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Electrodes of Conductive Metal Oxides: Part A

edited by SERGIO TRASATTI, *Laboratory of Electrochemistry, University of Milan, Italy*

STUDIES IN PHYSICAL AND THEORETICAL CHEMISTRY 11

The discovery by Beer in the second half of the sixties that the performances of anodes made of thermally prepared noble metal oxides were better than those of noble metals provoked something of a technological revolution in the large electrolytic industry. Since then an ever increasing number of fundamental studies have been published but the large amount of data has, until now, not been adequately assimilated.

This two-part work provides a general unifying introduction plus a state-of-the-art review of the physicochemical properties and electrochemical behaviour of conductive oxide electrodes (DSA). The text has been divided into two volumes – Part A dealing mainly with structural and thermodynamic properties and Part B, to be published in due course, dealing with kinetic and electrocatalytic aspects. This division came about due to the large amount of material to be treated and also because, in a rapidly developing field, difficulties arise in collecting all relevant material at one given moment.

The editor approaches the subject from a multidisciplinary angle, for example, the electrochemical behaviour of oxide electrodes is presented and discussed in the context of a variety of physicochemical properties – electronic structure, nonstoichiometry, crystal structure, surface structure, morphology and adsorption properties. For the first time the different groups of oxides are treated together in order to place emphasis on their similarities and differences.

This major reference work is mainly directed to electrochemists and those working or catalysis. It will also be useful to those in the fields of materials science, physical chemistry, surface and colloid chemistry and in areas where oxide surfaces may play a major role as in chromatography and photochemistry.

CONTENTS: Chapters. 1. Electronic Band Structure of Oxides with Metallic or Semiconducting Characteristics *(J. M. Honig)*. 2. Chemisorption and Catalysis or Metal Oxides *(A. Cimino and S. Carrà)*.. 3. Oxide Growth and Oxygen Evolution on Noble Metals *(L. D. Burke)*. 4. Electrochemistry of Lead Dioxide *(J. P. Pohl and H. Rickert)*. 5. Properties of Spinel-Type Oxide Electrodes *(M. R. Tarasevich and B. N. Efremov)*. 6. Physicochemica and Electrochemical Properties o Perovskite Oxides *(H. Tamura, Y. Yoneyama and Y. Matsumoto)*. 7. Properties of Conductive Transition Metal Oxides with Rutile-Type Structure *(S. Trasatti and G. Lodi)*.

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COMPUTER SIMULATION OF THE KINETICS OF ION EXCHANGE ACCOMPANIED BY COMPLEX FORMATION IN IONITES*

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SUMMARY

The kinetics of ion exchange accompanied by the formation of weakly dissociating compounds between counter ions and fixed ionite exchange groups has been studied by computer simulation of ion-exchange processes at different ratios of the individual diffusion coefficients and in the case of convex and concave exchange isotherms. The results show that exchange in ionites filled initially with less mobile ions is faster in the case of a convex than in the case of a concave isotherm. The distribution of counter ions and co-ions with particle radius is dependent on the relation between the equilibrium and kinetic parameters. The simulated ion distribution curves obtained for the exchange kinetics in carboxyl and vinylpyridinecarboxyl ionites are similar to the experimental ones.

INTRODUCTION

The formation of weakly dissociating compounds in an ionite or a solution phase is, in a number of cases, a necessary condition for effective chromatographic separation and controls both the equilibrium and kinetic separation parameters. The mechanism of the exchange when accompanied by the formation of a weakly dissociating compound of a counter ion with a fixed ionite exchange group has been discussed qualitatively by Helfferich[1]. An approximate solution of the exchange kinetics has been derived[2,3] for the complexing ion in the case of a rectangular isotherm, and equations together with a numerical solution have been presented[4] for an arbitrarily shaped isotherm with certain combinations of the individual ion diffusion coefficients.

The present paper discusses the kinetics of ion exchange in the presence of ion association in ionites for convex and concave isotherms, and considers various relations between the individual diffusion coefficients D_B, D_A, D_Y for the counter ions B^+, A^+ and co-ion Y^-.

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* Presented at the *6th International Symposium "Advances and Application of Chromatography in Industry", Bratislava, September 16–19, 1980.*

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THEORETICAL

The expression describing the kinetics of such a process[2,3]

$$\partial\,(a_i + c_i)/\partial\, t = -\mathrm{div}\,\bar{J}_i \tag{1}$$

where i = B, A or Y, the law of mass balance

$$\sum_{i=\mathrm{B,A,Y}} z_i \bar{J}_i = 0 \tag{2}$$

in the absence of an electric current, where z_i are the charges, and the condition for electroneutrality

$$z_\mathrm{B}\, c_\mathrm{B} + z_\mathrm{A}\, c_\mathrm{A} + z_\mathrm{Y}\, c_\mathrm{Y} = a_0 - z_\mathrm{B}\, a_\mathrm{B} - z_\mathrm{A}\, a_\mathrm{A} \tag{3}$$

generate a condition for equilibrium between the bound and free ions in the ionite which for singly charged ions is

$$K_\mathrm{RB} = c_\mathrm{B}\,(a_0 - a_\mathrm{A} - a_\mathrm{B})/a_\mathrm{B};\; K_\mathrm{RA} = c_\mathrm{A}\,(a_0 - a_\mathrm{A} - a_\mathrm{B})/a_\mathrm{A} \tag{4}$$

where c_B, c_A and a_B, a_A are the concentrations of the free ions and ion pairs formed by B^+, A^+ and fixed ionite exchange groups, respectively, a_0 is the concentration of the fixed exchange groups and K_RB, K_RA are the dissociation constants. The ion fluxes, J_i, are defined by Nernst–Planck relations

$$J_i = -D_i\left(\nabla c_i + \frac{F}{RT}\cdot z_i\, c_i\, \nabla\varphi\right) \tag{5}$$

where φ is the electric potential, F is the Faraday constant, R is the gas constant and T is the absolute temperature.

The system of eqns. 1–5 for the exchange kinetics of singly charged ions is computed at the initial and boundary conditions corresponding to the exchange between the ionite and continually renewed solution, when initially the ionite particle is filled with A^+

$$c_\mathrm{B}\,(r, 0) = 0;\; c_\mathrm{Y}\,(r, 0) = 0;\; 0 \leq r \leq r_0 \tag{6}$$

$$c_\mathrm{B}\,(r_0, t) = c_0;\; c_\mathrm{A}\,(r_0, t) = 0;\; \left(r^2 \cdot \frac{\partial c_i}{\partial r}\right)_{r=0} = 0,\; t > 0 \tag{7}$$

where r is the radial space coordinate, r_0 is the particle radius, t is the time and c_0 is the concentration of the surrounding solution.

In ref. 4 a numerical solution has been obtained for the ratio between the individual counter ion diffusion coefficients, $D_\mathrm{A}/D_\mathrm{B} = 0.1$, and $K'_\mathrm{RB} = 0.001$, $K'_\mathrm{RA} = 0.1$ where $K'_i = K/c_0$. This solution demonstrates that with time the well-defined boundary of the exchanging ion, $\mathrm{B}^+/\mathrm{A}^+$, shifts toward the particle centre, and ion

transfer toward and away from the exchange boundary involves the co-ion Y^- moving from the solution into the ionite particle.

RESULTS AND DISCUSSION

Numerical solutions of the four combinations of the individual diffusion coefficients D_B, D_A and dissociation constants K_{RB}, K_{RA} are shown in Figs. 1–5. To compare the curves of ion distribution with particle radius, times were adopted cor-

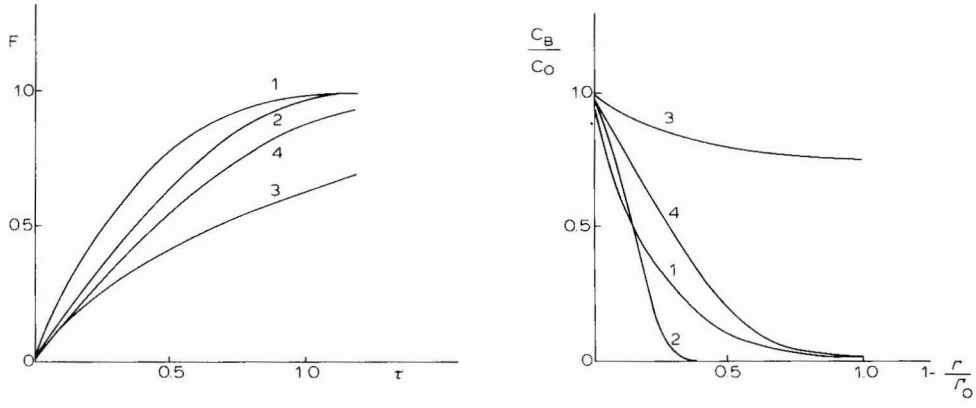

Fig. 1. Dependence of fractional conversion on the dimensionless time parameter $\tau = \sqrt{\bar{D}\, t\, c_0/(r_0^2\, a_0)}$, where $\bar{D}$ is the unit used for measuring D_i. Curves: 1, 2, $K'_{RB} = 0.001$, $K'_{RA} = 0.005$; 3, 4, $K'_{RB} = 0.005$, $K'_{RA} = 0.001$. $D_B = 1$, $D_A = 0.1$ for curves 1, 3; $D_B = 0.1$, $D_A = 1$ for curves 2, 4.

Fig. 2. Concentration distribution of the free B^+ for the time corresponding to $F = 0.58$. Curves numbered as in Fig. 1.

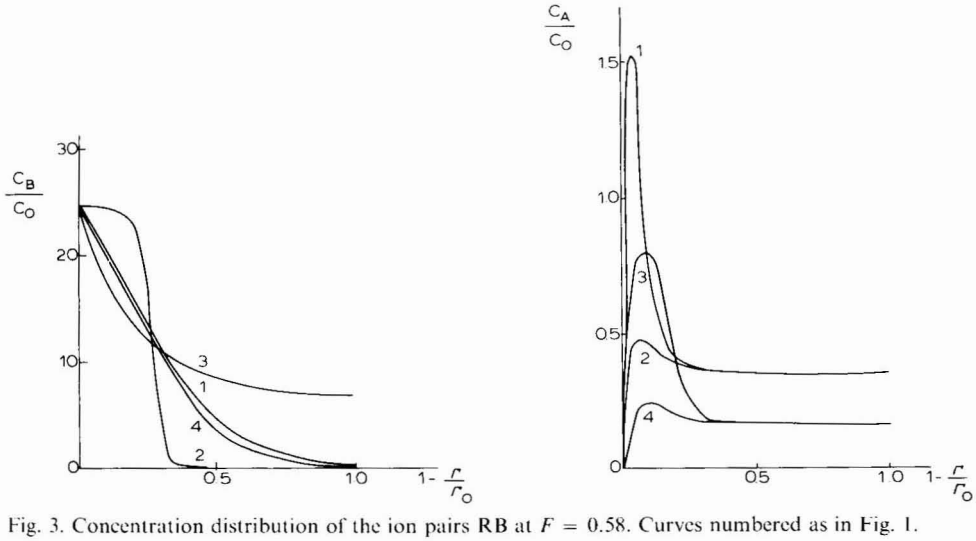

Fig. 3. Concentration distribution of the ion pairs RB at $F = 0.58$. Curves numbered as in Fig. 1.

Fig. 4. Concentration distribution of the free A^+ at $F = 0.1$. Curves numbered as in Fig. 1.

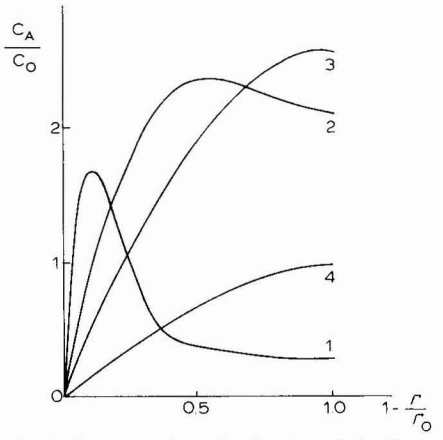

Fig. 5. Concentration distribution of the free A^+ with radius for the system with $K'_{RB} = 0.001$, $K'_{RA} = 0.005$, $D_B = 1$ and $D_A = 0.1$ at different fractional conversions F: 1, 0.2; 2, 0.5; 3, 0.7; 4, 0.9.

responding to fractional conversions $F = 0.58$ (Figs. 2 and 3) and $F = 0.1$ (Fig. 4). At these values of F the distribution curves exhibit the characteristic features of each of the four groups. Fig. 5 illustrates how the distribution of A^+ varies with radius during the process of exchange, for one of the combinations of the starting parameters.

Before discussing the results obtained it is necessary to note the difference between the kinetics of ion exchange accompanied by the formation of weakly dissociating compounds and that occurring in strong functional ionites. In the absence of ion association in the ionite phase, the rate of exchange from a continuously renewed solution is independent of its selectivity. It is governed by the value of and

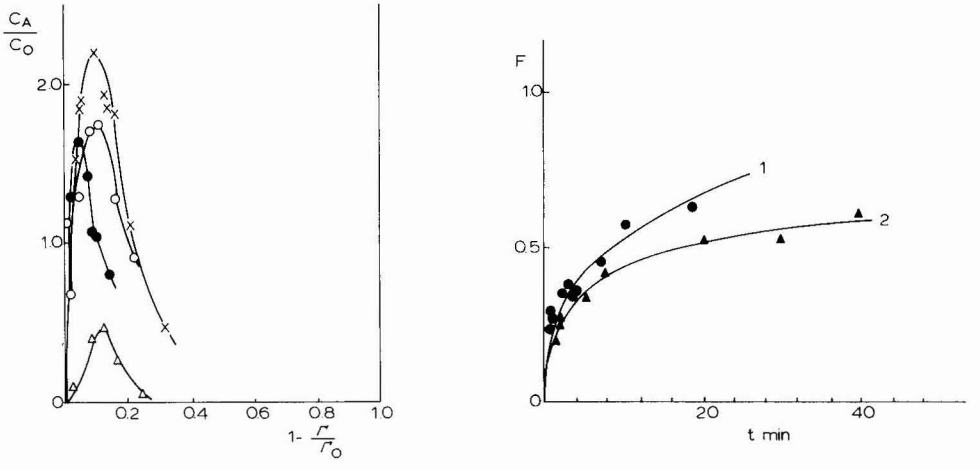

Fig. 6. Distribution of the free Ni^{2+}, Na^+ with radius as the Ni^{2+} are displaced from cationite KB-4 by H^+ (×), Na^+ from cationite KB-4 by H^+ (○, ●) and Na^+ from ampholyte VPC by Ni^{2+} (△). Fractional conversions: 0.16 (×); 0.22 (○); 0.1 (●); 0.2 (△).

Fig. 7. Exchange rates for Ni^{2+}-H^+ (convex isotherm) (1) and H^+-Ni^{2+} (concave isotherm) (2) on ampholyte VPC.

the ratio between the counter ion diffusion coefficients. The process is faster if initially the ionite contains more mobile ions[5].

In the case of exchange accompanied by ion association in the ionite phase, the exchange rate and ion distribution in the particle depend on the adopted combination of the starting equilibrium and kinetic parameters. With a convex isotherm ($K'_{RB} = 0.001$, $K'_{RA} = 0.005$) the exchange is faster than with a concave one ($K'_{RB} = 0.005$, $K'_{RA} = 0.001$) (see Fig. 1). Filling the starting ionite with less mobile ions results in a higher exchange rate in the case of the convex isotherm (Fig. 1, curves 1 and 2) and a lower one in the case of the concave isotherm (Fig. 1, curves 3 and 4).

The different effect of the D_B/D_A ratio on the exchange rate for the convex and concave isotherms can be visualized as follows. The low mobility of A^+ leads to its accumulation in the particle and lower relative free ion concentration, c_B/c_A. The relative variation of a_B as c_B/c_A varies is considerably smaller in the case of the convex exchange isotherm than with the concave isotherm, which, in the long run, may result in a variation of the exchange rate as suggested in Fig. 1.

Comparison of the F–τ curves in Fig. 1 with the kinetic dependence for isotope exchange[6] reveals an interesting feature of exchange accompanied by ion association in the ionite particle. The dependence F–τ for the convex isotherm and $D_B/D_A = 10$ is described by an equation of an in-sphere diffusion with a constant coefficient; on the other hand, for the concave isotherm and $D_B/D_A = 10$, this dependence formally corresponds to a model whose effective diffusion coefficient decreases in the course of exchange.

The effect of the ratio of the individual diffusion coefficients, and of the exchange selectivity, on the kinetic dependences in real systems has been studied for ion exchange in carboxylic (KB-4) and vinylpyridinecarboxylic (VPC) ionites. The exchange rate was measured in a thin ionite layer[3], and ion distribution was studied by using a spherical particle sector model[7]. The model comprised a cone made up of separate rings and filled with fine-ground swollen ionite. The cone, with a filter in its base, was immersed in a solution and after a period of ion exchange between the solution and the ionite in the cone it was removed from the solution, disassembled into separate layers and the concentration of the free and fixed group-bound ions in each layer was measured.

Fig. 6 compares the particle-radius distributions of the diplaced ions not bound to the fixed exchange groups, for ion exchange with different D_B/D_A ratios. The exchange isotherms in all three cases are strongly convex since the hydrogen ions in the carboxylic cationite and the nickel ions in the vinylpyridinecarboxylic ionite yield weakly dissociating compounds ($K_{RH} \approx 10^{-5}$, $K_{RNi} \approx 10^{-7}$). It is seen that as the relative mobility of the displaced ions decreases ($D_{Ni} < D_{Na} < D_H$) their maximum concentration increases, which is consistent with the results of the numerical solutions.

The experimental kinetic curves in Fig. 7 for the RH–Ni and RNi–H exchanges in VPC indicate a decrease in the exchange rate when passing from the convex to the concave isotherm and correspond to the calculated curves 2 and 3 of Fig. 1 where the exchange isotherm and ratio between the individual diffusion coefficients were used as starting parameters. It should be noted that the experimentally observed features of the kinetic exchange occur in the same sequence for ions having the same or different charges. This is not at variance with theory, since exchange isotherms of differently

charged ions and of singly charged ions, while having different analytical expressions, can be of a similar type. The value of qualitative comparison of experimental results and those from numerical solutions is thus demonstrated in all the present cases. Such a comparison is also valid for ion exchange processes in thin ionite layers and in the spherical particle sector model. The formation of the sharp boundary line of gegenions in the case of a square isotherm in ionite particles[2] and in the spherical particle sector model[7] shows that structural heterogeneity does not effect the formation of the profile a_i, c_i.

REFERENCES

1 F. Helfferich, *J. Phys. Chem.*, 69 (1965) 1178.
2 K. V. Chmutov, A. I. Kalinichev and T. D. Semenovskaya, *Dokl. Akad. Nauk SSSR*, 239, No. 3 (1978) 650.
3 A. I. Kalinichev, T. D. Semenovskaya and K. V. Chmutov, in *Sorption and Chromatography*, Nauka, Moscow, 1979, p. 144.
4 A. I. Kalinichev, E. V. Kolotinskaya and A. Ya. Pronin, *Zh. Fiz. Khim.*, 53 (1979) 506.
5 F. Helfferich, *Ionites*, Inostrannaya Literatura, Moscow, 1962, p. 254.
6 F. Helfferich, *Ionites*, Inostrannaya Literatura, Moscow, 1962, p. 244.
7 T. D. Semenovskaya, V. T. Avgyl, A. Ya. Pronin and K. V. Chmutov, *Zh. Fiz. Khim.*, 53 (1979) 1850.

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THE ROLE OF HYDROGEN BONDS IN CHROMATOGRAPHY

I. AMINO- AND HYDROXYANTHRAQUINONES

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(Received March 17th, 1981)

SUMMARY

The effect of hydrogen bonds on chromatographic separations is demonstrated on the example of amino- and hydroxyanthraquinones and their O- and N-methyl derivatives. The R_E values were used to choose a set of new stationary phases and to demonstrate the dependence between the shift in the R_F values for compounds with hydrogen bonds and the overall energy of the hydrogen bonds of all the donor and acceptor groups in the corresponding stationary phase.

INTRODUCTION

The importance of hydrogen bonds, both intermolecular and intramolecular, in chromatographic separations, especially in paper and thin-layer chromatography, is well established. Most workers, however, consider only the qualitative side of this phenomenon and point out the shift in R_F values on formation on internal hydrogen bonds compared with isomeric compounds that do not contain this type of bond[1–4].

It is obvious that the energies of these hydrogen bonds differ, depending on the nature of the atom bonded, the presence of other functional groups, steric hindrance, etc. So far, no one has attempted to quantify the energy of these bonds, although there have recently been attempts to employ various thermodynamic data in the study of processes in gas chromatography[5–7]. A single publication on paper chromatography considered the dependence of the magnitude of the R_F values of various aliphatic acids on the energy of the hydrogen bonds found from IR spectroscopy[8].

Attempts to express quantitatively the energy of hydrogen bonds using paper chromatography have already been described[10–18]. All of these works are based on data determined for the energy of the corresponding intramolecular hydrogen bonds by a physico-chemical method, and these data are compared with the magnitude of the shift in the R_F (R_M) values as a result of the formation of internal hydrogen bonds. In this connection, the "equivalent" of the hydrogen bond was introduced (R_E), defined as the energy of a hydrogen bond corresponding to a shift of 0.10 R_M. This equivalent is calculated from the equation

$$R_E = \frac{\Delta E}{\Delta R_M} \cdot 0.1$$

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where ΔE is the energy difference between isomers with and without intra-molecular hydrogen bonds and ΔR_M is the difference in the R_M values.

This paper is a continuation of an earlier study in which we attempted to use R_E values to characterize separation systems, to choose the best stationary phase and to predict the success of the separation. Simultaneously, it was possible to use completely new stationary phases in paper chromatography. This first attempt was carried out using derivatives of anthraquinones, 1- and 2-amino, 1- and 2-hydroxy, 1- and 2-dimethylamino and 1- and 2-methoxyanthraquinone.

EXPERIMENTAL

Survey of the mobile and stationary phases and sample substances used

Eight derivatives of anthraquinone, twenty-seven stationary phases and two types of mobile phase were employed.

The following model substances were used in the chromatographic separation: 1-aminoanthraquinone, 2-aminoanthraquinone, 1-N,N′-dimethylaminoanthraquinone, 2-N,N′-dimethylaminoanthraquinone, 1-hydroxyanthraquinone, 2-hydroxyanthraquinone, 1-methoxyanthraquinone and 2-methoxyanthraquinone.

For cyclohexane–pyridine (25:1) mobile phase, formamide, dimethylformamide, acetamide, acrylamide, ethylene glycol, propylene glycol, 2,3-butylene glycol, glycerine, polyglycol 300, ethanolamine, diethanolamine, monoisopropanolamine and methyl Cellosolve were used as stationary phases. For the ethanol–water mobile phase (in various ratios), methyl palmitate, butyl stearate, dimethylcyclohexyl fumarate, butylheptyl phthalate, dinonyl phthalate, didecyl phthalate, dilauryl phthalate, dioctyl adipate, dioctyl succinate, dioctyl sebacate, methyl myristate, ethyl myristate, butyl salicylate and dodecyl chloroacetate were used as stationary phases.

Chromatography

Chromatography of the anthraquinone derivatives was carried out by the descending technique using Whatman No. 1 paper. The paper was impregnated with the selected stationary phase, dissolved either in ethanol (amides, hydroxyamines, glycols) or in cyclohexane (esters) at concentrations of 20 % (w/w), except for formamide and dimethylformamide (30 %, w/w).

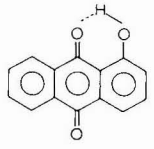
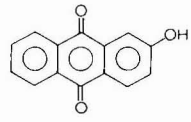
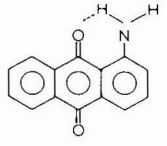
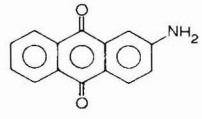
Chromatographic development was carried out at laboratory temperature (18–22°C). Separations in cyclohexane–pyridine took about 4 h and in ethanol–water 15 h or longer (overnight). Dectection was carried out under UV light without spraying.

RESULTS AND DISCUSSION

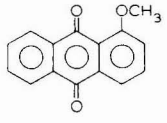
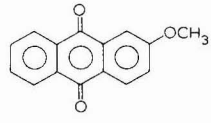
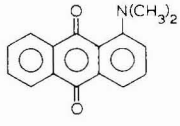
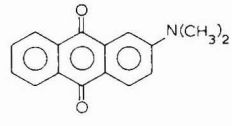
Much new information can be derived from the results given in Tables I–IV. Primarily, it is apparent that the number of possible stationary phases can be increased. For polar stationary phases, various glycols, *e.g.*, butylene glycol, propylene glycol, ethylene glycol and polyglycol 300, amides, in addition to formamide, dimethylformamide and acetamide and also acrylamide and some less volatile amines such as diethanolamine and monoisopropanolamine can be used successfully, and not only for separating anthraquinone derivatives. For non-polar stationary phases, various esters such as dinonyl phthalate, dilauryl phthalate, butylheptyl phthalate,

methyl palmitate, dimethylcyclohexyl fumarate, butyl stearate, dioctyl succinate, dioctyl adipate, dioctyl sebacate, butyl salicylate, ethyl myristate, methyl myristate, didecyl phthalate and dodecyl chloroacetate can be used.

The polar and non-polar stationary phases for separating amino- and hydroxyanthraquinones were chosen in order to demonstrate the effect of intermolecular and intramolecular hydrogen bonds on the course of chromatographic separations and in order to give a quantitative interpretation to this effect. For this purpose, only two mobile phases were chosen, one for polar stationary phases (cyclohexane–pyridine) and one for non-polar stationary phases (aqueous ethanol). It can be seen from the tables that the R_F value for 1-amino- or 1-hydroxyanthraquinone is always greater for a polar stationary phase than for 2-amino- or 2-hydroxyanthraquinone. The results are the opposite for non-polar stationary phases. The reason for this effect has already been discussed[17] and lies primarily in the formation of internal hydrogen bonds with the 1-derivatives:

To confirm this effect, the methyl derivatives were employed in addition to the amino- and hydroxyanthraquinones:

It follows from the R_F values obtained that the internal hydrogen bond O–H ··· O is as strong as if the hydrogen atom on the OH group were replaced by a methyl group and thus the R_F value is almost identical. In the O ··· H–N bond, the remaining hydrogen atom in the NH_2 group still has some energy, so that 1-amino and 2-aminoanthraquinone with a non-polar stationary phase have smaller R_F values than the corresponding methyl derivatives.

With the polar stationary phases, lower R_F values were found for 2-hydroxy-

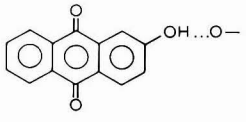
TABLE I

R_F, R_M and ΔR_M VALUES FOR AMINO- AND HYDROXYANTHRAQUINONES AND DIMETHYLAMINO- AND METHOXYANTHRAQUINONES ON A STATIONARY PHASE WITH BOTH DONOR AND ACCEPTOR GROUPS

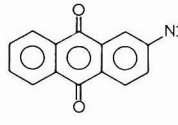
Anthraquinone	*Parameter*	*Stationary phase*				
		Dimethyl-formamide	*Formamide*	*Acetamide*	*Acrylamide*	*Ethylene glycol*
1-Amino-	R_F	0.10	0.34	0.32	0.11	0.44
	R_M	0.95	0.29	0.33	0.91	0.10
	ΔR_M	0.84	1.16	1.36	1.14	1.20
2-Amino-	R_F	0.016	0.034	0.02	0.009	0.049
	R_M	1.79	1.45	1.69	2.04	1.30
1-N,N-Dimethylamino-	R_F	0.41	0.76	0.72	0.37	0.83
	R_M	0.16	−0.50	−0.41	−0.23	−0.69
	ΔR_M	0.30	0.29	0.36	0.32	0.30
2-N,N-Dimethylamino-	R_F	0.26	0.62	0.55	0.22	0.71
	R_M	0.45	−0.21	−0.08	0.55	−0.39
1-Hydroxy-	R_F	0.57	0.84	0.83	0.59	0.89
	R_M	−0.12	−0.72	−0.70	−0.16	−0.83
	ΔR_M	1.37	1.58	1.42	1.86	1.62
2-Hydroxy-	R_F	0.056	0.12	0.16	0.02	0.14
	R_M	1.25	0.86	0.72	1.69	0.79
1-Methoxy-	R_F	0.51	0.81	0.66	0.58	0.80
	R_M	−0.02	−0.63	−0.29	−0.14	−0.60
	ΔR_M	0.04	0.10	0.06	0.085	0.03
2-Methoxy-	R_F	0.49	0.77	0.63	0.53	0.80
	R_M	0.02	−0.53	−0.23	−0.06	−0.63

anthraquinone than for 2-methoxyanthraquinone, indicating that the free OH group can form a hydrogen bond with the stationary phase.

The energies of the hydrogen bonds have been calculated to be approximately O–H · · · O 6 kcal/mol (25 kJ/mol), O · · · H–N 4.5 kcal/mol (18 kJ/mol) and H · · · H–N 3 kcal/mol (12.5 kJ/mol)[9]. Consequently, in a polar stationary phase 1-hydroxyanthraquinone has a higher R_F value than 1-aminoanthraquinone. With stationary non-polar phases, the opposite conditions prevail.



6 kcal/mol (25 kJ/mol)



4.5 kcal/mol (18.8 kJ/mol)
4.5 kcal/mol (18.8 kJ/mol)

Propylene glycol	*Glycerine*	*Polyglycol 300*	*Ethanol-amine*	*Diethanol-amine*	*Monoisopro-panolamine*	*Methyl Cellosolve*	*2,3-Butylene glycol*
0.25	0.67	0.073	0.78	0.56	0.47	–	0.11
0.48	−0.31	1.13	−0.55	−0.10	−0.055	–	0.91
1.08	1.31	0.97	1.05	1.38	1.20	–	1.04
0.027	0.001	0.008	0.24	0.052	0.056	–	0.011
1.56	1.00	2.10	0.50	1.28	1.25	–	1.95
0.67	0.85	0.62	0.90	0.88	0.84	0.68	0.46
−0.31	−0.76	−0.21	−0.95	−0.86	−0.72	−0.33	0.07
0.35	0.18	0.28	0.16	0.10	0.14	0.33	0.30
0.48	0.79	0.46	0.86	0.85	0.78	0.50	0.30
0.04	−0.58	0.07	−0.79	−0.76	−0.58	0.00	0.37
0.81	–	0.36	0.92	0.86	0.80	0.66	0.69
−0.63	–	0.25	−1.09	−0.79	−0.60	−0.29	−0.35
1.49	–	1.73	2.67	2.80	2.30	1.57	1.70
0.12	–	0.01	0.026	0.01	0.019	0.051	0.043
0.86	–	1.98	1.58	2.01	1.70	1.28	1.35
0.73	0.84	0.28	–	0.89	–	0.64	0.61
−0.43	−0.72	0.41	–	−0.91	–	−0.25	−0.19
0.10	0.03	0.00	–	0.05	–	0.07	0.05
0.68	0.83	0.28	–	0.88	–	0.60	0.58
−0.33	−0.69	0.41	–	−0.86	–	−0.18	−0.14

It again follows from these results that an important role in chromatographic separations is played by intermolecular and internal hydrogen bonds, provided that they can be formed in the substances to be separated and in the stationary phase.

It has already been demonstrated[10–18] that the effect of these hydrogen bonds can be quantified and one of the purposes of this work was to extend this information.

The chromatographic equivalent of a hydrogen bond, designated as R_E, was derived in the publications cited. This R_E value is calculated from the equation $R_E = 0.1 \cdot \Delta E/\Delta R_M$, where ΔE is the energy of the hydrogen bond and ΔR_M is difference in the R_M values between the compound with an internal hydrogen bond and an isomer without such a bond. The ΔE value, for example, corresponds to the difference between the heats of sublimation of the particular substances or other data, *e.g.*, the difference in the energies found from the IR spectra. For hydroxy- and aminoanthraquinones, the literature contains data on the heats of sublimation, from which it follows that the energy of the internal hydrogen bond of 1-aminoanthraquinone is 4.8 kcal/mol (20.2 kJ/mol) and of 1-hydroxyanthraquinone 7.8 kcal/mol (32.8 kJ/mol). If these values are taken as a basis for the calculation of R_E for the individual stationary phases, either polar or non-polar, then the order in

TABLE II

R_F, R_M AND ΔR_M VALUES FOR AMINO- AND HYDROXYANTHRAQUINONES AND DIMETHYL AMINO- AND METHOXYANTHRAQUINONES ON STATIONARY PHASES WITH ONLY ACCEPTOR GROUPS

Anthraquinone	*Parameter*	*Stationary phase**				
		Methyl palmitate (50% C_2H_5OH)	*Dimethylcyclohexyl fumarate (40% C_2H_5OH)*	*Butyl stearate (50% C_2H_5OH)*	*Butylheptyl phthalate (50% C_2H_5OH)*	*Dioctyl succinate (66% C_2H_5OH)*
1-Amino-	R_F	0.15	0.26	0.49	0.053	0.15
	R_M	0.77	0.45	0.02	1.27	0.76
	ΔR_M	0.44	0.40	0.25	0.55	0.45
2-Amino-2-	R_F	0.32	0.47	0.63	0.16	0.33
	R_M	0.33	0.05	−0.23	0.72	0.31
1-N,N-Dimethylamino-	R_F	0.20	0.30	0.60	0.061	0.28
	R_M	0.60	0.37	−0.17	1.21	0.41
	ΔR_M	0.15	0.08	0.17	0.24	0.07
2-N,N-Dimethylamino-	R_F	0.15	0.26	0.50	0.035	0.25
	R_M	0.75	0.45	0.00	1.45	0.48
1-Hydroxy-	R_F	0.064	0.13	0.13	0.023	0.088
	R_M	1.17	0.81	0.81	1.62	1.04
	ΔR_M	0.72	0.72	1.06	0.90	0.75
2-Hydroxy-	R_F	0.26	0.45	0.64	0.16	0.34
	R_M	0.45	0.087	−0.25	0.72	0.29
1-Methoxy-	R_F	0.058	0.13	0.26	0.019	0.09
	R_M	1.24	0.81	0.45	1.71	1.02
	ΔR_M	0.15	0.21	0.20	0.24	0.30
2-Methoxy-	R_F	0.079	0.20	0.36	0.032	0.16
	R_M	1.09	0.60	0.25	1.47	0.72

which the R_E values change can be found. More careful comparison of the individual R_E values has shown that their magnitude depends directly on the sum of the energies of all possible hydrogen bonds in the stationary phase, of both donor and acceptor functional groups.

The following review gives a summary of all possible intermolecular hydrogen bonds for polar and non-polar stationary phases.

If the R_E values are plotted as a function of the energy of possible hydrogen bonds, a set of points is obtained (Fig. 1), through which a straight line can be drawn,

Dioctyl adipate (50% C_2H_5OH)	*Dioctyl sebacate (66% C_2H_5OH)*	*Dodecyl chloroacetate (40% C_2H_5OH)*	*Methyl myristate (66% C_2H_5OH)*	*Ethyl myristate (66% C_2H_5OH)*	*Dinonyl phthalate (80% C_2H_5OH)*	*Didecyl phthalate (70% C_2H_5OH)*	*Dilauryl phthalate (80% C_2H_5OH)*	*Butyl salicylate (70% C_2H_5OH)*	*SiO_2 (30% toluene–dimethylformamide in C_2H_5OH)*
0.09	0.22	0.20	0.10	0.13	0.27	0.33	0.27	0.41	0.15
1.02	0.55	0.60	0.95	0.83	0.43	0.31	0.43	0.16	0.76
0.54	0.38	0.46	0.42	0.35	0.43	0.38	0.31	0.53	−0.87
0.25	0.40	0.42	0.23	0.25	0.50	0.54	0.43	0.70	0.023
0.48	0.17	0.14	0.53	0.48	0.00	−0.07	0.12	−0.37	1.63
0.15	0.31	0.18	0.12	0.16	0.32	0.37	0.25	0.35	0.059
0.76	0.35	0.66	0.86	0.72	0.33	0.23	0.48	0.27	1.23
0.22	0.08	0.13	0.13	0.11	0.15	0.22	0.10	0.02	0.21
0.095	0.27	0.14	0.093	0.13	0.25	0.26	0.21	0.34	0.09
0.98	0.43	0.79	0.99	0.83	0.48	0.45	0.58	0.29	1.02
0.032	0.13	0.068	0.051	0.066	0.068	0.17	0.10	0.095	0.38
1.48	0.82	1.16	1.28	1.18	1.12	0.69	0.95	0.97	0.21
0.85	0.70	1.06	0.75	0.63	0.76	0.79	0.78	0.95	−1.61
0.19	0.43	0.44	0.23	0.22	0.30	0.56	0.40	0.49	0.015
0.63	0.12	0.10	0.53	0.55	0.37	−0.10	0.17	0.02	1.82
0.046	0.10	0.067	0.056	0.069	0.072	0.14	0.12	0.10	0.37
1.32	0.95	1.17	1.24	1.16	1.10	0.79	0.86	0.95	0.23
0.15	0.26	0.22	0.14	0.21	0.28	0.21	0.17	0.16	−0.22
0.066	0.17	0.10	0.079	0.10	0.13	0.21	0.14	0.14	0.26
0.17	0.69	0.95	1.10	0.95	0.82	0.58	0.79	0.79	0.45

* (% C_2H_5OH) indicates the mobile phase used for the particular stationary phase. The R_F values are averages from several chromatograms.

expressed by the following equations (for the polar stationary phase):

$$R_E = -0.01\ X + 0.63$$

for the phase with both donor and acceptor groups, and

$$R_E = -0.01\ X + 1.25$$

for the phase with acceptor groups, where X is the overall energy of the hydrogen bonds to both donor and acceptor groups (Table V).

TABLE III

FOUND AND CALCULATED R_E VALUES FOR STATIONARY PHASE WITH DONOR AND ACCEPTOR GROUPS

Mobile phase: cyclohexane–pyridine (25:1). R_E (exp.) is the R_E value obtained from the experimental ΔR_M values using the relationship $R_E = 0.1\ \Delta E/\Delta R_M$. The column designated NH_2 contains R_E (exp.) values calculated from data obtained from chromatography of aminoanthraquinones, and the column designated OH contains R_E (exp.) values obtained for hydroxyanthraquinones. The R_E (calc.) values were obtained from the equation $R_E = f(x)$ found by linear regression.

Stationary phase	R_E *(exp.)*			R_E *(calc.)*	ΔR_E
	NH_2	*OH*	*Average*		
Dimethylformamide	0.57	0.57	0.57	0.58	+0.01
Formamide	0.41	0.49	0.45	0.50	+0.05
Acetamide	0.35	0.55	0.45	0.50	+0.05
Acrylamide	0.42	0.42	0.42	0.48	+0.06
Propylene glycol	0.44	0.52	0.48	0.43	−0.05
2,3-Butylene glycol	0.46	0.46	0.46	0.43	−0.03
Ethylene glycol	0.40	0.48	0.44	0.43	−0.01
Polyglycol 300	0.49	0.46	0.48	0.43	−0.05
Methyl Cellosolve	–	0.48	0.48	0.48	0.00
Glycerine	0.36	–	0.36	0.34	−0.02
Ethanolamine	0.46	0.32	0.39	0.45	+0.06
Diethanolamine	0.35	0.29	0.32	0.40	+0.08
Monoisopropanolamine	0.40	0.34	0.37	0.45	+0.08

TABLE IV

FOUND AND CALCULATED R_E VALUES FOR STATIONARY PHASE WITH ONLY ACCEPTOR GROUPS

Mobile phase: ethanol–water (in various ratios; see Table II). R_E values obtained as in Table III.

