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Chromatographic Reviews				141/1				141/2				141/3

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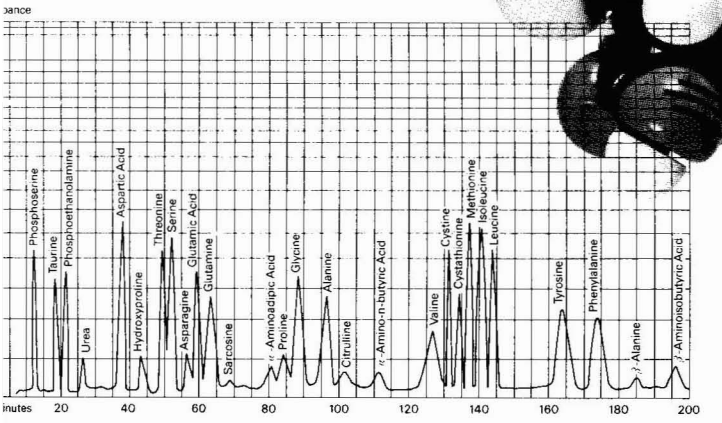

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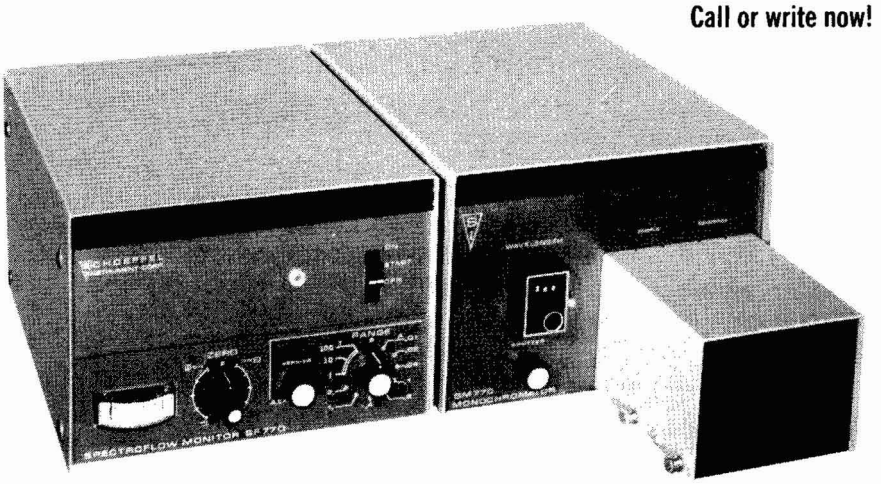

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CHROM. 9624

HYDROCARBON STATIONARY PHASES FOR GAS–LIQUID CHROMATOGRAPHY

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(Received July 9th, 1976)

SUMMARY

For the use of squalane as a standard stationary phase in gas–liquid chromatography, a preliminary clean-up step is suggested where the squalane, in a paraffin solvent, is passed successively through columns of activated carbon and alumina. A new standard non-polar stationary phase, produced by the catalytic hydrogenation of an Apiezon grease, is described. It has the advantage over squalane of a maximum operating temperature of 200°, allowing column polarities at high temperature to be evaluated.

INTRODUCTION

The polarity of a stationary phase, as defined by Rohrschneider[1], using the Kováts retention index system[2], and later by McReynolds[3] is based on the retention of various substances on the phase compared with their retentions on squalane. Although generally accepted as the standard non-polar phase, squalane suffers from the obvious disadvantage that, due to its volatility, it cannot be used at temperatures above 120°. Huber and Kováts[4] have stated that the molecular weight (MW) of a hydrocarbon to be used as a stationary phase should be higher than 1100, as the upper temperature limit for such a hydrocarbon would be around 300°. McReynolds has evaluated squalane, hexatriacontane, nujol, mineral oil, liquid paraffin, and Convoil-20 as paraffin hydrocarbon stationary phases, but they all have average MWs which are too low, all lying in the MW range of 400–900. In an earlier study on aromatic hydrocarbons as stationary phases[5], the behaviour of polystyrene and polybenzyl was compared with Apiezon L as a standard phase. The Apiezon greases have a sufficiently high MW to ensure thermal stability but their high degree of unsaturation will mean that retention parameters on Apiezon columns will depend upon the origin and nature of the stationary phase. It was therefore decided that an attempt to hydrogenate completely a sample of Apiezon grease would be undertaken. Average MWs, determined by differential vapour pressure determination, on samples of Apiezon L and M greases gave values of 3,970 and 2,340, respectively, consequently Apiezon M was chosen for the work.

It would be pointless to introduce a new standard stationary phase at this stage if such a phase rendered obsolete the enormous amount of data collected using squalane. As several papers[3,4] have demonstrated the dependence of the retention index (I) on the MW of the stationary phase, I values on such a new phase are highly likely to be higher than those obtained on squalane and a close comparison of the two, using the McReynolds system, is necessary to prove their interchangeability. During the study of squalane, it became apparent that I values far higher then those given by McReyolds were being obtained. The impurities in commercially available squalane and their effects upon I are also the subject of this paper, which claims that hydrogenated Apiezon M is a suitable material for standard stationary phase preparation and has the advantage of a max. operating temperature of 200°.

EXPERIMENTAL

All experiments were made on a Pye 104 gas–liquid chromatograph with flame ionization detector. The degree of hydrogenation of Apiezon grease was determined by UV spectrophotometry using a Unicam S.P. 800 spectrophotometer.

Hydrogenation of Apiezon grease —recommended method

10 g of Apiezon M and 5 g of freshly prepared Raney nickel catalyst in 200 ml of cyclohexane are hydrogenated at 260° for 24 h with stirring and using a hydrogen pressure of 100 atm. After removal of catalyst by filtration, the solvent is removed by rotary evaporation.

Degree of residual unsaturation

The UV spectra of both Apiezon L and M show clearly defined max. at 238 and 260 nm, being the absorbance max. of olefinic and aromatic unsaturation, respectively. Measurement of absorbances at these two wavelengths, (a) of a suitably diluted sample of the Apiezon M in cyclohexane, and (b) of the hydrogenation solution after removal of catalyst and re-adjustment of solution volume to 200 ml, will give the percentages of residual unsaturation of each type directly.

Clean-up procedure for commercial squalane

5 g of squalane are dissolved in 100 ml of petroleum ether (b. p. 60–80°). The solution is passed first through a column (15 × 1 cm I.D.) of activated carbon, then through a column (30 × 1 cm I.D.) of activated alumina. The columns are washed with a further 50 ml of petroleum ether (b.p. 60–80°) and the bulked solution is evaporated using a rotary evaporator.

I of benzene, 1-butanol, 2-pentanone, 1-nitropropane, and pyridine were determined on the hydrocarbon columns at a temperature of 120° and a 20% loading of stationary phase. I of *n*-butylbenzene, benzyl alcohol, acetophenone, nitrobenzene, and aniline were determined at 180° column temperature.

RESULTS AND DISCUSSION

From MW determinations on the Apiezon greases, Apiezon M —MW 2340— was selected as being sufficiently above the min. value of 1100 recommended by

Huber and Kováts[4] to ensure thermal stability. After hydrogenation, and the rehydrogenation necessary to bring residual olefinic and aromatic unsaturation down to values below 2%, the average MW of the product was found to be 2,680. Addition of hydrogen cannot account for such a large increase and it has been assumed that the increase is mainly due to the removal of low-molecular-weight material during the prolonged rotary evaporation treatment when the material, as a thin film, was subjected to high vacuum and a temperature of 100°.

Initial attempts to hydrogenate the grease involved the use of a catalyst consisting of active nickel in kieselguhr[6] but the residual unsaturation with this catalyst was very high (see Table I). Conditions under which Apiezon L had already been hydrogenated[7], involving the use of an active nickel catalyst prepared from Raney nickel alloy, were used on Apiezon M and, with increase in catalyst concentration and hydrogenation time, the product described in Tables I–III was produced with residual olefinic unsaturation and aromatic unsaturation percentages of 1.6 and 1.2, respectively.

TABLE I

HYDROGENATION OF APIEZON M

No.	*Hydrogenation conditions*	*Percentage residual unsaturation*	
		Olefinic	*Aromatic*
1	Ni-kieselguhr (30%) 260°, 100 atm H_2, 12 h	44	29
2	Raney Ni (25%) 260°, 100 atm H_2, 12 h	29	22
3	Rehydrogenation of No. 2 Raney Ni (50%) 260°, 100 atm H_2, 24 h	1.6	1.2

Thermogravimetric analysis of 20% Apiezon M hydrogenated (Apiezon MH) on silylated Celite conditioned at 100° revealed that the material is stable to 180°, whilst a similar column packing conditioned at 200° was subsequently stable at this temperature, and at 250° 3% of the phase was lost from the column packing.

From Table II, it can be seen from a comparison of the data on the two commercial squalane samples and McReynolds' values, that the squalanes are impure. Particularly high values are obtained for *I* of 1-butanol, indicating that the impurity is electron donating in character although high values for the electron donor compounds indicate electron acceptor properties on the squalane. The possibilities are that some squalene is present and that oxidation producing some ketone compound has occurred. The nature of these impurities is not, however, important. What matters is that these squalane samples, obtained at different times from different suppliers of materials for gas–liquid chromatography, should give *I* values showing such wide variation from the generally accepted values. Treatment of the second commercial sample by activated carbon and alumina can be seen to have reduced the variation to what may be considered as tolerable limits although this itself is debatable. The question which now arises is: How much work has been published giving polarity

data where the workers have assumed that their squalane was pure material? Chemical analysis will be of no help in this situation, where a little impurity goes a long way, however, a careful scrutiny of *I* on squalane of both the Rohrschneider and McReynolds compounds is urged. The clean-up procedure for squalane suggested here should be adopted as routine when working with this phase.

The *I* values on Apiezon MH, given in Table II, are higher than the value for purified squalane given by McReynolds and others. This is believed to be a stationary phase MW effect however, rather than retention due to residual unsaturation in the grease. Huber and Kováts have given theoretical max. for *I* of the McReynolds compounds on a paraffin hydrocarbon of infinite MW. These values are included in in Table II and it may be seen that the corresponding values for Apiezon MH are slightly lower or equal to the max. values. From the work of Huber and Kováts, it has been possible to approximate *I* of the McReynolds compounds on a stationary phase of MW 2,700, the value obtained for Apiezon MH. These values are also included in Table II and the close relationship between them and the experimental values on Apiezon MH is apparent. This grease is therefore strictly analogous to squalane in its retention behaviour, slight increases in *I* values being attributable to the MW of the phase.

