	$glc^{2,3}$-glc	glc
Rebaudioside C	glc^{2}-rham, ^{3}-glc	glc
Dulcoside A	glc^{2}-rham	glc

glc ; β-glucopyranosyl
rham ; α-rhamnopyranosyl

Fig. 1. Structures of stevioside, rebaudioside A and C and dulcoside A.

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separation and determination of eight *Stevia rebaudiana* diterpene glycosides. These methods have been applied either to plant materials[6,9,11] or sweetener products[7,8]. Two HPLC methods have been applied to actual foodstuffs[10,12], but included only two typical stevia glycosides, namely stevioside and rebaudioside A.

The simultaneous determination of the four stevia sweeteners including rebaudioside C and dulcoside A in foodstuffs is reported here. A clean up treatment for stevioside, rebaudioside A and C and dulcoside A in beverages, soy sauce, candy and pickled radish using a reversed-phase cartridge and an HPLC method for their determination is described.

EXPERIMENTAL

Stevioside, rebaudioside A and C and dulcoside A of analytical-reagent grade were supplied by Maruzenkasei (Hiroshima, Japan). Amounts of 25 mg of these standard sweeteners were accurately dissolved in 25 ml of water and diluted with the HPLC mobile phase to obtain final concentrations of 5, 10, 20, 50, 70 and 100 μg/ml.

The mobile phase for HPLC was prepared by mixing acetonitrile (HPLC grade, Kanto Chemical, Tokyo, Japan) and deionized and distilled water (80:20, v/v).

A Shimadzu Model LC-6A HPLC system (Shimadzu Seisakusho, Kyoto, Japan) equipped with a column oven (Shimadzu CTO-6A) was used to deliver the mobile phase at a flow-rate of 0.8 ml/min. A normal-phase LiChrosorb NH_2 (5 μm) column (250 × 4 mm I.D.) (Merck, Darmstadt, F.R.G.), thermostated at 50°C, and a Shimadzu SPD-6AV detector operated at 210 nm were used.

The sample solutions were prepared as follows.

Beverage and soy sauce

The sample (5 g) was treated in a Sep-Pak C_{18} cartridge (Millipore), which was pre-wetted with 5 ml each of acetone, methanol and water. The cartridge was washed with 3 ml of water and 10 ml of acetonitrile–water (80:20) solution and eluted with 2 ml of the HPLC mobile phase. The eluate was diluted to 5 ml and a 30-μl portion of the solution was subjected to HPLC analysis.

Candy

A 50-g portion of sample was crushed and mixed in a mortar. After 5 g of the mixture had been weighed into a 50-ml glass beaker, 20 ml of water were added and the mixture was heated on a hot-plate to dissolve the solid. The solution was filtered through No. 2 filter-paper (Toyo Roshi, Tokyo, Japan) and the beaker and the filter-paper were washed with 5 ml of hot water. After cooling, the combined filtrate was placed on a Sep-Pak C_{18} cartridge and treated in the same manner as for beverage and soy sauce.

Pickled radish

A 50-g portion of sample was finely sliced and mixed. After 10 g of the mixture had been weighed into a 50-ml volumetric test-tube, 35 ml of water were added and homogenized with a Polytron (Model PT10-35; Kinematica, Littau, Switzerland). The homogenizer was washed with 5 ml of water. The washings were transferred into the tube and the combined solution was diluted to 50 ml with water. After the solution had

been mixed and centrifuged at 12 600 *g* for 10 min, the supernatant was filtered through No. 2 filter-paper. The first 5 ml of the eluate were discarded and the subsequent 25 ml aliquot was placed on a Sep-Pak C_{18} cartridge and treated in the same manner as for beverage and soy sauce.

RESULTS AND DISCUSSION

Mixed aqueous solutions of stevioside, rebaudioside A and C and dulcoside A were treated on a Sep-Pak C_{18} cartridge for sample preparation, rinsed with 3 ml of water and 10 ml of 20% acetonitrile to remove co-extracts as much as possible, and then eluted with 2 ml of acetonitrile–water (80:20). These stevia sweeteners were never eluted from the cartridge with less than 10 ml of 20% acetonitrile.