Stationary phase	R_E *(exp.)*			R_E *(calc.)*	ΔR_E
	NH_2	*OH*	*Average*		
Dinonyl phthalate	1.11	1.03	1.07	1.05	−0.02
Didecyl phthalate	1.26	1.00	1.13	1.05	−0.08
Dilauryl phthalate	1.55	1.00	1.27	1.05	−0.22
Butylheptyl phthalate	0.88	0.87	0.88	1.05	+0.17
Butyl salicylate	0.91	0.81	0.86	1.05	+0.19
Dioctyl succinate	1.07	1.04	1.06	1.05	−0.01
Dioctyl adipate	0.89	0.92	0.91	1.05	+0.14
Dioctyl sebacate	1.26	1.12	1.19	1.09	−0.14
Methyl palmitate	1.09	1.08	1.09	1.17	+0.08
Butyl stearate	1.92	0.74	1.33	1.17	−0.16
Methyl myristate	1.14	1.04	1.09	1.17	+0.04
Ethyl myristate	1.36	1.24	1.30	1.17	−0.13
Dimethylcyclohexyl fumarate	1.20	1.03	1.14	1.05	−0.09
Dodecyl chloroacetate	1.04	0.74	0.89	1.17	+0.16

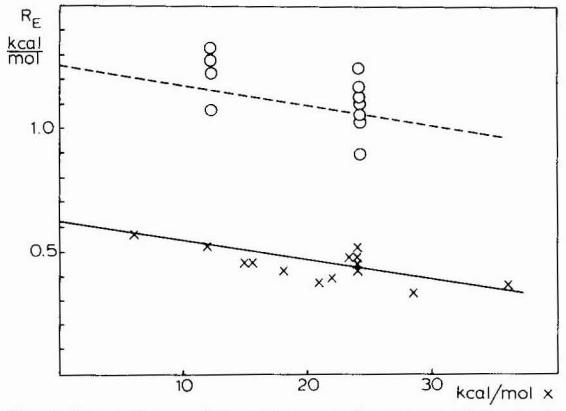


Fig. 1. Dependence of R_E values on the energy of the hydrogen bond. ×, Stationary phases with both donor and acceptor groups; ○, stationary phase with only acceptor groups (except for butyl salicylate).

A similar procedure was employed for the other stationary phases (Table VI).

It follows from this dependence that the greatest differences in the R_F values for substances with and without intramolecular hydrogen bonds will be found for stationary phases for which the sum of the energies of all possible hydrogen bonds is greatest.

For example, ΔR_M for 1-amino- and 2-aminoanthraquinone on dimethylformamide (where all possible hydrogen bonds have an energy of about 6 kcal/mol) is 0.84, whereas on diethanolamine (where all possible hydrogen bonds have an energy of 28.5 kcal/mol) ΔR_M is 1.38. Similarly, for 1- and 2-hydroxyanthraquinone ΔR_M = 1.37 and 2.80, respectively.

It therefore follows that the smaller the R_E value for the given system, the better is the separation of substances with donor functional groups. Thus the R_E values can be used for the quantitative evaluation of the separation of particular substances.

A further group of compounds studied were the esters of mono- and dibasic acids. As these stationary phases have no donor group, their separation ability is poorer.

If the linear equation given for the polar phases were valid, but with a slope of opposite sign, then it would follow for those stationary phases with an overall energy of possible hydrogen bonds of 12 kcal/mol that R_E = 0.89 and for those with an energy of 24 kcal/mol that R_E = 1.13. Consequently, this is in reasonable agreement, although it should be taken into consideration that the energy of the hydrogen bonds is also affected by the size of the alkyl groups in these stationary phases. It was calculated for the second group of stationary phases that the energy of the hydrogen bonds is 24 or 12 kcal/mol, depending on the number of carboxyl groups. It is necessary, however, to recall that the actual energy will differ as a result of the different sizes of the alkyl residues in the stationary phases, which was not taken into consideration in the rough calculation.

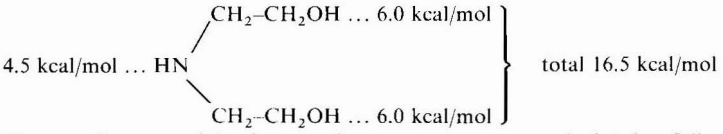
In this entire system, only one stationary phase has a donor group, butyl salicylate, which has an R_E value of 0.77.

If the R_E values are calculated and compared with the values found, then it

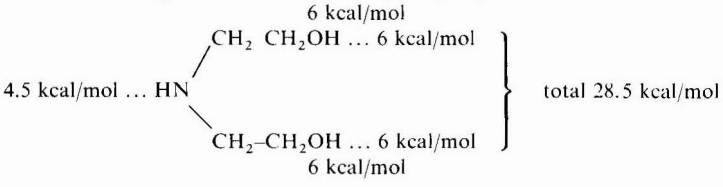
TABLE V

OVERALL ENERGIES OF POSSIBLE HYDROGEN BONDS

The energy of the donor groups, *e.g.* for diethanolamine, was calculated in the following manner:



The overall energy of the donor and acceptor groups was calculated as follows:



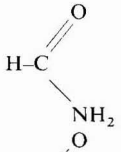
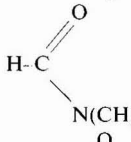
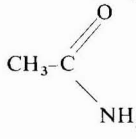
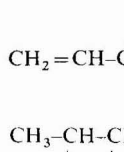
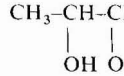
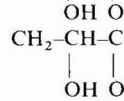
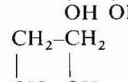
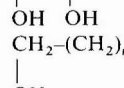
Stationary phase	*Energy of donor groups (kcal/mol)*	*Energy of donor and acceptor groups (kcal/mol)*
	9.0	15.0
	0.0	6.0
	9.0	15.0
	9.0	18.0
	12.0	24.0
	12.0	24.0
	12.0	24.0
	12.0	24.0

TABLE V *(continued)*

Stationary phase	*Energy of donor groups (kcal/mol)*	*Energy of donor and acceptor groups (kcal/mol)*
CH_2–CH_2 | | OH OCH_3	6.0	18.0
CH_2–CH–CH_2 | | | OH OH OH	18.0	36.0
CH_2–CH_2 | | NH_2 OH	15.0	21.0
CH_2–CH_2–NH–CH_2–CH_2 | | OH OH	16.5	28.5
CH_2–$CH(CH_3)$ | | NH_2 OH	15.0	21.0

follows that the differences are not large and, if they are, they can be attributed to the rough estimate of the bonding energy and the actual chromatographic evaluation, especially where the spots of the separated substances have R_F values close to the start or the front, where small differences in the R_F value have a large effect on the R_M value used for the overall calculation.

The R_L value for stationary phases with only acceptor groups is shifted by 0.63 kcal/mol (2.65 kJ/mol) compared with the R_L value for the phase with both donor and acceptor groups, *i.e.*, it is about twice as large for the same energy. This was found earlier and no satisfactory explanation has been found for whether this is accidental and the R_L value changes continuously or whether a real step change occurs on a change in the chromatographic separation conditions. Only further experiments can provide an explanation.

If, on the other hand, the R_L values are used to calculate the energy differences between individual compounds, then these values can be used to explain the chromatographic behaviour of the individual substances found experimentally. If, for example, the R_L values are used to calculate the energy differences of the hydrogen bonds between 2-hydroxyanthraquinone and 3-methoxyanthraquinone, an average difference of 7.2 kcal/mol (30 kJ/mol) is found for a polar stationary phase, corresponding to a single OH···O bond.

If the same calculations are carried out for 1-aminoanthraquinone and 1-dimethyl aminoanthraquinone, a difference of 3.6 kcal/mol (15.1 kJ/mole) is found. Consequently, the NH_2 group in 1-aminoanthraquinone is bonded through a single hydrogen bond to the oxygen of the anthraquinone skeleton and through another hydrogen bond to the stationary phase:

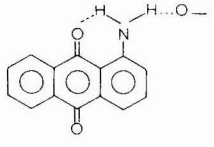
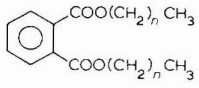
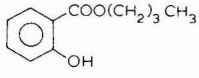
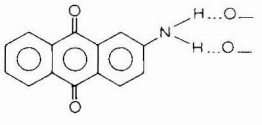


TABLE VI

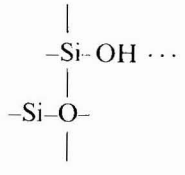
OVERALL ENERGIES OF POSSIBLE HYDROGEN BONDS FOR STATIONARY PHASES WITH ONLY ACCEPTOR GROUPS

Stationary phase	*Energy of possible hydrogen bonds (kcal/mol)*
 (dialkyl phthalate; n = 1, 2 ...)	24.0
 (butyl salicylate)	24.0
$CH_3(CH_2)_7OOCCH_2CH_2COO(CH_2)_7CH_3$ (dioctyl succinate)	24.0
$CH_3(CH_2)_7OOC(CH_2)_4COO(CH_2)_7CH_3$ (dioctyl adipate)	24.0
$CH_3(CH_2)_{14}COOCH_3$ (methyl palmitate)	12.0
$CH_3(CH_2)_{16}COO(CH_2)_3CH_3$ (butyl stearate)	12.0
$CH_3(CH_2)_{12}COOCH_3$ (methyl myristate)	12.0
$ClCH_2COO(CH_2)_{11}CH_3$ (dodecyl chloroacetate)	12.0
$CH_3OOC-C(C_6H_{11})=CH-COOCH_3$ (dimethylcyclohexyl fumarate)	24.0
$CH_3(CH_2)_7OOC(CH_2)_8COO(CH_2)_7CH_3$ (dioctyl sebacate)	24.0

Similarly, a difference of $\Delta R_M \approx 8$ kcal/mol is found for 2-aminoanthraquinone and 2-dimethylanthraquinone; this corresponds to the bonds shown below:



Apart from the chosen stationary phase, calculation of R_E was carried out for silica gel as a stationary phase with toluene–30% dimethylformamide in ethanol (10:2) as the mobile phase. Using the relationship given above, a value of R_E = 0.43 kcal/mol was calculated assuming that silica gel has both donor and acceptor groups:



A value of $R_E = 1.13$ was found (for both amino- and hydroxyanthraquinones). This behaviour corresponds to the presence of only acceptor groups with an overall energy of about 12 kcal/mol. This is also confirmed by the smaller differences in the R_F values for the 1- and 2-isomers. This phenomenon will be studied in greater detail.

The work of Chasar[4] should be mentioned here. He studied the chromatography of 1,8-dihydroxyanthraquinone and its methoxy derivatives on silica gel with a chloroform mobile phase and found the following R_F values: 1,8-dihydroxyanthraquinone, 0.57; 1-hydroxy-8-methoxyanthraquinone, 0.41; and 1,8-dimethoxyanthraquinone, 0.86. A value of $R_E = 1.12$ for a single hydrogen bond follows from the R_M values for this system. The difference (ΔR_M) for two hydrogen bonds indicates that the energy of the second bond is less than half as great. This is understandable, as the formation of the first bond decreases the electronegativity of the oxygen.

Schulz and Herrman[19] chromatographed some hydroxybenzoic acids on silica gel with dichloromethane–toluene–formic acid (5:4:1) as the mobile phase. If a hydrogen bond energy of 5 kcal/mol (21 kJ/mol)[9] is calculated for salicylic acid, it follows that $R_E = 1.16$.

Comparison of the R_E values for aminoanthraquinones[16] is also interesting, yielding $R_E = 0.65$; chromatography was carried out on a poured layer of aluminium oxide.

So far it is not possible to decide from these data whether the given relationships are generally valid for the calculation of R_E values. It is apparent that further measurements should be carried out with other types of substances in order to decide whether the relationship is generally valid or is valid in a given form only for a particular group of substances. A difficulty is encountered in that the literature generally contains thermodynamic data only for simple substances or for a single substance in a given series which is separated chromatographically.

Nonetheless, the importance of hydrogen bonds for chromatographic separations and the quantitative expression of their energies has been verified and contributes to the general clarification of the chromatographic process taking place and permits the prediction of the behaviour of substances during the separation, choice of a suitable stationary phase and, hopefully in the future, expression of the difference in the energies of intermolecular bonds.

REFERENCES

1 A. L. LeRosen, P. H. Carlton and P. B. Mosely, *Anal. Chem.*, 25 (1953) 666.
2 W. A. Schroeder, *J. Amer. Chem. Soc.*, 73 (1951) 1122.
3 J. K. Carlton, *Talanta*, 9 (1962) 39.
4 D. W. Chasar, *Chem. Ind. (London)*, (1973) 31.
5 R. V. Golovnya and T. A. Misharina, *J. Chromatogr.*, 190 (1980) 1.
6 R. V. Golovnya and T. A. Misharina, *Usp. Khim.*, 49 (1980) 171.
7 R. V. Golovnya, *Chromatographia*, 12 (1979) 533.
8 J. Sliwisk and T. Kowalski, *Microchem. J.*, 22 (1977) 226.
9 L. P. Pauling, *The Nature of the Chemical Bond*, Cornell University Press, New York, 3rd ed., 1960.
10 J. Franc, *Collect. Czech. Chem. Commun.*, 22 (1957) 995.
11 J. Franc, *Collect. Czech. Chem. Commun.*, 24 (1959) 250.
12 J. Franc, *Collect. Czech. Chem. Commun.*, 23 (1958) 1231.
13 J. Franc, *Collect. Czech. Chem. Commun.*, 24 (1959) 2102.

14 J. Franc, *Collect. Czech. Chem. Commun.*, 24 (1959) 2096.
15 J. Franc, *Collect. Czech. Chem. Commun.*, 25 (1960) 657.
16 J. Franc and M. Hájková, *J. Chromatogr.*, 16 (1964) 345.
17 J. Franc, in K. Macek and I. M. Hais, *The Stationary Phase in Paper and Thin-Layer Chromatography*, Elsevier, Amsterdam, 1965, p. 249.
18 J. Franc, *Z. Elektrochem.*, 61 (1957) 1069.
19 J. M. Schulz and K. Herrman, *J. Chromatogr.*, 195 (1980) 85.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF BIOGENIC AMINES

I. USE OF AQUEOUS ACIDIC MOBILE PHASES WITH SILICA COLUMNS

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(First received February 20th, 1981; revised manuscript received March 16th, 1981)

SUMMARY

The separation of catecholamines and indoles using aqueous acids as mobile phases with silica columns was investigated. The effects on retention of different acids, pH variation, ionic strength and organic solvents were studied. A retention model based on a quasi-reversed-phase partition at pH $\leq$ 2, supplemented by ionic interactions with silica at pH $>$ 2, is suggested. With the aqueous acid–silica system the columns showed high efficiency, high reproducibility and extraordinary long lifetimes.

INTRODUCTION

The development of high-performance liquid chromatographic (HPLC) methods for the determination of biogenic amines and their metabolites has resulted in several approaches. In addition to procedures using ion-exchange columns[1], more recent methods are usually based on reversed-phase chromatography with hydrophobic stationary phases[2–5], often in the presence of ion-pair reagents. The advantages of reversed-phase systems over ion exchangers are improved efficiency and reproducibility at higher speed. The ion-pair systems do, however, suffer from the same basic problem as the ion exchangers, i.e., the absence of reproducible retention as a function of load. In addition, the long equilibration times and high temperature sensitivity limit the use of ion-pair systems. Without trying to reduce the great potential of reversed-phase columns, the limitations to their use should also be acknowledged. No matter how well a column is kept and protected, using pre-columns, etc., a slow but continuous 'erosion' of the bonded stationary phase takes place. The gradual appearance of more active polar sites results in peak broadening, loss of resolution and poor retention reproducibility, especially with basic solutes. A column lifetime of only a few weeks is not uncommon.

Silica is a possible alternative packing, but not when adsorption mechanisms are involved, which creates more problems with basic solutes than can be solved. Persson and Karger[6] have shown straight-phase partition in the presence of ion-pair

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reagents to be one alternative. Wheals[7] made use of the ion-exchange capability of silica at pH 10, and found that the solubility of silica at high pH was no problem with large amounts of methanol in the aqueous mobile phase. The use of aqueous acids as the mobile phase with silica columns had not been studied until Crommen[8] considered it in a recent paper.

In this work the determination of biogenic amines using a silica column and aqueous acids as the mobile phase was examined, special attention being paid to the requirements for routine methods, such as high speed, high reproducibility and long column lifetimes. The results are discussed in relation to the recent work of Crommen with aqueous ion-pair reagents on silica.

EXPERIMENTAL

Apparatus

The HPLC equipment consisted of Waters Model 6000A solvent-delivery system, a Waters U6K valve-loop injector and a Waters Model 440 absorbance detector (280 nm). For the detection of alcohols a Waters R 401 differential refractometer was used.

One column (4.6 × 250 mm I.D.) was packed with 5-μm Spherisorb S5 silica (Phase Separations, Queensferry, Great Britain), which had a specific surface area of 220 m^2/g. Injection of epinephrine gave an HETP of 0.05 mm, with an asymmetry factor, $A_s = b/a$, of 2.0, measured at 10% of the peak height.

The other column (3.9 × 300 mm I.D.) was packed with 10-μm μPorasil-60A silica (Waters Assoc., Milford, MA, U.S.A.), which had a specific surface area of 500 m^2/g. The packing was deactivated (not silanized) by the manufacturer to be useful for gel filtration, but specific information on the deactivation procedure could not be obtained. With epinephrine, HETP values of 0.10 mm were obtained, with an asymmetry factor of 1.8.

The pH measurements were made with a Beckman H5 pH meter with an Ingold 405-M3 combined microelectrode or with a Metrohm E 396B pH meter.

Detection

UV detection was used throughout this work. A comparison of UV, electrochemical and fluorescence detection of catecholamines and indoles has been made and will be published separately[9].

Chemicals

Doubly distilled water was used. Dodecyl hydrogen sulphate, perchloric acid (70°C), sodium perchlorate, trichloroacetic acid, formic acid (98–100%) and sulphuric acid (95–97%) (all pro analysi grade) were obtained from Merck (Darmstadt, G.F.R.). Monochloroacetic acid (purum grade) and dichloroacetic acid (puriss grade) were supplied by Fluka (Buchs, Switzerland), acetic acid (99.5%) by Baker (Phillipsburgh, NJ, U.S.A.) (analyzed reagent grade) and methanol and acetonitrile by Rathburn (Walkerburn, Great Britain; HPLC grade). All the standard substances were obtained from Sigma (St. Louis, MO, U.S.A.).

Stock solutions of the standards in 0.01 *M* perchloric acid (*ca.* 1 mg/ml) were stored at −20°C. Owing to the limited stability of indoles and N-acetyldopamine, fresh

soutions were prepared every 3 weeks. The limited solubility of melatonin, N-acetylserotonin and methylated tryptamines was overcome by the prior addition of 25 μl of acetonitrile to the 0.01 *M* perchloric acid.

Chromatographic procedures

The mobile phases were degassed for 1 h in an ultrasonic bath. After storage the columns were washed with water for 20 min at 2 ml/min. After a change of mobile phase, equilibration for 10 min at 2 ml/min was sufficient, as controlled with a standard mixture of indoles. All separations were performed at room temperature. After use the silica columns were washed with water and stored in methanol.

RESULTS AND DISCUSSION

Effect of acids

In order to examine the effect of different acids, aqueous solutions of acetic acid, formic acid, monochloroacetic acid (MCA), dichloroacetic acid (DCA), trichloroacetic acid (TCA), sulphuric acid and perchloric acid were tested as mobile phases for chromatography of one group of ten catecholamines and one group of eight indoles on silica. The 0.1 *M* concentrations of the organic acids were chosen to ensure a large excess if any ion-pair effects were present. Decreasing the concentration of perchloric acid from 0.1 to 0.01 *M* had no effect on the retention, but could have a positive effect on the column stability by increasing the pH from 1 to 2, as the specific pore volume of porous silica is constant at pH 2[10]; 0.01 *M* sulphuric acid gave the same results as 0.01 *M* perchloric acid and therefore has not been included in Tables I and II. The dissociation constants of the organic acids and the pH of the aqueous solutions are given in Table III.

The retention data and the chromatograms showed that weak acids, such as acetic acid and to some extent also formic acid, produced highly retained, broad peaks of solutes with basic functions, especially the substituted catecholamines and tryptamines. The acidic compounds had lower retentions and sharp peaks. The retention was only weakly affected by the acid strength. The neutral compound melatonin gave broad, well retained peaks in all of the media. Generally, the best efficiency and resolution were obtained with perchloric and trichloroacetic acids. The lack of resolution between EPI. DA and NMET showed that the separation system had limitations.

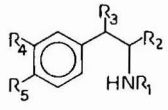
pH variation

In order to study the influence of pH on the retention at a constant concentration of anions, a group of catecholamines and indoles were chromatographed with perchlorate (Fig. 1) and trichloroacetate (Fig. 2) at different pH. The pH was varied by adding 6 *M* sodium hydroxide solution to 0.1 *M* solutions of the acids and measured with a pH meter. At pH > 3 the retention of NE, DA, EPI and EPI-Me increased to $k' > 20$ with both perchlorate and trichloroacetate, and the peaks became broad and asymmetric. For T, 5-HT, NMET and MET the increase in retention was significantly lower, and lower with perchlorate than trichloroacetate. For IAA, 5-HIAA, N-Ac-5-HT, MEL and HVA the retention decreased with increasing pH, especially at pH 4–5.

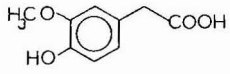
TABLE I

CAPACITY FACTORS OF CATECHOLAMINES AND HOMOVANILLIC ACID ON SPHERISORB SILICA (5 μm) WITH AQUEOUS ACIDS AS THE MOBILE PHASE

Catecholamines:



Homovanillic acid (HVA):



Compound	*Abbreviation*	R_1	R_2	R_3	R_4	R_5
Norepinephrine	NE	H	H	OH	OH	OH
Epinephrine	EPI	CH_3	H	OH	OH	OH
Dopamine	DA	H	H	H	OH	OH
Normetanephrine	NMET	H	H	OH	OCH_3	OH
Metanephrine	MET	CH_3	H	OH	OCH_3	OH
Epinephrine methyl ether	EPI-Me	CH_3	H	OCH_3	OH	OH
N-Acetyldopamine	N-Ac-DA	$COCH_3$	H	H	OH	OH
Dopa	DOPA	H	COOH	H	OH	OH

Compound	*0.01 M $HClO_4$*	*0.1 M TCA*	*0.1 M DCA*	*0.1 M MCA*	*0.1 M HCOOH*	*0.1 M HOAc*
NE	0.27	0.21	0.31	0.59	0.60	5.35
EPI	0.52	0.56	0.52	0.97	2.68	7.9
DA	0.52	0.56	0.52	0.97	2.68	7.9
NMET	0.52	0.56	0.52	0.97	2.84	8.0
MET	1.35	1.48	1.22	2.24	6.6	17.1
EPI-Me	1.29	1.22	1.06	1.92	6.0	16.1
HVA	0.94	0.76	0.65	0.68	0.90	0.68
N-Ac-DA	1.16	1.00	0.84	0.90	1.16	1.03
DOPA	0.05	0.21	—	0.37	—	—

Thus, different selectivities could be obtained by varying the pH. At pH 1–3 the type of anion was less important, while at pH > 3–4 more distinct effects could be obtained with trichloroacetate than with perchlorate.

Examples of changes in selectivity are shown in Figs. 3 and 4.

Ionic strength

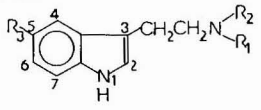
If the retentions (k') in 0.1 *M* acetic acid (pH 3) in Tables I and II are compared with those in 0.1 *M* perchloric acid (pH 3) and 0.1 *M* trichloroacetic acid (pH 3) in Figs. 1 and 2, a striking difference is observed. Solutes with basic functions were retained very strongly in 0.1 *M* acetic acid (pH 3) whereas acidic and neutral solutes were little affected. The cation (H^+) concentration in 0.1 *M* acetic acid was 0.001 *M*, whereas the cation (Na^+) concentration in the two other solvents was close to 0.1 *M*.

Apparently the cation-exchange capability of silica at this pH was sufficient to

TABLE II

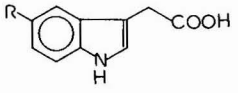
CAPACITY FACTORS OF INDOLES ON SPHERISORB SILICA (5 μm) WITH AQUEOUS ACIDS AS MOBILE PHASE

Indoleamines:



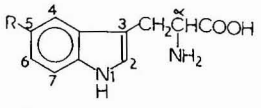
Compound	*Abbreviation*	R_1	R_2	R_3
Tryptamine	T	H	H	H
Serotonin	5-HT	H	H	OH
N-Acetylserotonin	N-Ac-5-HT	H	$COCH_3$	OH
Melatonin	MEL	H	$COCH_3$	OCH_3
N-Methyltryptamine	N-Me-T	H	CH_3	H
N,N-Dimethyltryptamine	N,N-Di-Me-T	CH_3	CH_3	H

Indolic acids:



Indole-3-acetic acid (IAA), R = H
5-Hydroxyindole-3-acetic acid (5-HIAA), R = OH

Tryptophans:



Tryptophan (TRP), R = H
5-Hydroxytryptophan (5-HTP), R = OH
5-Methyltryptophan (5-Me-TRP), R = CH_3

Compound	*0.01 M* $HClO_4$	*0.1 M TCA*	*0.1 M DCA*	*0.1 M MCA*	*0.1 M HCOOH*	*0.1 M HOAc*
5-HT	0.68	0.71	0.65	1.27	3.63	10.4
T	2.56	2.61	1.73	3.19	11.7	28
5-HTP	0.40	0.46	0.43	0.68	1.09	1.86
TRP	1.22	0.84	0.95	1.36	1.89	2.37
5-HIAA	0.45	0.37	0.33	0.33	0.40	0.33
IAA	1.06	0.78	0.65	0.70	1.15	0.80
N-Ac-5-HT	1.54	1.25	1.06	1.19	1.54	1.32
MEL	7.6	5.3	3.9	4.5	7.3	5.4

TABLE III

DISSOCIATION CONSTANTS AND pH VALUES IN AQUEOUS SOLUTIONS

Acid	pK_a	*pH in 0.1 M solution*
TCA	0.70	1.3
DCA	1.48	1.5
MCA	2.85	2.1
HCOOH	3.75	2.5
HOAc	4.75	3.0

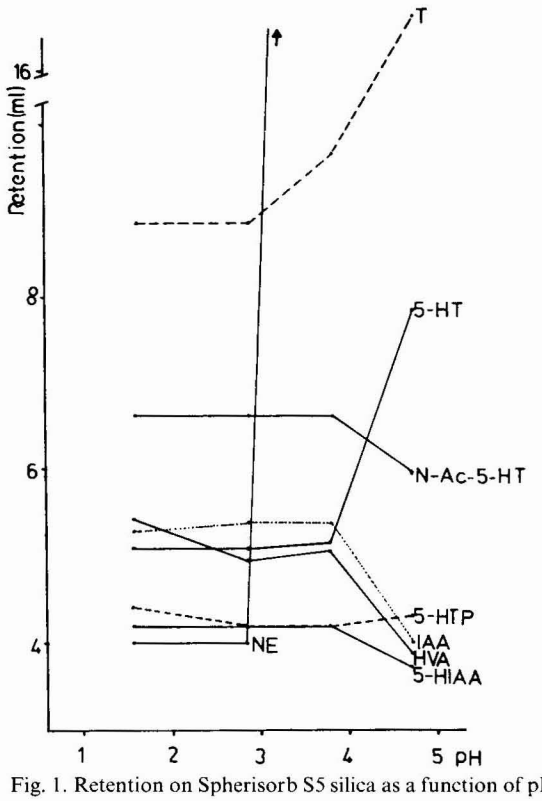


Fig. 1. Retention on Spherisorb S5 silica as a function of pH with 0.1 *M* $HClO_4$ + NaOH as mobile phase.

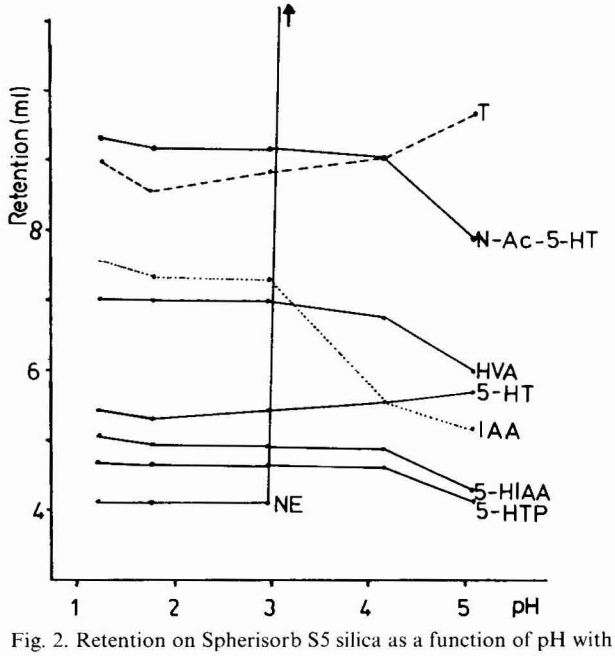


Fig. 2. Retention on Spherisorb S5 silica as a function of pH with 0.1 *M* TCA + NaOH as mobile phase.

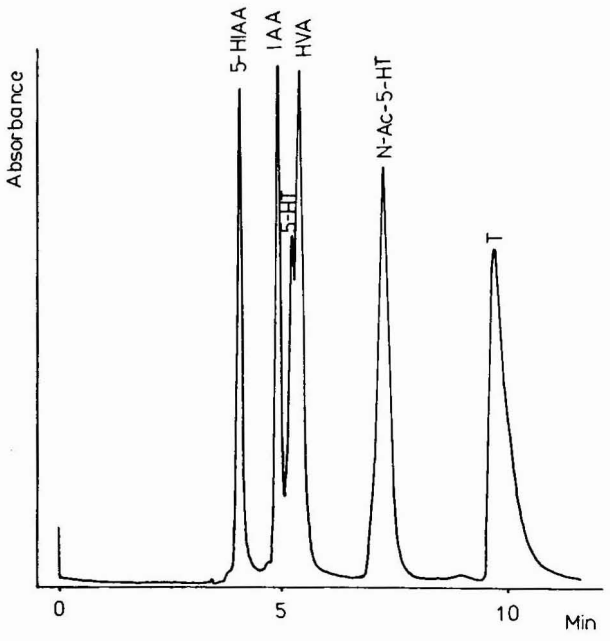


Fig. 3. Separation of six components involved in the metabolism of tryptophan with 0.1 *M* $NaClO_4$ (pH 5.05) as mobile phase.

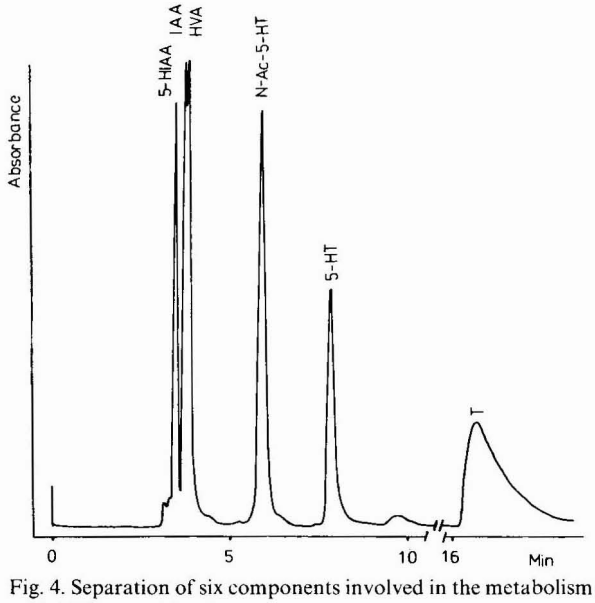


Fig. 4. Separation of six components involved in the metabolism of tryptophan with 0.1 *M* trichloroacetate (pH 4.70) as mobile phase.

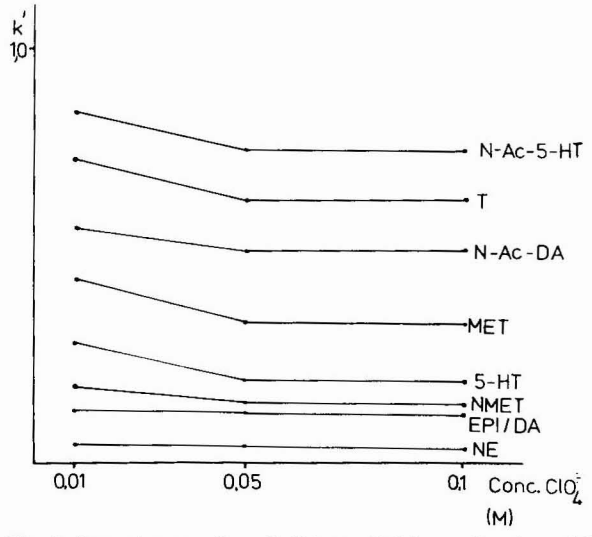


Fig. 5. Retention on μPorasil-60A at pH 2.0 as a function of ClO_4^- concentrations.

cause a high retention of basic solutes. As the charge density of silica at pH 3 is very low, large effects can be obtained by moderate changes in the cation concentration.

When 0.04–0.09 *M* sodium perchlorate was added to 0.01 *M* perchloric acid (pH 2), the retention was independent of or slightly decreased with increasing amount of perchlorate (Fig. 5). The addition of sodium perchlorate resulted in slightly sharper peaks and also in some small changes in selectivity (Fig. 6).

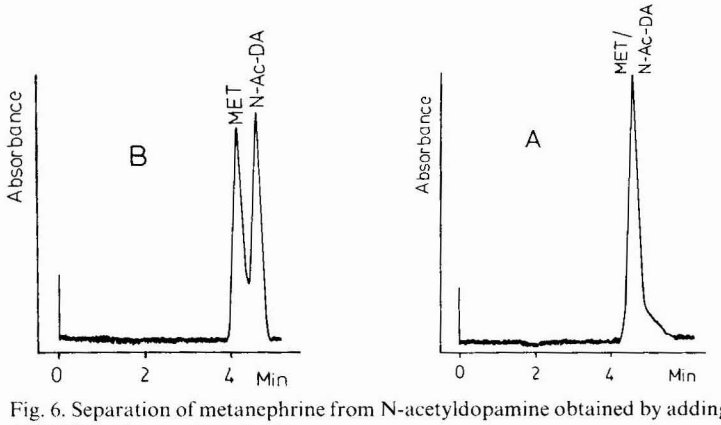


Fig. 6. Separation of metanephrine from N-acetyldopamine obtained by adding 0.04 *M* $NaClO_4$ (B) to 0.01 *M* $HClO_4$ (A) on μPorasil-60A.

Addition of organic solvents

Addition of small amounts of methanol or acetonitrile to the mobile phase strongly reduced the retention of the more hydrophobic components (Fig. 7). By adding 2% methanol to the perchlorate solvent, isomeric 7-Me-T, 5-Me-T and N-Me-T were resolved and separated from T, TRP and Me-TRP (Fig. 8). Acetonitrile

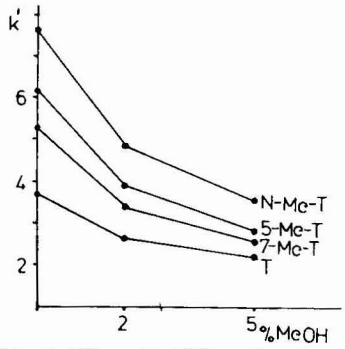


Fig. 7. Effect of addition of methanol to 0.04 *M* $NaClO_4$ + 0.01 *M* $HClO_4$ on the separation of tryptamines on Spherisorb S5 silica.

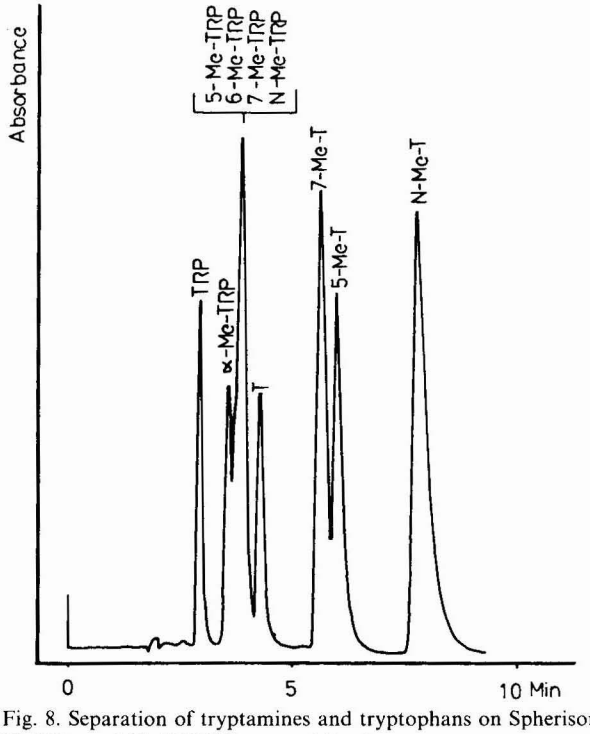


Fig. 8. Separation of tryptamines and tryptophans on Spherisorb S5 silica with 2% methanol in 0.04 *M* $NaClO_4$ + 0.01 *M* $HClO_4$ as mobile phase.

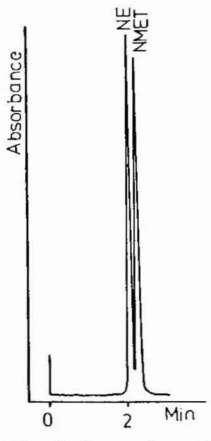
had a greater elution strength than methanol. Addition of 2% of acetonitrile resulted in decrease in resolution of 5-Me-T and 7-Me-T; otherwise the relative retentions were similar.

The retention of early eluting components, such as DA, NE and EPI, was increased by the addition of methanol; addition of 1% increased the retention more than addition of 20%.

These results are not easy to explain, but presumably indicate the existence of different retention mechanisms for the early and late eluting components.

Functional group effects

As a summary of Tables I and II and as illustrated by Figs. 9–15, the following effects were observed. Introduction of hydroxyl groups (aliphatic or aromatic) gave shorter retention. O- and N-methylation gave longer retention. N-Acetylation gave pH-dependent retention, longer at pH $\leq$ 2 and shorter at pH > 2. An increase in the alkyl chain length resulted in an increase in retention, as shown by the alcohols in Fig. 16. Carboxylic acids were eluted prior to the corresponding amides. Amino acids were eluted prior to the corresponding amines.



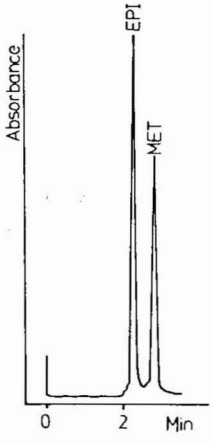
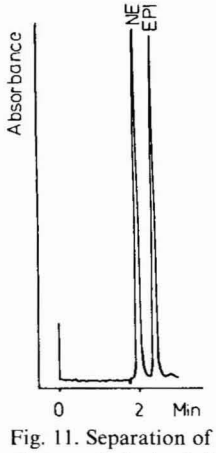


Fig. 9. Separation of the metabolite normetanephrine from its precursor norepinephrine with 0.04 *M* $NaClO_4$ + 0.01 *M* $HClO_4$ on μPorasil-60A.

Fig. 10. Separation of the metabolite metanephrine from its precursor epinephrine with 0.04 *M* $NaClO_4$ + 0.01 *M* $HClO_4$ as mobile phase on μPorasil-60A.



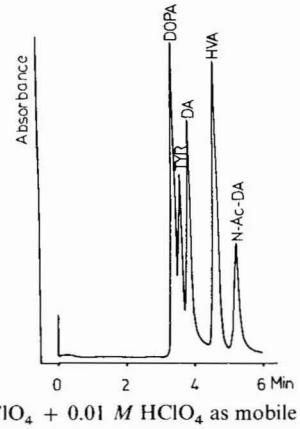


Fig. 11. Separation of norepinephrine and epinephrine with 0.04 *M* $NaClO_4$ + 0.01 *M* $HClO_4$ as mobile phase on Spherisorb S5 silica.

Fig. 12. Separation of precursors and metabolites of dopamine with 0.04 *M* $NaClO_4$ + 0.01 *M* $HClO_4$ as mobile phase on Spherisorb S5 silica.