TABLE II

RETENTION INDICES (*I*) OF THE McREYNOLDS COMPOUNDS AT 120°

Stationary phase	*I*				
	Benzene	*1-Butanol*	*Pentanone*	*Nitropropane*	*Pyridine*
Commercial squalane (1st sample)	769	740	694	659	757
Commercial squalane (2nd sample)	678	720	690	740	820
Purified squalane (2nd sample)	651	607	632	678	716
Squalane (McReynolds' values[3])	653	590	627	652	699
Infinite MW hydrocarbon (theoretical from ref. 4)	684	602	635	669	736
Hydrocarbon, MW 2,700 (theoretical from ref. 4)	678	597	632	665	730
Apiezon M hydrogenated	671	599	632	667	736
Apiezon L	687	627	651	700	770

Table III lists the *I* values for the aromatic compounds suggested as high temperature standard substances in earlier work[5], a column temperature of 180° having been selected as a reference. Values are given for retention data on the four Apiezon greases L, M, T, and W and it can be seen that the polarity of the greases increases in this order. Each of the greases had been hydrogenated under comparable conditions (Table I No. 2), and the lowering of *I* upon hydrogenation is clearly seen although the so-called hydrogenated greases in Table III in fact contained approximately 20% of residual unsaturation. The effects of hydrogenation under similar conditions upon the retention properties of the greases are quite unusual. Thus Apiezon T shows the smallest decreases in *I*, except for benzyl alcohol, where hydrogenation causes the largest decrease $-\Delta I$ being 110 units. Apiezon W shows the largest decrease for the electron donor acetophenone, ΔI on hydrogenation of this

grease, at 87 units, being twice the values obtained on Apiezons M and L. Finally, the comparatively small decreases for ΔI of aniline on M, T, and W are not in accordance with the large decrease of 80 units for this substance on hydrogenated Apiezon L.

It may be seen from Table III that the min. values for all retention data are obtained on Apiezon MH as would be expected. These values could be taken as the non-polar standards for the five substances at 180°. An interesting comparison is that made between Apiezon MH and polyethylene, where the MW effect of the stationary phase will be at a max. The values on polyethylene then must represent values on an infinite MW saturated phase, and, for the four substances containing a functional group, give increments of approximately 40 units over the values on Apiezon MH.

TABLE III

RETENTION INDICES (I) ON THE APIEZON GREASES, HYDROGENATED APIEZON GREASES, AND LINEAR POLYETHYLENE AT 180°

Stationary phase	*I*				
	n-Butylbenzene	*Benzyl alcohol*	*Acetophenone*	*Nitrobenzene*	*Aniline*
Commercial greases					
Apiezon L	1099	1120	1141	1147	1065
M	1098	1085	1123	1143	1000
T	1104	1176	1124	1156	1029
W	1144	1219	1297	1306	1117
Hydrogenated greases					
Apiezon L	1085	1041	1093	1128	985
M (Table I, No. 2)	1086	1036	1083	1112	988
T	1097	1067	1115	1150	1011
W	1130	1142	1210	1261	1101
Rehydrogenated greases					
Apiezon MH (Table I, No. 3)	1083	1032	1053	1105	986
Polyethylene	1095	1071	1096	1139	1001

Apiezon M grease may therefore be hydrogenated comparatively easily to procedure a stationary phase with reduced retention values. The I values obtained on such a phase compare closely with values obtained on squalane at 120° and on polyethylene at 180°. Apiezon MH grease, therefore, offers the means of obtaining standard values for column polarity evaluation without the temperature limits imposed by squalane. The new phase is stable initially to 180° but, after a conditioning period at the temperature, may be used successfully at 200°.

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CHROM. 9579

COUPLING OF A GEL-PERMEATION CHROMATOGRAPH AND AN AUTOMATIC CAPILLARY VISCOMETER

I. INFLUENCE OF THE COLUMN EFFICIENCY

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(Received July 7th, 1976)

SUMMARY

The causes of some anomalous effects observed while using an automatic capillary viscometer coupled to the separation system of a gel-permeation chromatograph have been investigated. Various systems of separation columns differing in their separation capacity have been used, but the effects have not been completely eliminated, even by means of a system having a very high resolution. The anomalous effects are explained in terms of zone-spreading of the chromatographic bands and of the experimental errors.

INTRODUCTION

The determination of the intrinsic viscosity of a polymer in the eluent leaving a separation system in gel-permeation chromatography (GPC) has been examined by several workers. Meyerhoff[1] described the application of a capillary viscometer coupled with GPC. In his later paper[2] he recommended the function 1 for the calibration of the GPC separation systems

$$[\eta] = f(V_e) \tag{1}$$

where $[\eta]$ is the intrinsic viscosity and V_e is the elution volume. The coupling of a capillary viscometer with GPC for the evaluation of the molecular-weight distribution of polymers, by means of a universal calibration based on the product $[\eta] \cdot M$ (where M is the molecular weight), has several advantages as shown by Benoit[3]. Goedhart and Opschoor[4] demonstrated the agreement between $[\eta]$ values and constants of the Mark–Houwink equation (determined by methods of classical viscometry) with the corresponding values calculated from data obtained by GPC coupled with an automatic capillary viscometer. A similar agreement was obtained by Meunier and Grubisic[5] for linear and branched polystyrene (PS) (however, $[\eta]$ values measured by classical viscometry seemed systematically higher by *ca.* 5%), and by Grubisic-Gallot *et al.*[6]. The same agreement between the $[\eta]$ values was also obtained by

Gallot *et al.*[7], who also pointed out the possible errors in the K and a values of the Mark–Houwink equation calculated from data obtained by use of the automatic capillary viscometer. A somewhat different system for the determination of $[\eta]$ of GPC fractions has been described by Ouano[8,9]. The fractions were not examined by means of a capillary viscometer, but by use of a detector which reacted to the pressure changes due to changes in the viscosity of the solvent in a system having a constant flow of eluent.

Brüssau[10] recently reported some anomalous effects observed with a coupled capillary viscometer. No agreement was obtained between calibration graphs constructed in the standard manner and those obtained from data from an automatic capillary viscometer coupled with a GPC separation system. Part of the present work is an attempt to explain such anomalous results.

EXPERIMENTAL

Gel-permeation chromatography

All of the GPC measurements were carried out with an apparatus constructed at the Institute of Macromolecular Chemistry of the Czechoslovak Academy of Sciences. The separation columns were thermostated to $25 \pm 0.1°$. Tetrahydrofuran (THF), distilled from cuprous chloride and potassium hydroxide in a nitrogen atmosphere, was used as the solvent. Its flow-rate was 0.375 ml/min. The elution volumes were measured by means of a calibrated siphon having a volume of 1.704 ml (measured at a flow-rate of 0.375 ml/min). The solutions of the samples were injected from a calibrated loop (volume 1.636 ml). The columns were packed with the silica gel Sphérosil (Produits Chimiques, Péchiney-Saint-Gobain, France), Types B, D and E, and connected in series, *i.e.*, two columns E, followed by two of D and two of B. A column packed with porous glass CPG-10-1000 (Electro Nucleonix, Fairfield, N.J., U.S.A.) was used independently.

Automatic capillary viscometer

The capillary viscometer was constructed according to published data[6] on the electronic and recording part of a Sofica automatic viscometer (ARL, Le Mesnil-Saint-Denis, France), and was connected behind the siphon and thermostatted to $25 \pm 0.005°$. The flow-time of an exactly defined amount of pure THF between two photocells was *ca.* 155 sec.

Polystyrene and poly(vinyl chloride) samples

Polystyrene (PS) standards (Waters Assoc., Milford, Mass., U.S.A.) having a very narrow distribution, several fractions of poly(vinyl chloride) (PVC) and unfractionated PVC samples were used in the investigation. The designations and molecular parameters of the samples are given in Table I. The polydispersity indices, $\bar{M}_w/\bar{M}_n$, of the PS standards were as supplied by the manufacturer; the $[\eta]$ values of the PS standards were measured in ref. 11. The $\bar{M}_w/\bar{M}_n$ and $[\eta]$ values for the PVC samples were measured in ref. 12.

TABLE I

MOLECULAR PARAMETERS ($\bar{M}_w/\bar{M}_n$) AND INTRINSIC VISCOSITIES [η] OF PS STANDARDS AND PVC SAMPLES MEASURED BY CLASSICAL VISCOMETRY AND BY A CAPILLARY VISCOMETER COUPLED WITH A GPC SEPARATION SYSTEM

Sample	$\bar{M}_w/\bar{M}_n$*	$[\eta]$* (dl/g)	$\bar{M}_w/\bar{M}_n$**		$[\eta]_{av}$***	
			A	*B*	*A*	*B*
PS standards						
PS 1	1.21	3.54	1.61	1.18	4.04	4.28
PS 2	1.12	2.07	1.21	1.11	2.33	2.32
PS 3	1.23	1.43	1.15	1.12		
PS 4	1.05	1.25	1.11	1.10	1.27	1.35
PS 5	1.06	0.67	1.07	1.09	0.70	0.69
PS 6	1.02	0.44	1.09	1.09	0.51	
PS 7	1.04	0.28	1.14	1.10	0.28	0.28
PS 8	1.01	0.14	1.24	1.17		
PS 9	1.06	0.09	1.35	1.30		
PS 10	1.09	0.05	1.47			
PVC samples (polymer, fraction)						
5, 2	1.76	1.10	1.41		1.06	
5, 4	1.54	0.81	1.29		0.86	
5, 6	1.37	0.64	1.23		0.70	
5, 8	1.21	0.50	1.21		0.56	
5, 10	1.04	0.35	1.24		0.40	
5, 12	1.29	0.28	1.24		0.27	
1, 0		0.57	2.03		0.57	0.57
3, 0		0.55	2.00		0.51	0.52

* For PS standards: $\bar{M}_w/\bar{M}_n$, manufacturer's data; [η] from ref. 11. For PVC samples $\bar{M}_w/\bar{M}_n$ and [η] from ref. 12.

** Calculated from GPC data.

*** Calculated from coupled capillary-viscometer data.

RESULTS AND DISCUSSION

The intrinsic viscosities, $[\eta]_{av}$, of the individual samples were calculated from data obtained from the capillary viscometer coupled to the GPC separation system according to the equation

$$[\eta]_{av} = \frac{\Sigma [\eta]_i \cdot c_i}{\Sigma c_i} \tag{2}$$

where $[\eta]_i$ and c_i are the intrinsic viscosity and concentration respectively of the individual fractions separated by the siphon. The $[\eta]_i$ values in eqn. 2 were calculated from the data obtained with the automatic capillary viscometer for the respective fractions according to the equation.

$$[\eta]_i = \frac{\eta_{r,i} - 1}{c_i} \tag{3}$$

where $\eta_{r,i}$ is the relative viscosity of the polymer solution of the respective fraction.

The polymer concentration, c_i, can be calculated from the refractometer data by using the equation

$$c_i = \frac{S_i}{S} \cdot \frac{m}{q} \tag{4}$$

where S and S_i are the areas of the whole chromatogram and of the corresponding fraction respectively, m is the weight of the injected polymer and q is the siphon volume. The approximation given by eqn. 3 is possible because the polymer concentrations in the eluent leaving the column are very low.