As shown in Fig. 2, the use of a LiChrosorb NH_2 normal-phase column with UV detection at 210 nm gave a sufficient baseline separation for the determination of these stevia sweeteners without interferences from other food components.

The method was applied to the analysis of beverage, soy sauce, candy and pickled radish. Typical chromatograms of the four kinds of food extracts are shown in Fig. 3. The peaks of stevioside in beverage, dulcoside A in soy sauce, stevioside and rebaudioside C in candy and rebaudioside A and C in pickled radish suffered slight interference with the appearance of unknown peaks close to the main peaks.

Plots of peak heights of the four stevia sweeteners showed a linear correlation over the concentrations range from 5 to 100 μg/ml. Table I shows the recoveries of the sweeteners fortified with standards at 20 and 100 ppm. The added standards were recovered in the range from 87.9 to 99.7% at the 20 ppm level, and from 93.2 to 97.8% at the 100 ppm level. The limit of detection for these stevia sweeteners in foods was 5 ppm.

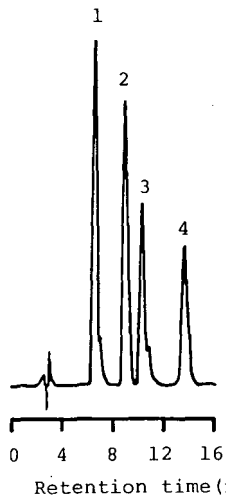

Fig. 2. Chromatogram of standard stevioside sweeteners (3 μg each). Peaks: 1 = dulcoside A; 2 = stevioside; 3 = rebaudioside C; 4 = rebaudioside A. Conditions: column, LiChrosorb NH_2 (5 μm) (250 × 4 mm I.D.); mobile phase, acetonitrile–water(80:20); flow-rate, 0.8 ml/min; detection, UV (210 nm); column temperature, 50°C.

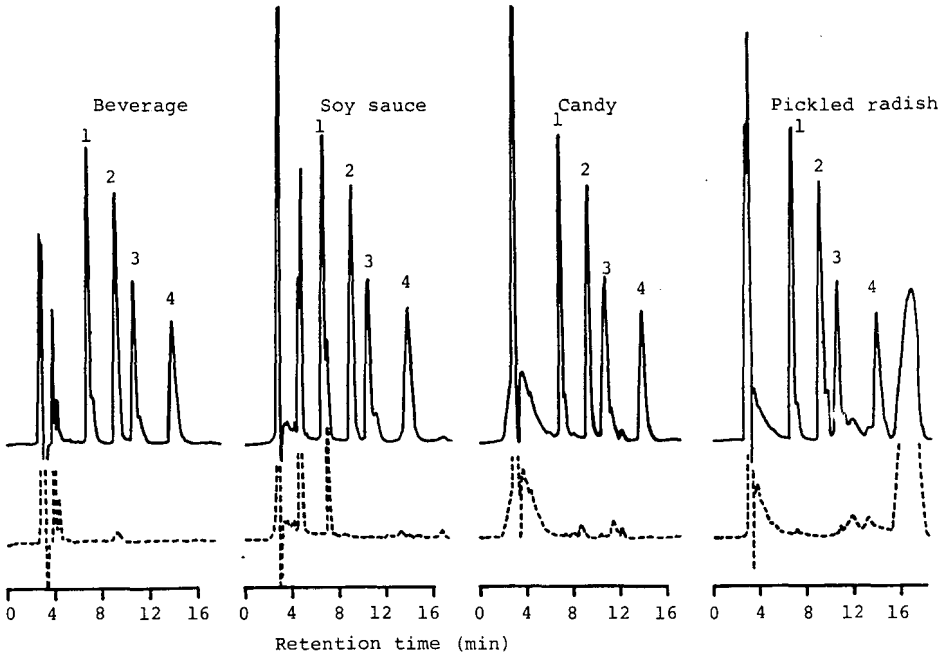

Fig. 3. Chromatograms of food samples without stevia sweeteners (broken curves), and the same samples after addition of 100 μg/g of stevia sweeteners (solid curves). Peak numbers as in Fig. 2.