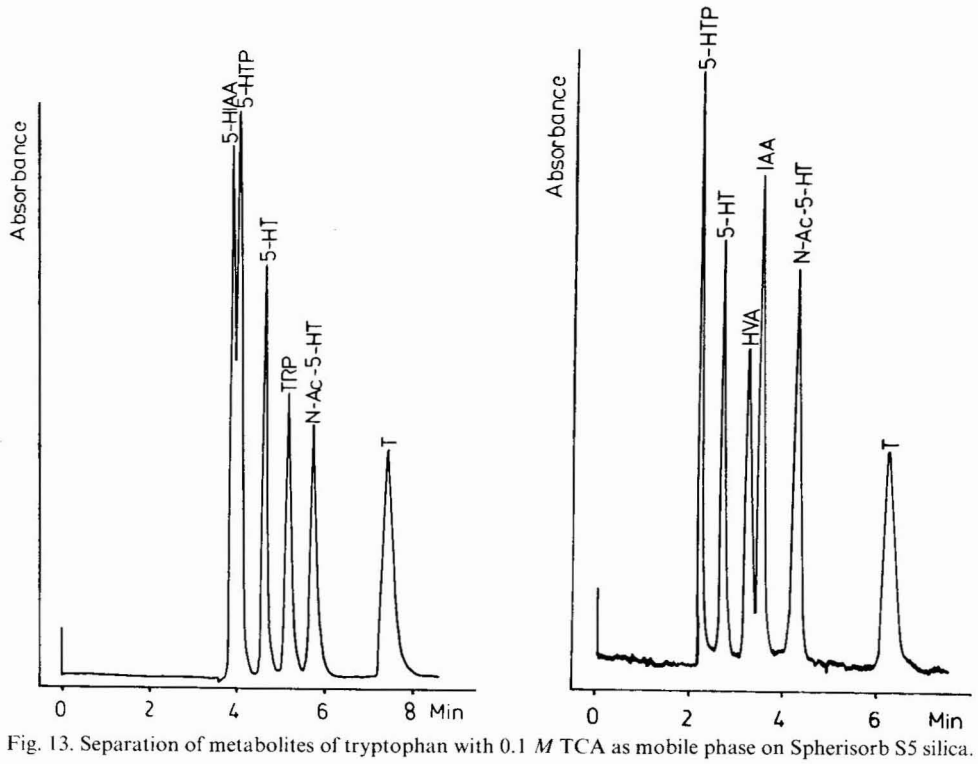


Fig. 13. Separation of metabolites of tryptophan with 0.1 *M* TCA as mobile phase on Spherisorb S5 silica.

Fig. 14. Separation of indoles and metabolites with 0.04 *M* $NaClO_4$ + 0.01 *M* $HClO_4$ as mobile phase on Spherisorb S5 silica.

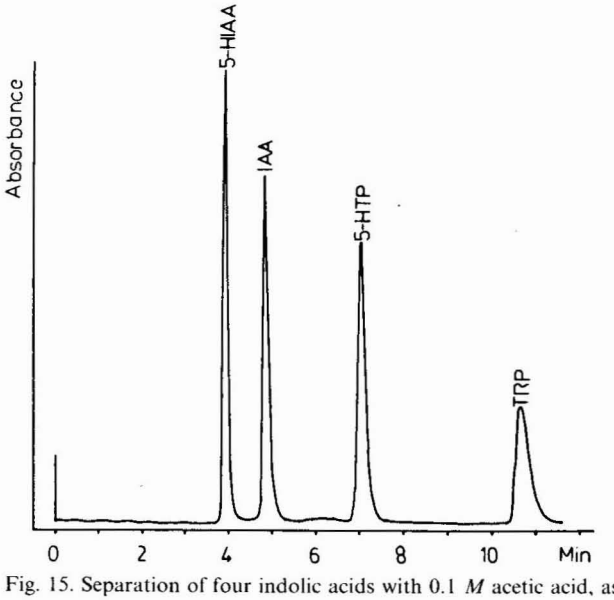


Fig. 15. Separation of four indolic acids with 0.1 *M* acetic acid, as mobile phase on Spherisorb S5 silica.

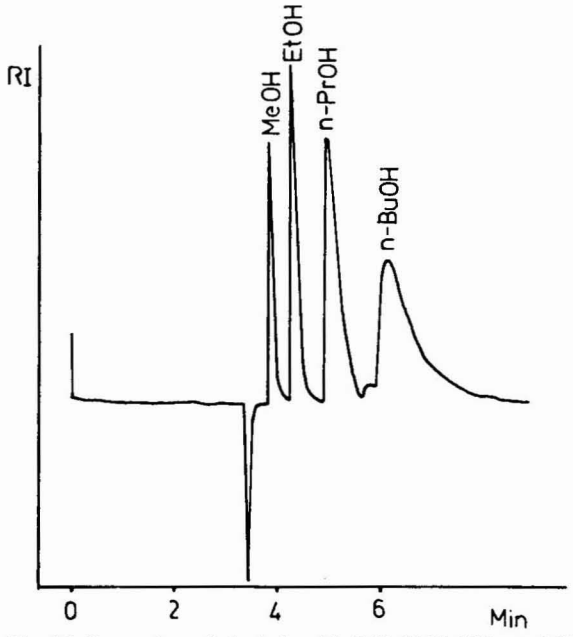


Fig. 16. Separation of alcohols with 0.04 *M* $NaClO_4$ + 0.01 *M* $HClO_4$ as mobile phase on Spherisorb silica.

Addition of dodecyl sulphate

In order to try to improve the selectivity of the early eluting catecholamines, mobile phases consisting of 0.01 *M* dodecyl hydrogen sulphate in 0.1 *M* formic acid at pH 2.5, 3.0 and 3.4 were tested on NE, DA and EPI. The retention was slightly lower than in 0.1 *M* formic acid alone, the peak shape at higher pH was only slightly improved and no separation of DA and EPI was obtained.

As the addition of an ion-pair reagent with a C_{12} chain did not increase the retention, the conclusion that can be drawn is either that NE, DA and EPI did not form ion pairs or that the formation of ion pairs had little effect on the retention.

A similar retention of the three components was actually obtained by Crommen[8] with camphor-10-sulphonate as the assumed ion-pairing reagent and yet the conclusion was drawn that ion-pair formation could be used to regulate (by increasing) the retention of the most hydrophilic biogenic amines on aqueous silica. Based on the experimental results this conclusion cannot be accepted.

Retention mechanisms

As can be seen from the effects of different functional groups, a reversed-phase elution order was generally observed, in agreement with the findings of Crommen[8]. A decrease in the retention of hydrophobic compounds as a result of adding organic solvents also supported the concept of a quasi-reversed-phase system. The pH effects were generally in line with, but could not be explained solely by, "reversed-phase partition".

As "reversed-phase partition" requires a stationary phase that is less polar

than the mobile phase, the postulated model contains a stationary phase of water adsorbed on silica and a mobile phase of the aqueous acid with or without an organic solvent. At low pH (1–2) the retention of moderately and highly retained components depends mainly on the partition coefficients between the two liquid phases, without direct contact with the silica surface. At higher pH coulombic forces can compete with and also overshadow the effect of Van der Waals forces, owing to direct contact with silica caused by a reduction in the amount of adsorbed water. The behaviour of components with low retentions cannot be satisfactorily explained by this model, which probably means that hydrophilic compounds can compete directly with water for the silica surface functions.

The concept of partition chromatography without direct interactions with the silica surface is supported by the fundamental studies of Scott and co-workers[11–13] on solute–solvent interactions on silica. The different mechanisms for hydrophilic components is also in full agreement with this work.

The appearance of ion-exchange effects on increasing the pH is supported by the fact that increased ionization of silanol groups reduces the amount of hydrogen-bonded adsorbed water[14]. The experimental evidence was found in the strong retention of basic solutes and the low retention of acidic solutes. As a natural consequence of the reduced volume of the stationary phase, the retention of neutral solutes decreased. The postulated model does not pretend to behave like a genuine reversed-phase system. Thus, the "salting-out effect" on adding salts to the mobile phase is not expected, and was not found, at least with the moderate concentrations examined.

Crommen[8] showed that the retention of a number of different amines on aqueous silica was increased by adding ion-pair reagents. The results of this study show that the same is generally not true for catecholamines and indoleamines. Except for a predominantly reversed-phase elution order, the retentions of amines, amino acids and acids on aqueous silica cannot be described by one general mechanism.

The present conclusion is that quasi-reversed-phase partition, adsorption/ion exchange and ion-pair formation may contribute to the retention, depending on the nature of the solute, the pH and the content of acids/ions in the mobile phase.

Silica

The sample retention is usually[8], but not necessarily, related to the specific surface area of the silica. Thus, the 10-μm μPorasil-60A packing with a surface area of 500 m^2/g generally gave lower retentions than the 5-μm Spherisorb S5 packing with a surface area of 220 m^2/g. The μPorasil-60A packing had been deactivated by the manufacturer, a procedure that is supposed to have reduced the density of silanol groups. Since water is adsorbed to the silanol groups and not to the hydrophobic siloxane surface[12], the reduction in the amount of adsorbed water should result in decreased retention. Owing to the higher retention, better resolution was normally obtained on the Spherisorb S5 column. With silica of higher surface area (600–800 m^2/g) improved resolution of the hydrophilic catecholamines is expected. A study of this and of the combination of silica with a cation exchanger is in progress.

Reproducibility and column lifetime

Based in 10–20 measurements on each component, the capacity factors in Tables I and II were calculated with a relative precision (percentage standard devia-

tion of the mean) of better than 2% over a 10-h measuring period and better than 5% over a 2-week measuring period at pH 2. At pH > 2 the short-term precision was lower (better than 10%).

Over an 18-month trial period with the same columns the retention of basic components decreased slowly, the greatest decrease being for the basic highly retained compounds, such as a 25% reduction per year for tryptamine. This is the reason why N-Ac-5-HT changed place with T in Fig. 14 compared with Fig. 1. After 1 year the void volume of the Spherisorb S5 column had increased by 5%.

The column efficiencies were maintained at the same high level over the 18-month trial period.

REFERENCES

1 C. Refshauge, P. T. Kissinger, R. Dreiling, L. Blank, R. Freeman and R. N. Adams, *Life Sci.*, 14 (1974) 311.
2 J. H. Knox and J. Jurand, *J. Chromatogr.*, 125 (1976) 89.
3 D. P. Wittmer, N. O. Nuessle and W. G. Haney, *Anal. Chem.*, 47 (1975) 1422.
4 I. Molnár and C. Horvath, *Clin. Chem.*, 22 (1976) 1497.
5 P. A. Asmus and C. R. Freed, *J. Chromatogr.*, 169 (1979) 303.
6 B. A. Persson and B. L. Karger, *J. Chromatogr. Sci.*, 12 (1974) 521.
7 B. B. Wheals, *J. Chromatogr.*, 187 (1980) 65.
8 J. Crommen, *J. Chromatogr.*, 186 (1979) 705.
9 H. Svendsen and T. Greibrokk, *J. Chromatogr.*, 213 (1981) in press.
10 K. K. Unger, *Porous Silica,—Its Properties and Use as Support in Column Liquid Chromatography*, Elsevier, Amsterdam, Oxford, New York, 1979.
11 R. P. W. Scott and P. Kucera, *J. Chromatogr.*, 149 (1978) 93.
12 R. P. W. Scott and P. Kucera, *J. Chromatogr.*, 171 (1979) 37.
13 R. P. W. Scott and P. Kucera, *J. Chromatogr.*, 196 (1980) 193.
14 R. K. Iler, *The Chemistry of Silica*, Wiley, New York, 1979.

Journal of Chromatography, 212 (1981) 167–177
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

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IMPROVED HIGH-FREQUENCY PERMITTIVITY DETECTOR FOR THE CHROMATOGRAPHY OF ALCOHOLS AND OTHER POLAR SPECIES

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SUMMARY

A permittivity detector for high-performance liquid chromatography (HPLC) and flowstream analysis employing three oscillators is described. The cell circuit is of the Franklin type, and the third oscillator allows operation over the complete range of permittivities and prevents locking. A new variable-spacing conical plate cell is described and the detector applied to amino acids and HPLC of alcohols.

INTRODUCTION

The principle of detecting an eluting solute by means of the change in permittivity of the carrier solvent is well known. The theory of such detectors has been developed by Haderka[1,2]. Most work published has involved low-permittivity, low-conductivity eluents with great emphasis on thermal considerations[3–5]. The permittivity detector offers universal sensitivity with a different sequence of sensitivities from the refractive index monitor together with no theoretical lower limit to the cell volume. A comparison of the relative changes of several bulk physical properties in Table I shows permittivity to be one of the most favourable.

Three techniques of measurement have been described, in all of which the flow cell forms a capacitor whose value varies with eluent permittivity. The heterodyne method[7] measures a change in the resonant frequency of an LC circuit with respect to a stable reference oscillator. Alternating current bridge methods[3] typically measure the out of balance signal of a bridge incorporating the flow cell in one arm. A time constant based detector that measures the resistance–capacitance product has been described[8], but it cannot distinguish between changes in these two properties. Heterodyne systems offer potentially the highest resolution which should be proportional to their frequency of operation.

The device described is a three oscillator heterodyne system, operating around 25 MHz designed to allow the use of solvents of any permittivity.

In the conventional two oscillator approach[7] the difference frequency between analyser and reference oscillator is passed to a frequency-to-voltage converter to produce a voltage output. Often the reference oscillator contains a second flow cell

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TABLE I

A COMPARISON OF BULK PHYSICAL PROPERTIES (P) IN TERMS OF RANGE AND FRACTIONAL CHANGE WITH TEMPERATURE, $(dP/P)/dT^6$

	Range P_{min} *to* P_{max}	P_{max}/P_{min}	*Range* 10^{-3} $(dP/P)/dt$
Static permittivity	1.9–80+	40	20
Thermal conductivity (mW cm^{-1} $°K^{-1}$)	1.0–6.1	6	3.0
Refractive index (5893 nm)	1.3–1.65	1.26	6.0
Viscosity (cP)	0.3–10	30	1.6
Density (g cm^{-3})	0.7–1.2	1.7	9.0
Ultrasonic velocity (km sec^{-1})	1.1–1.9	1.7	1.1
Magnetic susceptibility	0.99999–1.00001	–	–

through which a reference flowstream runs. Mounting both cells in the same block offers the advantage of thermal compensation[5], but the added complexity of maintaining two streams makes it unattractive. In the work described here a crystal controlled oscillator is used to provide the reference frequency; to permit frequency–voltage conversion the difference frequency is made less than 100 kHz.

Two oscillators of similar frequency will tend to lock such that the small potential change in frequency due to a small change in cell capacitance will not be observed at the output. This tendency to lock can be reduced by buffering and careful shielding, but locking occurred over several kHz with previous devices[9] and it is better to adopt a design which eliminates the problem totally.

If a single frequency quartz reference oscillator is employed the difference frequency will vary greatly with the eluent used, due to the wide change in cell capacitance. Such a system can only be optimised for a small range of solvent permittivities unless the plate spacing can be varied to achieve the desired capacitance — which, although possible, is not the best solution.

To overcome the "locking" problem it was initially arranged to run the reference and cell oscillators about 667 kHz apart. This first difference frequency was then mixed with a second crystal oscillator operating at 667 kHz and a second difference frequency obtained which was converted to a voltage output as before. (There is no scientific significance to the choice of difference frequency; anywhere in this region would be all right.) The operating restrictions of such a device were as severe as before and are shown in Fig. 1. The cell oscillator frequency must lie within ± 100 kHz (the range of the frequency–voltage converter) of $f_2 \pm 667$ kHz. By varying the plate spacing this can generally be achieved, but perhaps only at undesirably large cell volumes with consequent degradation of resolution.

To achieve greater flexibility, this fixed intermediate frequency oscillator was replaced by a frequency synthesizer allowing operation between $f_2 \pm 5$ MHz. This wider operating range is shown in Fig. 1 and allows the use of whatever plate spacing is best from the views of mechanical stability and minimum cell volume for any eluent. This work describes the development of an improved capacitance cell and

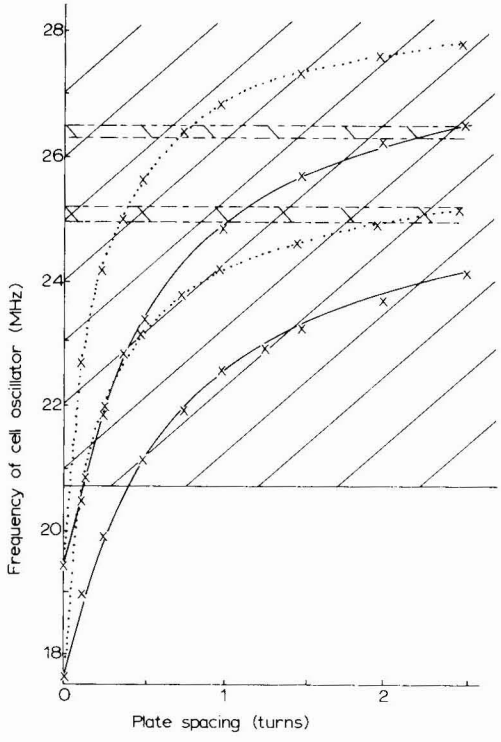


Fig. 1. Operating range of detector with crystal (cross-hatched) and frequency synthesizer (shaded) as intermediate frequency oscillator. ....., Propan-2-ol; ——, methanol–water (60:40).

measurement circuit for high-performance liquid chromatography (HPLC) and its application is shown for the chromatography of alcohols.

THEORETICAL

The theory of permittivity of mixtures (ε_m) is only fully understood for non-associating mixtures of non-polar liquids whose permittivities (ε_1, ε_2) are additive in terms of volume fractions (v_1, v_2):

$$\varepsilon_m = \varepsilon_1 v_1 + \varepsilon_2 v_2 \tag{1}$$

For dilute solutions of polar molecules such as the effluent from HPLC columns, ($v_x < 10^{-3}$), this may provide a reasonable approximation. If, however, the solute is a solid or changes on dissolution, *i.e.*, ionises, dimerises or forms zwitterions, no such theoretical approximation is justified. It is found in practice that at sufficiently low concentrations the increase in relative permittivity is linear[10]

$$\varepsilon_m = \varepsilon_1 + \delta m_2 \tag{2}$$

where m_2 is the solute molarity and δ is a constant, the dielectric increment of the

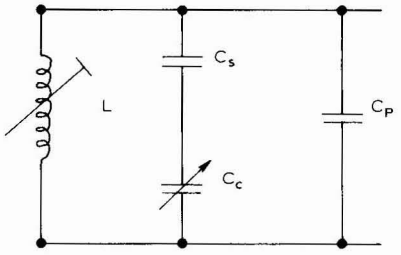


Fig. 2. Frequency determining components of an inductance–capacitance (LC) oscillator.

solute. Values for δ can be predicted, *e.g.*, Oster's equation[10] and the same reference gives a selection of values for amino-acids and other compounds in aqueous solution.

For the present study the predicted response of the detector, Δf, will be derived in terms of δ.

The frequency determining portion of the Franklin oscillator is shown in Fig. 2. The parallel capacitance, C_p, is the stray capacitance of leads, etc., amounting to about 0.5 pF. The frequency (f) will be given by

$$f = (1/2\pi)\,(LC_\tau)^{-\frac{1}{2}} \tag{3}$$

where L is the inductance and the total capacitance, C_τ, is given by:

$$C_\tau = C_p + \left(\frac{C_c C_s}{C_c + C_s}\right) \tag{4}$$

C_c is the cell capacitance with solvent in the cell and C_s the series capacitance. Rearranging eqn. 4 and substituting into eqn. 3 gives:

$$f = \frac{1}{2\pi} \times \left(\frac{C_c + C_s}{L\,[C_c(C_p + C_s) + C_s C_p]}\right)^{\frac{1}{2}} \tag{5}$$

When the cell is full of solvent of permittivity ε_1, $C_c = A\varepsilon_1$, $f = f_1$, where A = constant. When the cell is filled with the mixture, $f = f_m$ and:

$$C_m = A\varepsilon_m = A\varepsilon_1 + A\delta m_2 \tag{6}$$

Substituting eqn. 6 into eqn. 5 for the condition $f = f_m$ results in:

$$f_m = f_1\left(1 + \frac{A\delta m_2}{A\varepsilon_1 + C_s}\right)^{\frac{1}{2}} \left(1 + \frac{A\delta m_2(C_p + C_s)}{A\varepsilon_1(C_s + C_p) + C_s C_p}\right)^{-\frac{1}{2}} \tag{7}$$

If $m_2 \ll 1$ the latter two terms can be expanded by the binomial theorem:

$$f_m = f_1\left(1 - \frac{A\delta m_2 C_s^2}{2\,[A\varepsilon_1(C_s + C_p) + C_s C_p]\,(A\varepsilon_1 + C_s)}\right) \tag{8}$$

Subtracting f_1 to obtain the difference frequency (Δf) and replacing $A = C_c/\varepsilon_1$ yields:

$$\Delta f = \frac{f_1 \delta m_2 C_c C_s^2}{2\varepsilon_1 [C_c(C_s + C_p) + C_s C_p](C_s + C_p)} \tag{9}$$

Using eqn. 3 this gives:

$$\frac{\Delta f}{m_2} = \frac{f_1 \delta C_c C_s^2}{2\varepsilon_1 C_r (C_c + C_s)^2} \text{ Hz l mol}^{-1} \tag{10}$$

Defining a circuit constant $Z = C_c C_s^2/C_r(C_c + C_s)^2$ and converting into concentration (X) units where M_2 is the molecular weight of the solute such that $X = m_2 M_2/1000$ yields:

$$\frac{\Delta f}{\Delta X} = \frac{500 f_1 \delta Z}{\varepsilon_1 M_2} \text{ Hz ml g}^{-1} \tag{11}$$

To obtain a high sensitivity one requires high operating frequency, low permittivity solvents and maximum Z. This last condition means making cell capacitance, C_c, small compared with C_s and eliminating as much stray capacitance, C_p, as possible. A complication is that the cell capacitance is composed of two parts and is better represented by $C_c = C_1 + B = A\varepsilon_1 + B$ where C_1 is that due to the filling liquid and B is due to stray paths through the air. Substituting C_1 for C_c leads to a reduced value (Z') for the circuit constant Z. For the cell described $B = 4$ pF (measured on a 0.1% bridge at 1 MHz) and the typical value of $C_c = 17$ pF used means that the uncorrected value of Z is 24% high. Thus cell design must attempt to minimise stray capacitance.

EXPERIMENTAL

A block diagram of detector circuitry is shown in Fig. 3; the power supply used was a ±15-V encapsulated unit (R.S. Components Ltd.) with 0.1% voltage regulation. The cell oscillator, of the Franklin design[11], operated nominally at 25 MHz, but was able to swing between 17 and 31 MHz. The reference was a crystal oscillator working on the third harmonic of an 8.579-MHz crystal to give an output of 25.736 MHz. The frequency synthesizer was programmed from a set of binary-coded decimal-

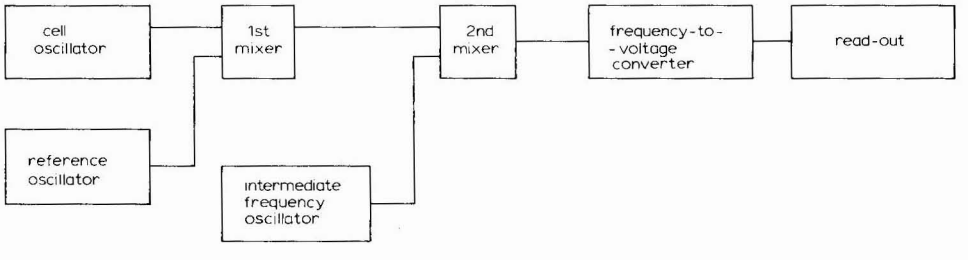


Fig. 3. Block diagram of three-oscillator detector.

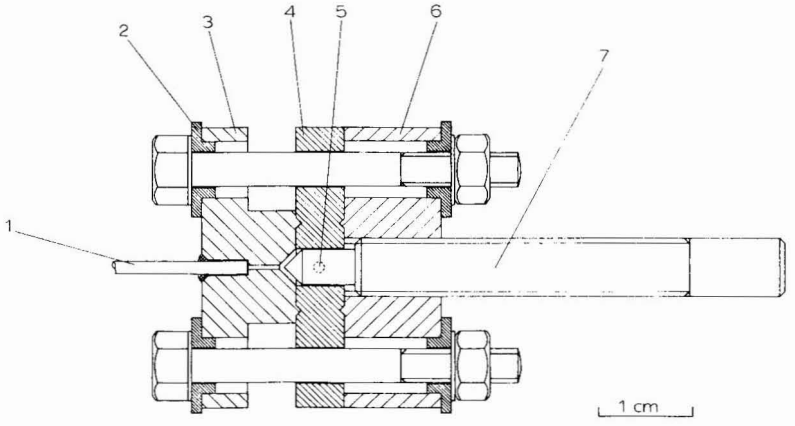


Fig. 4. Conical plate permittivity cell (section across diameter). 1 = Capillary inlet from column; 2 = insulating bush; 3 = ground plate; 4 = PTFE spacer; 5 = cell outlet and PTFE tubing; 6 = cell body; 7 = threaded rod forming moveable "high" plate.

encoded logic switches mounted on the front panel of the device. It produced a square-wave output between 3 kHz and 5 MHz in 1-kHz steps. The frequency-to-voltage converter (R.S. Components Ltd.) had a linearity of 0.1 % up to 100 kHz.

The pumps used were an HPL-HM (MPL Ltd.) metering pump, with a house-built pump damper for chromatographic studies and a Masterflex (Cole-Palmer Inc.) peristaltic pump with continuously variable pumping speed for development work. Solvents and chemicals used were of the highest purity obtainable and were degassed by boiling under reduced pressure to avoid bubble formation within the cell. All amino acids used were "chromatographically pure grade" (BDH, Poole, Great Britain).

The cell shown in Fig. 4 was of PTFE and stainless-steel construction. The column effluent entered through a short length of 0.006-in. or 0.010-in. I.D. stainless steel tubing (Phase Separations, Queensferry, Great Britain) into a conical cavity impinging onto a matching conical plate. The spacing could be varied by screwing-in this plate by 0.025-in. per turn. The volume between the conical plates was 5.5 μl per turn and the stray capacitance in air was 4 pF. The effluent passes out between the plates and a PTFE spacer into a PTFE exit tube.

In the chromatographic work a Specac 30-100 sampling valve (Specac-Sidcup) fitted with a 10-μl sample loop was used to introduce samples onto a 10-μm Li-Chrosorb RP-8 column (20 cm × 4.6 mm I.D.).

RESULTS

Experiments were performed to verify that response was proportional to δ/M (eqn. 11).

Amino acids were selected for their high δ/M values typically $0.1 < \delta/M < 0.6$ due to the formation of zwitterions. Solutions were made up in methanol–water (50:50) in the range 1–10 g l^{-1} and passed sequentially through the cell using a peristaltic pump, the solutions being first equilibrated in a thermostatted bath. The

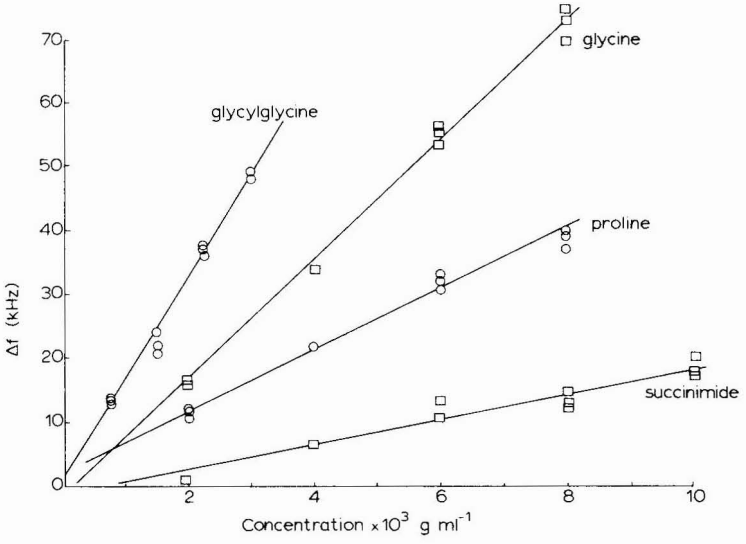


Fig. 5. Calibration curves for amino acids in methanol–water (50:50) (parallel plate cell, solvent as reference blank).

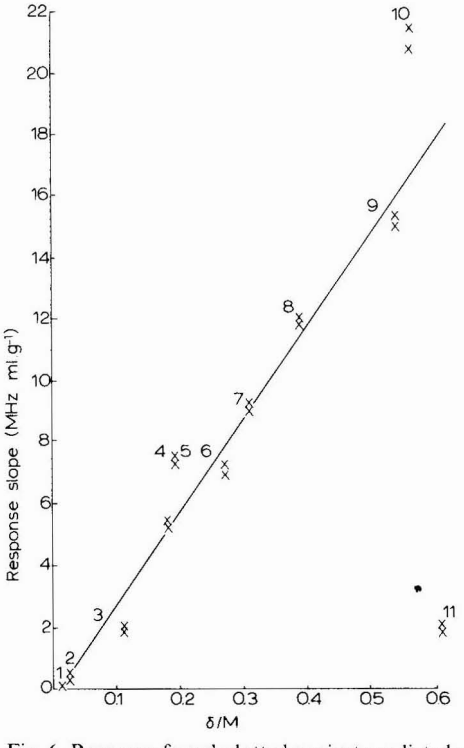


Fig. 6. Response found plotted against predicted sensitivity in methanol–water (50:50). 1 = Sorbitol; 2 = glucose; 3 = succinimide; 4 = proline; 5 = norvaline; 6 = alanine; 7 = glycine; 8 = β-alanine; 9 = glycylglycine; 10 = 6-amino-*n*-hexanoic acid; 11 = benzoic acid.

change in frequency, Δf, was measured with respect to the solvent blank. To avoid errors due to changes in the methanol–water ratio and hence carrier permittivity, the solvent was not heated to dissolve the acid but rather stirred under sealed conditions. It was not possible, generally, to make a stock solution in this way containing more than 10 g l^{-1}. The solvent was degassed beforehand whilst stirring under water pump vacuum. Over this limited concentration range linear calibrations of response were obtained as shown in Fig. 5 and the slopes of these are plotted against δ/M in Fig. 6 and presented in Table II together with values for glucose, sorbitol and benzoic acid.

TABLE II

LIST OF RESPONSES TO AMINO ACIDS AND OTHER COMPOUNDS IN METHANOL–WATER (50:50) (25.1 MHz)

All amino acids are BDH "chromatographically pure grade".

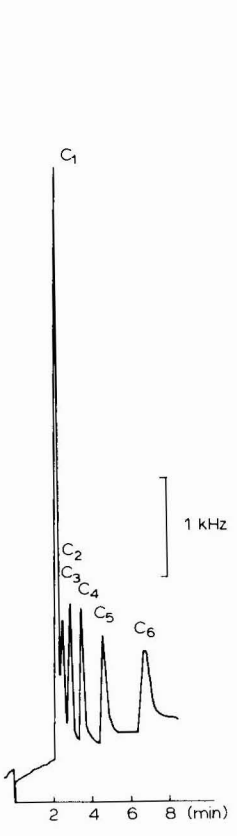
Compound	*Slope (MHz ml g^{-1})*	*T (°C)*	*Range (g l^{-1})*	*δ/M*
Succinimide	2.0	21.0	0–10	0.11
	1.9		0–10	
L-Tryptophan	4.0		0–5	
	4.9	22.8	0–3	?
β-Phenylalanine	4.3	22.6	0–3	
	4.4	23.1	0–3	?
L-Proline	5.4	25.1	0–10	0.18
	5.3		0–10	
L-Alanine (α)	7.0	21.4	0–10	0.27
	7.3		0–10	
Glycine	9.2	24.0	0–10	0.31
	9.0	25.6	0–10	
β-Alanine	12.0	20.1	0–10	0.39
	11.8	20.1	0–5	
Glycylglycine	15.3	25.0	0–3	0.54
	18.1		0–3	
6-Amino-	20.8	19.0	0–8	0.56
n-hexanoic acid	21.5	23.8	0–5	
Norvaline	7.5	19.2	0–5	0.19
	7.8	21.6	0–5	
Benzoic acid	1.9		0–10	0.61
	2.1	25.2	0–10	

It can be seen that with the notable exception of benzoic acid a reasonable straight line is obtained. Individual slopes were averaged over four runs using a least-squares fitting programme, run on a Nascom 2 microcomputer, to evaluate the relative standard deviation as between 9 and 16%.

The gradient of Fig. 6 is 30 MHz ml g^{-1} as against a predicted value (C_c = 17 pF, C_p = 7.5 pF, C_s = 15 pF) of 43 MHz ml g^{-1}. It is important to note that published values[10] of δ/M were obtained in aqueous solution while these results are for methanol–water (50:50). It has been shown[12] that the dielectric increment of the

amino acid α-butyrine is largely independent of solvent permittivity (between 35.4 and 134.9) or solution water content so that this approximation is of some value. The same source indicates that the increment is also nearly independent of temperature and concentration. This is presumably due to the fact that charge transfer is internal and complete so that solution effects are unimportant. Benzoic acid gives less response than expected, presumably since the ionisation (which gives rise to solvent ordering and hence a reduction of permittivity) is suppressed on the addition of methanol. The sugars give about a quarter of the predicted response, perhaps due to changes in equilibria between the various ring forms and conformational isomers in solution.

It is usual[13] to buffer solutions of amino acids to increase retention on reversed-phase columns but the conductivity of the buffer acts as a large admittance in parallel with the cell and causes spurious changes in the oscillator frequency. It was not possible to gain any significant separation of amino acids (as indicated by a fixed 254-nm UV detector operated in series with the permittivity detector) at concentrations below 0.025 *M* acetic acid which was found to be the maximum tolerable



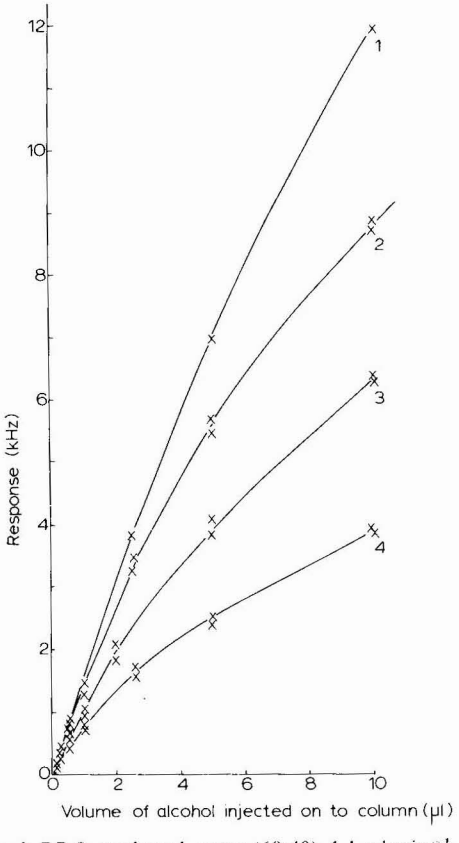


Fig. 7. Chromatogram of primary alcohols on LiChrosorb RP-8, methanol–water (60:40), 1.1 ml min^{-1}.

Fig. 8. Variation of peak height obtained in methanol–water (50:50) against volume injected (C_s = 30 pF). Curves: 1 = propan-1-ol; 2 = butan-1-ol; 3 = pentan-1-ol; 4 = hexan-1-ol.

level. This means that although there is sufficient sensitivity, the detector is unsuitable for the detection of amino acids in practical HPLC.

Primary alcohols are miscible with methanol–water mixtures and have a wide range of permittivities. Solutions were made up in the range 0.2–100% (v/v) and introduced via a 10-μl sampling valve into an eluent of methanol–water (60:40). The resulting chromatogram is shown in Fig. 7. Graphs of peak height against volume injected are shown in Figs. 8 and 9. The expected order of sensitivities is reversed due to the order of elution which spreads the less polar, higher alcohols more.

Since sensitivity should be linear at low concentrations the area under the peak should be proportional to the weight injected $\times$ δ/M. Solutions of alcohols (10%, w/v) were made up in methanol and 10-μl samples injected onto the column at a flow-rate of 1.5 ml min^{-1} of methanol–water (70:30 or 50:50). The area of the peak was found by cutting and weighing, and plotted against δ/M, a measure of predicted response (Fig. 10). (Relative standard deviation on cutting and weighing was 2.8%.)

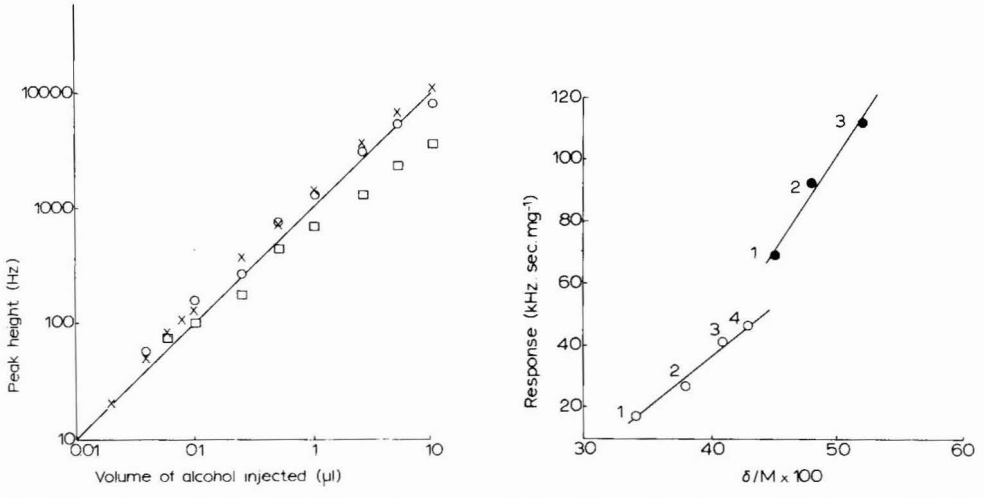


Fig. 9. Log–log graph of peak height against amount injected in methanol–water (50:50) (C_s = 30 pF). $\times$ = Propan-1-ol; $\bigcirc$ = butan-1-ol; $\square$ = hexan-1-ol. Line drawn has a slope of +1 for comparison.

Fig. 10. Peak area response against δ/M for several primary alcohols in methanol–water (70:30) (full circles) and methanol–water (50:50) (open circles). 1 = Propan-1-ol; 2 = butan-1-ol; 3 = pentan-1-ol; 4 = hexan-1-ol.

For the individual solvents the relationship is as might be expected; the reason for the different gradients between the two solvent systems is not obvious but is possibly connected with the differing degree of solvation of the alcohols in the more aqueous medium. (Hexanol was not adequately chromatographed in the lower permittivity medium to warrant plotting the point.)

CONCLUSIONS

The instrument described, employing a very stable Franklin type oscillator for the cell circuit and second intermediate frequency to prevent locking, is a significant

improvement over the earlier devices. The new cell employed has better stability characteristics than the parallel plate cell used earlier, is less prone to bubble formation and has minimal dead volume. The detection system has shown itself most useful for the sample alcohols and amino acids, the conductivity of the buffering media, however, prevents its exploitation at present.

ACKNOWLEDGEMENTS

We are indebted to Shell Biosciences, Sittingbourne, Great Britain for advice and the loan of equipment and to A. Thoër for helpful discussion. P. K. P. Drew is in receipt of a Science Research Council grant and P. R. Fielden is supported by the Ministry of Defence. This work was carried out in the Department of Chemistry, Imperial College, London, Great Britain.

REFERENCES

1 S. Haderka, *J. Chromatogr.*, 54 (1971) 357.
2 S. Haderka, *J. Chromatogr.*, 91 (1974) 167.
3 R. P. W. Scott, *Liquid Chromatography Detectors*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 69.
4 V. Slavík, *J. Chromatogr.*, 148 (1978) 117.
5 H. Poppe and J. Kuysten, *J. Chromatogr.*, 132 (1977) 369.
6 R. C. Weast (Editor), *Handbook of Chemistry and Physics*, Chemical Rubber Co., Cleveland, OH, 58th ed., 1977.
7 J. F. Alder and A. Thoër, *J. Chromatogr.*, 178 (1979) 15.
8 Y. Hasimoto, M. Morisayu, E. Kato, M. Endo, N. Miyamoto and H. Uchida, *Mikrochim. Acta*, 11 (1978) 159.
9 A. Thoër, *DIC Thesis*, Imperial College, London, 1979.
10 J. B. Hasted, *Aqueous Dielectrics*, Chapman & Hall, London, 1978, Ch. 7.
11 S. Garner, *Radio. Commun.*, 55 (1979) 32.
12 J. P. Greenstein and M. Winitz, *Chemistry of the Amino acids*, Vol. 1, Wiley, New York, London, 1961.
13 I. Molnár and Cs. Horváth, *J. Chromatogr.*, 142 (1977) 623.