We first determined the $[\eta]_{av}$ values for all of the samples using a series of six columns denoted by A. The results are summarized in Table I. The averages of the $[\eta]_{av}$ values are within $\pm 7.5\%$ of those measured by classical viscometry. The average reproducibility of the $[\eta]_{av}$ values was $\pm 5\%$ under the given experimental conditions, as verified by several measurements of the PS standards and PVC samples. By plotting $[\eta]_i$ *versus* the respective elution volumes, $V_{e,i}$, we found an anomalous dependence similar to that in ref. 10, although less pronounced. No agreement was obtained between these functions and a calibration graph constructed in the classical manner, *i.e.*, by plotting the $[\eta]$ values obtained by standard viscometry against the elution volume of the maximum of the chromatogram, $V_{e,max}$. This dependence is shown for some PS standards in Fig. 1. Similar behaviour was observed for the PVC fractions.

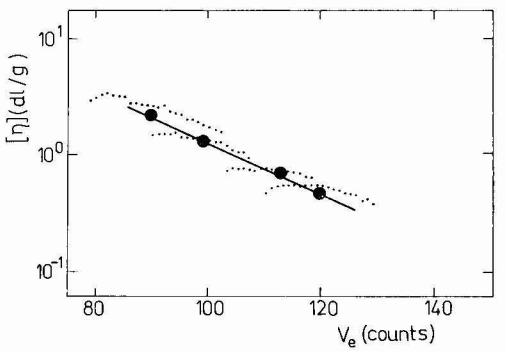


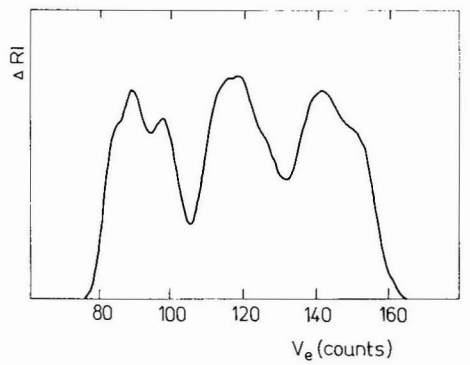
Fig. 1. The function $[\eta] = f(V_e)$ obtained for PS standards with system A. ····, $[\eta]_i = f(V_{e,i})$ measured by use of a capillary viscometer coupled with GPC; ●—●, the function $[\eta] = f(V_{e,max.})$, $[\eta]$ being measured in the classical way.

The anomalous behaviour of the $[\eta]_i$ *versus* $V_{e,i}$ functions may be due to spreading of the chromatographic zone. This is suggested by the smaller slope of these graphs compared with the calibration graph of $[\eta]$ against $V_{e,max}$. At high molecular weights the $[\eta]_i$ values are lower owing to spreading, while at low molecular weights they are higher, compared to the respective values from the calibration graph. Such hypothesis is also corroborated by the rather marked disagreement between the functions in the high-molecular-weight region, where the separation efficiency of the A system of columns is lower, as follows from a comparison of the $\bar{M}_w/\bar{M}_n$ values

supplied by the manufacturer for the PS standards with those calculated from the respective GPC chromatograms. The anomalies observed cannot be due to mixing in the siphon, because the moment of elution from the siphon is assumed to correspond to $V_{e,i}$, which means that in an ideal case of an infinitely high resolution all of the experimental points of the function $[\eta]_i$ against $V_{e,i}$ would lie above the calibration graph.

In order to verify the above hypothesis experimentally, we reconstructed the separation system A so that all of the dead volumes, which increase spreading, were minimized. This was achieved by shortening all of the connecting capillaries, especially that between the differential refractometer and the siphon. The separation system thus modified, containing the original six columns and denoted by B, was used for repeated measurements of the PS standards and unfractionated PVC samples. The separation efficiency of system B was somewhat higher than that of system A, as can be seen from a comparison of the $\bar{M}_w/\bar{M}_n$ values calculated from the GPC chromatogram (Table I) which in some cases are even lower than the manufacturer's data[13]. On the other hand, however, the change of the $[\eta]_i$ *versus* $V_{e,i}$ functions of the PS standards for the systems A and B was at the limits of experimental error. Use of the Huggins equation instead of eqn. 3 for calculating $[\eta]_i$ did not result in any important changes in the $[\eta]_i$ *versus* $V_{e,i}$ functions. The Huggins constant, $k_H = 0.362$, was calculated from our earlier experimental data[11]. The $[\eta]_{av}$ values also remained unchanged within the limits of experimental error.

Since a somewhat better agreement between the $[\eta]_i$ *versus* $V_{e,i}$ functions and the classical calibration graph was obtained for the PVC samples having a broader molecular-weight distribution, we prepared a model PS sample having a wide distribution by mixing approximately the same amounts of the PS standards 1, 2 and 4–10. The chromatogram of this sample is shown in Fig. 2, and the $[\eta]_i$ *versus* $V_{e,i}$ function is in Fig. 3. Both figures show that the $[\eta]_i$ values are affected by the precision of the measurements of time with the capillary viscometer ($\pm$ 0.02 sec) and by the precision with which the heights h_i may be read from the GPC chromatogram. For instance, in the region of 100–110 counts, inaccuracies appearing at a large change in the concentration cause undulation of the $[\eta]_i$ *versus* $V_{e,i}$ function. The



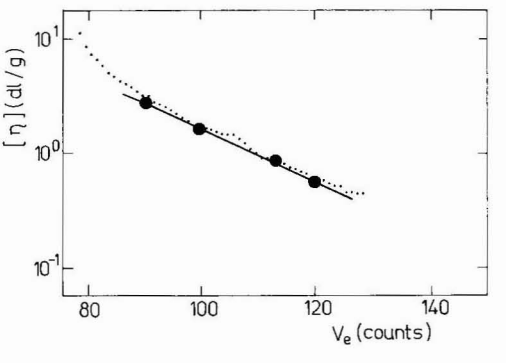


Fig. 2. Chromatogram obtained with system B of the model PS sample having a broad distribution.

Fig. 3. The function $[\eta] = f(V_e)$ obtained with system B for the model PS sample having a broad distribution. Other details as in Fig. 1.

calculation of the $[\eta]_i$ values at the end of the chromatogram is particularly subject to a large experimental error; we therefore eliminated values which had potentially higher errors than 10%. At the same time, it can be seen from Fig. 3 that a comparatively good agreement was achieved between the $[\eta]_i$ *versus* $V_{e,i}$ function and the original calibration graph for system B.

The results of measurements made with the use of only one column (CPG-10-1000), the separation efficiency of which is considerably lower than the systems A and B, are shown in Fig. 4. One can see clearly the large difference between the $[\eta]_i$ *versus* $V_{e,i}$ function and the original calibration graph compared with the systems A and B. The measurements were carried out at four concentrations of the injected standard PS 3, *i.e.*, at 0.4, 0.2, 0.1 and 0.05% (w/v). In this case too, however, $[\eta]_{av} = 1.57$ corresponds to $[\eta]$ measured by classical viscosimetry, even though a small decrease in $[\eta]_{av}$ was observed with decreasing concentration of the injected PS standard.

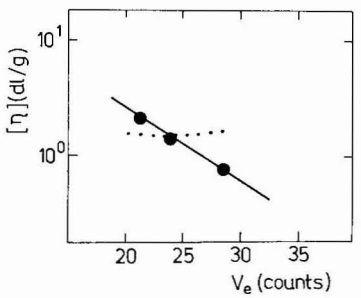


Fig. 4. The function $[\eta] = f(V_e)$ obtained with the CPG-10-1000 column for the PS standards. Other details as in Fig. 1.

Bearing in mind possible experimental errors, the calculation of $[\eta]_i$ from eqn. 3 or by means of the Huggins equation is justified. On the other hand, the determination of the concentration c_i or of the area S_i in eqn. 4 still remains a problem. Since the whole of the chromatogram is divided into constant-count segments, which we regard as equal to unity, the area S_i corresponding to the ith fraction can be calculated from the equation

$$S_i = \frac{h_i + h_{i-1}}{2} \tag{5}$$

where h_i is the height of the chromatogram in arbitrary units starting from the baseline in the ith count. The overall chromatogram area is then a sum of the areas of the individual fractions. In order to determine whether the use of eqn. 5 is justified, we used our earlier results obtained by the preparative GPC fractionation of the copolymer vinyl chloride–vinyl acetate[14]. The actual yield of the fractions obtained was in good agreement with the theoretically assumed yield calculated from the preparative chromatogram. Only the marginal high-molecular- and low-molecular-weight fractions were not in agreement, owing to material losses and the relatively higher errors in the calculation at generally lower yields.

Spreading and experimental errors lead to the observed anomalous behaviour of the $[\eta]_i$ *versus* $V_{e,i}$ functions constructed from the data obtained by GPC coupled with the automatic capillary viscometer compared with the classical calibration graph. On the other hand, the general eqn. 2 for calculations of $[\eta]_{av}$ does not suffer from spreading effects. This is why the $[\eta]_{av}$ values agreed within the limits of experimental error with the $[\eta]$ value measured in the classical way. The "limiting curve", which according to Brüssau[10] can be drawn through the low-molecular-weight ends of the individual $[\eta]_i$ *versus* $V_{e,i}$ functions, has probably no physical meaning. The extra-column contributions to spreading were very low for systems A and B. If columns having an extremely high separation efficiency were available, an even stronger suppression of the anomalous effects would be expected.

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PREPARATION OF PLATES WITH A PERMANENT ADSORBENT LAYER AND THEIR APPLICATION IN THE ANALYTICAL THIN-LAYER CHROMATOGRAPHY OF LIPIDS

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SUMMARY

Experimental conditions for preparing the thin-layer chromatographic plates with a permanent adsorbent layer (PAL) have been established; the particles of this layer are firmly bound to each other and to the glass support by means of fused glass powder. To prepare the PAL plates, a mixture of Woelm silica gel and glass powder (1:3, w/w) of particle size $7 \pm 1\ \mu$m was suspended in toluene and spread on the plates, yielding PALs of thickness 150–250 μm. The plates were heated in an electric furnace at 675° for 20 min. In the separation of neutral lipids, a PAL thus prepared is equal in efficiency and selectivity to silica gel layers prepared in the usual manner.