In conclusion, the simultaneous determination of stevioside, rebaudioside A and C and dulcoside A in foods by HPLC was satisfactorily achieved. These stevia sweeteners were effectively separated, identified and quantitated on LiChrosorb NH_2 with detection at 210 nm. The proposed method allows the simple, rapid and accurate determination of the four stevia glycosides in foods, and is suitable for routine analysis.

TABLE I

RECOVERIES OF STEVIA SWEETENERS ADDED TO BEVERAGE, SOY SAUCE, CANDY AND PICKLED RADISH

Sample	*Added (μg/g)*	*Recovery[a] (%)*			
		Dulcoside A	*Stevioside*	*Rebaudioside C*	*Rebaudioside A*
Beverage	20	92.5 ± 1.1	100.2 ± 3.1	99.4 ± 3.3	100.0 ± 1.4
	100	94.1 ± 0.8	97.5 ± 0.9	95.6 ± 2.1	96.3 ± 1.1
Soy sauce	20	91.1 ± 1.9	91.5 ± 2.8	87.9 ± 1.8	90.6 ± 1.4
	100	95.9 ± 0.7	96.7 ± 0.8	96.8 ± 1.9	97.3 ± 1.3
Candy	20	90.8 ± 2.2	99.2 ± 3.6	98.6 ± 1.5	96.8 ± 2.7
	100	94.2 ± 1.3	95.9 ± 0.8	95.6 ± 1.5	96.6 ± 1.0
Pickled radish	20	94.3 ± 2.4	94.5 ± 2.3	91.1 ± 2.2	94.1 ± 3.1
	100	94.6 ± 1.8	93.5 ± 1.4	97.8 ± 2.6	93.2 ± 1.5

[a] Average ± standard deviation of four determinations.

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CHROM. 21 498

Note

Separation by high-performance liquid chromatography of oligosaccharides obtained after mild acid hydrolysis of *Klebsiella pneumoniae* O_1K_2 (NCTC 5055) lipopolysaccharides

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Cell wall lipopolysaccharides (LPS) are characteristic components of all Gramnegative bacteria[1–4] and confer antigenic properties to the cell. The complete LPS are constituted of three distinct structural parts: the O-specific side-chain, the core oligosaccharide and the lipid A.

The lipopolysaccharides can easily be cleaved by mild acid hydrolysis with 1% acetic acid[5]. The free water-insoluble lipid A precipitates and can be separated by centrifugation. The acetic acid supernatant contains the water-soluble degraded polysaccharides. Gel permeation chromatography is the most commonly used method to fractionate these components into polysaccharides (O-specific side-chains) and oligosaccharides (core oligosaccharides)[6,7]. In this paper we describe a high-performance liquid chromatographic (HPLC) method for the separation of the water-soluble fraction components obtained by the mild acetic acid hydrolysis of *Klebsiella pneumoniae* O_1K_2 lipopolysaccharides.

EXPERIMENTAL

Materials

Bacterial culture of *Klebsiella pneumoniae* O_1K_2 (NCTC 5055) was carried out as described previously[8]. The lipopolysaccharides (LPS) were extracted by the hot phenol–water procedure[9] and purified by ultracentrifugation.

Mild acid degradation of lipopolysaccharides and fractionation of oligosaccharides

The lipopolysaccharides (334 mg) were dissolved in 1% acetic acid at a concentration of 10 mg/ml and heated at 100°C for 90 min. The precipitated lipid A was separated by centrifugation. The supernatant was freeze-dried and fractionated by HPLC on Magnum 9 SAX (10 μm) anion-exchange column (50 cm × 9.4 mm I.D.)

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(Whatman, Clifton, NJ, U.S.A.) with a Spectra-Physics Model 8700 liquid chromatograph equipped with a Model 8400 variable-wavelength detector and connected to a Model 4100 computing integrator.