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USE OF $F(ab')_2$ ANTIBODY FRAGMENTS IN THE SYNTHESIS OF IMMUNOADSORBENTS FOR PREPARING MONOSPECIFIC ANTIGEN

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SUMMARY

Class specific $F(ab')_2$ antibody fragments were prepared by pepsin digestion of the labile Fc immunoglobulin fragments in antigen–antibody precipitates. The $F(ab')_2$ fragments were covalently coupled to cyanogen bromide-activated Sepharose® 4B and the resultant immunoadsorbent used to isolate IgG from human serum with a single chromatographic step, in high yield and purity and negligible non-specific interaction. This technique affords a simple method for preparing an enriched source of class specific affinity-purified immunoglobulin antibodies suitable for many immunochemical applications.

INTRODUCTION

The use of affinity-purified antibodies in the preparation of immunoadsorbents should, theoretically, increase the specific binding capacity of the immunoadsorbents and minimize non-specific interactions. However, the conventional method for preparing affinity-purified antibodies involves a number of steps. The antigen–matrix immunoadsorbent is first prepared and used to isolate specific antibodies by suitable elution techniques. After subsequent dialysis, concentration and characterization, the antibodies are used to prepare an antibody–matrix immunoadsorbent. The degree of protein denaturation increases with the number of manipulations.

Pepsin hydrolysis of immunoglobulins has revealed that the $F(ab')_2$ fragment is the major undigested product resulting from the proteolysis of IgG[1,2]. Since this fragment possesses the two antigen binding sites of the parent molecule it is still an effective truncated antibody molecule capable of binding the complementary antigen. The class specific Fc region of the immunoglobulin molecule is the most immunogenic part of the molecule[3]. Consequently, antisera raised to immunoglobulins consist mostly of antibodies of the antigenic determinants in the class specific Fc region. Lachman[4] employed pepsin digestion of antigen–antibody precipitates at optimal proportion to prepare class specific anti-immunoglobulin antibodies as $F(ab')_2$ fragments. The application of this method to other antigens depends, however, on their susceptibility to pepsin digestion.

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The removal of the Fc portion of the immunoglobulin molecule should afford several advantages: (i) it should minimize the contribution to non-specific binding associated with the heterologous antibodies[5,6] and (ii) since complement binds primarily to the Fc region removal of this region should eliminate serum complement binding to solubilized antibody[7].

In this study we report the preparation of covalent conjugates of immunoglobulin $F(ab')_2$ fractions with cyanogen bromide-activated Sepharose® 4B and demonstrate that the $F(ab')_2$-matrix immunoadsorbent is more efficient than the intact antibody–matrix immunoadsorbent in the purification of class specific IgG from pooled, whole human serum. Truncation of the antibody molecule did not impair the capacity of the antibodies to bind reciprocal antigens. In addition, no severe loss in stability and activity of the cyanogen bromide-activated Sepharose 4B-$F(ab')_2$ immunoadsorbent was observed after five serial adsorption–desorption cycles. The antibody (sheep anti-human IgG) used in the present study for preparing the $F(ab')_2$ fraction and in previous studies for preparing immunoadsorbents[5,6] was obtained by ammonium sulphate precipitation of crude immunoglobulin from whole human serum followed by DEAE-cellulose anion-exchange chromatography of the dissolved precipitate. Only a small portion (about 10%) of the resultant IgG fraction is specific antibodies (most of the immunoglobulins are inert or non-specific). Nevertheless, the use of $F(ab')_2$ fragments derived from immune precipitates results in an approximately 10–20 fold purification of the initial antibody fraction.

MATERIALS AND METHODS

Cyanogen bromide-activated Sepharose® 4B and Sephadex® G-150 (40–120 μm beads) were obtained from Pharmacia (Uppsala, Sweden). Sheep anti-human IgG (batch Z511G, 24.7 mg ml^{-1}), human IgG (batch D152, 6.34 mg ml^{-1}) and NIRDL (normal human serum code number BR99) serum standard were purchased from Seward Laboratories (London, Great Britain). Chromatography columns GA 10 × 15 (1.0 × 15 cm) were obtained from Wright Scientific (Kenley, Great Britain). Pepsin was purchased from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.), agarose for immunochemical assays from Fisons (Loughborough, Great Britain). The materials used for preparing buffers and salt solutions were of AnalaR grade.

Preparation of antigen–antibody (human IgG–sheep anti-human IgG) precipitates

The titre (the amount in mg of antigen which reacts with a known amount of antiserum at the equivalence point) of the sheep anti-human IgG antibody was determined by a preliminary precipitin titration followed by manual nephelometric quantitation of the amount of precipitate[8]. The antigen–antibody precipitate was then prepared close to the equivalence point. Human IgG (4 ml, 5.93 mg ml^{-1}) dialysed against normal saline was added to sheep anti-human IgG (10 ml, 24.7 mg ml^{-1}) which was also dialysed against saline and adjusted to 0.1 *M* with respect to ethylenediaminetetraacetate (EDTA) in order to enhance precipitation. After thorough mixing, the solution was incubated at room temperature for 30 min followed by 5 min at 4°C to complete precipitation. The precipitate was then centrifuged for 10 min at 4°C, 2000 *g*, and after washing with cold saline it was dissolved in

distilled water (10 ml) acidified with a few drops of acetic acid (0.2 ml). The solution was finally adjusted to pH 3.1 with 0.2 ml acetic acid and the volume made up to 15 ml with distilled water. An aliquot (0.5 ml) of the solution was assayed for protein by the Folin Ciocalteu technique[9] and the remaining 14.5 ml were used for pepsin digestion.

Pepsin digestion of the antigen–antibody complex (human IgG–sheep anti-human IgG)

To the solution (14.5 ml) of the dissolved precipitate, freshly prepared pepsin solution (1 mg ml^{-1} in 0.2 *M* acetic acid) was added to yield a 2% (w/w) pepsin solution which was then incubated at 37°C for 3 h. The enzyme reaction was terminated by adjusting the solution to pH 8.0 with sodium carbonate solution (0.5 *M*), and the solution was incubated further at 37°C for 4 h.

Isolation of $F(ab')_2$ from pepsin digest

Anhydrous sodium sulphate (to 1.5 *M*) was added to the pepsin digest with stirring at 37°C. After standing at room temperature ($\approx$20°C) for 30 min, the mixture was centrifuged at 20°C, 2000 *g*, for 10 min. The precipitate was then redissolved in distilled water (5.0 ml) and an aliquot (0.5 ml) used for protein assay by the Folin Ciocalteu method[9]. The remaining 4.5 ml of the dissolved sodium sulphate precipitate were concentrated to 2.5 ml by negative pressure dialysis. To the 2.5-ml concentrate [11.1 mg $F(ab')_2$ equivalent to 16.7 mg antibody], Bence Jones protein marker (10 mg) was added and the mixture loaded on to a Sephadex G-150 column (2.2 × 81 cm) pre-equilibrated with a solution of sodium chloride (0.5 *M*) buffered at pH 7.2 with tris(hydroxymethyl)aminoethane–HCl (Tris, 0.1 *M*) containing 0.02 *M* sodium azide. Elution was effected with the sodium chloride–Tris buffer (pH 7.2) which allowed separation of $F(ab')_2$ from any soluble aggregates, the p Fc′ fraction and smaller peptide products. The eluted fractions (5 ml) were collected with the aid of an LKB (Selsdon, Surrey, Great Britain) automatic fraction collector at a flow-rate of 18 ml/h and monitored at 280 nm with an LKB Uvicord analyser.

Immunochemical analysis

The fractions for the major protein peaks resulting from the Sephadex G-150 gel filtration were pooled, dialysed against distilled water and concentrated by negative pressure dialysis using 18/32 in. Visking dialysis tubing. The purity of the pooled fractions was assayed by Ouchterlony double immunodiffusion[10] and one-dimensional immunoelectrophoresis[11] (i.e.p.) techniques. Immunoelectrophoresis was performed on glass plates (8 × 8 cm) coated with 1% agarose in barbitone buffer (0.06 *M*, pH 8.6). Electrophoresis was conducted for 80 min with a constant current of 10 mA or about 7 V per cm width (interelectrode distance) of the plate. The troughs were filled with antisera and diffusion allowed to occur while the plate was left in a moist atmosphere for about 12 h. The antigen binding capacity, *C*, of an antibody immunoadsorbent column was calculated as

$$C = (P - p)\,T$$

where *P* is the amount of antibody added to the actual gel; *p* the amount coupled and *T* is the titre of the antibody[5].

Coupling of sheep anti-human IgG F(ab')$_2$ to cyanogen bromide-activated Sepharose 4B

Cyanogen bromide-activated Sepharose 4B (1 g dry weight) was swollen in, and washed with 10^{-3} *M* HCl to remove dextran and lactose stabilizers, filtered on a No. 3 sintered Buchner funnel and equilibrated in sodium citrate buffer (0.2 *M*, pH 6.5). The F(ab')$_2$ solution (4 ml, 3.6 mg) predialysed against sodium citrate buffer was added to the Sepharose gel suspended in sodium citrate buffer (6 ml, approximately twice the volume of the swollen gel) in a universal vial and tumbled end over end for 3 h at room temperature. After filtering, the gel was washed in sodium citrate buffer and then lightly stirred magnetically in excess of freshly prepared aqueous ethanolamine solution (1 *M*, pH 9.5) for 2 h at room temperature to block residual active groups. After filtering, the gel was washed with sodium chloride–Tris buffer (0.5 *M* NaCl, 0.1 *M* Tris, pH 7.2) containing 0.5% (w/v) sodium azide. All the washings and filtrates were retained and pooled and an aliquot of the concentrate was monitored for uncoupled antibody by the reverse Mancini technique, *i.e.*, with the antigen instead of the antibody dispersed in the agarose gel mounted on a glass plate.

Immunoadsorbent column preparation

After degassing, the Sepharose 4B-F(ab')$_2$ immunoadsorbent was packed into a column (1.0 × 15 cm), washed with ammonia solution (0.5 *M*, pH 11.5) and equilibrated with NaCl–Tris buffer until the absorbance reading at 280 nm (1-cm cell) corresponded to that of the ambient buffer. Pooled whole human serum (0.1 ml, 1.12 mg IgG) was loaded onto the column which was then washed with NaCl–Tris buffer (0.5 *M*/0.1 *M*, pH 7.2) until the absorbance of the eluate at 280 nm was negligible. The immuno-adsorbed IgG was then eluted with ammonia solution (0.5 *M*, pH 11.5) in 4-ml fractions until the absorbance of the eluate at 280 nm was negligible (< 0.005). The column was re-equilibrated in NaCl–Tris buffer before another adsorption–desorption cycle commenced.

The eluates were monitored on an LKB Uvicord analyser at 280 nm and the fractions collected on an LKB automatic fraction collector. Both the pooled unadsorbed and pooled desorbed IgG fractions were quantitated by the Mancini technique[12] using NIRDL serum standard (11.6 mg IgG per ml) for calibration.

RESULTS

Yield of F(ab')$_2$ antibody fragments

In Fig. 1 is illustrated the gel filtration fractionation of the sodium sulphate precipitate resulting from the pepsin digested antigen–antibody precipitate. The yield of F(ab')$_2$ antibody fragments from the sheep anti-human IgG–human IgG precipitate is summarized in Table I. After Sephadex gel filtration, approximately 6 mg F(ab')$_2$ were obtained from the initial antigen–antibody precipitate (19.5 mg), a 30% yield. However, assuming that 6 mg F(ab')$_2$ are equivalent to 9 mg native antibody[4], some 44% of the immunoglobulin in the original precipitate was recovered. This accords favourably with the results reported by Lachman[4].

Activity of F(ab')$_2$ fragments

The activity of the F(ab')$_2$ fraction obtained by Sephadex gel filtration was

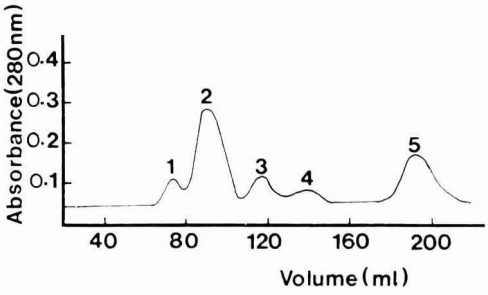

Fig. 1. Elution profile of the dissolved sodium sulphate precipitate from pepsin digested antigen–antibody precipitate. A solution (2.5 ml, 4.4 mg ml^{-1}) of the precipitate was applied to a column of Sephadex G-150 (81 × 2.2 cm) equilibrated with sodium chloride–Tris buffer (pH 7.2). Peaks: 1, soluble complexes; 2, $F(ab')_2$, the only peak to show antibody activity; 3, p Fc′ species (dimers of the C_H3 homology region of the antibody molecule); 4, polypeptides; 5, the Bence Jones protein marker.

TABLE I

SUMMARY OF THE QUANTITATIVE YIELD OF $F(ab')_2$ FRAGMENTS FROM SHEEP ANTI-HUMAN IgG–HUMAN IgG PRECIPITATE AND ITS PEPSIN DIGEST

Antigen–antibody precipitate (mg)	*Protein yield after pepsin digestion (mg)*	*Protein loaded on Sephadex column (mg)*	*$F(ab')_2$ yield (mg)*	*Yield of $F(ab')_2$ from antigen–antibody precipitate (%)*
19.5	12.5	11.1	5.9	30.0

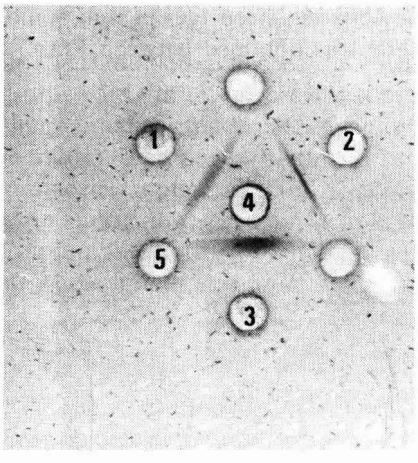

Fig. 2. Ouchterlony double immunodiffusion analysis of sheep anti-human $F(ab')_2$ antibody against human IgG. The central well (4) contained the $F(ab')_2$ fraction (1 μl, 6.3 μg) and wells 1, 2 and 3 contained 6.0, 2.5 and 60 μg human IgG respectively; well 5 contained 6 μg sheep IgG. The wells were punched in a 1% agarose gel on a glass plate (4 × 1 cm).

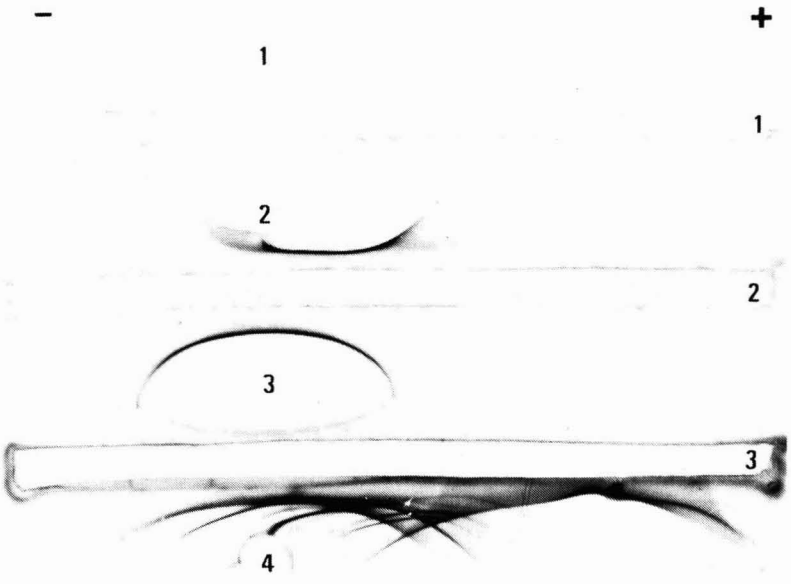

Fig. 3. Immunoelectrophoretic analysis of sheep anti-human $F(ab')_2$. Wells: 1 and 3, 2 μl human IgG, 6.34 mg ml^{-1}; 2 and 4, 1 μl NIRDL standard, 11.6 mg IgG per ml. Troughs: 1, $F(ab')_2$; 2, sheep anti-human IgG; 3, sheep anti-whole human serum.

determined by Ouchterlony double immunodiffusion (Fig. 2) and immunoelectrophoresis (Fig. 3). Pronounced precipitin lines were observed between the $F(ab')_2$ in well 4 and the various antigen concentrations in wells 1, 2 and 3. It is noticeable that the sharpest precipitin line was between wells 2 and 4, indicating optimal antigen–antibody concentrations. There was no precipitin line between sheep IgG in well 5 and $F(ab')_2$ in well 4. In Fig. 2 a distinct precipitin line was obtained between $F(ab')_2$

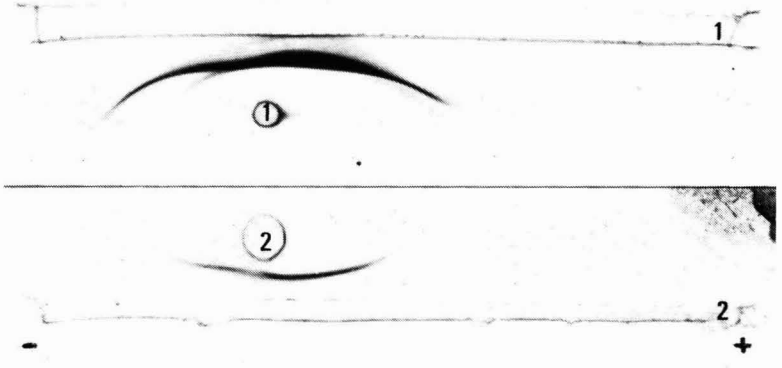

Fig. 4. Immunoelectrophoretic analysis of affinity-purified IgG using the cyanogen bromide-activated Sepharose 4B–sheep anti-human IgG $F(ab')_2$ immunoadsorbent. Wells: 1, 10 μl NIRDL standard; 2, 10 μl eluted IgG, 1 μg. Troughs: 1 and 2, sheep anti-whole human serum.

TABLE II

SUMMARY OF THE QUANTITATIVE PERFORMANCE OF THE CYANOGEN BROMIDE-ACTIVATED SEPHAROSE 4B–SHEEP ANTI-HUMAN $F(ab')_2$ IMMUNOADSORBENT COLUMN

ND = Not determined.

Description	*Cycle*				
	1	*2*	*3*	*4*	*5*
IgG applied (mg)	1.12	1.12	1.12	1.12	1.12
IgG unadsorbed (mg)	0.9	1.1	1.0	0.9	0.6
(%)	(80.4)	(93.7)	(89.3)	(80.4)	(53.6)
IgG adsorbed (mg)	0.22	0.07	0.12	0.22	0.52
(%)	(19.7)	(6.3)	(10.7)	(19.7)	(46.4)
IgG eluted (mg)	0.22	ND	ND	0.22	0.52
(%)	(100.0)			(100.0)	(100.0)
Potential binding capacity (mg IgG)	0.2	0.2	0.2	0.2	0.2
Purity	Very good			Very good	Very good

(trough 1) and human IgG (well 1). The $F(ab')_2$ precipitin line corresponded with similar IgG precipitin lines obtained with the human serum standard (NIRDL) against sheep anti-human IgG (trough 2) and against sheep anti-whole human serum.

Purification of human serum IgG with cyanogen bromide-activated Sepharose 4B-$F(ab')_2$ immunoadsorbent column

The performance of the immunoadsorbent column is summarized in Table II. The yield of IgG was consistently 100% from five serial adsorption–desorption cycles. It is noticeable that the amount of adsorbed IgG corresponded to the binding capacity of the column (0.2 mg IgG) for the first and fourth cycles. Nevertheless, some variation in adsorbed IgG was observed in cycles 2 and 3. The amount of adsorbed IgG in the fifth cycle was approximately twice the value of the potential column binding capacity. This could be attributed to non-specific retention of IgG by the matrix. The purity of the eluted IgG fraction was excellent as determined by immunoelectrophoresis (Fig. 4). The eluted IgG fraction (well 2) gave a single precipitin line against sheep anti-whole human serum. This line corresponded to the IgG precipitin lines formed by human serum standard (NIRDL) in well 1 against sheep anti-whole human serum (trough 2).

DISCUSSION

The separation of affinity-purified $F(ab')_2$ antibodies from immune precipitates by pepsin digestion of the labile Fc region of the immunoglobulin molecule allows the preparation of class specific antibodies. Some pitfalls of the technique are firstly the contamination of $F(ab')_2$ antibody peak with $F(ab')_2$ from the antigen if the antigen is incompletely complexed with antibodies. Since no precipitin line was detected between sheep anti-human IgG and the purified $F(ab')_2$ fraction, this situation did

not occur under the conditions described herein. Secondly, the possible occlusion of antibody binding sites was not a dominant feature in view of the well defined precipitin lines obtained. Although the yield of $F(ab')_2$ antibody is dependent on the pepsin lability of the Fc region, the yield (30%) obtained in this study is quite reasonable for the IgG–anti-IgG system.

The use of cyanogen bromide-activated Sepharose 4B-$F(ab')_2$ immunoadsorbent offers a simple, quicker approach for the purification of class specific antigens. Many conventional intermediate steps are eliminated. High binding capacity immunoadsorbents can be prepared, resulting in negligible non-specific adsorption and requiring minimum antigen loading. After five serial adsorption–desorption cycles there was no drastic change in the column performance with respect to protein yield and purity. This technique has the potential for isolating antigens present in low concentration in plasma, urine and tissue fluid.

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REFERENCES

1 A. Nisonoff, F. C. Wisler, L. N. Lipman and D. L. Woernely, *Arch. Biochem. Biophys.*, 89 (1960) 230.
2 A. Nisonoff, G. Markus and F. C. Wisler, *Nature (London)*, 189 (1961) 293.
3 E. Hurwitz, R. Maron, R. Arnon and M. Sela, *Cancer Biochem. Biophys.*, 1 (1976) 197.
4 P. J. Lachman, *Immunochemistry*, 8 (1971) 81.
5 J. F. Kennedy and J. A. Barnes, *Int. J. Biol. Macromol.*, 2 (1980) 289.
6 J. A. Barnes and J. F. Kennedy, *Immunochemistry*, (1981) in press.
7 D. R. Stanworth and M. W. Turner, in D. M. Weir (Editor), *Handbook of Experimental Immunology*, Blackwell, Oxford, 3rd ed., 1967, p. 6.1.
8 L. M. Killingsworth and J. Savory, *Clin. Chem.*, 18 (1972) 335.
9 O. H. Lowey, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
10 O. Ouchterlony, *Progr. Allergy*, 5 (1958) 1.
11 P. Grabar and C. A. Williams, *Biochim. Biophys. Acta*, 10 (1953) 193.
12 G. Mancini, A. D. Carbonara and J. F. Heremans, *Immunochemistry*, 2 (1965) 235.

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EVALUATION OF A LIQUID–LIQUID EXTRACTION TECHNIQUE FOR WATER POLLUTANTS

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SUMMARY

A simple liquid–liquid extraction technique was evaluated and judged to be useful for semiquantitative determination of 37 organic pollutants present in water at concentrations of 50 μg/l or better. Gas chromatographic analysis of hexane extracts showed detection limits of $\leqslant 5$ μg/l for 30 compounds, recoveries of $\geqslant 80\%$ for 20 compounds and, generally, peak area precision of better than $\pm 5\%$ for triplicate sample analyses. Storage of aqueous standard solutions at 4°C for 4 weeks did not significantly affect recovery values.

INTRODUCTION

A wide range of organic compounds has been found in potable water supplies[1–4] and there is concern that ingestion of water containing these compounds may pose a potential hazard to human health. To aid in the health hazard assessment, Canadian potable water supplies have been surveyed[5–7] for the occurrence of selected organic contaminants. An analytical method for the determination of semi-volatile organic compounds ranging in boiling point up to 200°C was required to complement the head space and XAD-2 macroreticular resin analytical techniques which were used for these surveys. As part of its master scheme for the analysis of 114 organic priority pollutants[8], the U.S. Environmental Protection Agency has proposed a number of methods utilizing liquid–liquid extraction (LLE) as the first step of the analytical procedure. Water samples are extracted three times with methylene chloride and the combined extracts are reduced to a small volume while the solvent is changed to hexane. Simple, one step, liquid–liquid extraction methods employing pentane[9–11], hexane[9,12–14], isooctane[9,11], methylcyclohexane[9,15], cyclohexane–diethyl ether[16] and benzene–hexane[17] as the extractant have been reported for the determination of trihalomethanes and a few other organics. We now report the evaluation of a single step, LLE method of analysis for a representative group of 41 semi-volatile organic pollutants. The compounds investigated have either been listed as compounds of concern[8,18] or have been found in potable water supplies[1–4].

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EXPERIMENTAL

Equipment

All analyses were performed on a Hewlett-Packard Model 5840 gas chromatograph equipped with a ^{63}Ni electron-capture detector (ECD), a flame ionization detector (FID) and a Tracor Model 310 Hall electrolytic conductivity detector (HElCD). The signal from the HElCD was processed by a Spectra-Physics 4000 chromatography data system. The HElCD was operated in the chloride mode with the furnace temperature at 840°C, a hydrogen flow-rate of 10 ml/min and a 2-propanol–water (50:50, v/v) electrolytic solvent flow-rate of 0.2 ml/min. The ECD and FID were held at 300°C and hydrogen and air flow-rates respectively for the flame were 20 ml/min and 250 ml/min. Columns were attached to a Hewlett-Packard capillary column inlet system, held at 180°C and operated in the splitless mode. An inlet nitrogen gas purge at 75 ml/min was initiated 0.25 min after each injection for FID analyses. Nitrogen passed through Oxiclear and molecular sieve traps was used as column carrier, inlet purge and detector makeup gases. Makeup gas flows of about 25 ml/min were used for the FID and ECD. A Varian Model 8020 autosampler was interfaced with the gas chromatograph and injected 4-μl sample aliquots for FID and 2-μl sample aliquots for ECD and HElCD analyses.

The GC columns and conditions were:

(i) an OV-17 support-coated open tubular (SCOT) stainless-steel column (15 m × 0.5 mm I.D.) with a carrier gas flow of about 4 ml/min. After each injection the column temperature was maintained at 60°C for 3 min and was then raised at a rate of 8°C/min to 150°C where it was maintained 18 min before the oven was cooled;

(ii) a nickel column (1.8 m × 2 mm I.D.) packed with 0.1% SP-1000 on Carbopack C (80–100 mesh), and with a carrier gas flow-rate of about 20 ml/min. After each injection the column temperature was maintained at 100°C for 3 min and was then raised at a rate of 15°C/min to 220°C where it was maintained for 28 min before the oven was cooled.

Confirmatory analyses were performed on a Model 4000 Finnigan mass spectrometer–gas chromatograph coupled with a Model 6000 data system and utilizing a glass column (1.8 m × 2 mm I.D.) packed with 3% OV-17 on Gas-Chrom Q (100–120 mesh). A Burrell Model 75 wrist action shaker set at 3° was used for mechanical agitation of containers during solvent extraction procedures. A Brinkmann 5-ml capacity Dispensette was used to deliver hexane aliquots with a precision of better than ±0.1%. Culture tubes (capacity 32 ml), amber glass bottles (capacities 120, 500, 1000 and 4000 ml), autosampler vials (capacity 2 ml) and other glass containers were heated at 400°C for several hours, cooled to about 50°C and then sealed with PTFE-coated silicon disks and screw caps with centered holes.

Reagents

Purified water (pH 5.9) was prepared by irradiating distilled, deionized water for 5 h at 254 nm in a 5-l capacity vessel[19]. Stock tap-water (pH 8.7) was allowed to stand 2 days in an open container before use. The purity of all organic compounds was determined by analysis of hexane solutions on at least two chromatographic systems.

Primary standard solutions in methanol (25.0 ml) and containing 0.20 g/l of the

compounds listed in Table I were prepared in sealed culture tubes. Such primary standard solutions containing one compound only (single component), a group of compounds (groups A, B, C, D, E, F, G and H) and all compounds (multicomponent, groups A–H) were used to prepare corresponding aqueous standard solutions and standard solutions in hexane. Aqueous standard solutions containing 1, 5, 20 and 100 μg/l of each compound in water were prepared in sealed glass bottles or a volumetric flask. Standard solutions containing 0.01, 0.04, 0.20, 0.80 and 4.0 mg/l of each compound in hexane were prepared in sealed culture tubes. All aqueous solutions were prepared in bottles containing thiosulphate so as to provide 8.3 mg $Na_2S_2O_3 \cdot 5\ H_2O$ per 100 ml aqueous solution.

Procedures

Appropriate blanks were prepared and analyzed during all tests. Duplicate samples (two bottles) were used during exploratory tests and triplicate samples were used for recovery tests. Single component solutions in hexane were used for peak identification during chromatographic analyses and, where necessary, gas chromatography–mass spectrometry (GC–MS) was applied for confirmation. Multicomponent aqueous standard solutions and the OV-17 SCOT column with the ECD were used for the exploratory tests.

Choice of extraction solvent. A 5-ml aliquot of the aqueous solution (20 μg/l, in purified water) was removed from each full, 120-ml capacity, bottle and a 3-ml aliquot of pentane, hexane, hexane saturated with methanol, 15% (v/v) acetone in hexane, isooctane or benzene was added to the bottle. Extractions were completed by shaking the bottles for 30 min, storing them for 3 days at 25°C and transferring the organic phase into autosampler vials.

Hexane–water ratio. A portion (4, 7, 7 and 15 ml respectively) of the aqueous solution (1 μg/l, in purified water) in full 120, 500, 1000 or 4000 ml capacity bottles was removed from and a measured volume (3, 5, 5 and 10 ml respectively) of hexane was added to each bottle. Extractions were completed by the foregoing procedure.

Extraction technique. Aqueous standard solutions (5 μg/l, in tap-water) were prepared in 120-ml capacity bottles and in a 2-l volumetric flask. Within 24 h of preparation the solution in the volumetric flask was distributed into 120-ml capacity bottles. The full bottles were stored 0, 24 or 72 h before a 5-ml aliquot of the aqueous solution was withdrawn from and a 3-ml aliquot of hexane was added to each container. Extractions were completed by shaking the bottles for 1, 4 or 24 h, storing them for 0, 24 or 72 h and then transferring the organic phase into autosampler vials. Selected containers were drained and the inner walls were rinsed with measured volumes of hexane which were subsequently analyzed.

Storage effect. Aqueous standard solutions (20 μg/l, in pure water) were prepared in six, 120-ml capacity, bottles. Three bottles were stored 24 h at 25°C before a 5-ml aliquot of the aqueous solution was removed from, and a 3-ml portion of hexane was added to each bottle. Extraction was completed by shaking each bottle for 24 h, storing it for 24 h and transferring the organic phase for analysis in an autosampler vial. The remaining three bottles were stored at 4°C for 4 weeks and stored at 25°C for 24 h before extraction and analysis of the extracts.

Recovery studies. Aqueous solutions, each containing a group of compounds at concentration levels of 1, 5, 20 or 100 μg/l in pure water were prepared and extracted

in 120-ml capacity bottles for recovery studies utilizing the OV-17 SCOT column. Solutions containing groups A + B + C, groups D + E + F, group H + chloroform + trichloroethylene + 1,1,1-trichloroethane or group G + isophorone at the 20-μg/l concentration level only were prepared in both pure and tap-water in 120-ml capacity bottles for recovery studies utilizing the 0.1 % SP-1000 column. Extractions were conducted as described for the storage effect test.

Quantitative determination. Calibration curves were constructed by plotting concentration against peak area obtained for analyses of standard solutions in hexane. Percent recovery of an extracted compound was determined by comparing the mean peak area from analyses of extracts from triplicate solutions with the calibration curve peak area corresponding to the concentration calculated from 100 % recovery of the compound in the organic layer. In some instances peak height was used for quantitation. Detection limits were estimated from the results of recovery studies.

RESULTS AND DISCUSSION

Since the FID, ECD and HElCD were used for compound identification, the choice of extractant was limited to those solvents which were compatible for use with all three detectors. Six solvent systems were evaluated for extraction of the compounds in groups A–H (Table I). It was found that their extraction efficiency decreased in the order pentane $\geqslant$ hexane $>$ hexane saturated with methanol $>$ isooctane $>$ 15 % (v/v) acetone in hexane $\geqslant$ benzene, as estimated by comparison of the sum of peak areas. Also, pentane and hexane peaks showed the least interference with standard peaks during gas chromatography. Hexane was selected as the extraction solvent since the volatility of pentane makes it difficult to handle, particularly when it is used with an automatic injector[13].

Although use of larger sample bottles permitted an increase in the water–hexane volume ratio and, hence, an increased compound concentration in the hexane extract, it was considered that the 120-ml capacity bottles would be most convenient for shipping, storage and extraction of large numbers of survey samples. Also, extractions utilizing the latter containers allowed detection on the ECD of many compounds when present in water at a concentration of 1 μg/l. In order to minimize the number of manipulations and the loss of analyte, the hexane extraction was conducted in the sample bottle. Removal of 5 ml of water from the sample bottle and addition of 3 ml hexane allowed adequate mixing of the liquid phases, easy removal of the hexane for subsequent GC analysis and a potential concentration factor of 115/3.

A variety of short-term storage conditions and extraction procedures were investigated for fortified (5 μg/l) water samples and some results are shown in Table II. Percent recoveries obtained for extractions conducted with 4-h and 24-h agitation periods were essentially the same for any particular compound, whereas only 1 h of agitation resulted in considerably lower recoveries. Storage of contents after 24 h of agitation did not significantly change recovery values. With the exception of the 1,1,2,2-tetrachloroethane results, percent recovery values for any particular compound were essentially the same, whether tap-water was spiked directly in the extraction vessel or in a volumetric flask from which the solution was distributed into

TABLE I

COMPOUNDS INVESTIGATED

Formula	*Compound*	*Formula*	*Compound*
Group A		*Group F*	
C_6H_6	Benzene	$C_{10}H_8$	Naphthalene
$C_6H_5CH_3$	Toluene	$(CH_3CH_2CH_2)_2NNO$	N-Nitrosodi-*n*-propylamine
$C_6H_5C_2H_5$	Ethylbenzene	$(CH_3)_2NNO$	N-Nitrosodimethylamine
o-$C_6H_4(CH_3)_2$	*o*-Xylene	$CH_2ClCH_2OCHCH_2$	2-Chloroethyl vinyl ether
m-$C_6H_4(CH_3)_2$	*m*-Xylene	$CCl_2CClCClCCl_2$	Hexachlorobutadiene
p-$C_6H_4(CH_3)_2$	*p*-Xylene		
		Group G	
Group B		C_6H_5OH	Phenol
C_6H_5Cl	Chlorobenzene	2-ClC_6H_4OH	2-Chlorophenol
o-$C_6H_4Cl_2$	*o*-Dichlorobenzene	2,4-$Cl_2C_6H_3OH$	2,4-Dichlorophenol
p-$C_6H_4Cl_2$	*p*-Dichlorobenzene	2,4,6-$Cl_3C_6H_2OH$	2,4,6-Trichlorophenol
1,2,4-$C_6H_3Cl_3$	1,2,4-Trichlorobenzene		
$C_6H_5NO_2$	Nitrobenzene	*Group H*	
		$(CH_2ClCH_2)_2O$	Bis(2-chloroethyl) ether
Group C		$(CH_2Cl)_2O$	Bis(chloromethyl) ether
CCl_2CCl_2	Tetrachloroethylene		
$CHCl_2CH_2Cl$	1,1,2-Trichloroethane	*Other*	
$CHCl_2CHCl_2$	1,1,2,2-Tetrachloroethane	CH_2Cl_2	Dichloromethane
CCl_3CCl_3	Hexachloroethane	$CHCl_2CH_3$	1,1-Dichloroethane
		CCl_3CH_3	1,1,1-Trichloroethane
Group D		$CHCl_3$	Chloroform
$CH_2ClCHClCH_3$	1,2-Dichloropropane	CH_2ClCH_2Cl	1,2-Dichloroethane
$CH_2ClCHCHCl$	1,3-Dichloropropene	CCl_2CHCl	Trichloroethylene
$CHCl_2CClCHCl$	1,2,3,3-Tetrachloropropene	$C_9H_{14}O$	Isophorone
$CCl_2CClCHCl_2$	1,1,2,3,3-Pentachloropropene	2-$ClC_{10}H_7$	2-Chloronaphthalene
Group E			
$CHBr_2Cl$	Chlorodibromomethane		
$CHBr_3$	Bromoform		
C_5Cl_6	Hexachlorocyclopentadiene		

extraction vessels. This indicated that sorption of the detected compounds onto glass walls of a flask containing an aqueous standard solution was negligible. Since rinses of used extraction vessels and the empty stock solution flask were shown to be devoid of tested compounds, significant retention of these organics on container walls is unlikely. The relatively low recovery of 1,1,2,2-tetrachloroethane from aqueous solutions prepared in the extraction vessel as compared to recovery from the stock solution distributed into the extraction vessel could not be explained. For the chosen extraction technique, *i.e.*, 24-h agitation followed by 24-h storage, precision of peak area values from duplicate sample analyses were usually within $\pm 5\%$ of the mean peak area value.

Since survey samples may be stored for some time before analysis, the effect of sample storage on recovery values was investigated. Table III reports the effect of storage on recovery values for fortified (20 μg/l) water samples stored for either 1 day at 25°C or 4 weeks at 4°C. Percent recoveries for the two sets of samples were

TABLE II

EVALUATION OF HEXANE EXTRACTION PROCEDURE

Duplicate sample extracts analyzed on OV-17 SCOT column and ECD.

Treatment details	*Time (h)*						
Stored	24*	24*	72*	24	24	24	24
Agitated	1	24	24	1	4	24	24
Stored	24	24	24	24	24	24	72
Compound	*Recovery (%)*						
$CH_2ClCHCHCl$	50	59	50	44	56	52	51
$CCl_2CCl_2 + CHCl_2CH_2Cl$	75	82	86	56	78	78	76
$CHBr_2Cl$	58	72	78	59	76	75	74
$CHBr_3$	48	63	68	51	70	68	69
$CHCl_2CHCl_2$	36	48	42	45	63	63	64
$CHCl_2CClCHCl$	42	62	66	40	65	66	65
p-$C_6H_4Cl_2$	28	44	53	24	43	49	50
$CCl_3CCl_3 + o$-$C_6H_4Cl_2 + CHCl_2CClCHCl$	47	69	75	45	69	70	68
$CCl_2CClCHCl_2$	50	73	76	48	72	74	72
1,2,4-$C_6H_3Cl_3 + CCl_2CClCClCCl_2$	45	68	73	43	69	68	67

* Fortified in bottle.

TABLE III

EFFECT OF STORAGE ON RECOVERY

Hexane extracts analyzed on OV-17 SCOT column and ECD.

Compound	*24 h storage (25°C)**		*4 weeks storage (4°C)**	
	Recovery (%)	*R.S.D. (%)*	*Recovery (%)*	*R.S.D. (%)*
$CH_2ClCHCHCl$	64	5.9	56	7.1
$CCl_2CCl_2 + CHCl_2CH_2Cl$	77	2.2	102	10.1
$CHBr_2Cl$	74	1.8	80	8.4
$CHBr_3$	70	1.3	73	7.3
$CHCl_2CHCl_2$	70	2.9	50	1.2
$CHCl_2CClCHCl$	74	2.4	63	9.3
$CCl_3CCl_3 + o$-$C_6H_4Cl_2 + CHCl_2CClCHCl$	89	1.3	91	4.8
$CCl_2CClCHCl_2$	76	2.8	80	12.0
1,2,4-$C_6H_3Cl_3 + CCl_2CClCClCCl_2$	88	0.8	83	13.7

* Three bottles.

generally in good agreement, thus indicating that storage at 4°C maintains the integrity of the water sample. It has previously been shown that bottled water samples may be stored for at least a few weeks without a significant change in trihalomethane[20] or total organic carbon[21] values.

TABLE IV

RETENTION TIMES AND DETECTION LIMITS FROM HEXANE EXTRACT ANALYSES OF 41 AQUEOUS POLLUTANTS

Retention times are relative to injection time. Detection limits refer to the concentration of compounds in purified water which gave clearly defined peaks upon analysis of the hexane extract. ND = Not detected at ≤ 100 μg/l; NA = not analyzed.