INTRODUCTION

At present, thin-layer chromatography (TLC) is a standard method for the qualitative separation of mixtures of organic compounds and the quantification of their individual components[1,2]. Although TLC has obvious advantages, it also has some serious drawbacks, in particular the fact that in most instances the worker himself is obliged to spread an adsorbent layer on plates for each chromatographic experiment. The uniform spreading of the adsorbent layer requires special equipment, certain technical skill and constant expenditure of time, and the chromatographic properties of the layers thus prepared are nevertheless difficult to standardize. In addition, the preparation of the plates in the laboratory in most instances involves the use of silica gel powder, which is harmful to health. Finally, many difficulties arise from the necessity of using binders while preparing the adsorbent layer. For example, if starch or poly(vinyl alcohol) is employed to fix the layer, the chromatographic zones cannot be rendered visible by means of strong mineral acids and heating[2,3]. If, on the other hand, gypsum is used as a binder, the separation of substances that form insoluble calcium salts, *e.g.*, acidic phospholipids, is impaired.[4]

Several workers have tried to reduce to the minimum these limitations of TLC. The first effort was made in 1968 by Taylor[5], who suggested a different approach to the fixation of the thin layer on a glass support. Employing a layer of highly porous

glass as an adsorbent, he fixed it on the plate by means of short-term heating. As a result, the powder and support fused to each other and the possibility arose of cleaning the plate with strong mineral acid mixtures after each chromatographic experiment so that repeated use would be feasible. The fixed layers thus obtained were used to separate standard dye mixture and also alkaloids. In 1972, Taylor's method was modified by Okumura and co-workers[6,7], who used silica gel mixed with powdered glass in definite proportions as the adsorbent. The mixture was spread on the plate, which was then subjected to heating. As a result, the silica gel particles became firmly bound to each other and to the surface of the plate by the fused glass powder.

Our attempt to prepare plates as decribed by Okumura *et al.*[7] failed because their description is incomplete. Therefore, we developed a method for the preparation of plates with a permanent adsorbent layer (PAL) that would be at least equal in its efficiency and selectivity in the separation of mixtures to the layers obtained in the usual manner. We examined the influence of the grade of silica gel, particle size of the glass powder, the weight ratio of the powder and silica gel in the mixture and also the heating conditions on the chromatographic parameters. Stahl's dyes, soybean seed oil and standard neutral lipids were used as test mixtures for fractionation. The results obtained indicate that the method developed can be used to prepare PAL plates that possess optimal efficiency and selectivity for the separation of lipids.

EXPERIMENTAL

Preparation of the mixture of silica gel and glass powder

Grossly crushed window glass (3–4 kg) was ground in a 5-l barrel of a ball-mill (LE-107, Hungary) for 32 h. The powder was screened, the $\leq$ 195-mesh fraction of particle size 5–30 μm was collected and additionally fractionated from the aqueous suspension by sedimentation. For this purpose, the 195-mesh fraction (1.3 kg) was suspended in 6 l of water and allowed to stand for 1.5 h; the supernatant was decanted and these operations were repeated until it became clear. After the last decantation, the whole of the supernatant was allowed to stand for a further 2.5 h and the residue suspended in a 2-l beaker filled with water; the residue was washed until the supernatant became clear. Sedimented glass particles were dehydrated with acetone and the powder of particle size 7 ± 1 μm was screened with a 195-mesh sieve in order to remove lumps. Silica gel (Silica Gel Woelm TLC Adsorbent, Woelm, Eschwege, G.F.R.) and glass powder were mixed in a weight ratio of 1:3.

Preparation of PAL plates

The apparatus used to prepare PAL plates was assembled as shown in Fig. 1. The "hot" end (1) of a chromel–alumel thermocouple was inserted into a muffle furnace (2) through a hole (3), while the „cold" end (6) was positioned at the bottom of a glass tube (8) inserted into a Dewar vessel (9) filled with ice–water (5) and covered with a plastic cap (7). The Dewar vessel was placed in a cylindrical support (4). The thermocouple was connected with the input of a potentiometer (10), used to record temperature changes in the furnace; a reading of 1 mV on the potentiometer scale corresponded to 25°. Plates of glass ($10 \times 20 \times 0.2$ cm) were placed in an apparatus for spreading[2]. The mixture of silica gel and glass powder (24 g) was suspended in 45 ml of purified toluene and a 350-μm thick suspension layer was spread by means

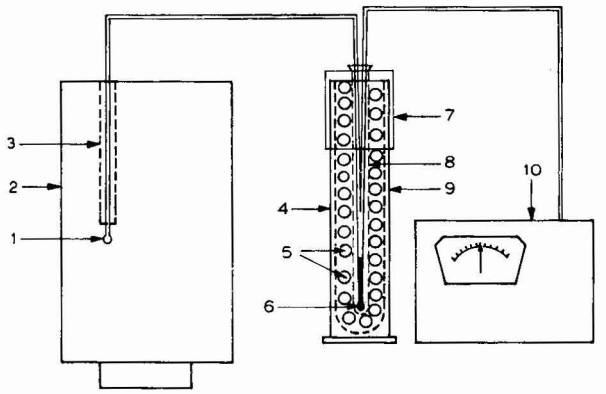

Fig. 1. Apparatus for the preparation of plates with a permanent adsorbent layer (PAL). 1 = "Hot" end of thermocouple; 2 = muffle furnace; 3 = hole for the thermocouple in the rear wall of the furnace; 4 = cylindrical support for Dewar vessel; 5 = pieces of ice; 6 = "cold" end of thermocouple; 7 = plastic cap, 8 = 45 × 2 cm glass tube; 9 = Dewar vessel; 10 = pH 340 potentiometer.

of a spreader[2]; the amount of the mixture mentioned above was sufficient to prepare five plates. After evaporation of toluene, the plate was placed on a graphite block (21 × 11 × 1 cm) lying on a stainless-steel support (Fig. 2,I), which was then inserted by means of a lever (Fig. 2,II) into a muffle furnace heated at 100–150°. The temperature was increased to 675°, the plate was left at this temperature for 20 min and then the furnace was gradually cooled to room temperature. Uniformity of the thickness of the PAL was controlled by means of a micrometer.

Immediately after preparation and also after each chromatographic experiment, the PAL plates were cleaned with concentrated sulphuric acid–nitric acid (9:1) at 100–120° for 30 min, then the plates were cooled, washed for 1 h with tap water and activated at 110° for 30 min.

Preparation of test mixture solutions for separation on PAL plates

A mixture of indophenol, Sudan Red G and Butter Yellow according to Stahl[8] (Test Mixture, Desaga, Heidelberg, G.F.R.) was used as a standard.

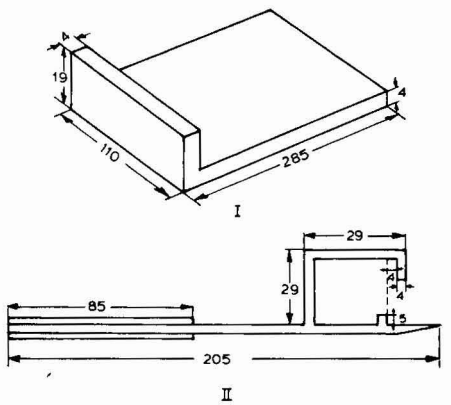

Fig. 2. Stainless-steel support and steel lever with a plastic handle. I = Support, general view; II = lever, side view. Dimensions are in millimetres.

Soybean seed oil was extracted as described previously[9] and dissolved in benzene to give a concentration of 100 mg/ml. To this solution, 0.01 % of butylated hydroxytoluene (BHT) was added.

Standard preparations of mixed monoglycerides of palmitic and stearic acids, mixed diglycerides of the same acids and also cholesteryl palmitate were used without further purification. Cholesterol was purified on an alumina column [activity II, benzene–acetone (1:1) used as eluent]. Triglycerides were extracted from soybean oil[9] and purified by the same procedure [*n*-hexane–benzene (3:2) used as eluent]. Free fatty acids were products of the saponification of soybean oil[10]; a proportion of these acids was converted into the methyl esters[9]. The purity of the preparations was checked by TLC on PAL plates with *n*-hexane–diethyl ether–acetic acid (80:20:1). In all instances the content of the major component in the preparations was more than 97 %. Standard neutral lipids were dissolved in benzene, the concentration of each class of lipid in this solution being 4 mg/ml.

Separation of substances on PAL plates

The separations were performed in 20.5 × 14-cm cylindrical chambers saturated with the vapour of the solvent to be used (saturation time 45 min) and covered with ground covers. To each solvent 0.01 % of BHT was added.

The standard dye mixture solution (2 μl) was applied on the layer by means of a Hamilton Microliter Syringe 10–12 mm above the lower edge of the plate; benzene was used as the solvent.

Soybean seed oil (100 μg) and a standard neutral lipid mixture (56 μg) were separated in *n*-hexane–diethyl ether–acetic acid (90:10:1 and 80:20:1). To render the lipid spots visible, the plates were treated with a 7 % solution of molybdophosphoric acid in 45 % aqueous isopropanol and heated at 120° for 5 min[2].

Standard lipids were also fractionated in a Wöelm silica gel layer without the glass admixture; the layers were spread on the plates by means of a spreader as described above.

Determination of chromatographic separation parameters

The relative retentions of the substances were expressed by R_M values[11], the selectivity of the separation by the number of chromatographic zones obtained after fractionation of a given mixture of substances and the separation efficiency by the number of theoretical plates (N) and HETP value (H)[12] for a given substance.

RESULTS AND DISCUSSION

Influence of the grade of silica gel on the chromatographic parameters of PAL plates

The chemical nature and the quality of the adsorbent are among the major factors that influence chromatographic separations[13]. In particular, the adsorption properties of silica gel depend largely upon the mode of its preparation[14]. Many grades of silica gel are available, manufactured by different firms with various degrees of purity, presence of binders, particle sizes, pore diameters, specific surface areas, inclusion of the fluorescent indicators, etc. Okumura *et al.*[7] did not specify the grade of silica gel they used, so it was essential to establish which grades were suitable for the preparation of the PAL plates to ensure the most efficient and selective separation of

TABLE I

INFLUENCE OF THE GRADE OF SILICA GEL ON THE RELATIVE RETENTION OF SUBSTANCES AND ON THE SELECTIVITY AND EFFICIENCY OF SEPARATION OF THE STANDARD DYE MIXTURE AND SOYBEAN OIL BY THIN-LAYER CHROMATOGRAPHY ON PAL

Silica gel grade	*Particle size (μm)**	R_M		*Number of chromatographic zones*		N		H *(mm)*	
		*TG** of soybean oil*	*Butter Yellow*	*Soybean oil*	*Dye mixture*	*TG** of soybean oil*	*Butter Yellow*	*TG** of soybean oil*	*Butter Yellow*
Woelm	15 ± 3	0.05	0.21	12	3	83	812	0.80	0.07
Merck	15 ± 10	0.24	0.37	8	3	13	324	3.24	0.14
Chemapol	20 ± 14	0.50	0.43	8	3	4	282	5.01	0.15

* Measured in the present work; $\bar{x} \pm S$ values are given, where $\bar{x}$ is the arithmetic mean of the particle size for 30 measurements and S is the absolute standard deviation[10].

** TG = triglycerides.

lipids. In our work, several common grades of silica gel were investigated, namely Woelm silica gel (see Experimental), LS-5/40 silica gel, (Chemapol, Prague, Czechoslovakia) and Kieselgel G (Merck, Darmstadt, G.F.R.); the last type was washed with hydrochloric acid until free from the binder (calcium sulphate) (these silica gels will be referred to below as Wöelm, Chemapol and Merck, respectively). For the preparation of the PAL, the glass fraction of particle size $7 \pm 1 \mu$m was used (see below).