The lyophilizate of the supernatant (198 mg) obtained by the 1% acetic acid hydrolysis of LPS was dissolved in 4 ml of water. The solution was filtered on a 0.45-μm Millipore filter and 1-ml fractions were injected on to the HPLC column using a water–0.5 *M* KH_2PO_4 gradient as follows: elution with distilled water for 30 min, then a linear gradient to 2.5% of 0.5 *M* KH_2PO_4 for 10 min; isocratic gradient with the latter buffer for 30 min; linear gradient to 5% of 0.5 *M* KH_2PO_4 for 10 min; isocratic gradient with the same buffer for 30 min and then a linear gradient to 100% 0.5 *M* KH_2PO_4 was applied for 50 min. The flow-rate was 2 ml/min and 2-ml fractions were collected. Each fraction was assayed for neutral sugar by the phenol–sulphuric acid method[10]. The sugar-containing fractions were pooled, desalted on a Sephadex G-10 column (100 cm × 1.5 cm I.D.) and freeze-dried.

Determination of monosaccharide composition

The molar composition of monosaccharides was determined by gas chromatography of trifluoroacetylated methyl glycosides according to Zanetta *et al.*[11] or of trimethylsilylated methyl glycosides according to Kamerling *et al.*[12] as modified by Montreuil *et al.*[13].

Chemical methods

The 2-keto-3-deoxy-D-mannooctonic acid (KDO) content was determined by the thiobarbituric acid method according to Karkhanis *et al.*[14]. The total phosphorus content was determined by the method described by Lowry *et al.*[15] with Na_2HPO_4 as a standard. The total protein content was determined by the Lowry method[16]. The total neutral carbohydrate content was determined by the orcinol–sulphuric acid method as described by Rimington[17].

RESULTS

Isolation of lipopolysaccharides

The lipopolysaccharides were extracted from *Klebsiella pneumoniae* O_1K_2 by the hot phenol–water procedure[9] and purified by repeated ultracentrifugation. Nucleic acids were removed by precipitation with cetyltrimethylammonium bromide (Cetavlon)[18]. After lyophilization, the lipopolysaccharides were obtained with a yield of 2% based on the dry bacterial weight.

As shown in Table I, the lipopolysaccharide fraction contained neutral sugars (62.6%), glucosamine (1.9%), KDO (5.6%), phosphate (3.6%) and fatty acids (7.7%). The LPS preparation was found to be free from proteins ($<0.5\%$) as estimated by the method of Lowry *et al.*[16].

Mild acid hydrolysis of lipopolysaccharides

The lipopolysaccharides were subjected to weak acid hydrolysis (1% acetic acid, 100°C, 1.5 h). The insoluble lipid A was separated from the water-soluble fraction by centrifugation. Starting from 334 mg of lipopolysaccharides, 85.7 mg (25.4%) of lipid A and 199 mg (59.5%) of water soluble materials were obtained. As shown in Table I,

TABLE I

COMPOSITION OF NATIVE LIPOPOLYSACCHARIDE, THE WATER-SOLUBLE FRACTION AND THE LIPID A FRACTION

Gal = galactose; Glc = glucose; Man = mannose; Hep = heptose; KDO = 2-keto-3-deoxy-D-manno-octonic acid; GlcN = glucosamine. –, Not detected.

Constituent[a]	*Concentration (%)*		
	Native LPS	*Water-soluble fraction*	*Lipid A fraction*
Gal	34.5	51.4	–
Glc	6.1	7.9	–
Man	Traces	Traces	–
Hep	7.2	8.9	–
KDO	5.6	1.1	–
GlcN	1.9	0.1	16.1
Phosphate	3.6	3.7	5.4
Fatty acids	7.7	–	52
Proteins	<0.5	–	–

glucosamine and fatty acids were absent from the water-soluble fraction but were present in the lipid A fraction. The KDO content decreased after acid hydrolysis. Phosphate was detected in both water-soluble and lipid A fractions. The presence of this compound with neutral sugars in the water-soluble fraction led us to fractionate negatively charged compounds by HPLC with an anion-exchange column.