Compound	*OV-17 SCOT column*				*0.1% SP-1000 column*		
	Retention time (min)	*Detection limit (μg/l)*			*Retention time (min)*	*Detection limit (μg/l)*	
		FID	*ECD*	*HElCD*		*FID*	*ECD*
CH_2Cl_2	2.2	ND*	ND*	<1	–	ND	ND
$CHCl_2CH_3$	2.4	ND*	ND*	<1	–	ND	ND
$CHCl_3$	3.2	ND*	<1	<1	1.4	ND*	<1
CCl_3CH_3	3.6	ND*	<1	10	2.0	ND*	<1
CH_2ClCH_2Cl	4.0	ND*	20	2	–	ND	ND
CCl_2CHCl	4.6	ND*	<1	2	3.9	ND*	2
C_6H_6	–	ND	ND	ND	–	ND	ND
$CH_2ClCHClCH_3$	5.0	50	5	5	3.1	ND*	20
$C_6H_5CH_3$	5.3	5	ND	ND	8.6	5	ND
$CH_2ClCH_2OCHCH_2$	5.3	50	ND	NA	–	ND*	–
CCl_2CCl_2	5.8	10	<1	5	7.8	ND*	<1
$CH_2ClCHCHCl$	5.9	20	5	5	3.4, 4.4**	ND*	5
$CHCl_2CH_2Cl$	6.0	20	<1	<1	4.4	ND*	10
$CHBr_2Cl$	6.4	50	<1	<1	4.5	ND*	<1
C_6H_5Cl	7.6	5	ND	1	9.0	10	ND
$C_6H_5(C_2H_5)$	7.6	2	ND	ND	10.3	5	ND
m-$C_6H_4(CH_3)_2$	7.7	2	ND	ND	11.6	5	ND
p-$C_6H_4(CH_3)_2$	7.7	2	ND	ND	11.9	5	ND
o-$C_6H_4(CH_3)_2$	8.4	2	ND	ND	11.9	5	ND
$CHBr_3$	8.9	50	<1	1	6.1	ND*	<1
$CHCl_2CHCl_2$	9.6	5	<1	<1	7.4	ND*	<1
p-$C_6H_4Cl_2$	11.4	2	5	<1	13.7	10	2
$(CH_2ClCH_2)_2O$	11.9	20	ND	1	9.5	20	20
CCl_3CCl_3	12.0	10	<1	<1	9.0	ND	1
o-$C_6H_4Cl_2$	12.1	2	5	<1	13.7	10	2
$CHCl_2CClCHCl$	12.1, 10.5**	10	<1	1	9.6, 8.8**	50	1
$CCl_2CClCHCl_2$	12.5	10	<1	<1	11.7, 12.5**	ND	5
$C_6H_5NO_2$	14.5	5	20	ND	12.8	10	5
$CCl_2CClCClCCl_2$	14.6	5	<1	NA	12.3	50	1
1,2,4-$C_6H_3Cl_3$	14.8	2	<1	<1	26.7	10	5
$C_{10}H_8$	15.5	2	ND	ND	34.8	20	ND
$(CH_3)_2NNO$	–	ND	ND	NA	9.1	50	ND
$(CH_3CH_2CH_2)_2NNO$	–	ND	ND	NA	11.7	50	ND
C_6H_5OH	–	ND	ND	ND	9.7	10	ND
2-ClC_6H_4OH	–	ND	ND	ND	11.5	20	20
2,4-$Cl_2C_6H_3OH$	–	ND	ND	ND	21.0	20	5
2,4,6-$Cl_3C_6H_2OH$	–	ND	ND	ND	56.4	ND	10
$C_9H_{14}O$	17.9	ND	ND	ND	14.5	10	20
C_5Cl_6	–	ND	ND	ND	–	ND	ND
$(CH_2Cl)_2O$	–	ND	ND	ND	9.0	ND	ND
2-$ClC_{10}H_7$	21.3	ND	ND	ND	–	ND	ND

* Interference by hexane peak.

** Two peaks; major peak listed first.

TABLE V

PERCENT RECOVERY AND R.S.D. OBTAINED FOR HEXANE EXTRACTION OF AQUEOUS STANDARD SOLUTIONS

Italicized values were obtained on the SP-1000 column and are listed only for instances when corresponding OV-17 SCOT column results were not available. Results were calculated for triplicate sample analyses. NA = Not analyzed; D = detected but not quantitated. Values obtained from peak height measurements are given in square brackets.

Compound	*Detector*	*1 μg/l*		*5 μg/l*		*20 μg/l*		*100 μg/l*	
		Recovery (%)	*R.S.D. (%)*	*Recovery (%)*	*R.S.D. (%)*	*Recovery (%)*	*R.S.D. (%)*	*Recovery (%)*	*R.S.D. (%)*
$CHCl_3$	ECD	NA	–	NA	–	*83*	3.3	NA	–
CCl_3CH_3	ECD	NA	–	NA	–	*91*	4.5	NA	–
CCl_2CHCl	ECD	NA	–	NA	–	*94*	5.3	NA	–
$CH_2ClCHClCH_3$	FID	–	–	–	–	–	–	36	16
	ECD	–	–	–	–	D	–	84	3.2
$C_6H_5CH_3$	FID	–	–	D	–	[83]	1.2	D	–
$CH_2ClCH_2OCHCH_2$	FID	–	–	–	–	–	–	[29]	12
CCl_2CCl_2	FID	–	–	D	–	D	–	[88]	1.8
	ECD	D	–	D	–	*78*	1.7	D	–
$CH_2ClCHCHCl$	FID	–	–	–	–	–	–	35	2.7
	ECD	–	–	–	–	43	9.9	41	2.4
$CHCl_2CH_2Cl$	FID	–	–	–	–	D	–	57	5.1
	ECD	D	–	D	–	*60*	25	D	–
$CHBr_2Cl$	FID	–	–	–	–	–	–	[90]	5.3
	ECD	D	–	68	3.1	70	1.9	80	0.5

C_6H_5Cl	FID	–	–	D	–	[91]	2.1	[94]	1.9
$C_6H_5C_2H_5$	FID	–	–	80	5.2	83 [85]	2.3, 2.3	81	1.4
m-$C_6H_4(CH_3)_2$ } *p*-$C_6H_4(CH_3)_2$ }	FID	–	–	85	1.7	85 [84]	2.2, 1.6	78	1.4
o-$C_6H_4(CH_3)_2$	FID	–	–	93	8.1	96 [85]	5.9, 1.5	80	1.3
$CHBr_3$	FID	–	–	–	–	–	–	29 [90]	12, 5.3
	ECD	30	1.0	70	3.0	77	2.4	68	1.6
$CHCl_2CHCl_2$	FID	–	–	–	–	49	4.4	68 [70]	2.3, 2.0
	ECD	75	36	70 [75]	3.6, 2.7	86	22	69	0.2
p-$C_6H_4Cl_2$	FID	–	–	99	7.0	89 [86]	1.7, 0.9	91 [92]	1.5, 2.2
	ECD	–	–	–	–	79 [93]	22, 4.7	92 [100]	2.4, 1.8
o-$C_6H_4Cl_2$	FID	–	–	77	18	94 [85]	4.3, 2.6	90 [92]	1.3, 2.8
	ECD	–	–	–	–	88 [92]	17, 6.9	94	2.3
$(CH_2ClCH_2)_2O$	FID	–	–	–	–	–		[16]	4.8
CCl_3CCl_3	FID	–	–	–	–	89	3.7	84 [87]	0.8, 1.5
	ECD	103	38	90	3.1	97	4.7	94	0.4
$CHCl_2CClCHCl$	FID	–	–	–	–	69	3.7	82 [84]	0.6, 2.3
	ECD	96	11	39	24	70	2.3	87	1.4
$CCl_2CClCHCl_2$	FID	–	–	–	–	72	5.3	92 [90]	5.2, 2.7
	ECD	102	9.0	46	18	82	2.0	90	1.4
1,2,4-$C_6H_3Cl_3$	FID	–	–	87	48	87 [90]	5.7, 2.6	85 [95]	6.0, 2.4
	ECD	76	16	86	21	94	13	93	2.1
$C_{10}H_8$	FID	–	–	54	14	69 [75]	0.9, 0.5	69	1.0
2,4-$Cl_2C_6H_3OH$	ECD	–	–	–	–	*20*	11	–	–
2,4,6-$Cl_3C_6H_2OH$	ECD	–	–	–	–	*18*	4.7	–	–
$C_6H_5NO_2$	FID	–	–	D	–	39 [40]	2.5, 4.7	36 [37]	6.1, 5.0
	ECD	–	–	–	–	*27*	0.7	38	31
$CCl_2CClCClCCl_2$	FID	–	–	D	–	68 [86]	6.1, 2.2	81	5.2
	ECD	133	21	93	2.7	97	1.7	99	1.9

Results listed in Table IV show that a detection limit of $\leqslant 5$ μg/l was obtained for many of the compounds in fortified water. Blank analysis results generally showed no significant interfering peaks for purified water. Percent recoveries obtained for hexane extraction of aqueous standard solutions fortified at 1, 5, 20 and 100 μg/l are reported in Table V for those compounds whose detection limit (FID or ECD) was below 100 μg/l. Benzene could not be quantitated since it coeluted with hexane on the GC columns. Hexachlorocyclopentadiene which is believed to degrade quickly in solution[22] could not be quantitated in the aqueous solution or in the hexane extract. Analyses conducted on the 0.1% SP-1000 column generally gave similar recovery values as those reported (Table V) for the OV-17 SCOT column. Recovery values calculated from peak height values were comparable to those calculated from peak areas and were usually more precise (Table V). When all results obtained at well above the detection limit, *i.e.*, usually at 20 and 100 μg/l, and with a precision of $\leqslant 6\%$ R.S.D. were considered, then twenty compounds showed recoveries of $\geqslant 80\%$. For the remaining compounds, 1,1,2-trichloroethane, 1,1,2,2-tetrachloroethane and naphthalene had recoveries of 57-75%, and 2-chloroethyl vinyl ether, bis(2-chloroethyl) ether, 1,3-dichloropropene, nitrobenzene, 2,4-dichlorophenol and 2,4,6-trichlorophenol had recoveries of $\leqslant 41\%$. For compounds which were also extracted from fortified tap-water, the recovery values were similar to those obtained for extractions from purified water.

The variations in recovery values for a particular compound at several concentration levels (Table V) was usually due to inconsistent and/or inaccurate integration of peak areas. This sometimes occurred for analyses at concentrations well above the compound detection limit and when the peak shape appeared to be clearly defined. These difficulties were primarily due to the fact that integration parameters had to be set at the beginning of the automated GC analyses and, therefore, could not be optimized for each sample and compound. This does not appear as a problem in those studies[9–17] where only a small number of compounds were being investigated and integration parameters and GC conditions could easily be optimized. For the 41 compounds investigated in this study GC conditions could not be optimized for each component. Among several columns tested, no single GC column could be found which would resolve all 41 compounds, but the use of two columns, an OV-17 SCOT column and a column packed with 0.1% SP-1000–Carbopack C, did permit resolution of most of the compounds. In addition the use of three different GC detectors also aided in compound identification and quantitation (Table V).

Compound identification and quantitation can be simplified by the use of concentration and fractionation procedures as reported for the U.S.A. priority pollutants master scheme[8] and/or by use of GC–MS. However, these procedures are less amenable for use in large scale surveys or for laboratories lacking the specialized equipment required.

CONCLUSION

The single step LLE procedure, although considered to give quantitative results for studies involving a limited number of compounds, has some limitations when applied to water samples containing large numbers of compounds. Analytical results for the latter type of sample are likely to be semiquantitative and require careful

evaluation. The hexane extraction procedure is, however, very useful as a simple, rapid, screening method which can be applied to complement other methods for the analysis of organic pollutants in potable water.

ACKNOWLEDGEMENTS

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REFERENCES

1 W. M. Shakelford and L. H. Keith, *Frequency of Organic Compounds Identified in Water, EPA-600/4-76-062*, U.S. Environmental Protection Agency, Springfield, VA, December 1976.
2 A. W. Garrison, *Ann. N.Y. Acad. Sci.*, 298 (1977) 2.
3 J. A. Cotruvo and C. Wu, *J. Amer. Water Works Assoc.*, 70 (1978) 590.
4 J. M. Symons, T. A. Bellar, J. K. Carswell, J. De Marco, K. L. Kropp, G. G. Robeck, D. R. Seeger, C. J. Slocum, B. L. Smith and A. A. Stevens, *J. Amer. Water Works Assoc.*, 67 (1975) 634.
5 D. T. Williams, R. Otson, P. D. Bothwell, K. L. Murphy and J. L. Robertson, in B. K. Afghan and D. Mackay (Editors), *Hydrocarbons and Halogenated Hydrocarbons in the Aquatic Environment*, Plenum, New York, 1980, p. 503.
6 F. M. Benoit, G. L. LeBel and D. T. Williams, *Bull. Environ. Contam. Toxicol.*, 23 (1979) 774.
7 D. T. Williams, F. M. Benoit, E. E. McNeil and R. Otson, *Pest. Monit. J.*, 12 (1978) 163.
8 *Guidelines establishing Test Procedures for the Analysis of Pollutants; Proposed Regulations, Fed. Reg.*, 44 (1979) 69464.
9 M. M. Varma, M. R. Siddique, K. T. Doty and A. Machis, *J. Amer. Water Works Assoc.*, 71 (1979) 389.
10 J. E. Henderson, G. R. Peyton and W. H. Glaze, in L. A. Keith (Editor), *Identification and Analysis of Organic Pollutants in Water*, Ann Arbor Sci. Publ., Ann Arbor, MI, 1976, p. 105.
11 J. J. Richard and G. A. Junk, *J. Amer. Water Works Assoc.*, 69 (1977) 62.
12 J. F. J. Van Rensburg, J. J. Van Huyssten and A. J. Hassett, *Water Res.*, 12 (1978) 127.
13 H. Norin and L. Renberg, *Water Res.*, 14 (1980) 1397.
14 R. Otson, D. T. Williams and P. D. Bothwell, *Environ. Sci. Technol.*, 13 (1979) 936.
15 J. P. Mieure, *J. Amer. Water Works Assoc.*, 69 (1977) 60.
16 G. J. Piet, P. Slingerland, G. H. Bijlsma and C. Morra, in B. K. Afghan and D. Mackay (Editors), *Hydrocarbons and Halogenated Hydrocarbons in the Aquatic Environment*, Plenum, New York, 1980, p. 69.
17 A. J. Bergasser and J. F. Colaruotolo, *Anal. Chem.*, 51 (1979) 1588.
18 *Priority Chemicals, The Canada Gazette*, Part 1, December 1, 1979, p. 7365.
19 M. Malaiyandi, M. H. Sadar, P. Lee and R. O'Grady, *Water Res.*, 14 (1980) 1131.
20 *National Survey for Halomethanes in Drinking Water, 77-EHD-9*, Health and Welfare Canada, Ottawa, 1977.
21 R. Otson, D. T. Williams, P. D. Bothwell, R. S. McCullough and R. A. Tate, *Bull. Environ. Contam. Toxicol.*, 23 (1979) 311.
22 F. M. Benoit and D. T. Williams, *Bull. Environ. Contam. Toxicol.*, in press.

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HYDROPHOBIC INTERACTION CHROMATOGRAPHY: A NEW METHOD FOR SUNFLOWER PROTEIN FRACTIONATION

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SUMMARY

Hydrophobic interaction chromatography has been exploited to fractionate saline-soluble sunflower proteins and the fractions obtained have been analysed further by SDS–polyacrylamide gel electrophoresis. Using octyl-Sepharose Cl-4B, the less hydrophobic fractions are bound only when the ionic strength is increased with a strong salting-out cation (NH_4^+) or anion (PO_4^{3-}). The strong hydrophobic fractions are normally bound to the matrix and could be eluted with a chaotropic agent. With 20% saturated $(NH_4)_2SO_4$ solution containing 10% NaCl and 0.02 *M* borate, pH = 7.4, about 98% of the salt-soluble proteins are bound to the matrix and most of the chlorogenic acid (98%) passes straight through the column. The elution of five low hydrophobic protein fractions is achieved by selective decrease of the ionic strength. Then, two strong hydrophobic fractions are eluted using $MgCl_2$ and 2-methoxyethanol as a polarity-reducing agent. Results obtained in SDS–polyacrylamide gel electrophoresis indicate that these seven protein fractions are composed partly of similar subunits and partly of different subunits.

INTRODUCTION

Few investigations have been conducted on the nature and biochemical properties of the globular proteins in sunflower flour[1–3]. High concentrations of chlorogenic, caffeic and quinic acids interact with proteins[4,5] and they must be eliminated in such studies[6–8]. Proteins of seeds are usually separated according to their solubility[9]. Most sunflower flour proteins are salt extractable[10] and protein fractions have been isolated by dialysis against water as salt-soluble proteins (globulins) and water-soluble proteins (albumins)[11]. Salt-soluble proteins from sunflower kernels are usually fractionated by gel filtration after chlorogenic acid has been removed by a preliminary Sephadex G-50[2], G-25[12] gel filtration or not[1]. Sephadex G-50 filtration permits at the same time a partial separation of sunflower proteins and the isolation of three main groups of proteins after dialysis against water: globulins, heavy albumins and light albumins[2].

The Osborne classification[9], although useful, is quite arbitrary and variable

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results are obtained unless the pH, salt concentration and extraction procedure are rigorously constant. On the other hand fractionations based on the differences in molecular weights are not related to the physico-chemical properties of seed proteins. The main fractions obtained by this method undergo dissociation under various experimental conditions and thus appear to be heterogeneous.

Despite the fact that vegetable proteins are known to have significantly different amino acid compositions to animal proteins, most of the usual fractionation methods used for animal proteins are applied to seed proteins. Although the distribution and the reactivity of apolar residues has been little studied[13], some experiments involving hydrophobic interaction chromatography have been performed[14–17]. The low water solubility of sunflower proteins suggests that some apolar interactions play an important rôle in the physical properties of these compounds.

In the present paper a new method for separating sunflower proteins is proposed, based on their hydrophobic binding properties. These investigations could lead to a new approach to seed protein fractionation and to an improved study of some of their properties. Chlorogenic acid is readily eliminated and seven protein fractions are isolated by a stepwise procedure using the octyl-Sepharose Cl-4B hydrophobic matrix.

METHODS

Protein extracts

Sunflower seeds (mirasol variety) were manually dehulled and were ground in a Wiley mill so as to pass a 40-mesh sieve. The flour was defatted by stirring with 10 volumes hexane for 48 h at room temperature, with three changes of solvent. Residual hexane was removed under vacuum and the flour was allowed to stand in order to reach a humidity equilibrium at 25°C. A 10-g amount of sunflower flour was then extracted for 30 min under stirring with 10 volumes 0.02 *M* borate buffer, pH 8.6, 10% NaCl and the supernatant was collected after centrifugation (15 min at 10,000 *g*). A second extraction was performed under the same conditions with the pellet and the supernatants were combined, so constituting the crude saline extract.

Materials

Octyl-Sepharose Cl-4B and phenyl-Sepharose Cl-4B were purchased from Pharmacia (Uppsala, Sweden). All other reagents were of analytical grade or the best available commercially. All chromatographic procedures were performed at room temperature, and the column effluents were monitored at 280 nm with an ISCO Type 6 optical unit and recorded with an ISCO Model UA 5 absorbance monitor.

Analytical methods

Protein (nitrogen × 6.25) in the crude extract was determined by the micro-Kjeldahl procedure. After the chromatographic step, protein was determined simultaneously from the absorbance at 280 nm and by a modified biuret method for vegetable proteins[18]. Chlorogenic acid was determined from the absorbance at 345 nm.

SDS–polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS)–polyacrylamide-gel electrophoresis was per-

formed at 220 V (ISCO 492 current supply) in an electrophoresis apparatus constructed in our laboratory by using slabs of gel (180 nm × 150 nm × 1.5 mm). Samples were dissolved in a solution of 15% (v/v) glycerol, 2.5% (w/v) SDS, 6 *M* urea, 5 m*M* 2-mercaptoethanol or not and 50 m*M* Tris–borate, pH 8.8, and then heated for 5 min at 100°C. SDS–polyacrylamide gel electrophoresis was carried out on gels containing 12% (w/v) acrylamide (0.5% bisacrylamide). The gels were stained overnight in 0.003% Coomassie Brillant Blue R250 and destained in methanol–acetic acid–water (40:5:55, v/v/v). The molecular weights were estimated by comparing the mobilities of the different proteins with those of markers of known molecular weights.

RESULTS

Fractionation of sunflower proteins on octyl-Sepharose Cl-4B

Preliminary studies. Hydrophobic chromatography on octyl-Sepharose Cl-4B was performed with a column (10 × 2.5 cm) equilibrated with 10% NaCl, 0.02 *M* borate, pH 7.4. Protein extracts (250 mg in 5 ml at pH 7.4) were placed on the column which was washed with the equilibration buffer. Unbound material, which contains most of the proteins and all of the chlorogenic acid, is washed out. Elution with 10^{-3} *M* borate, pH 7.4, gave a small protein peak. The use of a chaotropic solvent [50% aqueous methyl Cellosolve (2-methoxyethanol), MCS] permits the elution of another protein fraction (Fig. 1A) which represents about 15% of the starting material. We name it the "strong hydrophobic fraction". Thus, under the previous starting conditions (0.02 *M* borate, 10% NaCl, pH 7.4) only a minor part of the applied protein is retained by the hydrophobic matrix.

Increasing the salt concentration with salting-out ions results in an increase in hydrophobic interactions between the proteins and the matrix.

Tests were performed by adding to the initial crude extract three concentrations of ammonium sulphate (10%, 15% and 20% in saturation), the octyl-Sepharose column being equilibrated with 10% NaCl, 0.02 *M* borate, pH 7.4, containing 10, 15 or 20% saturated $(NH_4)_2SO_4$. As shown in Fig. 1B, C, D the progressive increase in ammonium sulphate concentration leads to a parallel increase in the amount of bound proteins. These proteins can be desorbed by washing the column with 10^{-3} *M* borate, pH 7.4.

Thus in 20% $(NH_4)_2SO_4$, 95–98% of the proteins are retained on the hydrophobic matrix, and washing with the starting buffer only permits elimination in the void volume of the chlorogenic acid, whose absorbance at 280 nm is always important. Elution with 10^{-3} *M* borate, pH 7.4, gives an important protein fraction (Fig. 1D) which we name the "low hydrophobic fraction" because it is not normally bound on octyl-Sepharose and is only retained when the hydrophobic interactions have been artificially increased with salting-out ions. The low hydrophobic fraction represents about 85% of the protein extract.

The unbound material is heterogeneous as shown by spectrophotometric analysis (Fig. 2). The beginning of the non-adsorbed peak possesses an absorption spectrum whose maximum near 260 nm is probably due to nucleic acids. The subsequent material comprises a chromogenic compound which possesses an absorption spectrum close to that of chlorogenic acid. So chlorogenic acid appears to be at least partly delayed on the column, but not bound. This is important because if too much ma-

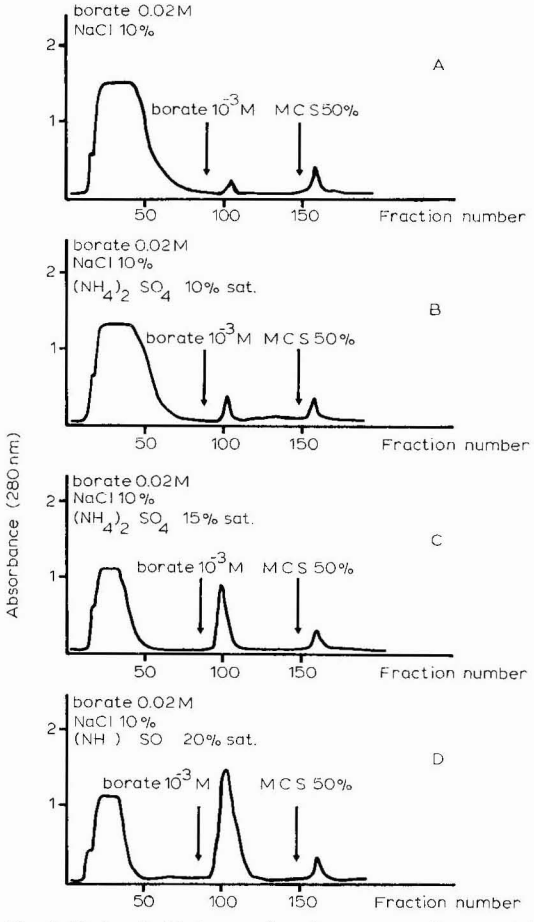

Fig. 1. Hydrophobic interaction chromatography on octyl-Sepharose Cl-4B column (10 × 2.5 cm). Buffer: 0.02 *M* borate, 10% NaCl, pH 7.4 (A), containing 10% (B), 15% (C) or 20% (D) saturated $(NH_4)_2SO_4$. Sample: 250 mg of saline-soluble sunflower proteins in 5 ml. Fractions: 1 ml. Flow-rate: 7.5 ml/cm^2 · h.

terial is used and if the column is short, a partial competition occurs between proteins and chlorogenic acid and the presence of too much pigment could disturb the protein fixation. To avoid this we recommend that the column be filled step by step and partially washed after each step.

Gradient elution studies. The fractionation of sunflower proteins could be increased if the elution of the low hydrophobic and strong hydrophobic fractions were gradually performed. In one experiment, 5 ml (250 mg of protein) of crude extract in 10% NaCl, 20% saturated $(NH_4)_2SO_4$ and 0.02 *M* borate, pH 7.4, were loaded on the octyl-Sepharose column. After washing with the starting buffer (Fig. 3), elution was carried out with a decreasing linear gradient of NaCl from 10 to 0% in 20% saturated $(NH_4)_2SO_4$, 0.02 *M* borate, pH 7.4. A single protein peak (peak 1) was eluted. Then a gradient decreasing, in ionic strength from 20% $(NH_4)_2SO_4$, 0.02 *M* borate, pH 7.4, to 10% NaCl, 0.02 *M* borate, pH 7.4, gave a second protein fraction (peak 2). Another decreasing gradient, from 10 to 0% NaCl (0.02 *M* borate, pH 7.4) permitted

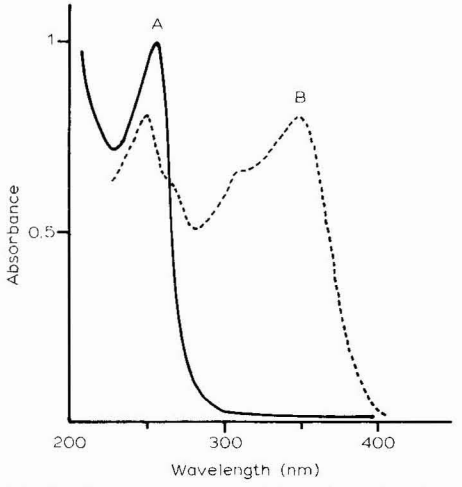

Fig. 2. Absorption spectra of the unbound material: A, beginning of the peak (——); B, following fractions (– – –).

the elution of a third single protein peak. A fourth fraction (peak 4) was eluted when the molarity of borate was reduced from 0.02 *M* to 10^{-3} *M*, pH 7.4, and a final protein fraction (peak 5) was obtained with water. So the low hydrophobic fraction could be fractionated into five separate peaks.

The strong hydrophobic fraction could also be fractionated. A linear increasing gradient of $MgCl_2$ from 0 to 0.2 *M* gave a minor protein fraction (peak 6) at 0.1 *M*

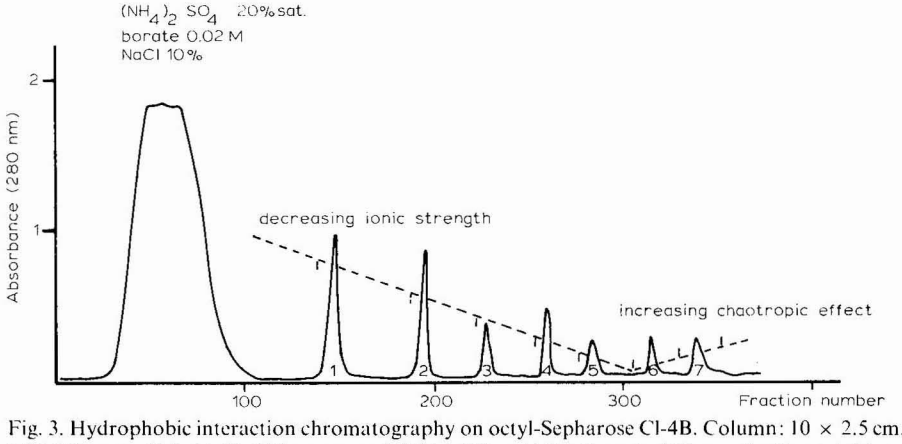

Fig. 3. Hydrophobic interaction chromatography on octyl-Sepharose Cl-4B. Column: 10 × 2.5 cm. Buffer: 0.02 *M* borate, 10% NaCl, 20% saturated $(NH_4)_2SO_4$, pH 7.4. Sample: 250 mg of saline-soluble sunflower proteins in 5 ml. The elution was performed as follows: 1, decreasing linear gradient in NaCl, from 0.02 *M* borate, 10% NaCl, 20% saturated $(NH_4)_2SO_4$, pH 7.4, to 0.02 *M* borate, 20% saturated $(NH_4)_2SO_4$, pH 7.4; 2, decreasing linear gradient in ionic strength, from 0.02 *M* borate, 20% saturated $(NH_4)_2SO_4$, pH 7.4, to 0.02 *M* borate, 10% NaCl, pH 7.4; 3, decreasing linear gradient in NaCl, from 0.02 *M* borate, 10% NaCl, pH 7.4, to 0.02 *M* borate, pH 7.4; 4, decreasing linear gradient from 0.02 *M* borate, pH 7.4, to 10^{-3} *M* borate, pH 7.4; 5, decreasing linear gradient from 10^{-3} *M* borate, pH 7.4, to water; 6, increasing linear gradient in $MgCl_2$ (0 to 0.2 *M*); 7, increasing linear gradient from 1% to 50% MCS. Fractions: 1.5 ml. Flow-rate: 7.5 ml/cm^2 · h.

$MgCl_2$ due to the chaotropic effect of the Mg^{2+}, and 50% MCS solution gave a final protein fraction (peak 7).

Stepwise elution studies. Since the different protein fractions were eluted near the end of the gradients (except for fraction 6 which is eluted by 0.1 *M* $MgCl_2$) a stepwise procedure was used in the last experiment. On a column (15 × 2.5 cm) of octyl-Sepharose equilibrated with 10% NaCl, 20% saturated $(NH_4)_2SO_4$ and 0.02 *M* borate, pH 7.4, 40 ml of crude extract (under the same starting conditions) were loaded by a step by step procedure (10 ml × 4). When all the chlorogenic acid had been removed by washing the column with the starting buffer, elution was performed with 20% $(NH_4)_2SO_4$ and 0.02 *M* borate, pH 7.4 (peak 1), then with 10% NaCl and 0.02 *M* borate, pH 7.4 (peak 2), with 0.02 *M* borate, pH 7.4 (peak 3), with 10^{-3} *M* borate (peak 4) and finally water (peak 5). The strong hydrophobic fractions were then eluted with 0.1 *M* $MgCl_2$ (peak 6) and 50% MCS solution (peak 7). The resulting chromatographic profile is exactly the same as in Fig. 3. Low hydrophobic proteins represent 86% (peak 1, 52%; 2, 19%; 3, 7%; 4, 5%; 5, 3%) and strong hydrophobic fractions 14% (peak 6, 3%; 7, 11%) of the adsorbed substances.

When hydrophobic chromatography on octyl-Sepharose was performed without ammonium sulphate under the starting conditions (10% NaCl, 0.02 *M* borate, pH 7.4), the unbound material was much more important (Fig. 4). Only three low hydrophobic fractions were eluted (with 0.02 *M* borate, pH 7.4, then 10^{-3} *M* borate, pH 7.4, and finally water) followed by two strong hydrophobic fractions. On the other hand, the same elution profile was obtained when NH_4^+ (high salting-out cation) was replaced by PO_4^{3-} (high salting-out anion). Thus, potassium phosphate is able to replace ammonium sulphate at a concentration of 0.6 *M* in the starting buffer (0.02 *M* borate, pH 7.4, 10% NaCl). Under these conditions, 90% of the proteins are bound to the matrix and the elution was performed with 0.6 *M* K_2HPO_4, 0.02 *M* borate, pH 7.4, and successively with the six other solutions in the same manner as before (results not shown).

Phenyl-Sepharose Cl-4B is also suitable, and the sunflower proteins are adsorbed at a lower ammonium sulphate concentration. In this case, however, the proteins are bound so firmly that elution at decreasing ionic strength and with a

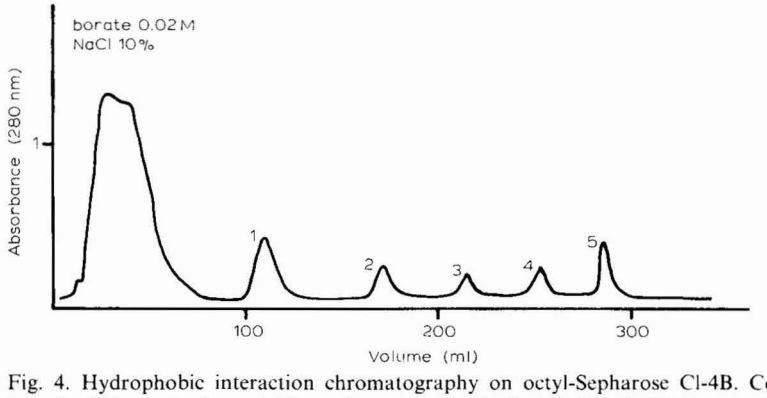

Fig. 4. Hydrophobic interaction chromatography on octyl-Sepharose Cl-4B. Column: 10 × 2.5 cm. Sample; 250 mg of saline-soluble sunflower proteins in 5 ml. Buffer: 0.02 *M* borate, 10% NaCl, pH 7.4. The elution was performed as follows: 1, 0.02 *M* borate, pH 7.4; 2, 10^{-3} *M* borate, pH 7.4; 3, water; 4, 0.1 *M* $MgCl_2$; 5, 50% MCS. Fractions: 2.5 ml. Flow-rate 7.5 ml/cm^2 · h.

polarity-reducing agent give a lower resolution than those obtained with the octyl-Sepharose matrix (results not shown).

SDS–polyacrylamide gel electrophoretic analysis

The seven separated fractions were analysed by SDS–polyacrylamide gel electrophoresis in order to establish the compositional differences among these fractions.

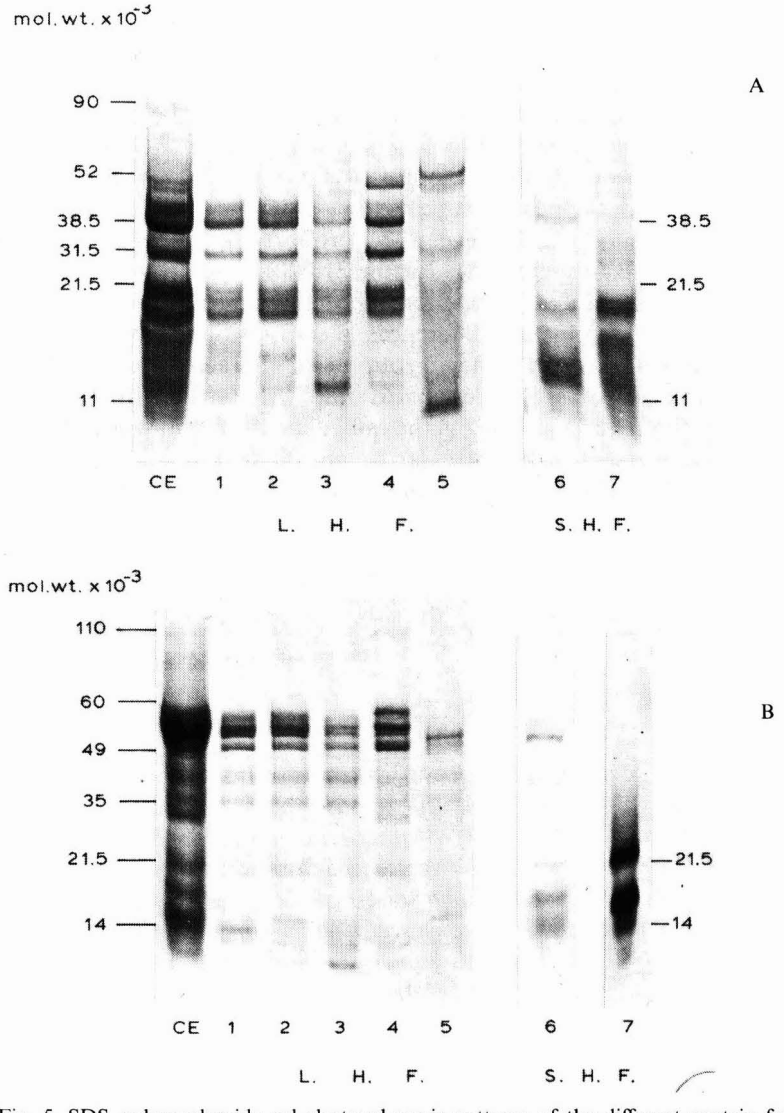

Fig. 5. SDS–polyacrylamide gel electrophoresis patterns of the different protein fractions obtained by hydrophobic chromatography on octyl-Sepharose Cl-4B according to the procedure described in Fig. 3. CE = Crude extract. The seven eluted fractions were analysed and are numbered 1–7 from the lowest to the strongest hydrophobicity. A, Non-reduced samples; B, reduced samples. One hundred and twenty micrograms (fraction CE), 50 μg (fractions 1, 2, 3, 4, 6, 7) and 30 μg (fraction 5) of protein were loaded. L.H.F. = Low hydrophobic fractions; S.H.F. = strong hydrophobic fractions.

Without prior reduction with 2-mercaptoethanol, the SDS electrophoresis pattern (Fig. 5A) shows that the proteins of the crude extract are separated into several subunits with molecular weights ranging from 60,000 to 49,000 and only a few with lower molecular weights. With previous reduction with 2-mercaptoethanol, the highest-molecular-weight protein bands disappear and simultaneously some smaller-molecular-weight polypeptides (52,000 to 10,000) appear (Fig. 6B).

The unbound protein material is essentially composed of low-molecular-weight peptides. The patterns obtained in non-reduced form for the first and second fraction were essentially the same (Fig. 5A). Upon SDS gel electrophoresis, these fractions appear to consist of the main subunits found in the crude extract. However, there are obvious differences in the intensity of the stained bands in the region below molecular weight 35,000. On the other hand, reduced fractions 1 and 2 (Fig. 5B) also showed differences in their electrophoretic patterns. As in the case of the crude extract, some smaller polypeptides appear after reduction. Fractions 1 and 2 seem to be identical in the number of subunits, however their proportions are different, particularly in the lower-molecular-weight range (below 21,000).

Compared with fractions 1 and 2, the electrophoretic patterns of fractions 3, 4 and 5 (Fig. 5A, B) show more important differences in composition as well as in the proportions of the subunits, and each protein group has a characteristic pattern.

The electrophoretic patterns of fractions 6 and 7 (the strong hydrophobic fractions) are also very different. Fraction 7 is the only eluted fraction which doesn't possess any polypeptidic band of high molecular weight (60,000 to 49,000) and the pattern of reduced fraction 7 shows a large number of low-molecular-weight polypeptides.

The fractions obtained when the chromatographic procedure was conducted without NH_4^+ (see Fig. 4) were also submitted to electrophoretic analysis. As shown in Fig. 6 the exclusion peak contains most of the subunits found in the crude extract.

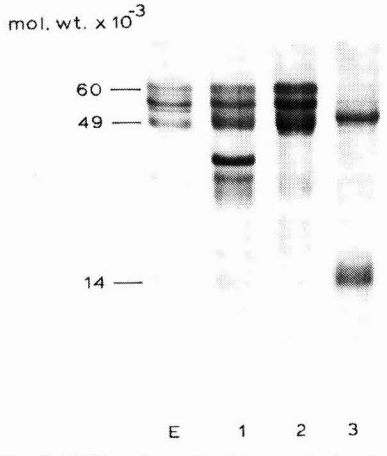

Fig. 6. SDS–polyacrylamide gel electrophoresis patterns of eluted protein fractions from octyl-Sepharose Cl-4B without prior addition of $(NH_4)_2SO_4$ as described in Fig. 4. E = Exclusion fraction. Fractions 1, 2 and 3 were respectively eluted with 0.02 *M* borate, pH 7.4, 10^{-3} *M* borate, pH 7.4, and water. A 50-μg amount of protein in non-reduced form was loaded.