It can be seen from Table I that the plates obtained from the various silica gels have different chromatographic properties, the lowest relative retention (R_M) and the highest selectivity and efficiency of separation of the standard mixtures being shown by PAL prepared from the Wöelm silica gel. It can be that in this instance the result obtained is due to the differences in the particle sizes, which is supported by the well known property in "conventional" TLC that the smaller the particles used for the preparation of the adsorbent layer and the narrower the range of their size variations, the higher is the efficiency of separation of compound mixtures[12]. It can be seen from Table I that of the grades of silica gel examined the Woelm silica gel demonstrated these properties most effectively; it is not unlikely that its particle size did not change upon heating, as its melting point was not reached. We used only this grade of silica gel in subsequent work.

Influence of the original size of the glass powder particles and of the glass powder to silica gel weight ratio in the layer on the chromatographic parameters of PAL plates

Okumura and co-workers[6,7] claimed that, in order to prepare the silica gel layer fixed on the plate by means of fused glass powder, the particles of glass and the adsorbent must have similar particle sizes, but no recommendation was given of the exact size of these particles. To check this claim and to determine the optimal particle size of the glass for the preparation of PAL, the "original" $\leqslant$ 195-mesh (11 ± 10-μm) glass fraction was used in our preliminary experiments. However, PAL prepared from the mixture of this fraction and Wöelm silica gel ($15 \pm 3 \mu$m) did not give a sufficiently good separation of the minor components of soybean seed oil (Table II). We considered that the chromatographic properties of the layer might be improved by a further decrease in the particle size of the glass and a simultaneous narrowing of

TABLE II

INFLUENCE OF THE GLASS PARTICLE SIZE ON RELATIVE RETENTIONS AND ON THE SELE TIVITY AND EFFICIENCY OF SEPARATION OF THE STANDARD DYE MIXTURE AND SOYBEA OIL BY THIN-LAYER CHROMATOGRAPHY ON PAL

*Glass powder fractions in PAL**	*Particle size (μm)*	R_M		*Number of chromatographic zones*		*N*		*H (mm)*	
		TG of soybean oil	*Butter Yellow*	*Soybean oil*	*Dye mixture*	*TG of soybean oil*	*Butter Yellow*	*TG of soybean oil*	*Butter Yellow*
"Original"									
(≤195 mesh)	11 ± 10	0.20	−0.01	10	3	23	1024	1.96	0.08
1	21 ± 11	−0.01	−0.05	9	3	35	717	1.78	0.12
2	7 ± 1	0.05	0.08	12	3	83	1837	0.80	0.04
3	5 ± 1	−0.08	0.30	5	3	39	218	1.81	0.22

* Glass to silica gel weight ratio in PAL, G/SG = 3:1.

the range of their sizes. In fact, when fraction 2 (7 ± 1 μm) was used, the selectivity and efficiency of separation of the components of both soybean oil and the standard dye mixture were considerably improved compared with the original fraction. This size seems to be optimal, since an increase or decrease from this value adversely affected the chromatographic parameters of PAL (Table II, fractions 1 and 3). Therefore, only 7 ± 1-μm glass particles were used in subsequent experiments.

The results reported by Okumura and Kadono[6] suggest that the R_F values of steroids depend on the weight ratio of glass to silica gel (G/SG) in the layer; according to these authors, the optimal range of this ratio is from 2:1 to 5:1. To investigate the possible influence of this factor on the relative retention (R_M) and separation efficiency, we fractionated standard mixtures of dyes and neutral lipids in PAL having G/SG ratios of 1:1, 3:1 and 5:1.

Table III shows that in most instances the R_M value decreases and the separation efficiency increases with an increase in this ratio. At the same time, the maximal amount of lipids that can be separated without a loss of linearity of the adsorption isotherm decreases because, with a decrease in the silica gel content in the PAL, the linear capacity[12] of the layer is reduced. Therefore, the use of layers with G/SG ≥ 5:1 would lead to a considerable decrease in the maximal weight of the sample that can be separated. Apparently, the linear capacity of the layer can be enhanced if the silica gel content in the PAL is increased; however, at G/SG < 3:1 the content of glass in the layer becomes insufficient to ensure the necessary mechanical durability of the PAL. Consequently, to prepare PAL plates with a maximal linear capacity, adequate durability and acceptable chromatographic efficiency, the G/SG ratio of 3:1 must be used. Our results for the optimal particle size of the glass and the G/SG ratio are not in conflict with those of Okumura and co-workers[6,7], but they give much more precise information.

After being spread on the surface of a glass plate, the thin layer of silica gel–glass mixture is highly porous, which enhances rapid and efficient separations. To prevent deterioration of porosity during heating, the temperature at all points in the layer must be the same at all times during both heating and cooling of the plate. The

TABLE III

INFLUENCE OF THE GLASS TO SILICA GEL WEIGHT RATIO IN PAL ON RELATIVE RETENTIONS AND THE EFFICIENCY OF SEPARATION OF THE STANDARD DYE MIXTURE AND NEUTRAL LIPIDS BY THIN-LAYER CHROMATOGRAPHY ON PAL

*Substance separated**	*Glass to silica gel weight ratio*								
	1:1			*3:1*			*5:1*		
	R_M	N	H (mm)	R_M	N	H (mm)	R_M	N	H (mm)
Indophenol	1.65	36	0.08	1.38	44	0.11	1.24	39	0.18
Sudan Red G	1.06	144	0.08	0.69	467	0.06	0.62	334	0.10
Butter Yellow	0.40	225	0.20	0.08	1837	0.04	−0.10	1708	0.05
MG	1.76	16	0.13	1.54	36	0.08	1.59	16	0.19
DG	0.79	196	0.11	0.46	747	0.10	0.48	747	0.05
Cholesterol	0.58	272	0.12	0.37	455	0.11	0.36	494	0.10
FFA	0.13	625	0.12	−0.17	1283	0.08	−0.23	1044	0.10
TG	−0.29	743	0.15	−0.45	1681	0.07	−0.55	2329	0.06
FAME	−0.54	1600	0.08	−0.64	3003	0.05	−0.71	4153	0.04
SE	−1.10	2844	0.07	−1.06	4869	0.03	−1.14	6561	0.03

* Neutral lipids: MG = monoglycerides; DG = diglycerides; FFA = free fatty acids; TG = triglycerides; FAME = fatty acid methyl esters; SE = sterol esters.

optimal temperature and duration of heating to ensure the preparation of a highly porous and sufficiently strong PAL should be determined experimentally. They depend on the melting point of the glass used and on its content in the layer; in our experiments, the plate with the applied layer (G/SG = 3:1) was heated to 675° and left at this temperature for 20 min.

Separation of neutral lipids on PAL plates

Fig. 3 shows the chromatogram of soybean seed oil (I and II) and a standard mixture of neutral lipids (III and IV) obtained on PAL (G/SG = 3:1) and on a thin layer of Wöelm silica gel without the admixture of glass; *n*-hexane–diethyl ether–acetic acid (80:20:1) was used as the developing solvent. It can be seen that on PAL both mixtures are separated fairly well, while no adequate separation of minor components of the oil was achieved on the silica gel layer obtained in the usual manner, and during fractionation of the standard mixture of neutral lipids on this layer diglycerides were not separated from cholesterol.

Table IV gives the values of R_F, R_M, N and H during the separation of neutral lipids on PAL and on a "loose" Woelm silica gel layer. It can be seen that PAL gives a higher efficiency of lipid fractionation than the silica gel layer obtained in the usual manner. It is possible that one of the reasons for this difference is that, provided the same solvent is employed, lipids have a lower relative retention (R_M) on PAL than on a "loose" silica gel layer. The same reasoning applies to the different selectivities of the separation of diglycerides and cholesterol on these layers.

A comparison of our results with those for lipid separations using conventional TLC systems reported by other workers (see Table V and also Fig. 130 in ref. 18) leads to the general conclusion that, as regards the selectivity and efficiency of separation of both standard and natural mixtures of neutral lipids, PAL is comparable to

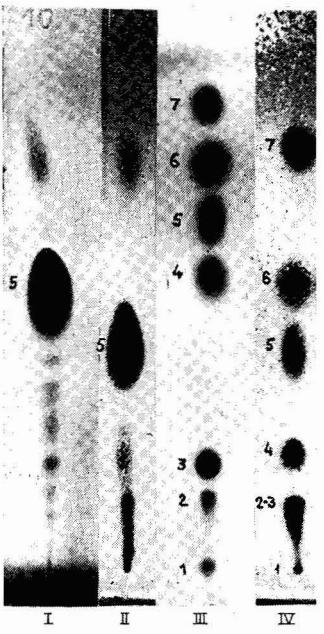

Fig. 3. Chromatograms of soybean seed oil and a standard mixture of neutral lipids. I and II = soybean oil; III and IV = standard mixture of neutral lipids. I and III = PAL, G/SG = 3:1; II and IV = Woelm silica gel layer containing no glass. Spots: 1 = monoglycerides; 2 = diglycerides; 3 = cholesterol; 4 = free fatty acids; 5 = triglycerides; 6 = fatty acid methyl esters; 7 = sterol esters. Components of soybean oil other than triglycerides were not identified.

TABLE IV

INFLUENCE OF THE PROPERTIES OF THE SILICA GEL ADSORBENT LAYER ON RELATIVE RETENTIONS AND THE EFFICIENCY OF SEPARATION OF THE STANDARD MIXTURE OF NEUTRAL LIPIDS BY THIN-LAYER CHROMATOGRAPHY

Neutral lipid	*PAL (G/SG = 3:1)*				*Woelm silica gel layer without glass admixture***			
	hR_F*	R_M	N	H (mm)	hR_F*	R_M	N	H (mm)
MG	3	1.54	36	0.08	—	—	—	—
DG	26	0.46	747	0.10	—	—	—	—
Cholesterol	30	0.37	455	0.11	10	0.96	455	0.04
FFA	60	−0.17	1283	0.08	18	0.65	314	0.10
TG	74	−0.45	1681	0.07	37	0.11	476	0.13
FAME	81	−0.64	3003	0.05	48	0.04	868	0.09
SE	92	−1.06	4869	0.03	72	−0.41	3782	0.03

* $hR_F = R_F \times 100$ (ref. 15).

** — indicates that hR_F, R_M, N and H were not measured because DG were not separated from cholesterol and MG were on the origin of the chromatogram (see Fig. 3, IV).

silica gel layers that contain no glass. In the work of Itoh *et al.*[16], plates prepared according to Okumura were used for lipid separation. The separation pattern of the standard mixture of neutral lipids obtained in our work agrees well with that of Itoh *et al.* (see Table V).