Fractionation of oligosaccharides by HPLC

As shown in Fig. 1, six fractions (S_1–S_6) were obtained. The fractionation of 198 mg of the water-soluble materials obtained from the mild acid hydrolysis of LPS gave 55.2 mg of fraction S_1, which was not retained by the column and was eluted at the void volume with a retention time of 9 min; 98.9 mg of retained fractions (S_2–S_6) were eluted by the water–0.5 *M* KH_2PO_4 gradient. The overall chromatographic yield was 73.6%.

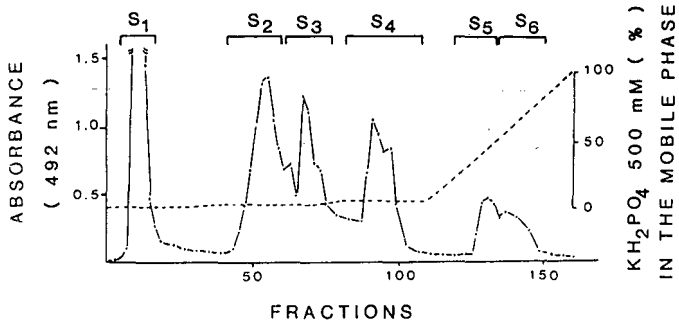

Fig. 1. HPLC on a Magnum 9 SAX anion-exchange column of the acetic acid-soluble fraction obtained by mild acid hydrolysis of *Klebsiella pneumoniae* O_1K_2 lipopolysaccharides.

TABLE II

COMPOSITION AND WEIGHTS OF FRACTIONS OBTAINED BY PREPARATIVE HPLC OF THE WATER-SOLUBLE FRACTION OBTAINED BY MILD ACID HYDROLYSIS OF *KLEBSIELLA PNEUMONIAE* O_1K_2 LIPOPOLYSACCHARIDES

–, Not detected.

Fraction	*Weight (mg)*	*Yield (%)*	*Retention time (min)*	*Phosphate (%)*	*Molar ratios*[a]			
					Gal	*Man*	*Glc*	*Hep*[b]
Water-soluble fraction	198	73.6	–	3.7	6.52	Trace	1	0.96
S_1	55.2	27.8	9	–	M[c]	–	–	–
S_2	26.8	13.4	55	1.7	14.10	–	1	0.67
S_3	19.3	9.6	68	2.2	5.28	–	1	1.00
S_4	28.0	14.1	89	2.9	0.34	–	1	1.09
S_5	7.4	3.6	130	4.3	2.98	–	1	0.80
S_6	9.2	4.6	140	4.5	0.78	0.16	1	0.69

[a] On the basis of one glucose residue.
[b] Hep = heptose.
[c] M = Main component.

Monosaccharide compositions

The monosaccharide compositions of the fractions obtained by HPLC are given in Table II. Fraction S_1 contained only galactose residues whereas all the other fractions contained variable amounts of galactose and approximately the same heptose:glucose ratios (0.7:1 to 1:1). Phosphate was absent from S_1 but was present in all of the other fractions. Fraction S_6 also contained mannose, which was detected in a very small amount in native LPS.

DISCUSSION

The oligosaccharides released from LPS by mild acid hydrolysis have usually been fractionated by gel permeation chromatography. However, with this method we did not obtain a good separation of oligosaccharides released from the *Klebsiella pneumoniae* O_1K_2 LPS. By using HPLC with an anion-exchange column we obtained six distinct fractions, S_1–S_6.

Fraction S_1 contained only neutral sugar (galactose) and was not retained by the anion-exchange column. In comparison with the structure described by Kenne and Lindberg[19], this fraction corresponds to the O-antigen polysaccharide of *Klebsiella* serogroup O_1 lipopolysaccharide.

The other fractions (S_2–S_6) were retained on the column by their negative charges. Their fixed glucose and heptose composition for variable amounts of galactose can be explained by the length heterogeneity of the O-antigen[20–22] and by the incomplete cleavage of this O-antigen from the core oligosaccharide. Structural studies are currently being undertaken that should shed light on the nature of the LPS architecture.