Fractions 1, 2 and 3 respectively eluted with 0.02 *M* borate, pH 7.4, then 10^{-3} *M* borate, pH 7.4, and finally water have similar electrophoretic patterns to those of fractions 3, 4 and 5 previously described.

SDS–polyacrylamide gel electrophoresis shows that all the eluted fractions exhibit similar patterns despite the fact the chromatographic procedure has been conducted in the presence of NH_4^+ or PO_4^{3-} (results not shown).

The described procedure allowed us to fractionate proteins from other vegetable origins. For example, electrophoretic patterns of soybean and broadbean protein fractions obtained by this procedure are shown in Fig. 7A and B.

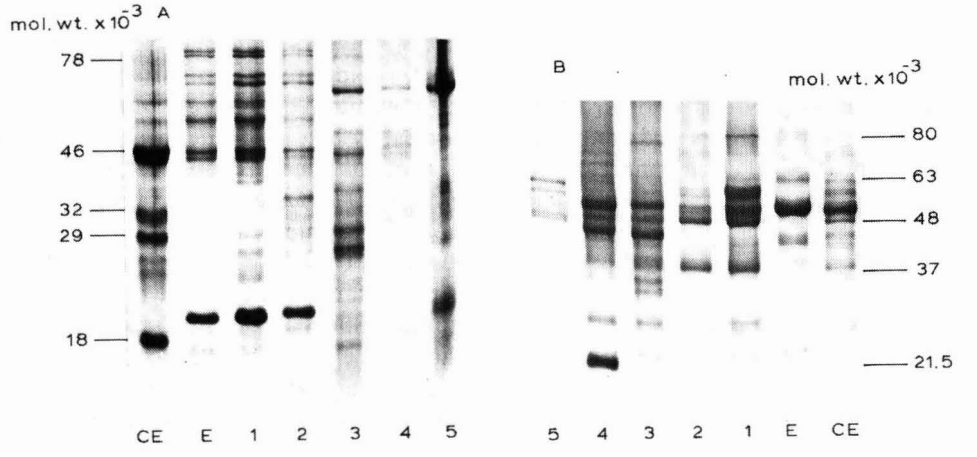

Fig. 7. SDS–polyacrylamide gel electrophoresis patterns of the different protein fractions obtained from soybean (A) and broadbean (B) using hydrophobic chromatography, by the procedure described for sunflower proteins. CE = Crude extract; E = exclusion fraction. Only the five low hydrophobic fractions (1–5) were analysed in non-reduced form.

DISCUSSION

The hydrophobic properties of sunflower proteins seem to be provide an interesting approach to seed protein fractionation. Using octyl-Sepharose Cl-4B as matrix, hydrophobic chromatography permits the separation of two main protein species: the low hydrophobic proteins which are not normally bound to the matrix, and the strong hydrophobic proteins which are retained and only eluted with the help of a chaotropic agent which decreases the hydrophobic interactions between the protein and the hydrophobic groups of the gel. The strong hydrophobic protein fraction represents about 15% of the starting material.

The hydrophobic interactions are known to increase upon increasing the ionic strength of the medium[16]. In this way, our results show that in 20% saturated $(NH_4)_2SO_4$, only about 1% of the crude proteins are not bound to the hydrophobic matrix. Under these conditions chlorogenic acid is readily eliminated in a non-adsorbed fraction. Thus, by this procedure, it is possible to remove chlorogenic acid and prepare almost pure chlorogenic-free protein samples. The elimination of this phenolic acid is of great interest because its presence causes considerable discoloration of sunflower meals, restricting their application in the food industry.

This chromatographic procedure not only removes chlorogenic acid but also permits the fractionation of the salt-soluble protein extract adsorbed on the hydrophobic matrix. The separation is based on the fact that a progressive decrease in the ionic strength of the elution buffer may promote selective desorption of proteins by decreasing their hydrophobic interactions with the gel. Our studies have shown that a stepwise procedure using five different solutions of decreasing ionic strength yields five low hydrophobic protein fractions.

The strong hydrophobic proteins may also be separated in two different fractions by the use of chaotropic agents such as Mg^{2+} and MCS.

Like other seed proteins[19,20], sunflower proteins are heterogeneous and composed of different subunits[1–3]. Thus, seed proteins may be considered as an associating protein system[21,22]. Results obtained on SDS–polyacrylamide gel electrophoresis indicate that the seven protein fractions are partly composed of similar subunits (but in different proportions) and partly of different subunits. The existence of polypeptide chains in these subunits and connected at least partly by disulphide bridges could be proved by SDS gel electrophoresis of reduced proteins. The hydrophobic amino acid content of these different subunits and/or their spacial arrangement in the quaternary structure may explain their hydrophobic chromatographic behaviour.

It has been shown in this study that fixation of the low hydrophobic proteins may be achieved by raising the ionic strength in the starting buffer. The use of salting-out anions or cations (NH_4^+ or PO_4^{3-}) leads to the same fixation effectiveness. On the other hand, the presence of NH_4^+ seems to have an effect upon the fixation of only some specific proteins since the electrophoretic patterns of fractions 3, 4 and 5 are similar to those of fractions 1, 2 and 3 obtained when the chromatographic procedure is carried out without prior addition of ammonium sulphate.

Preliminary results in our laboratory have shown that seed proteins of other species such as broadbean and soybean exhibit, under the described conditions, similar behaviour when they are submitted to octyl-Sepharose chromatography. For these two proteins about 20% of the starting protein material is not retained on the column. The importance of this "hydrophilic" fraction can be related to the high water solubility of legume seed proteins.

The analytical procedure described permits a new approach to the fractionation of sunflower storage proteins which is based on their hydrophobic properties. Octyl-Sepharose seems to be a very suitable matrix for most of the seed proteins studied and further investigations are now being performed to substantiate this fact.

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REFERENCES

1 M. A. Sabir, F. W. Sosulski and S. L. Mac. Kenzie, *J. Agr. Food Chem.*, 21 (1973) 988–993.
2 J. Baudet and J. Mossé, *J. Amer. Oil Chem. Soc.*, 54 (1977) 82A–86A.
3 E. H. Rahma and M. S. Naratinga Rao, *J. Food Sci.*, 44 (1979) 579–582.

4 J. V. Pomenta and E. E. Burns, *J. Food Sci.*, 36 (1971) 490–492.
5 C. M. Cater, S. Gheysuddin and K. F. Mattil, *Cereal. Chem.*, 49 (1972) 508–514.
6 F. W. Sosulski and C. W. Mac. Cleary, *J. Food Sci.*, 37 (1972) 253–256.
7 F. W. Sosulski, M. A. Sabir and S. E. Fleming, *J. Food Sci.*, 38 (1973) 468–470.
8 T. Y. Fan and F. W. Sosulski, *Cereal Chem.*, 53 (1976) 118–125.
9 T. B. Osborne and G. F. Campbell, *J. Amer. Chem. Soc.*, 19 (1897) 487–494.
10 S. Gheyasuddin, C. M. Cater and K. F. Mattil, *J. Food Sci.*, 35 (1970) 453–456.
11 A. Girault, J. Baudet and J. Mossé, *Improving Plant Protein by Nuclear Techniques, Proceedings of Symposium*, International Atomic Energy Agency and Food and Agriculture Organization, Vienna, 1970, pp. 275–285.
12 K. D. Schwenke, B. Raab, J. Unhlig, H. Trocz, J. Behlke, M. Böttger and V. Freimuth, *Nahrung*, 17 (1973) 791–795.
13 C. Tanford, *J. Amer. Chem. Soc.*, 84 (1962) 4240–4247.
14 Z. Er-el, Y. Zaidenzaig and S. Shaltiel, *Biochem. Biophys. Res. Commun.*, 49 (1972) 383–390.
15 T. Peters, H. Taniuchi and C. B. Anfinsen, *J. Biol. Chem.*, 248 (1973) 2447–2451.
16 S. Hjertén, *J. Chromatogr.*, 87 (1973) 325–331.
17 K. A. Caldwell, *J. Sci. Food Agric.*, 30 (1979) 185–196.
18 J. S. Noll, D. H. Simmonds and W. Bushuk, *Cereal Chem.*, 51 (1974) 610–616.
19 C. Simard, Y. Dupont and M. Boulet, *Can. Inst. Food Sci. Technol. J.*, 11 (1978) 38–44.
20 W. J. Wolf, in A. K. Smith and S. J. Circle (Editors), *Soybeans: Chemistry and Technology*, Vol. 2, Avi Publishing Company, Westport, CT, 2nd ed., 1978, pp. 93–143.
21 K. D. Schwenke, *Nahrung*, 19 (1975) 69–82.
22 K. D. Schwenke, W. Pähtz, K. J. Linow, B. Raab and M. Schultz, *Nahrung*, 23 (1979) 241–245.

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REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY FOR THE SEPARATION AND ANALYSIS OF ECDYSTEROIDS

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SUMMARY

Reversed-phase thin-layer chromatography plates of different types and different manufacture are all suitable for the chromatography of ecdysteroids (insect moulting hormones). A number of solvent systems have been examined, and elution with methanol–water mixtures from C_{18}-coated plates provides a suitable general system. The detection limit of ecdysteroids on reversed-phase plates is 10^{-7} g, using fluorescence quenching or vanillin–sulphuric acid spray. The recovery of ecdysteroids, which is inefficient from normal silica plates, is much more efficient from reversed-phase plates, especially when residual silanol groups are "capped" with organosilane reagents.

INTRODUCTION

A variety of chromatographic techniques have been used in the isolation, identification and analysis of ecdysteroids, the moulting hormones of insects and crustaceans. The existence of 17 zooecdysteroids and over 50 phytoecdysteroids, all of closely related structure[1], makes heavy demands upon the separation powers available to an investigator. Adsorption columns, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC) have all been used. TLC has been a popular method with ecdysteroids because it is simple to use, inexpensive, gives good resolution and R_F properties can be varied widely by exploiting the choice of solvent systems available[2]. TLC in this area suffers from two disadvantages, first, relatively poor sensitivity of detection compared with GC and HPLC, and secondly, losses of compounds due to irreversible adsorption on silica may be high when small quantities of compound are available[3]. Now that reversed-phase (RP) TLC plates are available from a number of manufacturers and can be made easily in the laboratory, we decided to explore their usefulness for ecdysteroids. RP-TLC is highly suitable for such polar compounds, and problems of irreversible adsorption should be reduced or eliminated. It provides another system with different

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separation properties for the qualitative analysis and identification of ecdysteroids in biological systems.

We have here examined the separation of several ecdysteroids on commercially available RP-TLC plates and the recovery of ecysteroids from them, and conducted some preliminary experiments with "homemade" RP-TLC plates.

EXPERIMENTAL

TLC plates

Absorption TLC plates were prepared by coating 5 × 20 cm glass plates with a 0.3-mm layer of silica gel G (E. Merck, Darmstadt, G.F.R.). The plates were allowed to dry in air and stored over $CaCl_2$.

RP-TLC plates were obtained from three commercial sources and were made in the laboratory. Precoated plates (10 × 10 cm) containing short, medium and long carbon chains bonded to the silica surface (RP-2, RP-8 and RP-18 respectively) were obtained from Merck. One type of plate (20 × 5 cm), KC_{18}, precoated with C_{18} carbon chains was obtained from Whatman (Springfield Mill, Great Britain). Three types of Macherey, Nagel & Co. (Düren, G.F.R.) plates were used, which have different degrees of silanization with octadecylsilane. These plates (10 × 10 cm) are 50, 75 or 100% silanized.

RP-TLC plates were prepared in the laboratory by immersing precoated silica TLC plates (10 × 10 cm, Whatman high-performance silica, Type HP-K) in a solution of 2% trichlorooctadecylsilane in toluene (dried, molecular sieves). The plates were sonicated three times for 30-sec intervals in the first 15 min of immersion and left in the bath overnight, then washed for 10 min in each of toluene, acetone and methanol. Plates were dried at 110°C for 1 h before use. Capping was carried out in the same way using trimethylchlorosilane or dimethyldichlorosilane.

All plates had an acid-stable fluorescent indicator incorporated.

Use of the plates

Ecdysone (95% pure by HPLC with UV detection) and 20-hydroxyecdysone were obtained from Simes (Milan, Italy). Solvents were of analytical grade.

Amounts of ecdysteroid from 10^{-6} to 10^{-7} g were spotted on the plates using a 10-μl SGE syringe. The reversed-phase plates were conditioned for 30 min in the atmosphere of the tank before use. They were then developed in 95% ethanol–water (1:1) for quantitative studies or the appropriate solvent for R_F studies. The adsorption plates were developed in chloroform–methanol (8:2).

After development, the plates were visualized under a UV lamp at 254 nm. Fluorescence was induced in ecdysteroids by heating for 1 h at 110°C in the presence of solid ammonium carbonate, and viewing under a UV lamp at 366 nm. Vanillin spray reagent was prepared by dissolving vanillin in a mixture of concentrated sulphuric acid and 95% ethanol (5:70:25, w/v/v). After spraying with the reagent, plates were heated to 100–120°C for 10 min and observed in normal light.

Quantification studies

Where recovery from the plates was measured, after visualization in UV light, the appropriate R_F bands were scraped off. The silica was transferred to a test-tube

and extracted with methanol (1 ml). The silica was separated by centrifugation and the clear supernatant liquid decanted into a Reacti-vial (Pierce & Warrner, Chester, Great Britain), where it was evaporated to dryness with a stream of warm nitrogen. The residue was re-dissolved in a known volume of methanol for quantification by HPLC, using a Pye LC3 XP pump, a Pye gradient elution system and Chromatronix 220 fixed-wavelength (254 nm) absorption detector. The analysis was carried out on a Shandon Southern 100 × 5 mm column packed with 5-μm particles of Hypersil ODS under isocratic conditions (methanol–water, 45:55) at a flow-rate of 1.0 ml min^{-1}. Samples of 10 μl were injected onto the column. The quantity of compound present was calculated from a standard curve obtained by plotting peak area against quantity of ecdysone injected. The least detectable amount was 10 ng.

RESULTS AND DISCUSSION

At present, a range of RP-TLC plates are available commercially. Merck produce plates with ethyl (C_2), *n*-octyl (C_8) or *n*-octadecyl (C_{18}) groups chemically bonded to the silica surface, gibing a range of partitioning properties. Macherey, Nagel & Co. produce a similar range of properties by controlling the degree of silanizing to produce plates which are 50, 75 and 100% covered with C_{18} groups. Whatman also manufacture a C_{18}-coated plate.

We have determined the R_F values of ecdysone and 20-hydroxyecdysone, the commonest ecdysteroids encountered, on each of the Merck plates, using a range of organic solvents (methanol, ethanol, 2-propanol, acetone and acetonitrile) together with water. The variation of R_F value with solvent composition on the three types of plate are shown in Table I. Only the upper and lower limits of R_F values measured are given.

Good resolution of ecdysone and 20-hydroxyecdysone was possible using all the solvent systems tested. The eluotropic series formed by this group of organic

TABLE I

VARIATION OF R_F VALUES OF ECDYSONE (α) AND 20-HYDROXYECDYSONE (β) ON MERCK RP-TLC PLATES, IN DIFFERENT SOLVENT SYSTEMS

Solvent	*Proportions*	C_2		C_8		C_{18}	
		α	β	α	β	α	β
Methanol–water*	50:50	0.38	0.53	0.28	0.44	0	0
	80:20	0.88	0.88	0.75	0.82	0.76	0.82
Ethanol–water	40:60	0.38	0.53	0.34	0.52	0.41	0.57
	70:30	0.89	0.89	0.88	0.88	0.88	0.88
2-Propanol–water	20:80	0.18	0.46	0.19	0.46	0.24	0.46
	60:40	0.87	0.87	0.83	0.83	0.81	0.81
Acetonitrile–water	30:70	0.44	0.60	0.33	0.53	0.35	0.50
	60:40	0.86	0.86	0.90	0.90	0.90	0.90
Acetone–water	30:70	0.35	0.58	0.16	0.53	0.26	0.45
	70:30	0.79	0.87	0.86	0.86	0.84	0.91

* With methanol–water (60:40) on C_{18} plates, $\alpha = 0.39$ and $\beta = 0.54$. With methanol–water (90:10), R_F values were 0.9 for both compounds on all three types of plate.

solvents is as follows: isopropanol > acetonitrile > acetone > ethanol > methanol. An R_F of approximately 0.5 (on all three types of plate) was obtained with the following solvent compositions: isopropanol–water (30:60); acetonitrile–water (35:65); acetone–water (40:60); ethanol–water (45:55) and methanol–water (65:35). The largest separations between ecdysone and 20-hydroxyecdysone were achieved with the minimum possible organic modifier which still allowed solvent migration. The best separation, of 0.29 R_F units was obtained on a C_2 plate using isopropanol–water (20:80).

The effect of alcohol chain length on the quantity of organic modifier required to achieve the same separations is illustrated in Fig. 1. Differences resulting from the length of the carbon chain bonded to the silica were most noticeable at low R_F, where less organic modifier was present. With larger proportions of organic modifier in the solvent R_F values on all three types of plate were similar. There was no trend with chain length, the R_F values on the C_8 plates sometimes being lower than on the C_2 or C_{18} types. Bearing in mind that the greater the proportion of the organic component, the less viscous the mixture, and the shorter the elution time, none of the systems showed any distinct advantage over methanol–water mixtures, and for further work, attention was confined to this solvent system.

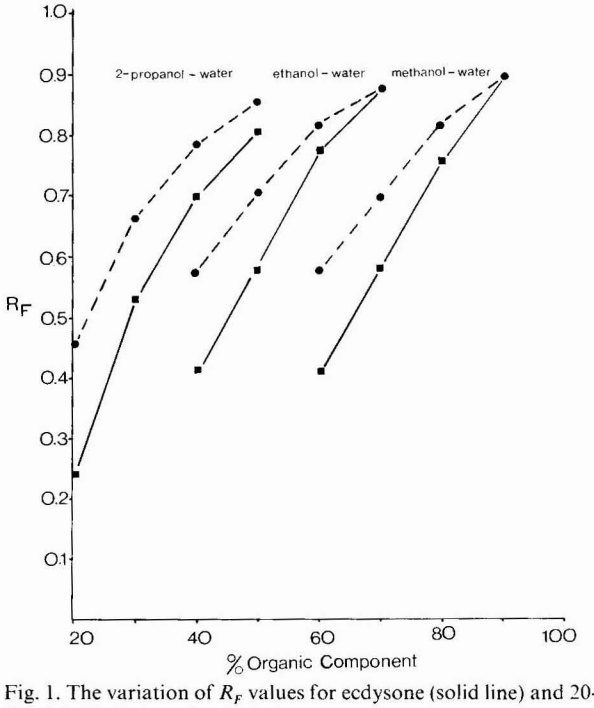

Fig. 1. The variation of R_F values for ecdysone (solid line) and 20-hydroxyecdysone (dashed line) on Merck C_{18} RP-TLC plates in alcohol–water solvent systems.

Examination of the Macherey, Nagel & Co. plates, coated with 50, 75 and 100 % of C_{18} hydrocarbon chains, showed that their R_F properties were very similar to those of the Merck plates (Table II). There was greater separation between ecdysone and 20-hydroxyecdysone at low proportions of methanol (methanol–water, 50:50), and

TABLE II

VARIATION OF R_F VALUES OF ECDYSONE (α) AND 20-HYDROXYECDYSONE (β) ON MACHEREY, NAGEL & CO. AND WHATMAN RP-TLC PLATES

Proportions of methanol–water	*Extent of coating, Macherey, Nagel & Co., C_{18}*						*Whatman C_{18}*	
	50%		*75%*		*100%*			
	α	β	α	β	α	β	α	β
50:50	0.25	0.38	0.20	0.34	0.13	0.19	*	*
90:10	0.84	0.84	0.89	0.89	0.86	0.86	0.78	0.78

* Not wetted by solvent; in methanol–water (60:40), R_F = 0.38 (α) and 0.5 (β).

R_F values of approximately 0.5 were achieved with solvent proportions 55:45 on the 50% coated plates and 65:35 on the 75% and 100% coated plates. A trend towards lower R_F values is seen, the greater the extent of coverage by the organic phase. The Whatman C_{18} plates had similar R_F values (Table II), but like the Merck C_{18} plates, were not wetted by methanol–water (50:50).

One of the factors which limits the use of RP-TLC plates is their hydrophobic nature, which limits the choice of solvent in which the sample can be applied, as well as limiting the choice of mobile phase. RP-TLC plates with a non-absorbent application zone, of the type already available for silica plates would be helpful where it is desired to apply aqueous solutions (*e.g.*, insect haemolymph or extracts containing a high proportion of water).

The quantity of water which can be accommodated in the mobile phase depends not only on the organic phase but also on the process of manufacture, Macherey, Nagel & Co. C_{18} 100% plates can be used with methanol–water (50:50) whereas there is no solvent migration with this mixture on Merck plates, and it causes adsorbant to come away from the glass backing of the Whatman plates.

The high cost of the commercial RP-TLC plates encouraged us to explore the preparation of our own plates in the laboratory. Normal high-performance silica plates were silanized in a bath of toluene and trichlorooctadecylsilane overnight[4]. The plates prepared in this way compared favourably with commercial plates, however, the fluorescent marker in the silica is lost during silanizing. The plates were also "capped", by causing remaining surface silanol groups to react with either dimethyldichlorosilane or trimethylchlorosilane. Some R_F values are given in Table III. It is noteworthy that the plates treated with trimethylchlorosilane gave the highest R_F values, and also had the most non-polar surface (see *Recovery of ecdysteroids from reversed-phase plates*).

Separation of a large number of ecdysteroids

The separations achieved on Merck C_2, C_8 and C_{18} plates for a range of twelve ecdysteroids, in three solvent systems are given in Table IV. It is interesting that significant changes in selectivity are apparent between alcohol–water and acetonitrile–water based solvent systems. In particular poststerone and cyasterone appear in alcohol–water to be more polar than ecdysone, whilst in acetonitrile–water they are

TABLE III

R_F VALUES OF ECDYSONE (α) AND 20-HYDROXYECDYSONE (β) ON LABORATORY-MADE REVERSED-PHASE PLATES

Solvent system: ethanol–water (60:40). DMCS = Dimethyldichlorosilane; TMCS = trimethylchlorosilane.

	Uncapped	*DMCS treated*	*TMCS treated*
α	0.75	0.81	0.84
β	0.78	0.85	0.89

less polar. It is perhaps noteworthy that these two compounds differ from all the other ecdysteroids examined in that they posess two, rather than one keto group. Muristerone and ajugasterone C (which are unusual in possessing a hydroxyl at C-11), also exhibit some differences in behaviour, relative to ecdysone, in different solvent systems. It should therefore be possible to manipulate both the type of plate used, and the solvent system employed to obtain a wide range of separations amongst the ecdysteroids, as both polarity and structural details seem to be important in their RP-TLC.

Detection of ecdysteroids on reversed-phase plates

The 7-en-6-one group common to the ecdysteroids strongly absorbs UV light at 240 nm. Consequently, on TLC plates incorporating a fluorescent marker, the ecdysteroids are readily visible as dark spots against the fluorescent background. By this method, the smallest amount of ecdysteroid we could detect was $1 \cdot 10^{-7}$ g on RP-TLC plates, and about the same quantity on normal silica TLC plates (*cf.*, ref. 2).

On silica the ecdysteroids have customarily been identified with the aid of a number of visualizing reagents. The one most widely used has been vanillin–sulphuric acid spray. The colour produced (blue to green to violet) can vary with the ecdysteroid structure. We have examined the usefulness of this reagent on RP-TLC plates. After spraying and heating, the typical colours were produced with ecdysone and hydroxyecdysone, discolouration of the plates was minimal and the detection limit was again about 10^{-7} g.

The formation of fluorescent derivatives can be used to lower detection limits in chromatography. Ecdysteroids form fluorescent derivatives in ethanol–sulphuric acid[5–7] but we have found the fluorescence intensity is variable with conditions, and therefore not reliable for quantitation. An alternative is to heat the plate in an atmosphere of ammonium carbonate at 110° C. When viewed under UV light of 366 nm, the ecdysteroids show a blue-white fluorescence, detectable to $5 \cdot 10^{-8}$ g, moreover the fluorescence appears to be stable for several days, but the fluorescence is rather non-specific[8], so ecdysteroids could not be detected in only partially purified biological samples.

Recovery of ecdysteroids from reversed-phase plates

It has been demonstrated in a number of cases that polar organic compounds are not recovered efficiently from silica or alumina chromatographic materials because of irreversible adsorption[3]. This loss through adsorption is negligible in most

TABLE IV

R_F VALUES OF VARIOUS ECDYSTEROIDS CHROMATOGRAPHED ON THREE TYPES OF MERCK RP-TLC PLATES, USING THREE SOLVENT SYSTEMS

Solvent systems: PrOH = 2-propanol; MeOH = methanol; ACN = acetonitrile; in each case the other component is water.

*Compound**	C_2			C_8			C_{18}		
	30% PrOH	*60% MeOH*	*40% ACN*	*30% PrOH*	*60% MeOH*	*40% ACN*	*30% PrOH*	*60% MeOH*	*40% ACN*
Ecdysone	0.38	0.52	0.66	0.39	0.38	0.61	0.43	0.31	0.64
20-Hydroxyecdysone	0.53	0.65	0.75	0.58	0.53	0.72	0.59	0.47	0.75
2-Deoxy-20-hydroxyecdysone	0.36	0.53	0.62	0.42	0.32	0.6	0.39	0.33	0.57
Inokosterone	0.49	0.67	0.71	0.53	0.53	0.72	0.55	0.50	0.73
Muristerone A	0.27	0.57	0.56	0.28	0.38	0.57	0.29	0.30	0.55
Makisterone A	0.45	0.63	0.67	0.5	0.47	0.72	0.51	0.39	0.71
Cyasterone	0.45	0.66	0.60	0.53	0.53	0.65	0.57	0.47	0.64
Poststerone	0.36	0.63	0.57	0.47	0.47	0.61	0.49	0.40	0.59
Ponasterone A	0.14	0.44	0.41	0.13	0.21	0.41	0.14	0.16	0.39
Polypodine B	0.52	0.71	0.70	0.63	0.55	0.78	0.61	0.48	0.77
Ajugasterone C	0.2	0.60	0.60	0.26	0.36	0.57	0.26	0.31	0.59
Carpesterol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

* For structures, see ref. 1.

TABLE V

RECOVERY (%) OF ECDYSONE FROM RP-TLC PLATES COMPARED WITH NORMAL-PHASE SILICA ADSORPTION TLC PLATES, IN QUANTITIES NEAR THE LIMIT OF DETECTION

Results were single determinations for laboratory-made plates, others are the average of two determinations.

Type of TLC plates	*Amount applied (g)*		
	$1 \cdot 10^{-6}$	$5 \cdot 10^{-7}$	$1 \cdot 10^{-7}$
Adsorption TLC	56	56	51
Merck C_2	84	84	79
Merck C_8	82	79	80
Merck C_{18}	87	84	82
Macherey, Nagel & Co. 100% C_{18}	88	83	75
Whatman C_{18}	76	76	71
Laboratory-made, not capped	82	69	*
capped DMCS	87	83	*
capped TMCS	91	80	*

* Not tested.

cases, but working in the μg and ng range, this loss can become significant to serious. With a non-polar surface bonded to silica this irreversible adsorption loss should be reduced, and RP-TLC plates should be more suitable for the quantitative separation and recovery of ecdysteroids.

We have therefore compared the recovery of small samples of ecdysone in the range 10^{-6}–10^{-7} g (near the limit of detection) from normal-phase silica TLC plates with that from various kinds of RP-TLC plates. The results are given in Table V. While recoveries varied from 50 to 60% on normal phase, all the RP-TLC plates showed much higher recovery, up to 90%. Recoveries are slightly better in all cases for the larger amounts. Doubtless larger errors would be found for still smaller quantities. The longer the carbon chain (see the Merck plates) the better the recovery, but the difference is small. The recovery from plates coated with C_{18} groups in the laboratory was improved by "capping" the unreacted surface silanol groups with dimethyldichlorosilane or trimethylchlorosilane. Both Merck and Macherey, Nagel & Co. plates are available with or without a fluorescent indicator added, and we have examined the effect of the indicator on recovery. No difference was found in recovery of ecdysone for the same manufacturer's plates with or without fluorescer, but the indicator plates contain elutable components absorbing UV light, which give a number of peaks when the sample is analysed later by HPLC.

We draw attention again to the poor recovery of such compounds as the ecdysteroids from an activated silica surface, whether column, thin layer or HPLC column is used. Clearly, RP-TLC is preferable for these compounds when all other considerations are equal. The best material for this chromatography is non-fluorescent plates, made by coating silica plates in the laboratory and then "capping" them with trimethylchlorosilane.

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REFERENCES

1 C. Hetru and D. H. S. Horn, in J. A. Hoffmann (Editor), *Progress in Ecdysone Research*, Elsevier-North Holland, Amsterdam, 1980, p. 13.
2 E. D. Morgan and C. F. Poole, *Advan. Insect Physiol.*, 12 (1976) 17.
3 E. D. Morgan and I. D. Wilson, in J. A. Hoffmann (Editor), *Progress in Ecdysone Research*, Elsevier-North Holland, Amsterdam, 1980, p. 29.
4 M. Ericsson and L. G. Blomberg, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 3 (1980) 345.
5 M. W. Gilgan and M. E. Zinck, *Steroids*, 20 (1972) 97.
6 R. T. Mayer and J. A. Svoboda, *Steroids*, 31 (1978) 139.
7 J. Koolman, *Insect Biochem.*, 10 (1980) 381.
8 R. Segura and A. M. Gotto, Jr., *J. Chromatogr.*, 99 (1974) 643.

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LIQUID CHROMATOGRAPHY MEASUREMENT OF CORTISOL IN METHYLENE CHLORIDE EXTRACTS OF AQUEOUS SOLUTIONS

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SUMMARY

Variable and incomplete analytical recovery of cortisol (*i.e.*, range 59–101 %) was observed from aqueous standards prepared for high-performance liquid chromatography (HPLC) by extraction into methylene chloride, solvent evaporation and residue reconstitution in our mobile phase. Analytical recoveries of cortisol following solvent evaporation in glass tubes without extraction were low (49 %) and showed no improvement by using containers of different composition. Comparison of a radiochromatogram for tritium-labelled cortisol and a chromatogram for unlabelled cortisol following sample preparation for HPLC indicated that low analytical recoveries were not the result of cortisol degradation during sample preparation. Adding polyethylene glycol (mol. wt. 20,000) to methylene chloride before solvent evaporation dramatically improved the analytical recovery of cortisol.

INTRODUCTION

Numerous methods for measuring cortisol in serum and plasma utilizing high-performance liquid chromatography (HPLC) have been reported[1–11] since Wortmann *et al.*[1] described the first method in 1973. Sample preparation, prior to chromatography, is an integral part of the HPLC method and typically consists of extracting the sample with an organic solvent, evaporating the extract to dryness and reconstituting the residue with the HPLC mobile phase. Van den Berg *et al.*[2] have shown that methylene chloride has a higher extraction efficiency for cortisol than either diethyl ether or ethyl acetate and has been the solvent selected by subsequent workers. The low analytical sensitivity of the various methods at physiological concentrations of cortisol requires that reconstitution volumes be substantially smaller than the original sample volume, with previously reported concentration factors (*i.e.*, sample volume/reconstitution volume) ranging from 5- to 333-fold.

While examining the stability and degradation products of cortisol in aqueous solutions using a recently published HPLC method[3], we observed variable and incomplete analytical recovery of cortisol during sample preparation. Garg *et al.*[4] previously observed only 72 % recovery of cortisol from aqueous standards and Scott

and Dixon[5] reported that the analytical recovery of cortisol from 30 g/l bovine albumin was approximately 10% higher than from water (*i.e.*, 76%). Other workers using analogous sample preparation protocols have observed higher recoveries from human sera (*e.g.*, 78%[6], 83%[7] and 96%[3]), but have bypassed potential problems encountered with aqueous standards by either preparing more concentrated standards for direct injection into the instrument without extraction or adding standard to a portion of the serum specimen for simultaneous work-up.

In this study, we examine the analytical recovery of cortisol after solvent evaporation and reconstitution with our mobile phase. The evaporation step is performed in a variety of containers and over a wide range of cortisol concentrations. Radioactively labelled cortisol is used to verify the low analytical recovery and to check for possible degradation of cortisol during sample preparation. Finally, addition of a macromolecular matrix material (*i.e.*, polyethylene glycol, mol. wt. 20,000) to the methylene chloride before solvent evaporation is examined as a technique for improving the analytical recovery and precision of cortisol measurement.

MATERIALS AND METHODS*

Chemicals

Cortisol, Standard Reference Material 921, was from the National Bureau of Standards (NBS) (Washington, DC, U.S.A.). [1,2,6,7-$^3H_4$]cortisol, 1 Ci/l (specific activity 93.1 Ci/mol), was from New England Nuclear (Boston, MA, U.S.A.). Methylene chloride and methanol (both distilled in glass) were from Burdick & Jackson Labs. (Muskegan, MI, U.S.A.). Ethanol (absolute) was from U.S. Ind. Chem. (Tuscola, IL, U.S.A.). Polyethylene glycol (PEG), mol. wt. 20,000, was from Fisher Scientific (Pittsburgh, PA, U.S.A.). Ready-Solv HP scintillation cocktail was from Beckman (Fullerton, CA, U.S.A.). Water (resistivity $\geqslant$ 10 MΩ) was charcoal filtered, deionized and filtered through a membrane of pore size 0.45 μm before use.

Equipment

The chromatographic system included: an SP8000 liquid chromatograph and data system from Spectra-Physics (Santa Clara, CA, U.S.A.); a μBondapak C_{18} reversed-phase column (30 cm $\times$ 3.9 mm I.D.) from Waters Assoc. (Milford, MA, U.S.A.) and a Varichrom variable wavelength detector from Varian (Palo Alto, CA, U.S.A.). An Isocap 300 liquid scintillation system from Searle Analytic (Des Plaines, IL, U.S.A.) was used for measuring tritium.

Procedures

Extraction. A 1-ml volume of aqueous cortisol solution was extracted with 10 ml of methylene chloride by shaking at 280 strokes per min on a mechanical shaker for 10 min. After shaking, the layers were allowed to separate completely and 8.0 ml of the methylene chloride layer were transferred to a 15-ml glass centrifuge tube for evaporation.

Evaporation. Methylene chloride was evaporated by partially immersing the

* Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

centrifuge tube in a water-bath at 40°C under a stream of nitrogen gas. An apparatus constructed of stainless-steel tubing directed the nitrogen flow into the tube, and the gas flow was maintained between 1 and 3 cm above the surface of the methylene chloride during evaporation.

Reconstitution and chromatography. Residues after evaporation were reconstituted by adding 100 μl of the HPLC mobile phase (methanol–water, 55:45, v/v), immediately replacing the container cap and vortexing the solvent over the interior surface of the tube for 15 sec. A 50-μl volume of solution was injected for chromatographic analysis. The mobile phase was pumped at a flow-rate of 1.0 ml/min producing a back pressure of 9 MPa (1300 p.s.i.). Column eluent was monitored at 242 nm at detector settings between 5 and 20 milliabsorbance units full scale.

Recovery studies

Standards: Stock solutions containing 500 μmol/l and 640 μmol/l of cortisol were prepared in absolute ethanol, stored at 4°C, and used within 1 month. Appropriate dilutions of stock solutions were made into methylene chloride, our mobile phase, and water as described in the studies below.

Calculations. Analytical recoveries were calculated by dividing the peak area of absorbance or radioactivity measured in a sample prepared following our protocol (*i.e.*, solvent evaporation and reconstitution) by the peak area of absorbance or radioactivity measured in a standard diluted directly in our mobile phase corresponding to the theoretical concentration of the reconstituted sample (*i.e.*, assuming complete analytical recovery). The dividend was then multiplied by 100.

Concentration effect. Solutions containing 0.04, 0.12, 0.40, 1.20, 4.0 and 8.0 μmol/l of cortisol in methylene chloride were prepared and 8-ml portions transferred to a corresponding centrifuge tube, evaporated to dryness and reconstituted with 100 μl of our mobile phase. We prepared the various concentrations of cortisol by sequentially diluting our 640 μmol/l standard four-fold with methylene chloride and then to the cortisol concentrations used in the study, *e.g.*, a 40-fold dilution to yield a cortisol concentration of 4.0 μmol/l. Chromatographic peak areas for the reconstituted samples were compared to the peak area of a 3.2, 8.0 or 9.6 μmol/l standard prepared by diluting our 640 μmol/l standard with mobile phase. After reconstituting samples with 100 μl of mobile phase, the two solutions with the lowest concentrations of cortisol (*i.e.*, 0.04 and 0.12 μmol/l) were injected directly into the HPLC system and compared to a standard corresponding to complete analytical recovery (*i.e.*, 3.2 and 9.6 μmol/l). Reconstituted solutions from the four remaining samples were diluted further with mobile phase as described in Table I.

Tubes for evaporation. A 40 nmol/l solution of cortisol in methylene chloride was prepared and 8 ml transferred to each of five different types of tubes: a Pyrex brand glass, 15-ml screw-capped centrifuge tube, Corning, (Corning, NY, U.S.A.); a Ray-Sorb, 1.8 × 15 cm, low-actinic glass tube, Kimble Products (Vineland, NJ, U.S.A.); a Falcon No. 2070, 50-ml conical polypropylene tube, Becton-Dickinson (Oxnard, CA, U.S.A.); a Tefzel ETFE (ethylenetetrafluoroethylene), 12-ml tube, Nalgene (Rochester, NY, U.S.A.); and a Pyrex brand glass, 15-ml tube rinsed in a 10 g/l solution of Dow-Corning 360 fluid (Dow Chemical, Midland, MI, U.S.A.), a clear liquid containing dimethyl polysiloxane which dries to a chemically inert, water repellant surface after heating to 300°C for 30 min. The cortisol solution was prepared by

sequentially diluting our 640 μmol/l standard 16,000-fold with methylene chloride, *i.e.*, a 200-fold dilution followed by an 80-fold dilution. The solution was evaporated to dryness and the residue reconstituted with 100 μl of our mobile phase. Chromatographic peak areas measured for the reconstituted specimens were compared to the peak area of a 3.2-μmol/l standard prepared by diluting 50 μl of our 640-μmol/l standard to 10.0 ml with mobile phase.

Tritiated cortisol. A 50-nmol/l solution of cortisol in methylene chloride containing approximately 6.25 μCi/l of tritiated cortisol was prepared and an 8-ml portion was transferred to a glass centrifuge tube, evaporated to dryness and reconstituted with 100 μl of our mobile phase. During chromatography, fractions were collected at the detector outlet into scintillation vials at 30-sec intervals beginning at the time of injection. Then 10 ml of scintillation fluid was dispensed into each vial, thoroughly mixed with each fraction and counted for 10 min.

Polyethylene glycol study. Solutions containing 50 nmol/l of cortisol and various concentrations of polyethylene glycol in methylene chloride were prepared and 8-ml portions transferred to centrifuge tubes, evaporated to dryness and reconstituted with 100 μl of mobile phase. Cortisol was added to the solutions by initially preparing a 100-fold dilution of our 500 μmol/l standard in methylene chloride and then dispensing 100-μl portions into eleven 10-ml volumetric flasks. Methylene chloride and ten solutions containing 0.004, 0.01, 0.10, 0.40, 1.0, 2.0, 5.0, 10, 20 and 50 mg/l of polyethylene glycol in methylene chloride were added to dilute the 100 μl of cortisol solution to 10.0 ml. Chromatographic peak areas for the reconstituted specimens were compared to the peak area of a 4.0-μmol/l standard prepared by diluting 80 μl of our 500-μmol/l standard to 10.0 ml with mobile phase.