TABLE V

hR_F VALUES OF NEUTRAL LIPIDS AFTER SEPARATION ON PAL AND SILICA GEL THIN LAYER WITHOUT GLASS ADMIXTURE

Solvent systems: *n*-hexane–diethyl ether–acetic acid (80:20:1) (this work); light petroleum–diethyl ether–acetic acid [90:10:1, refs. 16 (a) and 19, and 80:20:1, ref. 16 (b)]; benzene–diethyl ether–ethyl acetate-acetic acid (80:10:10:0.2) (ref. 19); *n*-heptane–diisopropyl ether–acetic acid (60:40:4) (ref. 20). In ref. 16, silica gel plates prepared according to Okumura *et al.*[7] were employed and in refs. 17–20 the silica gel layer without glass admixture was used.

Neutral lipid	hR_F						
	This work	*Data from the literature*					
		Ref. 16(a)	*Ref. 16(b)*	*Ref. 17*	*Ref. 18*	*Ref. 19*	*Ref. 20*
MG	3	—	15	8	2	0	5
1,2-DG	26	7	61	59	15	8	25
1,3-DG	26	11	68	72	21	8	40
Cholesterol	30	15	57	48	19	10	30
FFA	60	43	82	20	39	18	50
TG	74	52	89	80	60	30–40	82
FAME	81	75	—	—	77	65	—
SE	92	90	—	100	94	90	90

Hence the application of PAL plates makes it possible to overcome the limitations of TLC mentioned above. Moreover, these plates have certain advantages over the usual plates, the major one being that the same layer can be used many times for the analysis of mixtures without changes in its properties. It is clear that the selection of optimal conditions for the separation of a given mixture can thus be considerably accelerated. Moreover, application of PAL plates facilitates the impregnation of the adsorbent with different substances, because it can be immersed in any liquid except hydrofluoric acid and concentrated alkali solutions without any damage to the layer. Impregnation has been employed for the modification of layers prior to separation, and also for rendering visible the chromatographic zones formed[2,21]; up to now, spraying has been used for this purpose, but it does not ensure an even distribution of the substance on the surface of the adsorbent and can contaminate the laboratory with corrosive compounds or due stuffs. Finally, the application of PAL plates will make it possible to facilitate and to accelerate considerably the standardization of a densitometric determination of separated substances because, while performing a series of experiments, chromatographic analysis of the mixture can be effected repeatedly with the same PAL.

Various firms (Chemapol, Merck, Applied Science Laboratories and others) produce plates with a ready-made silica gel layer under such trade-names as Silufol, PSC-Fertigplatten and Prekotes. These plates are used by many workers because of the superior uniformity of the layer thickness compared with those of plates prepared in the laboratory. However, it should be stressed that the pre-coated plates suffer from some of the drawbacks mentioned above. In both instances the plates can be used only once; precautions should be taken while using the plates in order to prevent damage of the layer, and commercially available TLC plates on glass supports are expensive and cumbersome to handle. When a metal foil serves as the support, the plates cannot be used for densitometry in transmitted light. The difficulties that arise owing to the

presence of organic or mineral binders in the layers, and also while performing the impregnation of such layers with certain substances, have already been noted. It seems that in the future, plates with a ready-made layer will be replaced by PAL plates, which are free from the above limitations.

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ISOTACHOPHORESIS IN NARROW-BORE TUBES

INFLUENCE OF THE DIAMETER OF THE SEPARATION COMPARTMENT

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SUMMARY

The importance of the inside diameter of the separation compartment of electrophoretic equipment in which tubes are used in achieving optimal stabilization against conventive disturbances and for limiting the temperature increase is demonstrated. Both from a theoretical point of view and experimentally, smaller inside diameters than are commonly used are recommended. Results obtained with thermometric, conductivity and UV absorption detectors in narrow-bore tubes with inside diameters of *ca.* 0.2 mm are compared.

INTRODUCTION

In electrophoretic experiments, optimal stabilization against convective disturbances is nearly always desired. This stabilization can be achieved by, amongst other methods, decreasing the ratio of the cross-section of the separation compartment to its surface area, assuming that other disturbing phenomena can be well controlled.

Obviously, circular cross-sections, because of their high symmetry, are preferable to rectangular cross-sections. This is especially important when dealing with quantitative aspects. Annular cross-sections have limited applicability from a practical point of view and we shall therefore confine ourselves to circular cross-sections only.

The dimensions of the narrow-bore tubes are determined mainly by the material of which they are constructed and the detection systems applied. So far these dimensions have hardly been discussed in the literature. At the time the first reproducible isotachophoretic experiments were carried out in our laboratory[1] the only suitable material available was Pyrex glass. The optimal diameters, which are still applied in almost all instruments[2–5], were found to be I.D. 0.4–0.6 mm and O.D. 0.7–1.0 mm. No semi-permeable membrane was used. In order to prevent a hydrodynamic flow between the electrode compartments, the viscosity of the electrolyte in the separation compartment had to be increased.

A small diameter of the narrow-bore tube therefore necessitates a high pressure

for rinsing and refilling the separation compartment. Moreover, the electroendosmotic flow at low (centimolar) concentrations was found to be far from negligible when using glass tubes with inside diameters smaller than 0.4 mm. If the concentration of the electrolytes is increased, relatively high current densities can be applied and the electroendosmotic flow can be neglected, even in narrow-bore tubes with a very small inside diameter[6]. The diameter of the narrow-bore tube can thus be decreased without the necessity for taking special precautions except as regards the pressure for rinsing and refilling the separation compartment. From an analytical point of view, however, high concentrations of the electrolytes are less important, because the absolute amounts of the sample compounds increase and many components are no longer soluble.

In early work only a thermocouple was used as the detector, the resolution and response of which is determined mainly by the wall thickness[7] and the thermal conductivity of the material of which the narrow-bore tube is made. The use of Pyrex glass narrow-bore tubes made it impossible to decrease the thickness of the wall significantly because thin-walled tubes are very fragile. The ratio of the cross-section to the surface area also could not be decreased significantly, because for a full qualitative and quantitative analysis using a thermometric detector scarcely any signal remains at the current density chosen.

In order to permit a better understanding of the resolution, response and sharpness of a zone boundary in isotachophoretic analyses, we shall first summarize a few basic features. In isotachophoretic experiments, consecutive zones all have a characteristic temperature, potential gradient, pH and conductivity, once the steady state has been reached. Apart from the so-called transition boundary between the zones, in which two ions of consecutive zones are mixed, we always have to cope with the profiles of the zone boundaries. The transition boundary is determined mainly by the operational parameters such as the difference in the effective mobilities of the constituents involved. Convective disturbances may increase this transition boundary. The profiles of the zone boundaries are due mainly to the temperature differences between the centre of the tube and the wall of the tube and between the zones mutually. The viscosity of the electrolyte[8] and the additive used to increase this viscosity also play a role. In specific cases, the contributions of the electroendosmotic flow, adsorption on the wall and diffusion into the wall on the profile cannot be neglected. Suppose the zone boundary has an "infinite" sharpness, *i.e.*, the transition boundary can be neglected. The final registration may be poor, however, because the resolution of the detector is low, the response of the detector is low (*e.g.*, thermal *versus* conductivity detection) or the profile of the zone boundary is large. The various profiles (concentration, temperature and pH) need not be identical, because they are affected by different parameters.

A decrease in the inside diameter, at a constant current density, will decrease the temperature difference between the centre of the tube and the wall, and the temperature profile will thus also decrease. Moreover, the absolute increase in temperature is smaller, which reduces the convective disturbances. The resolution therefore increases, assuming that the response is sufficiently high. The addition of non-ionic soluble polymers reduces the convective disturbances, which are caused mainly by the difference in temperature. By this means an increase in resolution is obtained, again assuming that the response is sufficiently high. The addition of surface-

active compounds decreases the electroendosmotic flow, if any, and therefore also increases the resolution. Surface-active compounds may also improve the quality of the material of the instrument that is in direct contact with the electrolytes. By this means, adsorption on the wall and/or diffusion into the wall is reduced, which increases the resolution.

The availability of PTFE tubing made it easier to control the dimensions of the narrow-bore tubes more effectively. Together with the development of detectors with higher resolution and response than those of the thermometric detector, a better choice of dimensions of the narrow-bore tube is possible.

It is obvious that the temperature inside a conductivity probe made of a cylinder (8 mm diameter) of acrylic is lower than that in a narrow-bore tube made of PTFE with a similar inside diameter and a much smaller outside diameter, surrounded by air. The properties of the gas surrounding the narrow-bore tube have a great influence on the final temperature of the electrolytes in the separation compartment. The exact temperature inside the conductivity probe is difficult to measure, but we can state that the temperature profile is blurred.

EXPERIMENTAL AND RESULTS

The basic equipment has been described extensively by Everaerts *et al.*[9]. For experiments with thermometric detectors and comparative temperature measurements, PTFE narrow-bore tubing (Habia, Breda, The Netherlands) with approximate I.D.s of 0.4, 0.2 and 0.1 mm and corresponding O.D.s of 0.7, 0.4 and 0.2 mm was used. The diameters were found to be more constant if the narrow-bore tubes were prepared by stretching a tube with a larger inside diameter over a wire that has the inside diameter required for the tube. The inside diameter obtained was determined by measuring the length of a mercury thread at various points in the tube and weighing the mercury used.

The thermocouples (30 μm copper, 25 μm constantan) were made by electrically welding the twisted wires over a length of *ca.* 0.5 mm using a spark discharge from a capacitor (1000 μF, 25 V). The twisted section was kept under a reducing medium (*e.g.*, methanol) in order to prevent oxidation of the wires by oxygen in the air during the discharge. The junction obtained was round (diameter *ca.* 40 μm). After stretching the thermocouple, it was mounted around a narrow-bore tube with only one turn, making use of an elastic glue to which some solvent had been added in order to decrease its viscosity. Care was taken to ensure that besides the measuring point hardly any contact was made between the thermocouple wires and the narrow-bore tube. The reference junction was mounted on a probe of a quartz crystal thermometer (Hewlet-Packard, Avondale, Pa., U.S.A., Type 2801 A) and was protected with a heat-sink compound. By this means, absolute temperatures at the narrow bore tube could always be measured.

The narrow-bore tubes were clamped between an injection block and a counter electrode compartment, provided with a cellulose polyacetate semi-permeable membrane. These compartments are basically made of Perspex (acrylic) and are described in detail elsewhere[9]. The narrow-bore tube was surrounded by a Perspex tube (I.D. 30 mm) for thermostating the air around the tube. This Perspex tube was further surrounded by a weak PVC tube in which thermostated oil was circulated. The

ambient gas temperature could be kept constant to better than 0.1°. In all experiments the mode of operation was vertical.

The conductivity and UV absorption detectors have been discussed elsewhere[9]. The construction of conductometric cells of I.D. 0.2 mm with equiplanar-mounted electrodes is rather difficult. Moreover, the cell constant varies considerably. Therefore, conductimetric probes of the potential gradient type[9] have been constructed. The distance between the electrodes was 0.1 mm, the material of construction being platinum–iridium (9:1). The linearity of the electronics in the range 10 kΩ–10 MΩ (the normal operating condition for experiments with centimolar solutions) was ensured with detectors with I.D. 0.4 and 0.2 mm; the accuracy was better than 0.2%.