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Note

Application of an immunoaffinity column sample clean-up to the determination of aflatoxin M_1 in cheese

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Aflatoxin M_1 the hydroxylated metabolite of aflatoxin B_1 is found in milk and dairy products of animals that have consumed contaminated feed. Although Regulations control aflatoxin B_1 levels in the feeding stuffs there is still a need to monitor milk to check the effectiveness of these controls particularly concerning imported products. When aflatoxin M_1 contaminated milk is used to make other dairy products, the toxin is not destroyed and being associated with the casein fraction it is found for example at a 3–4 fold concentrated level in cheese[1]. Although recent analysis[2,3] of aflatoxin M_1 levels in European cheeses has shown a low incidence as well as generally low levels of contamination, it is nevertheless prudent from time-to-time for Regulatory Authorities to carry out monitoring of cheeses for aflatoxin M_1 as part of on-going food safety surveillance programs.

Analysis of cheese for aflatoxin M_1 involves solvent extraction, filtration and then a number of column or cartridge chromatographic clean-up stages prior to a high-performance liquid chromatographic (HPLC) or thin-layer chromatographic (TLC) determination[4,5]. Although these established methods are effective showing both good recoveries and precision, with detection limits of the order of 0.01 μg/kg, the sample preparation stage is time-consuming and may depend on some prior experience of the analysis to obtain consistent results. As part of our evaluation of new techniques for sample preparation we have recently reported the use of immunoaffinity columns for sample clean-up in the analysis of aflatoxin M_1 in liquid and powdered milk[6]. This approach offered rapid sample throughput, good recovery and with HPLC as the determinative step a sample extract that was significantly cleaner than could be obtained with more conventional approaches. In this paper we report an extension of the use of immunoaffinity columns to the analysis of aflatoxin M_1 in cheese where similiar advantages were obtained and with an even more significant time-saving.

EXPERIMENTAL

Materials

"Aflatoxin M_1 Easi-extract" immonoaffinity columns type TD 120 were obtained from Oxoid (Basingstoke, U.K.). Acetonitrile and methanol were purchased

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from Rathburn (Walkerburn, U.K.). All water used was deionised distilled and, for HPLC, passed through a Milli-Q (Millipore, London, U.K.) purification system. Aflatoxin M_1 was from Sigma (Poole, U.K.) and buffer salts from BDH (Poole, U.K.).

Sample preparation

A sample of cheese (20 g) chopped into small pieces was weighed accurately into a 250-ml beaker to which was added chloroform (75 ml), saturated sodium chloride solution (1 ml) and Celite 545 (5 g). The mixture was homogenized with an IKA-Ultra-Turrax blender at high speed for 2–3 min to produce a slurry, which was subsequently filtered through a Whatman 113V filter paper into a round bottomed flask. The beaker was washed with chloroform (50 ml) and the washings filtered, finally squeezing the filter paper against the funnel to obtain maximum yield of extract. The chloroform extract was evaporated to dryness under vacuum at 30°C and to the residue was added methanol (1 ml), water (30 ml) and hexane (50 ml). After gentle swirling, the mixture was transferred to a separating funnel with washing (2 × 10 ml of water) and shaken for 10–15 s. The lower layer was collected and used in the affinity column stage of the sample clean-up.

Immunoaffinity column clean-up

The immunoaffinity column was washed with distilled water (10 ml) using a syringe at a flow-rate of about 2–3 ml/min. This was followed by the sample extract prepared as above (50 ml) and a further washing of distilled water (10 ml). The aflatoxin M_1 was then slowly eluted from the column with acetonitrile (2 ml) into a glass vial. The solvent was evaporated to near dryness by blowing-down with a gentle nitrogen stream, and was then redissolved in acetonitrile–water (1:1) to give a final volume of 250 μl. The extract was finally filtered through a 0.2-μm membrane before HPLC analysis.