Extracted aqueous cortisol. Seven 1-ml aliquots of a 500-nmol/l solution of cortisol in water were each extracted with 10 ml of methylene chloride. The methylene chloride layers were combined, mixed and dispensed in 8-ml portions into seven glass centrifuge tubes. A 160-μl volume of a 1.0-g/l solution of polyethylene glycol in methylene chloride was pipetted into each tube. The extracts were evaporated to dryness, and reconstituted with 100 μl of mobile phase. Chromatographic peak areas for the reconstituted specimens were compared to the peak area of a 4.0-μmol/l standard prepared by diluting 80 μl of our 500-μmol/l standard to 10.0 ml with mobile phase.

RESULTS AND DISCUSSION

In a preliminary study, we observed analytical recoveries ranging between 59 and 101 % upon repeated analysis ($n = 6$) of an aqueous solution containing cortisol (500 nmol/l) in which sample preparation consisted of extraction with methylene chloride, solvent evaporation and reconstitution of the residue with the mobile phase. Additional studies in which cortisol standard (50 nmol/l) was dissolved directly in methylene chloride (*i.e.*, bypassing sample extraction) showed analytical recoveries ranging from 47 to 75 %. These observations suggested to us that cortisol was either decomposing during sample preparation or was dissolving incompletely from the side of the tube during reconstitution.

Data in Table I show that as the concentration of cortisol in methylene chloride is increased, there is a small concomitant increase in the analytical recovery.

TABLE I

ANALYTICAL RECOVERY AT VARIOUS CONCENTRATIONS OF CORTISOL

An 8-ml volume of cortisol solution in methylene chloride was evaporated to dryness and reconstituted with 100 µl of mobile phase

Solution evaporated	*Analytical recovery of cortisol*		
	Experimental (µmol/l)	*Theoretical (µmol/l)*	*%*
0.04	1.4	3.2	44
0.12	5.1	9.6	53
0.40	20.6*	32.0	64
1.20	62.8**	96.0	65
4.00	246***	320	77
8.00	454 §	640	71

* Reconstituted sample (50 µl) diluted four-fold with mobile phase and peak area compared to 8.0-µmol/l standard.

** Reconstituted sample (50 µl) diluted ten-fold with mobile phase and peak area compared to 9.6-µmol/l standard.

*** Reconstituted sample (50 µl) diluted 40-fold with mobile phase and peak area compared to 8.0-µmol/l standard.

§ Reconstituted sample (50 µl) diluted 80-fold with mobile phase and peak area compared to 8.0-µmol/l standard.

Chromatograms showed no extraneous peaks at 242 nm, following sample preparation, only a reduction in peak height compared to a cortisol standard prepared in our mobile phase. The relationship between recovery and concentration in Table I is inconsistent with a solubility problem associated with the reconstitution step, since concentrations exceeding the limit of solubility of cortisol would show reduced recoveries with increasing concentrations. Similarly, solubilities reported[12] for cortisol in water (0.77 mmol/l) and methanol (17 mmol/l) are well above the largest concentration examined in Table I. Unfortunately, the analytical recovery observed for the 0.04-µmol/l solution of cortisol in methylene chloride which showed the lowest value

TABLE II

ANALYTICAL RECOVERY OF CORTISOL FROM VARIOUS CONTAINERS

Type of tube	*Analytical recovery**	
	µmol/l	*%*
Pyrex glass	1.57	49
Low-actinic glass	1.94	61
Siliconized glass**	1.40	44
Polypropylene	0.98	31
Ethylenetetrafluoroethylene	1.71	53

* The theoretical concentration of a reconstituted specimen with 100% recovery is 3.20 µmol/l.

** Pyrex glass treated with Dow-Corning 360 fluid.

in Table I corresponds most closely to a typical physiological concentration in human serum before extraction with a ten-fold volume of solvent.

Table II shows that low analytical recovery of cortisol (*i.e.*, 31–61 %) was also observed in a variety of containers used while evaporating the methylene chloride prior to measurement by HPLC. Again no extraneous peaks were observed in the chromatogram at 242 nm. The analytical recovery of cortisol in a low actinic glass tube was only slightly higher than in a clear glass tube, suggesting the loss of steroid is not the result of photolysis to a non-absorbing compound during sample preparation. Assuming that a portion of the cortisol becomes irreversibly bound to the surface of the container during solvent evaporation, data in Table II suggests that the composition of the surface of the container has a negligible influence on the apparent binding. For example, coating the surface of the glass tube with dimethyl polysiloxane (*i.e.*, Dow-Corning 360 fluid) and the use of polypropylene and ethylenetetrafluoroethylene tubes failed to improve the analytical recovery compared to the untreated glass tube.

We further investigated the possible decomposition of cortisol during sample preparation by use of tritiated cortisol. Fig. 1 shows that the retention time of tritiated cortisol measured by radioactive counting in fractions collected during chromatography coincides with the retention time for cortisol measured by absorbance. There were no other peaks in the radiochromatogram and the calculated recovery of radioactivity in the eluted fractions was 71 %. These findings reaffirm the analytical loss of cortisol during sample preparation and suggest that the portion of cortisol

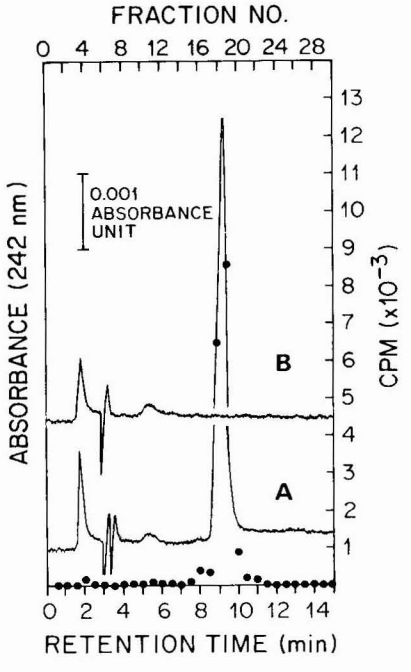

Fig. 1. Chromatogram (A, solid line) and a radiochromatogram (●) for cortisol and tritiated cortisol following sample preparation for HPLC. Chromatogram B shows a blank, consisting only of methylene chloride without cortisol, treated the same as the sample in A.

TABLE III

ANALYTICAL RECOVERY OF CORTISOL AT VARIOUS CONCENTRATIONS OF POLYETHYLENE GLYCOL

Solvent: methylene chloride. Cortisol concentration: 50 nmol/l.

Concentration of polyethylene glycol (mg/l)	*Analytical recovery**	
	μmol/l	*%*
0.000	2.16	54
0.004	2.27	57
0.01	1.70	42
0.10	2.34	58
0.40	3.48	87
1.00	3.62	90
2.00	3.67	92
5.00	3.85	96
10.0	3.96	99
20.0	3.95	99
50.0	3.96	99

* The theoretical concentration of a reconstituted specimen with 100% recovery is 4.00 μmol/l.

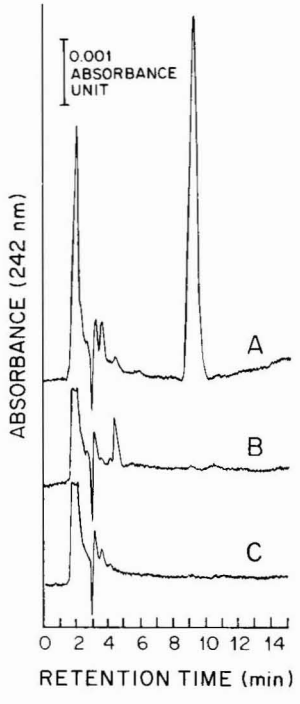

Fig. 2. Chromatogram A shows cortisol and polyethylene glycol following sample preparation for HPLC. Chromatograms B and C show blanks (no added cortisol) consisting of methylene chloride with and without polyethylene glycol prepared the same as the sample in A.

which redissolves in our mobile phase has not undergone chemical decomposition, *i.e.*, unless the product of such a decomposition has the same chromatographic retention time as cortisol and does not contain the radioactive portion of the molecule.

Collectively, the data presented above suggest that a portion of the cortisol becomes bound to the surface of the container during the evaporation step of sample preparation so that it is not readily redissolved in our mobile phase. We investigated the possibility of competing with the binding to the container by adding a material to the methylene chloride which would provide a matrix for cortisol in the residue produced during solvent evaporation. We selected polyethylene glycol as a candidate matrix material because it is soluble in methylene chloride, water and methanol and it is commonly used as a large molecular weight matrix component for lyophilized products. Table III shows that analytical recoveries of cortisol are increased with increasing concentrations of polyethylene glycol to a maximum of 99%, *i.e.*, even at a concentration of 1 mg/l of polyethylene glycol in methylene chloride the recovery increased to 90%. At a polyethylene glycol concentration of 10 mg/l and greater, values for the analytical recovery appeared to plateau at essentially the theoretical value.

Fig. 2 shows the effect of adding polyethylene glycol on the chromatogram for cortisol. Although several small extraneous peaks arising from polyethylene glycol are introduced in the beginning of the chromatogram, none of them coincide with the peak for cortisol.

As a final experiment, polyethylene glycol added to methylene chloride extracts prepared from an aqueous standard (500 nmol/l) gave analytical recoveries of cortisol ranging between 92 and 99% ($n = 7$, mean 96%). Although we have not examined different commercial sources of polyethylene glycol or the long-term effects of this polymer on our reversed-phase column, these results indicate that adding a suitable macromolecular matrix material to the methylene chloride before solvent evaporation substantially improves the analytical recovery of cortisol and lowers the variability of results.

REFERENCES

1 W. Wortmann, C. Schnabel and J. C. Touchstone, *J. Chromatogr.*, 84 (1973) 396.
2 J. H. M. Van den Berg, C. R. Mol, R. S. Deelder and J. H. H. Thijssen, *Clin. Chim. Acta*, 78 (1977) 165.
3 G. E. Reardon, A. M. Caldarella and E. Canalis, *Clin. Chem.*, 25 (1979) 122.
4 D. C. Garg, J. W. Ayres and J. G. Wagner, *Res. Commun. Chem. Pathol. Pharmacol.*, 18 (1977) 137.
5 N. R. Scott and P. F. Dixon, *J. Chromatogr.*, 164 (1979) 29.
6 F. K. Trefz, D. J. Byrd and W. Kochen, *J. Chromatogr.*, 107 (1975) 181.
7 J. Q. Rose and W. J. Jusko, *J. Chromatogr.*, 162 (1979) 273.
8 P. M. Kabra, L. Tsai and L. J. Marton, *Clin. Chem.*, 25 (1979) 1293.
9 F. J. Frey, B. M. Frey and L. Z. Benet, *Clin. Chem.*, 25 (1979) 1944.
10 T. Kawasaki, M. Maeda and A. Tsuji, *J. Chromatogr.*, 163 (1979) 143.
11 G. Cavina, G. Moretti, R. Alimenti and B. Gallinella, *J. Chromatogr.*, 175 (1979) 125.
12 P. G. Stecher (Editor), *The Merck Index*, Merck and Co., Inc., Rahway, NJ, 8th ed., 1968, p. 542.

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Note

Ion-exchange and straight-phase partition high-performance liquid chromatography of the opiates on silica

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Liquid chromatographic systems containing acidic amine salt buffers and using silica columns have recently been reported[1–3]. These systems were originally designed for simultaneous separations of primary, secondary and tertiary amines as well as quaternary ammonium salts. Recently one of these systems has been used for the determination of morphine in opium[4]. All the systems published can be described as straight-phase partition chromatography.

It is known that silica gel may also act as a cation exchanger in water-rich systems[5].

The opiates have previously been separated by high-performance liquid chromatography (HPLC) using straight-phase adsorption[6–9], straight-phase partition[4,10,11], reversed-phase[11–16] and ion-exchange chromatography[17]. In this study the influence on the retention of some opium alkaloids of a gradual change in the polarity of the mobile phase from a straight-phase system containing 1 % water to an ion-exchange system containing 90 % water is investigated using a silica column.

EXPERIMENTAL

Apparatus

A liquid chromatograph comprised of a Waters 6000 pump, a Rheodyne 7120 loop-injector, a Waters 440 UV-absorbance detector (254 nm) and an Omniscribe 5117-5 recorder was used.

Chemicals

All chemicals used were of analytical reagent grade, and the amines were distilled before use.

Chromatography

The chromatographic column (150 × 4.65 mm I.D.) was packed with LiChrosorb SI 60 5-μm particles as previously described[1]. The efficiency of the column was 6000 plates for thebaine (capacity ratio, $k' = 2.0$) measured with acetonitrile–water–glacial acetic acid–diethylamine (10:90:0.5:0.5) as the mobile phase.

A column identical to the chromatographic column was installed between the pump and the loop-injector in order to saturate the mobile phase with silica.

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RESULTS AND DISCUSSION

The basis for this investigation was a straight-phase partition chromatographic system with silica as the solid phase and tetrahydrofuran–methanol–glacial acetic acid–diethylamine–water (80:20:0.5:0.5:1.0) as the mobile phase.

The polarity of the mobile phase was gradually increased by substituting methanol for tetrahydrofuran (Fig. 1). This resulted in a decreased k' value for normorphine, but increased k' values for the other opiates tested. The k' value for morphine increased less than the values for codeine and thebaine.

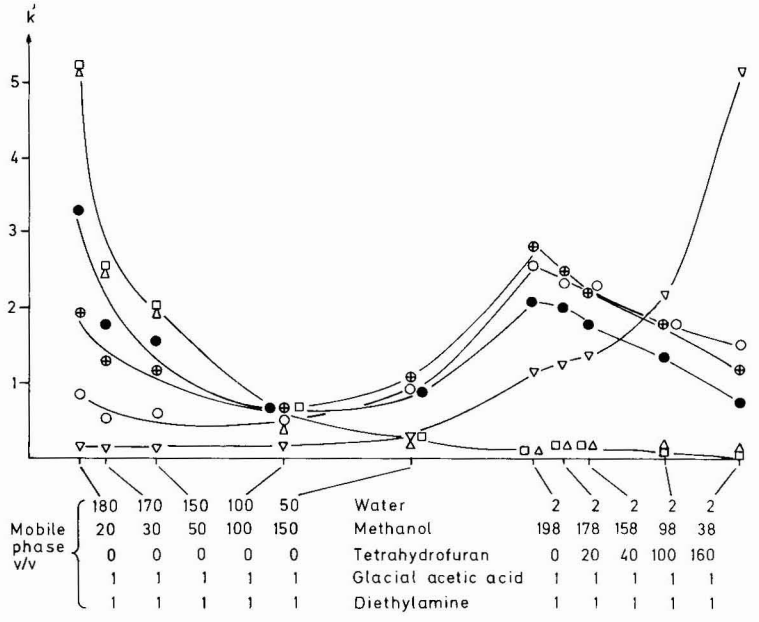

Fig. 1. Plots of the k' values of opiates obtained on unmodified silica *versus* the polarity of the mobile phase. Opiates: ⊕, codeine; ○, morphine; ▽, normorphine; □, noscapine; △, papaverine; ●, thebaine.

This behaviour may be due to solvation of the solutes and the diethylammonium acetate. Solvation of the solutes results in decreased k' values, the more polar substances being affected the most. Therefore a drastic change in retention is seen for normorphine. On the other hand, at the higher methanol concentration, less diethylammonium acetate will be adsorbed to the silica, which gives the solutes more access to the silica surface. This results in an increase in the retention of the less solvated semipolar compounds, thebaine and codeine.

When the polarity of the mobile phase was further increased by substituting water for methanol, the k' values for all the opiates decreased as a consequence of the deactivation of the silica. However, when the water content increased beyond *ca.* 50%, the k' values again began to increase, and the order of elution became the exact opposite of what was seen with the straight phase system at 80% tetrahydrofuran.

The retention of the opiates using mobile phases with from 90 to *ca.* 50% water may be ascribed to an ion-exchange effect, while the retention mechanism when using

mobile phases containing from *ca.* 50 down to 1 % water seems more complex, probably being a mixture of several mechanisms.

The influence of the pH of the mobile phase on the retention of the opiates in the cation-exchange system was investigated in the pH range 4–6 (Table I). As expected, an increase in retention is seen with increasing pH due to the increase in ionization of the silica gel.

TABLE I

k' VALUES FOR OPIATES OBTAINED ON SILICA

Eluent: methanol–0.05 *M* diethylamine buffer (15:85); the pH of the buffer was adjusted with acetic acid. Opiates: NM = normorphine; M = morphine; C = codeine; T = thebaine; N = noscapine; P = papaverine.

pH	*k'*					
	NM	*M*	*C*	*T*	*N*	*P*
4.0	0.0	0.2	0.6	1.0	1.6	1.8
5.0	0.1	0.4	0.7	1.4	2.3	2.4
5.5	0.2	0.8	1.3	2.1	3.2	3.3
6.0	0.4	1.6	1.9	3.2	4.5	4.6

Increasing the polarity of the amine resulted in increased *k'* values (Table II) probably due to the decreased affinity (increased solvation) of the more polar ammonium ions for the ion-exchange sites on the silica. The competition between the amines and the opium alkaloids for the ion-exchange sites is therefore in favour of the alkaloids. The pH values given in Table II were measured in the eluent containing methanol. Besides the increase in retention, the most polar amines gave rise to some peak asymmetry.

TABLE II

k' VALUES FOR OPIATES OBTAINED ON SILICA IN THE PRESENCE OF DIFFERENT AMINES

Eluent: water containing 15 % methanol, 0.088 *M* acetic acid and 0.05 *M* amine; the pH value is given in brackets.

Amine	*k'*					
	NM	*M*	*C*	*T*	*N*	*P*
Triethylamine (5.2)	0	0.4	0.7	1.2	1.7	1.7
Dibutylamine (5.2)	0.1	0.6	0.9	1.3	1.8	1.8
Diisopropylamine (5.1)	0.1	0.6	0.9	1.3	2.0	2.0
Diethylamine (5.1)	0.1	0.6	1.2	1.8	2.4	2.3
n-Butylamine (5.2)	0.2	0.8	1.7	2.2	3.2	3.1
Isopropylamine (5.1)	0.2	0.9	1.8	2.7	3.6	3.6
Diethanolamine (5.0)	0.2	0.9	2.0	2.9	4.3	4.3
Ethanolamine (5.0)	0.4	1.2	2.6	3.7	5.3	5.3

When another acid was substituted for acetic acid (Table III), it was seen that stronger acids gave smaller k' values. This is probably due to the change in pH since the adsorption of cations to silica decreases with decreasing pH.

TABLE III

k' VALUES FOR OPIATES OBTAINED ON SILICA IN THE PRESENCE OF DIFFERENT ACIDS

Eluent: water containing 15% methanol, 0.088 *M* acid and 0.05 *M* diethylamine; the pH value is given in brackets.

Acid	k'					
	NM	*M*	*C*	*T*	*N*	*P*
Perchloric (1.0)	0	0.2	0.7	1.0	1.3	1.5
Methanesulphonic (1.2)	0	0.4	0.8	1.2	1.7	1.9
Formic (3.6)	0	0.4	1.0	1.5	2.0	2.2
Acetic (5.1)	0.1	0.6	1.2	1.8	2.4	2.3
Propionic (5.5)	0.2	0.7	1.4	2.1	3.1	2.7

A change in the nature of the organic solvent in the mobile phase led to decreased k' values with decreasing polarity (Table IV). This behaviour is similar to that observed in other ion-exchange experiments[18,19], but the changes in k' values ongoing from isopropanol to acetonitrile to tetrahydrofuran were very small on the silica. A change in selectivity was seen when using tetrahydrofuran.

TABLE IV

k' VALUES FOR OPIATES OBTAINED ON SILICA IN THE PRESENCE OF DIFFERENT ORGANIC SOLVENTS

Eluent: water containing 10% organic solvent, 0.088 *M* glacial acetic acid and 0.05 *M* diethylamine.

Solvent	k'					
	NM	*M*	*C*	*T*	*N*	*P*
Methanol	0.2	0.9	2.0	3.2	5.3	5.2
Isopropanol	0.2	0.7	1.4	1.9	2.7	2.8
Acetonitrile	0.2	0.7	1.3	1.9	2.7	2.8
Tetrahydrofuran	0.1	0.6	0.9	1.7	2.1	2.5

The overall conclusion is that it is possible to separate the opium alkaloids in the straight-phase partition as well as in the ion-exchange mode on unmodified silica. The ion-exchange system behaves very much like a reversed-phase system and has the same advantages. This gives the analyst more possibilities for selection of the appropriate chromatographic system without changing the column.

REFERENCES

1 S. H. Hansen, *Arch. Pharm. Chem. Sci. Ed.*, 5 (1977) 19.
2 L. Nordholm and S. H. Hansen, *Analyst (London)*, 105 (1980) 629.
3 S. H. Hansen and L. Nordholm, *J. Chromatogr.*, 204 (1981) 97.
4 S. H. Hansen, Å. M. Hansen and B. Poulsen, *Arch. Pharm. Chem. Sci. Ed.*, 8 (1980) 181.
5 K. K. Unger, *Porous Silica —Its Properties and Use as Support in Column Liquid Chromatography*, Elsevier, Amsterdam, 1979, p. 130.
6 T. H. Beasley, D. W. Smith, H. W. Ziegler and R. L. Charles, *J. Ass. Offic. Anal. Chem.*, 57 (1974) 85.
7 R. Verpoorte and A. Baerheim Svendsen, *J. Chromatogr.*, 100 (1974) 227.
8 H. W. Ziegler, T. H. Beasley and D. W. Smith, *J. Ass. Offic. Anal. Chem.*, 58 (1975) 888.
9 P. G. Vincent and B. F. Engelke, *J. Ass. Offic. Anal. Chem.*, 62 (1979) 310.
10 B. B. Wheals, *J. Chromatogr.*, 122 (1976) 85.
11 K. E. Rasmussen, F. Tønnesen, B. Nielsen, B. Lunde and J. Røe, *Medd. Norsk. Farm. Selsk.*, 40 (1978) 117.
12 V. D. Gupta, *J. Pharm. Sci.*, 65 (1976) 1697.
13 I. Luris, *J. Ass. Offic. Anal. Chem.*, 60 (1977) 1035.
14 C. Olieman, L. Maat, K. Waliszewski and H. C. Beyerman, *J. Chromatogr.*, 133 (1977) 382.
15 C. Y. Wu and J. J. Wittick, *Anal. Chem.*, 49 (1977) 359.
16 C. Y. Wu, M. S. Michailidis and J. J. Wittick, *Anal. Chim. Acta*, 108 (1979) 233.
17 I. H. Knox and J. Jurand, *J. Chromatogr.*, 82 (1973) 398.
18 O. Samuelson, *Ion Exchange Separations in Analytical Chemistry*, Almqvist & Wiksell, Uppsala, 1963.
19 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979.

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Note

High-performance liquid chromatographic analysis of derivatized hypocholesteremic agents from fermentation broths

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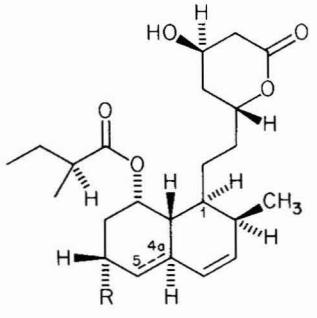
Substances which reduce cholesterol synthesis in humans are of great interest because of the possible connection between high blood cholesterol and atherosclerosis[1,2]. The fermentation metabolites of *Aspergillus terreus*, mevinolin (I)[3] and dihydromevinolin (II)[4], inhibit cholesterol biosynthesis at the level of the microsomal enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase). The analysis of these hypocholesteremic agents in fermentation broths by high-performance liquid chromatography (HPLC) is of great interest.

The determination of low concentrations of mevinolin in broth extracts by HPLC is conveniently carried out using ultraviolet (UV) detection at 237 nm. However, dihydromevinolin lacks the diene chromophore and has no appreciable absorption at this wavelength. An additional problem is that mevinolin and dihydromevinolin exist in the lactone and free acid forms which makes the chromatographic separation of all four substances from impurities difficult.

The formation of benzoate esters for UV detection in HPLC has been reported for hydroxylated compounds such as hydroxy steroids[5–8] and carbohydrates[9]. In this paper we report a method for the HPLC analysis of mevinolin and dihydromevinolin by the efficient formation of the 4-nitrobenzoate derivative. The method we employ yields the reaction products in the lactone form which reduces chromatographic difficulties. The derivatives can be readily separated from the reagents and broth impurities by reversed-phase HPLC with detection limits in the ng range. The method as presented in this paper demonstrates a simple derivatization procedure for fermentation broth extracts with minimal sample preparation before HPLC analysis.



I	Mevinolin	R = CH_3, $\Delta^{5,4a}$
II	Dihydromevinolin	R = CH_3
III	Compactin	R = H, $\Delta^{5,4a}$
IV	Dihydrocompactin	R = H

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The utility of forming 4-nitrobenzoate derivatives is not limited to the quantitative analysis of known hydroxylated compounds. This method can also be employed for observing hydroxylated compounds which cannot be readily detected because of the lack of a significant UV chromophore. When the procedure was applied to the fermentation broth of *Penicillium citrinum* which produces compactin (III)[10,11], the previously undetected compound dihydrocompactin (IV) was discovered[12].

EXPERIMENTAL

Reagents

4-Nitrobenzoylchloride (practical grade, Eastman, Rochester, NY, U.S.A.) was recrystallized from hexanes (b.p. 65.8°–68.8°C; Fisher, Pittsburgh, PA, U.S.A.). 4-Dimethylaminopyridine was obtained from Aldrich (Milwaukee, WI, U.S.A.). All solvents for extraction and derivatization were ACS certified (Fisher). The HPLC solvents were all HPLC Grade (Fisher).

Instrumentation

For HPLC analyses the following system was employed: a Micromeritics Model 725 autoinjector with a 6-μl injection loop, a Spectra-Physics SP 4100 Computing Integrator, a Laboratory Data Control Spectromonitor III detector set at 260 nm, a DuPont Zorbax C_8 column thermostatted at 60°C and a Spectra-Physics 740B pump set a 2.0 ml/min for the solvent system: acetonitrile–methanol–water (69:2:29).

Extraction and derivatization

Fermentation broth (2 ml), adjusted to pH 4.0 with HCl, was extracted with an equal volume of ethyl acetate. A 1-ml volume of the ethyl acetate was separated and evaporated to dryness under a stream of nitrogen. The sample was redissolved in 1 ml of methylene chloride and reacted with 50 μl of a 500 mg/ml solution of 4-nitrobenzoylchloride and 100 μl of a 200 mg/ml solution of 4-dimethylaminopyridine in the same solvent. After 5 min at room temperature, 100 μl of methanol were added followed by the addition of 1 ml of 0.75 *M* ammonium phosphate, pH 3.0. After mixing, the sample was then centrifuged at 1200 *g* for 30 sec and the lower methylene chloride phase was placed in an autoinjector vial. A 6-μl volume of sample was injected into the HPLC system. Injection of larger volumes caused peak splitting in the chromatography, apparently due to the incompatibility of the methylene chloride with the HPLC solvent system.

Preparation of standards

Standards were prepared by dissolving a known quantity of mevinolin and dihydromevinolin in methylene chloride. A 1-ml volume of the standard solution was nitrobenzoylated as described above for the fermentation extracts.

RESULTS AND DISCUSSION

Fig. 1 illustrates the chromatographic separation of the 4-nitrobenzoate derivative of mevinolin and dihydromevinolin for a standard and a fermentation broth

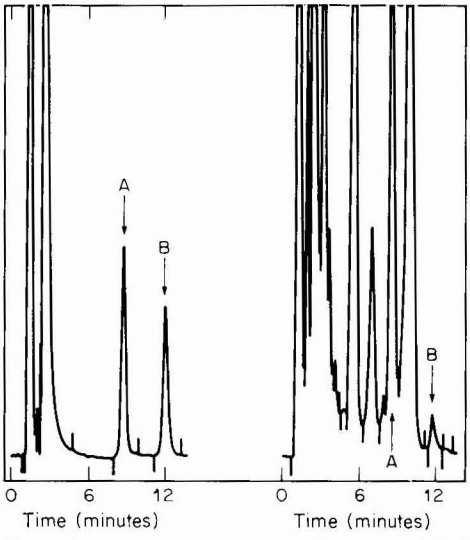

Fig. 1. Typical chromatograms of a standard (left) and a fermentation broth extract (right). A = Mevinolin; B = dihydromevinolin. The concentrations of the injected solutions are: A = 298 μg/ml, B = 268 μg/ml (left); A = 823 μg/ml, B = 62 μg/ml (right).

extract. The capacity factors (k') are 9.0 and 12.4 for mevinolin and dihydromevinolin nitrobenzoates, respectively. As shown in the figure most of the impurities, including excess reagents, side products, etc., elute very close to time = 0. Analysis time for each sample is less than 20 min and resolution of the mevinolin and dihydromevinolin nitrobenzoates is satisfactory for quantitative analyses by peak height measurements.

Derivatization of the acid forms of mevinolin and dihydromevinolin yielded the lactonized 4-nitrobenzoate forms of these compounds. The mechanism of this lactonization reaction is most likely to occur via the mixed anhydride. The concomitant lactonization is advantageous, when assaying fermentation broths which can contain both the acid and lactone forms of mevinolin and dihydromevinolin, because the quantitation and separation of two components from a complex mixture is more precise than four components. In addition, the analysis can be carried out isocratically in a reasonable time whereas the separation of four components could require a gradient analysis and increased time.

Linearity and precision

The calibration curves for the derivatives of mevinolin and dihydromevinolin are linear within the concentration range of 0–4 mg/ml and have zero intercepts.

The precision of the benzoylation procedure was determined by repeatedly assaying a single fermentation broth extract. The relative standard deviation for mevinolin is 2.2% and for dihydromevinolin is 2.7%.

Stability of the derivatized products

For this family of compounds, the hydroxyl group is located β to the lactone carbonyl and ideally poised for elimination when derivatized. For mevinolin, the

elimination of the 4-nitrobenzoate group is quite rapid in the reaction solution at room temperature (94% in 5.5 h). Extensive studies were performed to determine the best method for stabilizing the derivatization products. Interestingly, dihydromevinolin was more stable with only a 10% loss in 5.5 h. The additional $\Delta^{5,4a}$ double bond in mevinolin which flattens the decalin system, possibly interacts with the transition state to facilitate the β elimination. Various other acylating agents were explored; *i.e.*, 4-fluoro- and 4-bromobenzoylchloride and benzoyl chloride. The products were more stable (a 17% loss of the bromobenzoate group in 7 h for mevinolin) but the reaction times were slower. For the 4-fluoro- and 4-bromobenzoylchloride, the reaction times at room temperature were approximately 20 min and for benzoyl chloride much greater than 20 min. The most satisfactory method to prevent β elimination involved the addition of methanol after the desired reaction was complete, followed by extraction with pH 3.0 ammonium phosphate buffer. The 4-nitrobenzoate derivatives were stable for days at room temperature in the methylene chloride solution. Buffer extraction also removed other materials which resulted in improved chromatograms.

Discovery of dihydrocompactin

When the procedure was applied to the fermentation broth of *P. citrinum*, which produces compactin (III), a new peak was observed. A comparison of the relative retention times of mevinolin and dihydromevinolin as compared with compactin and this unknown peak suggested that the unknown peak was dihydrocompactin (IV). Isolation and structure determination of the material corresponding to this derivatized compound indeed proved this identification to be correct[12].

CONCLUSION

The formation of 4-nitrobenzoate derivatives of the HMG-CoA reductase inhibitors in the mevinolin family has been demonstrated as a practical method for sensitive HPLC analysis. The compounds of interest can be derivatized in a fermentation broth extract in a short time, at room temperature. Separation of the complex mixture can be achieved isocratically on a microparticulate C_8 column. Compounds previously with only low UV absorption can now be quantitatively observed at 260 nm with detection limits in the ng range.

There are numerous practical applications of this method. As demonstrated by the discovery of dihydrocompactin, compounds which were previously undetected can now be observed. The method can also be applied to analyses for fermentation development studies and drug metabolism studies. The 4-nitrobenzoylchloride derivatization technique should be applicable to many organic extractable hydroxylated compounds which occur in fermentation broths.

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REFERENCES

1 W. B. Kannel, W. P. Castelli, T. Gordon and P. M. McNamara, *Ann. Intern. Med.*, 74 (1971) 1.
2 J. Stamler, *Arch. Surg.*, 113 (1978) 21.
3 A. W. Alberts, J. Chen, G. Kuron, V. Hunt, J. Huff, C. Hoffman, J. Rothrock, M. Lopez, H. Joshua, E. Harris, A. Patchett, R. Monaghan, S. Currie, E. Stapley, G. Albers-Schönberg, O. Hensens, J. Hirshfield, K. Hoogsteen, J. Liesch and J. Springer, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 3957.
4 G. Albers-Schönberg, H. Joshua, M. Lopez, O. Hensens, J. Springer, J. Chen, S. Ostrove, C. Hoffman, A. Alberts and A. Patchett, *J. Antibiot.*, 34 (1981) 507.
5 F. A. Fitzpatrick and S. Siggia, *Anal. Chem.*, 45 (1973) 2310.
6 F. Nachtmann, H. Spitzy and R. W. Frei, *J. Chromatogr.*, 122 (1976) 293.
7 F. Nachtmann, H. Spitzy and R. W. Frei, *Anal. Chem.*, 48 (1976) 1576.
8 J. W. Higgins, *J. Chromatogr.*, 121 (1976) 329.
9 F. Nachtmann and K. W. Budna, *J. Chromatogr.*, 136 (1977) 279.
10 A. G. Brown, T. C. Smale, T. J. King, R. Hasenkamp and R. H. Thompson, *J. Chem. Soc. Perkin I*, (1976) 1165.
11 A. Endo, M. Kuroda and Y. Tsujita, *J. Antibiot.*, 29 (1976) 1346.
12 Y. K. Lam, V. Gullo, R. T. Goegelman, D. Jorn, H. Huang, C. DeRiso, R. L. Monaghan and I. Putter, *J. Antibiot.*, 34 (1981) 614.

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Note

High-performance liquid chromatography of naturally occurring estrogens

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(Received April 13th, 1981)

High-performance liquid chromatography (HPLC) of steroids has recently been reviewed[1]. Systematic studies of the behavior of natural estrogens in HPLC which have appeared since then include two on equine estrogens[2,3], and one on catechol estrogens[4]. In connection with our studies on steroid metabolism in plants[5,6], we have also developed a chromatographic method for the separation of free naturally occurring estrogens. By combining both adsorption and reversed-phase partition HPLC, the identification of most of the metabolites of interest to us has become possible[6]. The present study deals with 24 estrogens.

EXPERIMENTAL*

The HPLC apparatus was assembled from commercially available components. The adsorption column was a 250 × 4.6 mm I.D. stainless-steel chromatography tube (Altex, Berkeley, CA, U.S.A.), packed with Zorbax BP-SIL (7–8 μm; DuPont, Wilmington, DE, U.S.A.). The reversed-phase column had the same dimensions, but it was packed with Zorbax BP-ODS (7–8 μm, DuPont). The columns were packed in our laboratory. The packing method, detector, recorder, solvents[7], pump and sample injection valve[8] were as previously described. The chromatographic conditions are given in the figure legends.

RESULTS AND DISCUSSION

Our results are summarized in Table I. Being phenolic steroids, the estrogens were detected at 280 nm near their λ_{max}. The 24 estrogens in Table I were arranged in order of increasing polarity in adsorption chromatography and were divided into four groups: monools, diols, triols and tetraols. The number and locations of hydroxyl groups in estrogens play the most important role in their separation by HPLC. Addition of a keto group or double bond to an estrogen molecule does not increase

* Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

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TABLE I

RETENTION TIMES OF ESTROGENS

Conditions: 1: see Fig. 1; 2: see Fig. 3; 3: see Fig. 4; 4: see Fig. 2.

Compounds	*Retention time (min)*			
	1	*2*	*3*	*4*
Monools				
17-Deoxoestrone		3.25	>75	
Estrone		9.5	32	
6-Dehydroestrone		9.5	28.5	
Equilin		9.5	28.5	
Equilenin		10.5	25.5	
Diols				
Estradiol-17α		16.25	25.5	
Estradiol-17β	4.75	17.75	23	
2-Hydroxyestrone		19.5*	16	
6-Dehydroestradiol		20.25	18.5	
17α-Dihydroequilin		22.5	18.5	
3,16α-Estradiol		22.5	21	
17α-Dihydroequlenin		24.25	16	
16-Ketoestradiol		27.5	6.75	22.5
16α-Hydroxyestrone		30.75	7.25	25.5
6-Ketoestradiol	6.75	32	7.25	25.5
Triols				
16-Epiestriol	8.25		9.25	
17-Epiestriol	8.25		13.5*	
2-Hydroxyestradiol	8.25*		13.5	
Estriol	11		4.25	13
6α-Hydroxyestradiol	11		4.25	10
16,17-Epiestriol	12.75		4.25	13
6-Ketoestriol	16.75 (15)**		2.5	6.25
Tetraols				
2-Hydroxyestriol	19.25*		3.5	7.75
6α-Hydroxyestriol	28		2.5	4

* Broad peaks.

** Sample also gave a minor peak at 15 min.

the polarity in adsorption chromatography as much as in reversed-phase partition chromatography. On the other hand, epimeric estrogens are generally better resolved in adsorption than in reversed-phase systems. The fact that the elution sequence of estrogens in reversed-phase partition chromatography is not exactly the reverse of that in adsorption chromatography makes the two HPLC systems complement each other even more.

For adsorption chromatography of the more polar estrogens, *n*-hexane–ethanol (9:1) was used as the eluent (Fig. 1). This system resolved three of the four epimeric estriols: 16-epiestriol, estriol and 16,17-epiestriol. However, it did not separate 16-epiestriol from 17-epiestriol. This pair was resolved by reversed-phase partition chromatography with acetonitrile–water (35:65) as eluent (Table I), but the

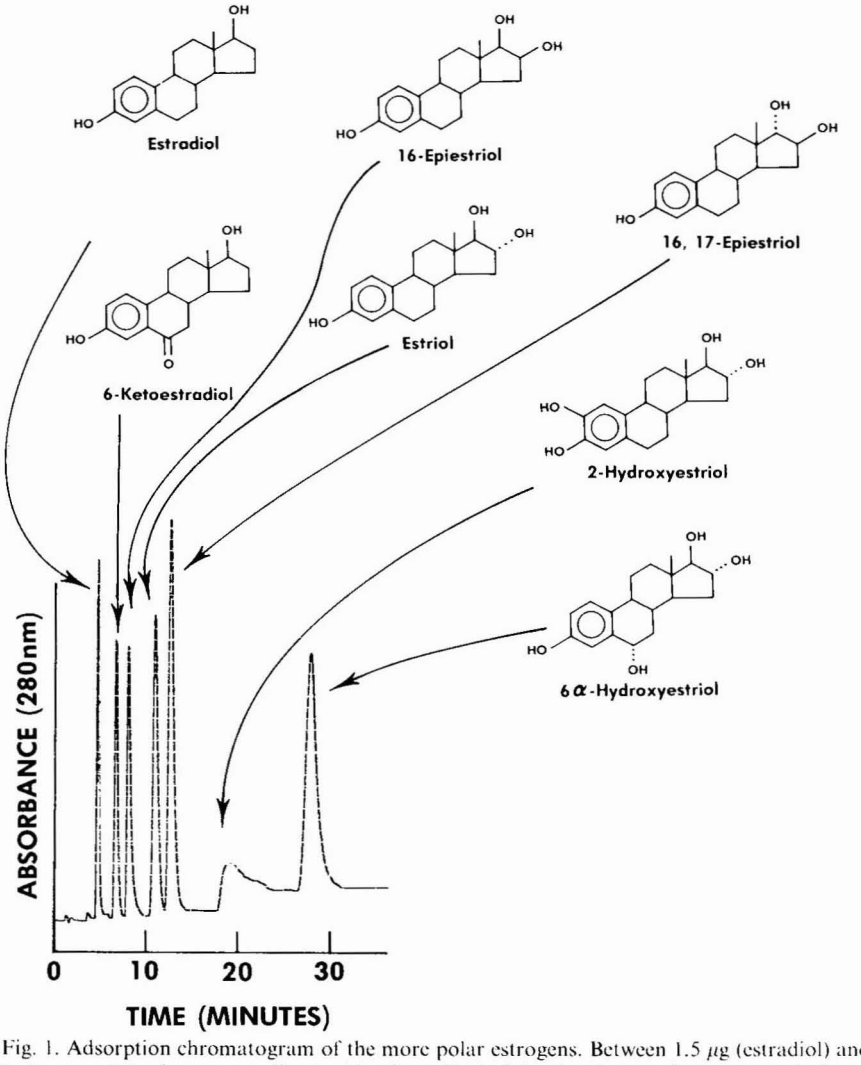

Fig. 1. Adsorption chromatogram of the more polar estrogens. Between 1.5 μg (estradiol) and 10 μg (6α-hydroxyestriol) of estrogens, dissolved in about 50 μl of the eluent, were chromatographed on a column of Zorbax BP-SIL, 250 × 4.6 mm I.D. Eluent, *n*-hexane–ethanol (9:1); flow-rate, 2 ml/min; pressure, 200 p.s.i. Detector at 280 nm; range, 0.05; time constant, 1.0. Recorder speed, 12 cm/h; span, 10 mV.

other epimers could not be separated this way. On the other hand, the position isomers, estriol and 6α-hydroxyestradiol, which could not be separated by adsorption chromatography, were resolved by reversed-phase partition chromatography (Fig. 2). 16-Epiestriol, 17-epiestriol and 2-hydroxyestriol are less polar than the other triols in Table I. Apparently, vicinal hydroxyl groups can form hydrogen bonds when they have the same orientation, and this makes the molecule less polar.