The UV absorption detector was equipped with a slit of diameter 0.3 mm for the narrow-bore tube of I.D. 0.4 mm and diameter 0.1 mm for the narrow-bore tube of I.D. 0.2 mm. All experiments were carried out in the operational system listed in Table I.

TABLE I

OPERATIONAL SYSTEM AT pH 6, SUITABLE FOR ANIONIC SEPARATIONS

MES = morpholinoethanesulphonic acid; TRIS = tris(hydroxymethyl)aminomethane; HEC = hydroxyethylcellulose.

	Electrolyte	
	Leading	*Terminating*
Anion	Chloride	MES*
Concentration	0.01 *N*	*ca.* 0.01 *N*
Counter ion	Histidine	TRIS
pH	6	*ca.* 7
Additive	0.3% HEC**	None

* Purified by recrystallization.

** Purified by shaking the 2% solution with a mixed-bed ion exchanger and filtering.

Thermal step heights of several components were measured in narrow-bore tubes of different sizes over a wide range of current densities. Some of the results, carried out with chloride and morpholinoethanesulphonic acid (MES) anions are shown in Fig. 1. A survey of some other anions is given in Table II, where the correct dimensions of the narrow-bore tube are also given.

Assuming a thermal balance of the parabolic type, a plot of log ΔT against log J (J, current density) should give a straight line with a slope of 2. The experimental values, shown in both Fig. 1 and Table II, are in good agreement with theory, but the slope differs significantly from the theoretical value of 2, for several reasons: (1) there is a cooling effect due to the thermocouple mounted; (2) there is a change in the effective mobility of the ions, due to the difference in temperature of a zone at different current densities; and (3) there is a thermal expansion of the narrow-bore tube, especially at high current densities and with low effective mobilities of the ions involved.

To establish the significance of the first factor in a narrow-bore tube of I.D. 0.45 mm and O.D. 0.81 mm for chloride–histidine (Table I) at 80 μA (J = 0.050

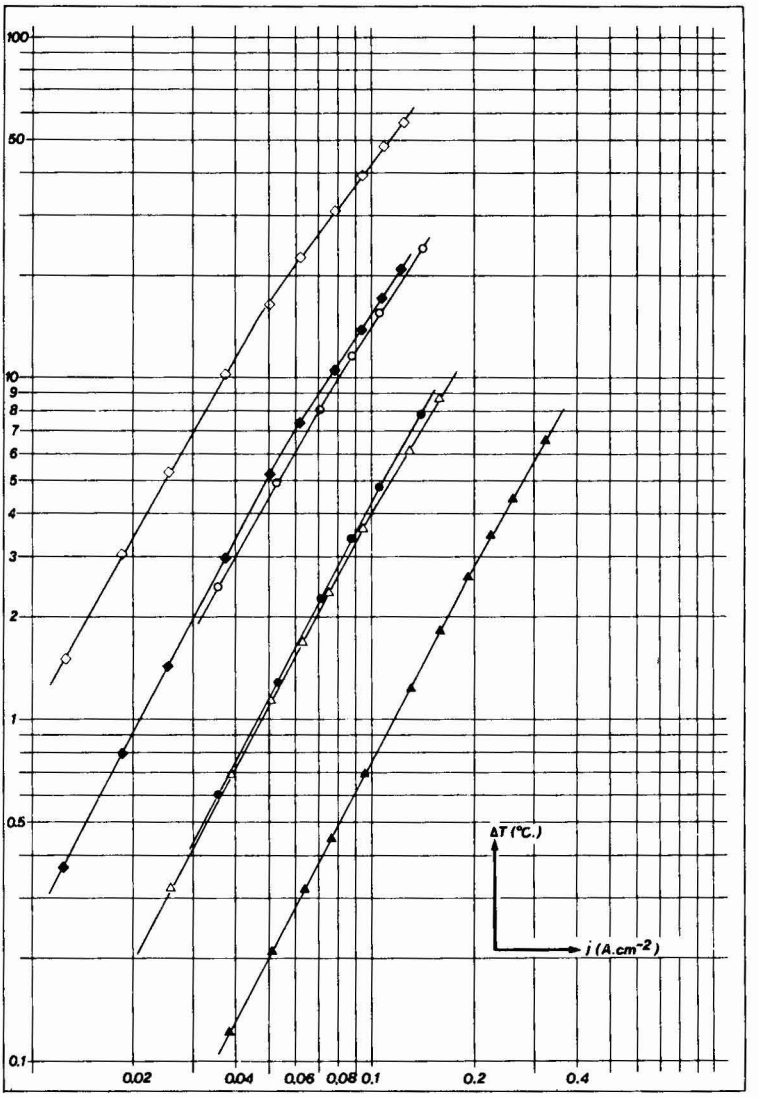

Fig. 1. Relationship between the temperature, measured by a thermocouple at the outside of a narrow-bore tube, and the current density applied. ▲, Chloride (0.10 mm I.D.; 0.20 mm O.D.), ●, chloride (0.19 mm I.D.; 0.40 mm O.D.), ■, chloride (0.45 mm I.D.; 0.81 mm O.D.); △, MES (0.10 mm I.D.; 0.20 mm O.D.); ○, MES (0.19 mm I.D., 0.40 mm O.D.); □, MES (0.45 mm I.D.; 0.81 mm O.D.). The experiments were carried out in the operational system listed in Table I.

$A \cdot cm^{-2}$), the following experiment was performed. After the current had been switched on and equilibrium of the thermal signal had been attained, a hydrodynamic flow was permitted in the direction of the counter electrode compartment. The increase in the thermal signal, due to the fact that leading electrolyte with a higher temperature (not cooled by the thermocouple wire) displaces the leading electrolyte with a lower temperature, was about 3.8%. Hence the thermal capacity of the thermocouple

TABLE II

SLOPES OF THE STRAIGHT LINES OBTAINED IF LOG J IS PLOTTED AGAINST LOG ΔT FOR VARIOUS COMPONENTS MIGRATING ISOTACHOPHORETICALLY IN NARROW-BORE TUBES WITH DIFFERENT DIMENSIONS

J is the current density and ΔT is the difference between the temperature of the wall of the narrow-bore tube and that of the ambient gas, measured with a thermocouple mounted on the outside of the narrow-bore tube.

Component	*Slope*		
	I.D. = 0.10 mm, O.D. = 0.20 mm	*I.D. = 0.19 mm, O.D. = 0.40 mm*	*I.D. = 0.45 mm, O.D. = 0.81 mm*
Chloride	1.92	1.92	1.90
Acetate	1.92	1.92	1.76
Glutamate	1.92	1.90	1.72
MES	1.90	1.80	1.68
ACES*	—	1.78	1.60

* ACES = Acetaminoethane sulfonic acid.

mounting influences the final temperature of the various zones. The correction for the temperature dependence of the mobilities for this case is *ca.* 3% for the chloride zone. The correction for the thermal expansion of the narrow-bore tube ($J = 0.050$ A·cm^{-2}) is only about 0.8% if the terminator MES is used, and can therefore be neglected. The influence of temperature on the pK values, the activity coefficients and the thermal conductivity of the solvent has not been taken into consideration.

The deviations in the straightness of the lines at high current densities are thus caused mainly by the temperature dependence of the mobilities and the cooling effect of the thermocouple mounting.

The influence of the inside diameter of the narrow-bore tube on the final temperature of the various zones is considerable, as shown in Fig. 1. Theory now predicts that for a constant current density and constant wall thickness, ΔT can be expected to be a square function of the internal diameter. For example, $J = 0.095$ A·cm^{-2} in a narrow-bore tube of I.D. 0.1 mm gives an increase in temperature of *ca.* 0.75°, while in a narrow-bore tube of I.D. 0.45 mm it will give an increase of 15°. Experi-

TABLE III

MEASURED AND CALCULATED[10] TEMPERATURES FOR THE LEADING AND TERMINATING ELECTROLYTES (TABLE I) IN TWO NARROW-BORE TUBES, INDICATING THE TEMPERATURE PROFILE, AT A CURRENT DENSITY OF $J = 0.095$ A·cm^{-2}

T_s = temperature of the surrounds (ambient gas temperature); T_m = temperature as measured by the thermocouple at the outside of the narrow-bore tube; T_w = calculated temperature at the inside of the wall of the narrow-bore tube; T_c = calculated temperature at the centre of the narrow-bore tube.

I.D. of narrow-bore tube (mm)	*Composition of electrolyte*	T_s	T_m	T_w	T_c
0.2	Cl^- (histidine H^+)	22.0	26.3	26.4	26.5
	MES^- (histidine H^+)	22.0	34.0	34.2	34.7
0.45	Cl^- (histidine H^+)	22.0	35.0	35.2	35.7
	MES^- (histidine H^+)	22.0	64.0	64.8	66.8

mentally, in the last example a difference in temperature of 14° was determined. For further information, Fig. 1 should be examined. These results indicate that, as far as possible, a reduction in the diameter of the electrophoretic separation column should be sought.

Apart from the gain in the temperature profile (some relevant temperatures are summarized in Table III), a smaller bore is preferable for many other purposes, such as quantitation, theoretical studies, times of analysis and the fact that slower terminators (for many thermally labile components such as proteins) can be applied. Although it is preferable to have a smaller difference in temperature between the various zones, it may occur that a given pair of ions in an operational system can be separated only as a result of the difference in temperature. So far little attention has been paid to temperature programming in electrophoretic analyses.

The influence of temperature on the conductivity of the various zones in isotachophoretic experiments is also far from negligible, as shown in Fig. 2. The effect

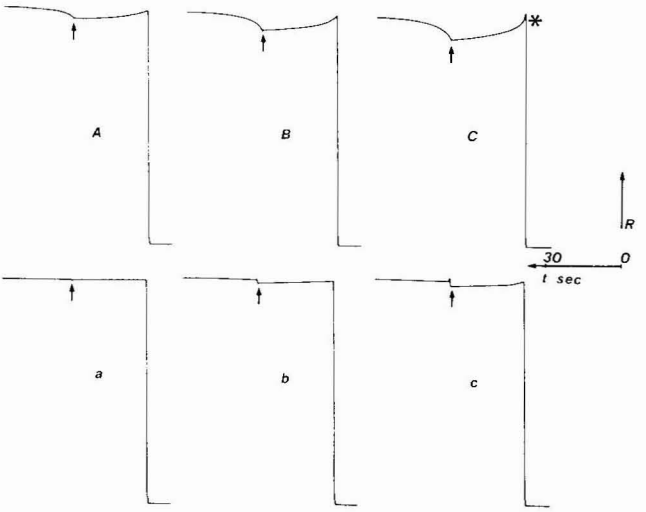

Fig. 2. Isotachopherograms (leading electrolyte/terminating steps) obtained from an a.c. conductivity detector with a potential gradient probe of I.D. 0.4 mm (A, B, C) and I.D. 0.2 mm (a, b, c) at various current densities. A, a, J = 0.064 A·cm^{-2}; B, b, J = 0.080 A·cm^{-2}; C, c, J = 0.095 A·cm^{-2}. *R* = increasing resistance; *t* = increasing time. The small arrows indicate the moment at which the electric current is switched off. *, overshoot.

of temperature on the conductivity is especially noticiable for the transition between the leading and terminating electrolytes. If sample zones are present, sandwiched between the leading and terminating zones, more time is available for the detector to warm up and hence the overshoot (marked with an asterisk in Fig. 2) is not as clearly visible, although the influence on the qualitative information is present.