Chromatography

The HPLC system consisted of a Varian 5500 ternary pump, a Rheodyne 7125 injector, and a Perkin Elmer LS-4 fluorescence detector set at 364 nm excitation and 434 nm emmission. The detector was linked to a Trivector Trilab 2000 data station. A Spherisorb ODS 1 column (5 μm particle size, 250 × 4.9 mm I.D.) was employed, thermostatted at 35°C with a mobile phase of water–acetonitrile–methanol (60:10:30) at 0.7 ml/min. Sample extract (50 μl) was injected using a fixed loop.

RESULTS AND DISCUSSION

The immunoaffinity column used in the work in this paper was derived from monoclonal antibodies and was originally developed for reactivity against the B and G aflatoxins. The column was however found to have a good retention of aflatoxin M_1, and the manufacturer's data for cross-reactivity were B_1, 100%; B_2, 75%; M_1, 50%; and G_1, 38%.

Preliminary work on the extraction of the cheese samples was carried out using the AOAC extraction procedure for aflatoxin M_1 in dairy products (Sect. 26.090)[7] based on a solvent mixture of acetone–water. Without further clean-up the crude

sample extract was directly loaded onto the immunoaffinity column. Although this simple approach was effective for detecting the presence of aflatoxin M_1, the HPLC chromatograms showed more extraneous peaks than might reasonably have been expected and aflatoxin recoveries were highly variable. This suggested that in attempting to analyse very crude sample extracts containing a high solids content, some entrained or bound aflatoxin was being carried through the column, thus not being available for antibody binding. This would however only explain the variable recoveries and not the chromatographic interferences.

The approach finally adopted was based on the AOAC First Action Method (Sect 26.095)[7] using a chloroform extraction and some liquid–liquid partitioning for a preliminary sample clean-up overcame both these initial difficulties. The method was found to take slightly longer than that for the analysis of aflatoxin M_1 in milk, but nevertheless represented a considerable time saving over the conventional approach to aflatoxin M_1 analysis in cheese. The recovery of the method for five replicate samples of one type of cheese spiked at 0.1 μg/kg averaged 75% with a standard deviation of 10%. Analyses of eight other cheese types, which varied considerably both in fat and water content, gave recoveries after spiking at 0.1 μg/kg in the range of 66 to 80%, but with one sample giving a recovery of only 55%. Calibration curves over the range 0 to 8.0 μg/kg were linear with a correlation coefficient of 0.9989. The limit of detection of the procedure was demonstrated to be 0.005 μg/kg at a signal-to-noise ratio of 5:1, by spiking a series of cheese samples with aflatoxin M_1 in the range 0.005 to 0.05 μg/kg.

In view of the large compositional differences in cheese types and the potential presence of interferences that might arise from artifically moulded cheeses, the performance of the columns was evaluated with a number of different varieties of cheeses from different countries of origin. In all cases the presence of aflatoxin M_1 could be detected in the cheeses and the naturally occurring levels which are shown in Table I were found to range from < 0.01 to 0.08 μg/kg. The HPLC chromatograms were in all cases essentially clean and no significant differences in chromatography were detected between the different cheese types. A typical chromatogram for a naturally

TABLE I

RECOVERIES AND RESULTS OF SMALL SURVEY OF AFLATOXIN M_1 IN RETAIL SAMPLES OF CHEESE

Cheese type	*Country of origin*	*Aflatoxin M_1 (μg/kg)*	*Recovery (%) (at 0.1 μg/kg spike)*
Danish Blue	Denmark	0.03	75
Tilister	F.R.G.	0.08	72
Emmenthal	Switzerland	<0.01	55
Gouda	The Netherlands	0.05	80
El Mancho	Spain	<0.01	–
Gorgonzola	Italy	0.04	70
Gjetust	Norway	0.04	80
Jarlesberg	Norway	<0.01	80
Raclett	Switzerland	0.02	66
Red Leicester	U.K.	0.01	–
Cheddar	U.K.	0.04	–