For adsorption chromatography of the less polar estrogens *n*-hexane–ethanol (97:3) was used. This eluent separated monools and diols very well (Fig. 3). However, this system was incapable of separating estrone from 6-dehydroestrone and 17α-

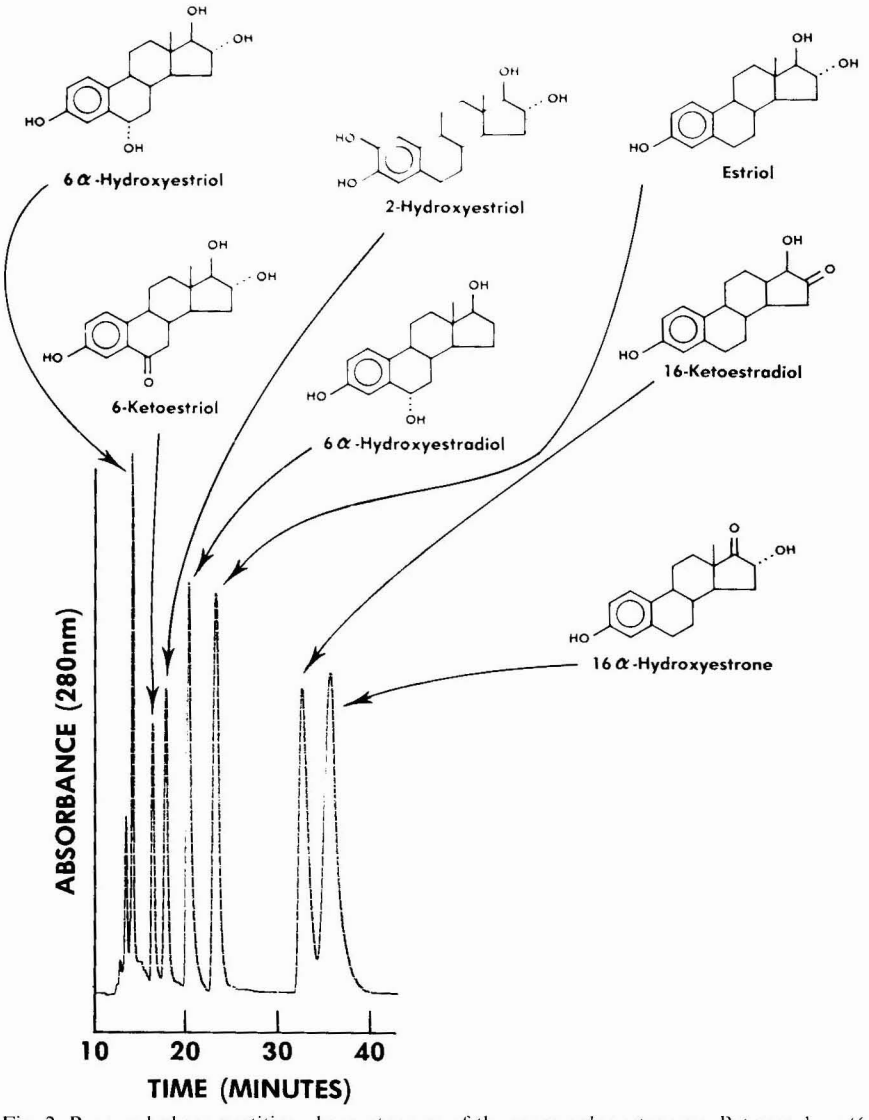

Fig. 2. Reversed-phase partition chromatogram of the more polar estrogens. Between 1 μg (6α-hydroxyestriol) and 5 μg (16α-hydroxyestrone) of estrogens, dissolved in about 50 μl of the eluent, were chromatographed on a column of Zorbax BP-ODS, 250 × 4.6 mm I.D. Eluent, acetonitrile–water (25:75); pressure, 700 p.s.i. Other conditions as in Fig. 1.

dihydroequilin from 3,16α-estradiol. These two pairs of estrogens could be separated by reversed-phase partition chromatography with acetonitrile–water (35:65) as the eluent (Table I). On the other hand, this reversed-phase partition system did not separate the following three pairs of estrogens: equilenin from estradiol-17α, 6-dehydroestradiol from 17α-dihydroequilin and 16α-hydroxyestrone from 6-ketoestradiol, whereas these three pairs could be resolved by the adsorption system (Fig. 3).

Generally, reversed-phase partition chromatography was superior to adsorp-

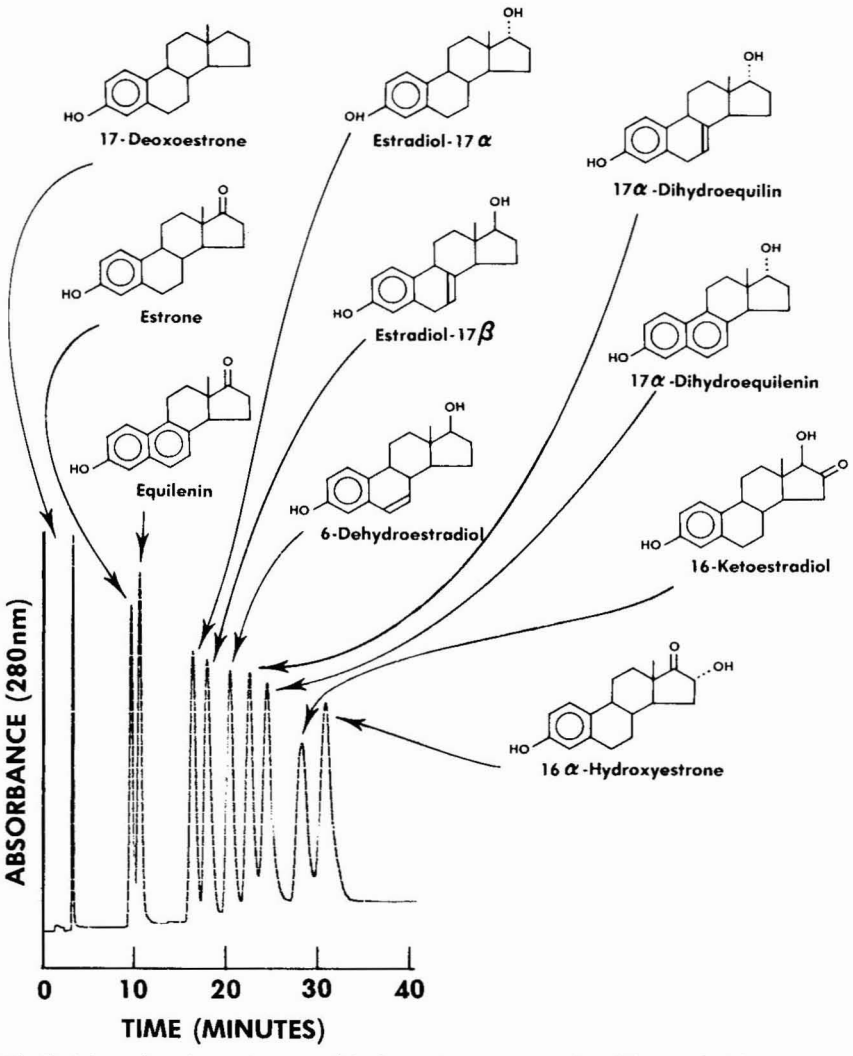

Fig. 3. Adsorption chromatogram of the less polar estrogens. Conditions as in Fig. 1, except between 1 μg (17-deoxoestrone) and 10 μg (16α-hydroxyestrone) of estrogens and an eluent of *n*-hexane–ethanol (97:3) were used.

tion chromatography in the separation of estrogens differing from each other by a double bond, *e.g.*, estrone and equilin (or 6-dehydroestrone) (Fig. 4). As for the relative polarity of the hydroxyl groups at C-16 and C-17, previously published generalizations[9] seem to hold. Estradiol-17β was found to be more polar than estradiol-17α and 3,16α-estradiol was more polar than estradiol-17β. Since 16,17-epiestriol is more polar than estriol, we predict that 3,16β-estradiol will be found to be more polar than 3,16α-estradiol. Thus, the polarity of hydroxyl groups at C-16 and C-17 increases in the order: 17α < 17β < 16α < 16β. 6-Ketoestradiol is more polar than 16-ketoestradiol in adsorption chromatography, but less polar in reversed-phase partition chromatography.

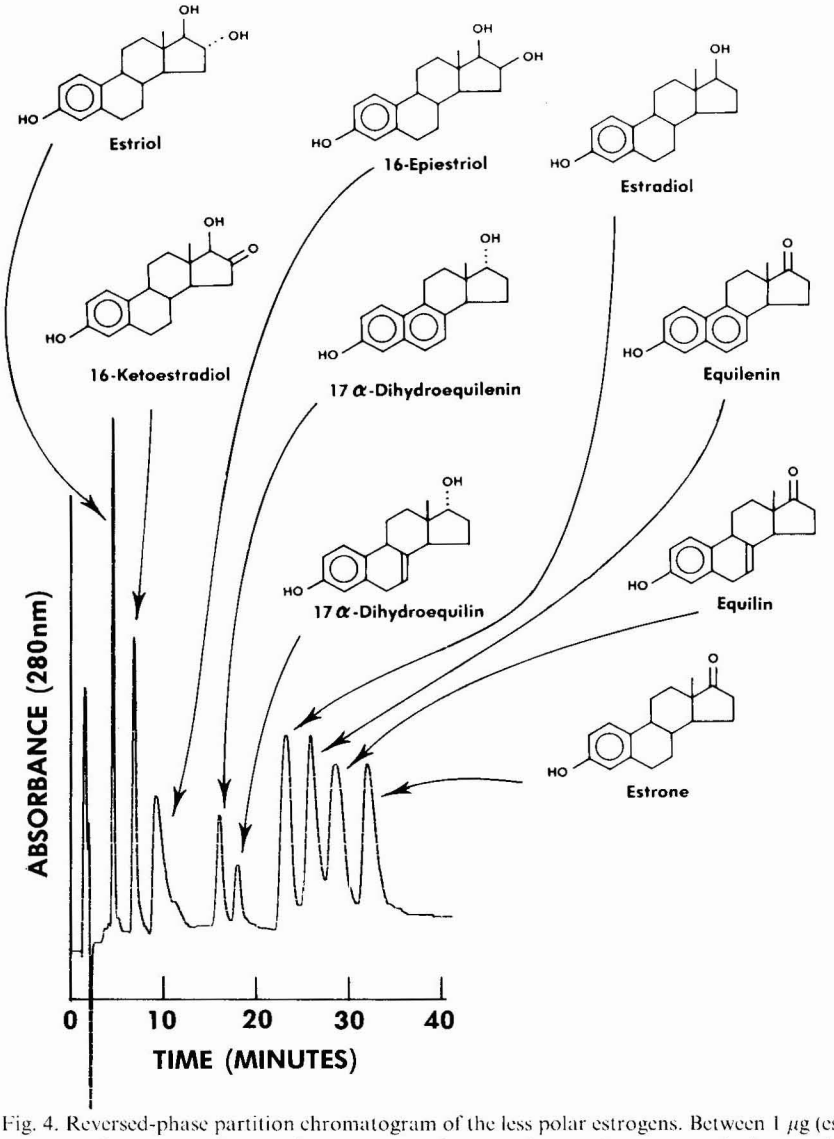

Fig. 4. Reversed-phase partition chromatogram of the less polar estrogens. Between 1 μg (estriol) and 3 μg (estrone) of estrogens, dissolved in about 50 μl of methanol, were chromatographed on a column of Zorbax BP-ODS, 250 × 4.6 mm I.D. Eluent, acetonitrile–water (35:65); pressure, 700 p.s.i. Other conditions as in Fig. 1.

REFERENCES

1 E. Heftmann and I. R. Hunter, *J. Chromatogr.*, 165 (1979) 283.
2 G. Capitano and R. Tscherne, *J. Pharm. Sci.*, 68 (1979) 311.
3 R. W. Roos, *J. Ass. Offic. Anal. Chem.*, 63 (1980) 80.
4 K. Shimada, T. Tanaka and T. Nambara, *J. Chromatogr.*, 178 (1979) 350.
5 J.-T. Lin, W. M. Proebsting and E. Heftmann, *Phytochemistry*, 18 (1979) 1667.
6 J.-T. Lin and E. Heftmann, *Phytochemistry*, in press.
7 J.-T. Lin, E. Heftmann and I. R. Hunter, *J. Chromatogr.*, 190 (1980) 169.
8 J.-T. Lin, W. D. Nes and E. Heftmann, *J. Chromatogr.*, 207 (1981) 457.
9 E. Heftmann, *J. Liquid Chromatogr.*, 2 (1979) 1137.

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Note

Liquid chromatography with UV absorbance and polarographic detection of ethylenethiourea and related sulfur compounds

Application to rat urine analysis

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Ethylenethiourea (ETU) is a major degradation product of ethylenebisdithiocarbamate fungicides that has been shown to be teratogenic in the rat[1]. Several urinary metabolites of ETU have been tentatively identified in the rat by thin-layer chromatography with radioscanning[2]. One of the metabolites, identified in this manner was the 4-imidazolone, which may be formed from ethyleneurea, the oxygen analogue of ETU via sulfur replacement. The occurrence of the imidazolone suggests that its sulfur-containing analogue, thioimidazole, might also be formed. The thiohydantoin was also included in this study since it has been reported[3] that the analogous N-methyl compound is a metabolite of methimazole, an anti-thyroid compound structurally related to ETU. High-performance liquid chromatography (HPLC) has much potential for the analysis of traces of organic compounds present in biological fluids and tissues. This report illustrates the use of HPLC for the separation and detection of ETU and related sulfur-containing compounds and application of the technique to the confirmation of the metabolite, thioimidazole, in the urine of rats fed ETU.

EXPERIMENTAL

Reagents

Table I shows the structures of the compounds studied. ETU, methimazole and thiohydantoin were obtained from Aldrich (Montreal, Canada). The thiomidazole and N-methyl ETU were obtained as gifts. Stock solutions of these were prepared at 1.0 mg/ml in methanol and were used directly for spiking purposes and recovery studies. The same solutions were diluted as required with liquid chromatography mobile phase for use as chromatographic standards. All organic solvents were distilled-in-glass grade materials. Distilled deionized water was used throughout.

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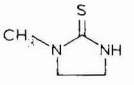
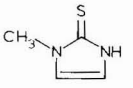
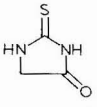
TABLE I

STRUCTURES OF THE COMPOUNDS STUDIED

Compound	*Structure*
ETU	
N-Methyl ETU	
Methimazole	
Thioimidazole	
Thiohydantoin	

High-performance liquid chromatography

Two different chromatographic systems were employed for the analyses. Normal-phase chromatography was carried out with a Waters Model 6000A solvent delivery pump, a Valco loop injector (25-μl loop) and a LiChrosorb Si 60 (10 μm) column (25 cm $\times$ 3.2 mm I.D.). The mobile phase was 15% ethanol + 0.5% NH_4OH in hexane at a flow-rate of 1.0 ml/min. Detection was carried out with a Schoeffel Model 770 variable-wavelength detector at 254 or 264 nm. The absorbance range was set to 0.01 absorbance units full scale. Reversed-phase chromatography was carried out with a Waters Model 6000A pump, a Valco loop injector (100-μl loop) and a Supelco LC-18 (5 μm) column (25 cm $\times$ 4.6 mm I.D.). The design of the dropping mercury electrode (DME) polarographic detector has been described elsewhere[4]. The mobile phase consisted of 0.1 *M* KNO_3 adjusted to pH 3. The detection mode chosen was sampled current polarography at $E = +200$ mV.

Sample preparation

Urine was obtained from male rats given an oral dose of 5.0 mg/kg ETU[2]. For normal-phase chromatography with UV detection, 1 ml of the urine was added to a 15-ml screw-capped culture tube. Following this about 250 mg of solid NaCl and 5.0 ml of ethyl acetate were added. The mixture was shaken vigorously for *ca.* 30 sec; then the tube was centrifuged at 1000 *g* to aid separation of the phases. A 25-μl aliquot of the organic layer was injected into the HPLC system for analysis.

Less sample preparation was required for reversed-phase chromatography with polarographic detection due to the detector selectivity. A 1-ml volume of urine was passed through a 0.45-μm Millipore filter and 100 μl of the filtrate injected directly into the HPLC system for analysis.

RESULTS AND DISCUSSION

Chromatography and detection

Fig. 1 shows the normal-phase separation of the compounds studied. Methimazole, besides being of interest in the ETU studies, is of some clinical importance since it has therapeutic applications and is the active metabolite of carbimazole. An HPLC method for methimazole in plasma has been reported earlier[5]; however the system described was not suitable for the compounds included herein in samples of rat urine.

Table II lists the detection limits by UV absorbance and polarographic detection and response ratios (254/264 nm) for the sulfur compounds studied. The response ratios were of some help in identifying the compounds in urine. Fig. 2 illustrates the different responses obtained at 264 nm for the same standard solution that was analysed in Fig. 1.

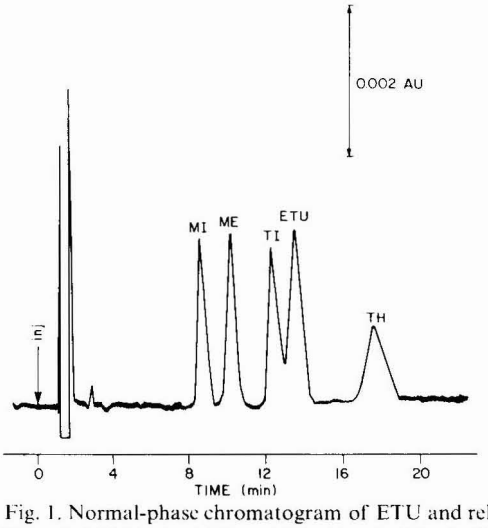


Fig. 1. Normal-phase chromatogram of ETU and related compounds. MI = methimazole (20 ng), ME = N-methyl ETU (5 ng), TI = thioimidazole (10 ng), ETU = ethylenethiourea (20 ng), TH = thiohydantoin (30 ng). Chromatography conditions as described in the text. Detection at 254 nm.

TABLE II

DETECTION LIMITS AND UV ABSORBANCE RESPONSE RATIOS

Compound	*Detection limit (ng)**		*Response ratio 254/264 nm*
	254 nm	*Polarography*	
ETU	2	1	25
N-Methyl ETU	1	–**	2.1
Methimazole	0.5	2	16
Thioimidazole	1	5	2.1
Thiohydantoin	4	15	1.4

* At signal-to-noise ratio of 3:1.
** Not determined.

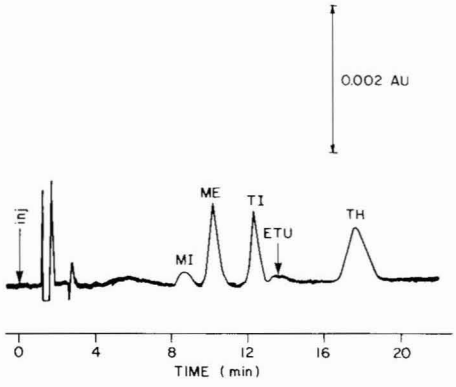


Fig. 2. Normal-phase chromatogram of ETU and related compounds. Conditions as in Fig. 1 except that detection was made at 264 nm.

The polarographic detector was found to be more selective than UV absorbance although detection limits for the compounds studied were similar, being 1–15 ng (Table II). The detection mechanism is based on a complexation between the thiourea and the mercury surface according to the reaction

$$Hg^0 + 2\,TU \rightarrow Hg\,(TU)_2^{2+} + 2\,e$$

where TU = thiourea. No such reaction occurs for the oxygen analogues. Optimum response was obtained under acidic conditions. Half-wave potentials were typically in the range of +50 to +250 mV. The advantage of operating in the positive voltage range reduces oxygen and trace metal interferences thus affording considerable selectivity for the thiourea compounds.

Urine analysis

The recoveries of all of the thiourea compounds from rat urine spiked in the range of 1–15 μg/ml urine were greater than 90% with the ethyl acetate extraction employed for normal-phase chromatography.

Fig. 3 compares UV absorption results (254 and 264 nm) of a 24-h pooled urine sample obtained from ETU-treated rats, compared to a control urine. The ETU is clearly evident. Also present is a peak corresponding to thioimidazole and one near where the thiohydantoin would be expected to elute. The response ratios (254/264 nm) correlate well with ETU and thioimidazole but not for the peak (indicated by an arrow) in the region of the thiohydantoin. Since a peak appears in the control urine in that same region further doubt is placed on any suggestion that the thiohydantoin is present in the treated rat urine.

Fig. 4 shows a reversed-phase chromatogram with polarographic detection of similarly treated urine samples. A peak corresponding to the thioimidazole confirms the findings of the normal-phase system. No thiohydantoin was detected by polarography in the treated samples.

It is interesting to note that in both HPLC systems, the thioimidazole elutes before ETU. It was found that depending on pH of the mobile phase the order of

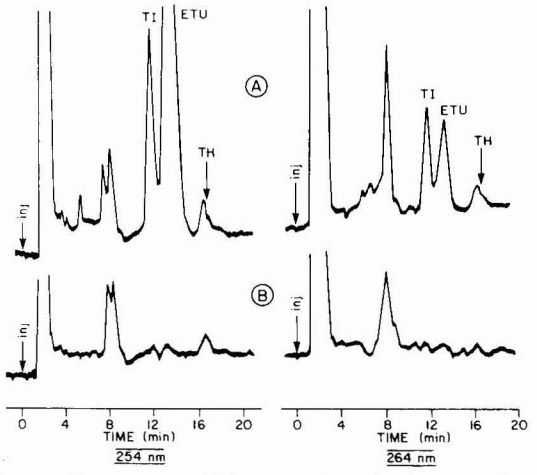


Fig. 3. Comparison of 254 and 264 nm for the analysis of ETU and metabolites in rat urine. A = Urine from treated rats, B = control urine. The arrow indicates the retention time of standard thiohydantoin (TH). Conditions as described in the text.

elution would reverse. The thiohydantoin was also significantly affected by mobile phase pH. These effects appear to be due to the combination of hydrophobicity and base strength of the compounds.

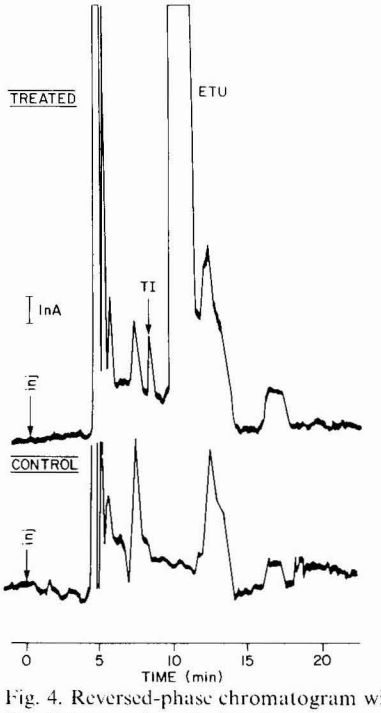


Fig. 4. Reversed-phase chromatogram with polarographic detection of rat urine sample. $E = +200$ mV. Conditions as described in the text.

CONCLUSION

Both normal-phase and reversed-phase HPLC has been found to be useful for the separation of ETU and related thiourea compounds. Both UV absorbance and polarographic detection are suitable for application to urine determinations. Because of the selectivity of the DME it is particularly suited to confirmation as well as rapid analysis with little or no sample preparation. The confirmation of thioimidazole as an ETU urinary metabolite enables the further understanding of its metabolic route in rats and other mammalian systems.

ACKNOWLEDGEMENT

Dr. J. Ruddick and Dr. W. H. Newsome of the Food Directorate, Health Protection Branch, Ottawa are thanked for the gifts of N-methyl ETU and the thioimidazole.

REFERENCES

1 J. A. Ruddick, W. H. Newsome and L. Nash, *Teratology*, 13 (1976) 263.
2 F. Iverson, K. S. Khera and S. L. Hierlihy, *Toxicol. Appl. Pharmacol.*, 52 (1980) 16.
3 P. W. Lee and R. A. Neale, *Drug Met. Disp.*, 6 (1978) 591.
4 H. B. Hanekamp, P. Bos and R. W. Frei, *J. Chromatogr.*, 186 (1979) 489.
5 G. G. Skellern, B. I. Knight and J. B. Stenlake, *J. Chromatogr.*, 124 (1976) 405.

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journal of chromatography news section

CONFERENCE AND EXHIBITION REPORT

THE 32nd PITTSBURGH CONFERENCE ON ANALYTICAL CHEMISTRY AND APPLIED SPECTROSCOPY

The 32nd Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy was held this year from March 9–13, in Atlantic City, NJ, U.S.A. The Conference is organized annually by the Society for Analytical Chemistry and the Spectroscopic Society of Pittsburgh. At the 32nd Pittsburgh Conference, 917 papers were presented in 103 sessions, compared to the 835 papers presented last year. Not only the number of papers presented at the conference is growing, but also the exhibition of modern laboratory equipment associated with the conference requires more space every year.

The continued growth of the Pittsburgh Conference in importance as a meeting place for analytical chemists and spectroscopists from all over the world, and as a showplace for new scientific equipment means that every year more conference rooms and exhibition space are required. For this reason, the organizing committees have twice moved the conference to more spacious conference and exhibition centres. The first move was made in 1968 from the 'birthplace' of the conference, Pittsburgh, PA, to Cleveland, OH. In the late '70's, a second move was necessary, and so in 1980, the Pittsburgh Conference was held for the first time in the spacious but rather old convention hall in Atlantic City, NJ. In 1980 this hall offered more space and more conference rooms than was required by the organizing committees. However, this year there was nearly a shortage of conference rooms; the traditional film festival of the conference had to be moved to a room improvised on the stage in the enormous hall; the rooms for mass spectrometry and infrared spectroscopy sessions were too small for the purpose, and participants had to listen to the presentation of papers while standing.

Nearly all available exhibition space was used by the 499 exhibiting manufacturers. The number of exhibitors rose from 1980 by 14.7%. The number of participants rose above the 17,000 mark, and some had trouble in finding suitable hotel accommodations and transport facilities.

The exhibition was open on the first four conference days and on Friday morning the huge hall was a turmoil of fork lift trucks, pick-ups and a vast number of wooden crates, in contrast to the 1980 situation when the exhibition closed at noon on Friday.

As usual, the Perkin-Elmer Corporation from Norwalk, CT, had the largest booth this year, occupying 35 standard booth areas at the exhibition. The Leco Corporation, which specializes in instruments for the determination of sulphur, carbon, nitrogen and oxygen in a variety of matrices, displayed their products on 20 standard booth areas; Bausch&Lomb/ARL had 19, Varian Associates 15, and Hewlett-Packard had a crowded 12-booth area.

In the chromatographic field in general there were few novel developments introduced. The most important in gas chromatography (GC) was the SP-7100 series of chromatographs freshly developed by Delta Electronics and produced by Spectra-Physics's Autolab Division in San Jose, CA, U.S.A. The SP-7100 is microprocessor based and available in various stages of sophistication. The central control and data processing unit is equipped with a printer/plotter unit and a video tube display for easy programming and methods development. The memory can store up to 10 methods. The SP-7100 has a

dedicated keyboard; the operator does not need any typing skill. The central unit can be coupled with a number of oven units. The SP-7100 system offers a spacious standard oven unit, prepared for several types of GC work. The central unit can also be coupled with units from other manufactures. The system comes with FID and TCD detectors; and others will become available, soon, according to the promises made at the conference by Spectra-Physics.

Varian's Aerograph Division showed the relatively new VISTA-series of chromatographic equipment and introduced the new GC Models 6000 and 6500. The Varian Model 6000 can be coupled to the VISTA 401 data system, but the 6000 is in fact an independent microprocessor-based instrument. The hardware looks very much like that of the 5000-series HPLC instruments. The 6000 is equipped with a video tube for easy programming and offers easy dedicated keyboard operation with the analytical possibilities of the well-known 3700-series. The Varian 6000 can also be coupled to a simple satellite gas chromatograph, the new Model 6500; the microprocessor electronics of the 6000 can handle the control and data processing for both analytical units.

Packard-Becker, the GC division of United Technologies. introduced their new models 437 and 438. These new microprocessor-controlled chromatographs complete the Packard program on the low cost side. The Model 437 offers a dual-column oven and dedicated keyboard operation. This instrument is aimed at the fields of routine analysis for quality control and of analytical education. The more expensive Model 438 has the same type of keyboard operation but offers the well-known principle of the analytical modules, fast and easy replacement of columns and detectors, built in one unit. The 438 can be programmed and used for multidimensional GC with the Packard MDSS modules.

Perkin-Elmer showed the SIGMA 115 system, an extension of the SIGMA series, especially in regard to data processing and methods programming.

HNU Systems, Inc., offered a new version of the photoionization detector. This new version is said to be more suitable for high temperature work.

Tracor Instruments displayed a photoionization detector, Model 703, and a modular detector, Model 700A HECD, specific for either halogen-, nitrogen-, sulphur- or nitrosamine-containing compounds. In the field of high-performance liquid chromatography (HPLC) Du Pont showed the 8800 system with extended multigradient elution possibilities, based on the three-pumping heads system introduced last year.

The Japanese manufacturer, Jasco, displayed a new HPLC system, the Familic-100N, equipped with a new two-head (TWINCLE) pump.

Beckman's Altex division showed a nearly completely innovated program for HPLC, primarily the microprocessor-based control unit, Model 421; a variable-wavelength detector, Model 165; a refractive-index detector, Model 156; and a fixed-wavelength detector, Model 160.

Micromeritics exposed the new automatic sampler Model 725, and a number of smaller innovations and changes in the sales program. Perkin-Elmer introduced the new LC-85 UV–VIS detection and control unit with double-beam optics and with an optional 2.6-μl flow cell. The older LC-75 Auto-control unit remains available from the manufacturer.

Shimadzu also exposed a double-beam UV detector, Model SPD-2A. Waters Associates, exposing under the Millipore banner, showed a variable-wavelength detector, Model 441; Kratos, Inc., exposed HPLC system components from the Schoeffel division; new was a system for post-column reactions.

IBM Instruments, Inc., a new exhibitor on the Pittsburgh Conference, surprised everyone with an extended program of scientific instruments, consisting mainly of OEM versions of other manufacturer's products, painted in an off-white color. Hewlett-Packard's new HP 3390 integrator was one of these products. IBM also showed an HPLC system, already shown on the Bruker-Franzen booth at last year's Interkama in Düsseldorf, G.F.R.

Spectra-Physics introduced the Model SP-8100 modular liquid chromatograph, which consists of SP-8400 and SP-8700 components and completed with a new integrated auto sampler. The sample information is read into the system with a laser-based bar code reader.

Hewlett-Packard displayed a Model 1084B liquid chromatograph coupled with the well-known HP-85 personal computer for control and data handling purposes.

In the field of GC–MS systems, the most important news was the acquisition of Varian's MAT group by the Finnigan Corp. The instruments from Bremen, G.F.R., were displayed in a joint Finnigan-MAT booth.

A new system for mass spectrometry (MS) based on ion cyclotron spectrometry, was introduced by the Nicolet Instrument Corp. from Madison, WI, U.S.A. This FT–MS system has promising properties for GC–MS work.

The Pittsburgh Conference 1981 showed the continued trend towards the use of microprocessors in all kinds of laboratory equipment. Many exhibitors displayed instruments already known which had extended and updated software, offering new possibilities for analysis, data processing, and combinations with other instruments. Most of the microprocessor-based systems are available in a version programmable in BASIC by the user. New was the strong trend towards the use of rather inexpensive personal computers, such as the HP-85, Apple II and PET for control and data processing purposes. In chromatography there was much interest in the dedicated forms of chromatography, such as multidimensional work and 'heart cutting', both in the fields of GC and HPLC.

Now that nearly all manufacturers have their own solutions for work with ternary and multi-solvent gradients, interests seem to be concentrated on special detection systems and column protection devices.

During the 32nd Pittsburgh Conference, the Elsevier Scientific Publishing Company introduced a new journal, TrAC (short for "Trends in Analytical Chemistry"). "TrAC" is an international magazine which publishes short critical reviews and news on trends and developments in analytical chemistry. The major aim of the magazine is to make the readers aware of techniques and possibilities outside their field of specialization and to inform them of what can be achieved by these techniques. The first issue contained a feature article on the career of Professor I.M. Kolthoff, who won the 1981 Analytical Chemistry Special Award, presented to him during a special session of this year's Pittsburgh Conference.

Klaas H. Broers

ANNOUNCEMENTS OF MEETINGS

PITTSBURGH CONFERENCE AND EXPOSITION ON ANALYTICAL CHEMISTRY AND APPLIED SPECTROSCOPY

The 33rd Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy will convene March 8–12, 1982, in Atlantic City, NJ, U.S.A. Atlantic City was chosen as the meeting site for the 3rd consecutive year because of the very successful 1981 Conference and Exposition.

Preliminary announcement – call for papers

Symposia have been organized as described below: Advances in thin-layer chromatography; Surface analysis – an important interface; Industrial hygiene monitoring of chemical hazards; Computerized methods in GC–MS analysis; Process analyzers; New developments and applications of energy dispersive X-ray fluorescence; Electroanalytical techniques in the horizon; State of the Art of the hyphenated techniques; Preparation and analysis of derivatives in chromatography; Analytical chemists as inventors and problem solvers; Practical advances in liquid chromatography; Advanced techniques and standards in environmental measurements; Dal Nogare Award Symposium; Coblentz Society Award Symposium; ASTM E13.06 – New directions in molecular luminescence; Spectroscopy Society of Pittsburgh Award; Society for Analytical Chemists of Pittsburgh Award.

A short course on Approaches to Laboratory Computerization, Part II, is under study and planned to be offered on Friday and Saturday, March 12 and 13, 1982.

General papers <u>are not</u> restricted to the symposia topics. Papers may be contributed in all areas of the disciplines of Analytical Chemistry and Applied Spectroscopy. Those authors wishing to present papers in the 1982 Pittsburgh Conference should submit four (4) copies of a 300-word abstract to:

Mrs. Linda Briggs, Program Secretary
Pittsburgh Conference, Department J-057
437 Donald Road
Pittsburgh, PA 15235, U.S.A.

EXPOSITION OF MODERN LABORATORY EQUIPMENT

In 1981, the modern laboratory equipment presented at the Conference totalled 498 exhibitors occupying 1,199 booths and 34 seminar rooms in which was displayed the latest equipment available in the areas of Analytical Chemistry and Spectroscopy. Anyone desiring to reserve exhibit space or to obtain additional information regarding the 1982 Exposition should contact:

Mr. Ralph M. Raybeck, Exposition Chairman
Pittsburgh Conference
437 Donald Road
Pittsburgh, PA 15235, U.S.A.

SYMPOSIUM ON "DETECTION IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY"

A symposium on the topic "Detection in High Performance Liquid Chromatography", co-sponsored by the Free University of Amsterdam, Hewlett-Packard GmbH, Waldbronn, G.F.R., and Hewlett-Packerd, B.V., Amstelveen, The Netherlands, will take place at the Free University of Amsterdam on January 19 and 20, 1982, in the Department of Analytical Chemistry.

Emphasis will be placed on diode-array detectors, LC–MS (MS), luminescence, reaction detectors, electrochemical detectors as well as on radiochemical, conductivity, refractive index and light scattering detection.

Presentations will be in the form of discussion papers as well as poster sessions. English and German may be used at the author's discretion.

The following scientists have already expressed their interest in actively participating in the symposium: J.H.M. van den Berg, U.A.Th. Brinkman, C. Burgess, R.S. Deelder, G. Dielmann, D. Dixon, R. Frei, H. Hanekamp, J.F.K. Huber, M. Martin, A. Maute, H. Poppe, J. Rocca and G. Schill. Further contributions are welcome. Please submit title and one page abstract, 300 words, to Professor R. Frei, Free University of Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands (Phone: 020-548911), before August 15.

For registration information please contact Hewlett-Packard Nederland B.V., Analytical Department, Mrs. Peschier, van Heuven Goedhartlaan 121, 1181 KK Amstelveen, The Netherlands (Phone: 020-472021).

ILMAC 81 MIT 6. SCHWEIZERISCHEM TREFFEN FÜR CHEMIE

Vom 29. September bis 2. Oktober 1981 findet in den Hallen der Schweizer Mustermesse in Basel die ILMAC 81, 8. Internationale Fachmesse für Laboratoriums- und Verfahrenstechnik, Messtechnik und Automatik in der Chemie, statt. Im Rahmen der Fachmesse werden einige der im Patronatskomitee vertretenen Institutionen, Verbände und Gesellschaften unter dem Titel "6. Schweizerisches Treffen für Chemie" mehrere Begleitveranstaltungen durchführen.

Am 30. September veranstaltet die Schweizerische Gesellschaft für Automatik eine Tagung über "Aspekte der Mensch–Maschinen-Kommunikation zur Führung automatisierter chemischer Prozesse". Die Schweizerische Gesellschaft für Thermoanalytik und Kalorimetrie befasst sich während zwei Tagen (30. September und 1. Oktober) mit dem Thema "Chemische Thermodynamik in der angewandten Forschung". Am 1. Oktober führen ferner die Fachgruppe für Verfahrens- und Chemieingenieur-Technik des Schweizerischen Ingenieur- und Architekten-Vereins eine Tagung über Verfahrens- und chemische Reaktionstechnik und die Schweizerische Gesellschaft für Mikrobiologie eine solche über Biotechnologie durch. Dem Problem der Qualitätssicherung und Produktehaftung schliesslich widmet sich am 2. Oktober die Schweiz. Vereinigung diplomierter Chemiker HTL.

NEW BOOKS

Katalytische Methoden in der Spurenanalyse, by H. Müller, M. Otto and G. Werner, Geest & Portig, Leipzig, 1980, 168 pp., price G.D.R. M 45.00.

Analytical chemistry of polycyclic aromatic compounds, by M.L. Lee, M.V. Novotny and K.D. Bartle, Academic Press, New York, 1981, XII + 462 pp., price US$ 60.00, ISBN 0-12-440840-0.

PUBLICATION SCHEDULE FOR 1981

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

MONTH	N 1980	D 1980	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography			203 204 205/1 205/2	206/1 206/2 206/3	207/1 207/2 207/3	208/1 208/2 209/1	209/2 209/3 210/1	210/2 210/3 211/1	211/2 211/3 212/1 212/2	212/3 213/1 213/2	The publication schedule for further issues will be published later.			
Chromatographic Reviews							220/1							
Biomedical Applications	221/1	221/2	222/1	222/2	222/3	223/1	223/2	224/1	224/2					

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. A free reprint can be obtained by application to the publisher)

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

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

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

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

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

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

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Journal of Chromatography Library, 19.

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Analytical Isotachophoresis

Proceedings of the 2nd International Symposium on Isotachophoresis, Eindhoven, September 9-11, 1980.

F. M. Everaerts, Eindhoven The Netherlands (Editor)

Analytical Chemistry Symposia Series, 6

"A new twig on the tree of differential separation methods": Thus has isotachophoresis been described. It is proving a valuable technique in clinical (bio)chemistry, pharmacy, physical and inorganic chemistry, as well as in the industrial and environmental field.

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