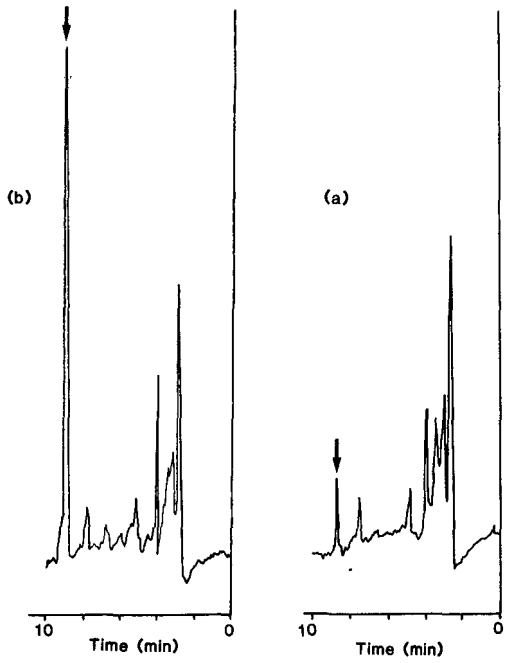

Fig. 1. HPLC of samples of cheese naturally contaminated with 0.02 μg/kg aflatoxin M_1 (a) and spiked with an additional 0.1 μg/kg (b). Detection: fluorescence at 364 nm excitation and 434 emmission. Column: Spherisorb ODS 1 with a mobile phase of water–acetonitrile–methanol operated at 0.7 ml/min.

contaminated cheese is shown in Fig. 1 illustrating the presence of low levels of aflatoxin M_1 and the effect of spiking with an additional 0.1 μg/kg. Confirmation of the presence of aflatoxin M_1 was carried out by reacting half the final extract from the cheese with trifluoracetic acid leading to the loss of the M_1 peak in the chromatogram at a retention time of 8.8 min and the appearance of a new derivative peak at a retention time of 6.4 min.

CONCLUSION

An extension of use of affinity columns for aflatoxin M_1 for the analysis of cheese has been demonstrated as being effective with considerable time-saving over a more lengthy sample preparation. Further work is required to evaluate the potential application of these columns to other sample matrices such as eggs and animal tissue, and to assessing their potential for cross-reactivity with other aflatoxin metabolites with close structural similarities.

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Letter to the Editor

Comments on a paper describing elution order shifts of halogenated compounds

Sir,

We noticed the shifts in the order of elution of the 1,1,1,2-tetrabromoethane and the trichloromethane graphed in Figs. 6 and 7 in the article by Castello *et al.*[1], entitled "Gas Chromatographic Separation of Halogenated Compounds on Non-Polar and Polar Wide Bore Capillary Columns". We wondered how the elution order of chemically similar compounds could be affected so markedly by temperature.

We were disturbed by the apparent similarity of retention of 1,1,1,2-tetrabromoethane and trichloromethane. In researching their boiling points further, we found that the listed boiling point of the 1,1,1,2-tetrabromoethane in Table II is its reduced pressure boiling point, although this fact is not stated in the table. According to the data on page C-265 in the 66th edition of the *CRC Handbook of Chemistry and Physics*, the boiling point of 1,1,1,2-tetrabromoethane at 18 mmHg is 112°C, the value listed in Table II. By analogy with the 1,1,2,2-isomer (atmospheric pressure boiling point 243.5°C as certified by the same reference, which also listed a boiling point of 114°C at 10 mmHg), the atmospheric pressure boiling point of 1,1,1,2-tetrabromoethane is expected to be above 200°C, and it should not elute in the vicinity of chloroform. This is despite recognizing that the Supelcowax-10 column is not a boiling point column.

We noticed that Table II indicates that 1,1,2,2-tetrabromoethane was apparently not eluted within the chromatographic timeframe on this column (see Table II). We would expect the chromatographic behavior of the two tetrabromoethane isomers to be similar on this column. Therefore, we believe that compound No. 1 is not eluting from their column, and the peak they are ascribing to it may be an impurity, chromatographic decomposition product, or altogether unrelated compound.

This observation bears on the understanding of the underlying mechanism for the elution order shift described in Figs. 6 and 7. The peak ascribed to the tetrabromoethane may in fact have totally different functionality, making its elution shift with respect to the chloroform perfectly reasonable.

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INFORMATION FOR AUTHORS

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