In Fig. 2, isotachopherograms (chloride–MES boundaries) obtained from a potential gradient probe (a.c. mode) with a bore of I.D. 0.4 mm (A, B, C) and a bore of I.D. 0.2 mm (a, b, c) under identical *J* values: 0.064 (A, a), 0.080 (B, b) and 0.095 A·cm^{-2} (C, c) are compared. At the lowest *J* value a difference of about 4% can still be found if the probe of I.D. 0.4 mm is used.

Hardly any difference is found with the probe of I.D. 0.2 mm. It needs no further explanation that especially for theoretical studies of, for example, complex formation and mobility determination or the influence of activity or solvation, this result is very important. Assuming that the analyses are carried out at current densities of up to 0.064 $A \cdot cm^{-2}$, the qualitative information is no longer a function of the current density and the sample injected (warming up of the detector) and is better than 1% (electronics of the a.c. conductivity detector).

Apart from the diameter, the gas surrounding the narrow-bore tube was also found to be important. If the narrow-bore tube was surrounded by hydrogen an inititial temperature difference from the ambient gas temperature was found to be *ca.* 1.9° for the leading electrolyte and *ca.* 7.0° for the terminating electrolyte (Table I). J was 0.050 $A \cdot cm^{-2}$ and the dimensions of the narrow-bore tube were I.D. 0.45 mm and O.D. 0.81 mm. Under similar conditions, a temperature difference of 5.2° for the leading electrolyte and 16.0° for the terminating electrolyte were measured when nitrogen (or air) surrounded the narrow-bore tube. If kerosene was used as the medium surrounding the narrow-bore tube, negligible signals remained for a complete qualitative (and/or quantitative) determination with thermometric detection. Carbon dioxide (which has an even smaller thermal conductivity than nitrogen) cannot be applied, because it easily passes through the PTFE wall. Carbon dioxide dissolves in the electrolytes and disturbs the isotachophoretic analysis, migrating as HCO_3^- or CO_3^{2-}, assuming that the pH of the leading electrolyte is sufficiently high (even at pH 6).

Because of the above effects, more care must be taken if operational systems are applied at high pH, not only during the preparation of the various electrolytes, under nitrogen, and with the addition of, *e.g.*, barium hydroxide to the terminating electrolyte.

Another effect was examined visually during experiments with the narrow-bore tubes of I.D. *ca.* 0.4 and 0.2 mm. Poly(vinyl alcohol) (Mowiol), purified by running a concentrated solution over a mixed-bed ion exchanger, was added to the electrolyte at a concentration of 0.05%. In the narrow-bore tubes of I.D. *ca.* 0.4 mm a clear convective disturbance appears at about 130 μA ($J = 0.081$ $A \cdot cm^{-2}$), for instance at the zone boundary of bromophenol blue-morpholinoethane sulphonate in the operational system at pH 6 (Table I). These disturbances could be suppressed by addition of 0.3% of hydroxyethylcellulose (Polysciences, Warrington, Pa., U.S.A.), also purified by using a mixed-bed ion exchanger and filtering*, at this current density.

These convective disturbances did not appear at comparable current densities in narrow bore-tubes of I.D. *ca.* 0.2 mm, owing to the smaller differences in temperature between the various zones, the gain in temperature profile and the stabilization by the decrease in the ratio of the cross-section to surface area.

Some isotachopherograms are shown of analyses carried out in narrow-bore tubes smaller than those conventionally used. Fig. 3 shows an isotachophoretic separation of a standard mixture of anions in the operational system listed in Table I, carried out in a narrow-bore tube of I.D. *ca.* 0.1 mm. An ordinary thermocouple (copper–constantan) was used as the detector ($J = 0.114$ $A \cdot cm^{-2}$).

* The polymer solution must be filtered in order to remove solid particles, which migrate under the influence of the electric field and disturb the registration, especially with a UV absorption detector.

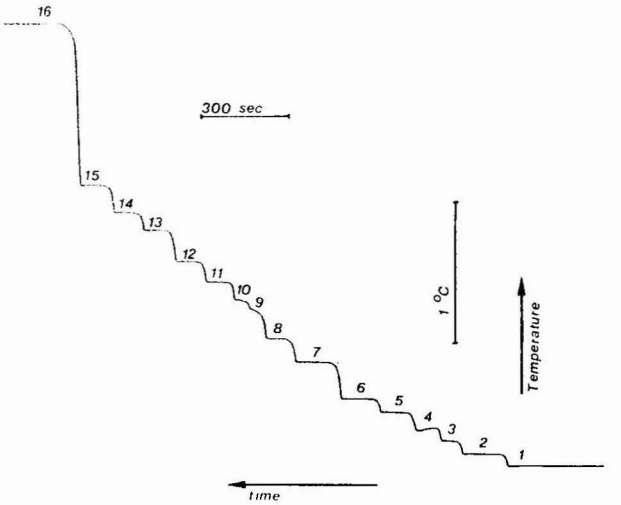

Fig. 3. Isotachopherogram of a test mixture of anions obtained using the operational system listed in Table I. The registration was effected with a thermocouple, mounted around a narrow-bore tube of O.D. 0.2 mm and I.D. 0.1 mm, $J = 0.114$ A·cm^{-2}. 1 = Chloride; 2 = sulphate; 3 = chlorate; 4 = chromate; 5 = malonate; 6 = pyrazole-3,5-dicarboxylate; 7 = adipate; 8 = acetate; 9 = β-chloropropionate; 10 = propionate; 11 = benzoate; 12 = naphthalene-2-sulphonate; 13 = glutamate; 14 = enanthate; 15 = benzyl-*dl*-aspartate; 16 = morpholinoethanesulphonate.

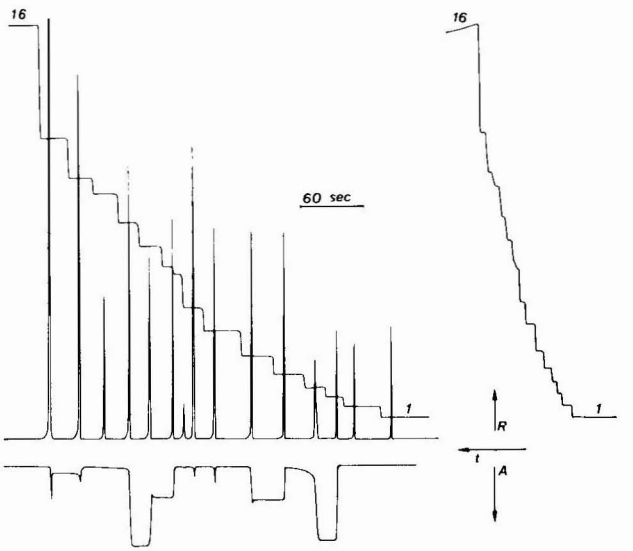

Fig. 4. Isotachopherogram of the test mixture of anions, as shown in Fig. 3, obtained using the operational system listed in Table I. The left-hand isotachopherogram was obtained in a narrow-bore tube of I.D. *ca.* 0.2 mm, and the right-hand isotachopherogram in a narrow-bore tube of I.D. *ca.* 0.4 mm. A conductivity detector (a.c. mode) and an UV absorption detector were applied. Equal amounts of sample were injected. R = increasing resistance; A = increasing UV absorption; t = increasing time. $J = 0.080$ A·cm^{-2}.

It is rather difficult to effect a satisfactory thermometric registration for this mixture of anions, making use of a narrow-bore tube of I.D. *ca.* 0.4 mm. Up till now no set of ions could be found, assuming that the concentration ratio was chosen correctly, that could be detected via a conductivity or UV detector once separated isotachophoretically, if the separation could not be registered with a thermometric detector.

Fig. 4 shows the test mixture of anions (Fig. 3) separated isotachophoretically in the operational system listed in Table I, using conductivity detectors (a.c. mode) with potential gradient probes of 0.2 mm (left-hand side) and 0.4 mm (right-hand side) as detectors. In the left-hand analysis, a registration is also shown that was obtained with a UV absorption detector (254 nm) with a slit width of 0.1 mm (in both experiments, $J = 0.080\ \mathrm{A \cdot cm^{-2}}$). The total amounts of all ions in the test mixture were identical in each experiment, which indicates once more the importance of reducing the inside diameter of the narrow bore tube. For both the conductivity and UV absorption detectors the resolution is improved if a smaller diameter of the narrow-bore tube is chosen (more spikes due to impurities are visible). The reproducibility of the step heights (qualitative information) found in the linear trace from the conductivity detector was better than 1 % and was no longer a function of J.

Fig. 5 shows the standard mixture of anions separated isotachophoretically in the operational system listed in Table I ($J = 0.064\ \mathrm{A \cdot cm^{-2}}$) using a conductivity detector (a.c. mode) with a potential gradient probe of I.D. 0.2 mm. The registration was effected with a potentiometric recorder with zero suppression. The total scale could be enlarged five times. The reproducibility of the linear trace, which shows that the conductivity in the various zones is not exactly the same throughout a zone (for instance, in the zone of chromate and benzyl-*dl*-aspartate), was found to be almost 100 %.

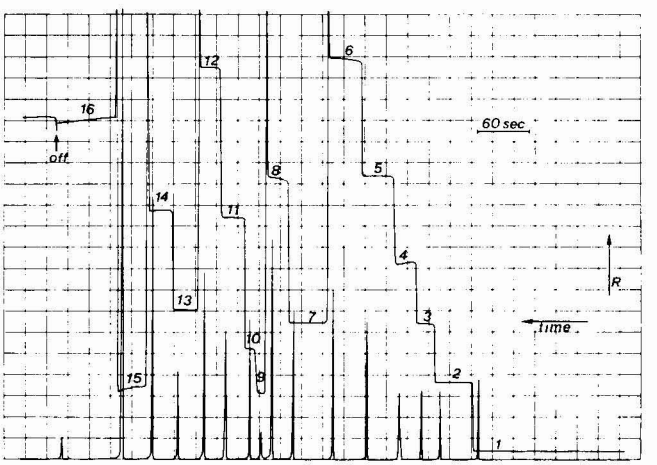

Fig. 5. Isotachopherogram of the test mixture of anions (Fig. 3) obtained using the operational system listed in Table I, carried out in a narrow-bore tube of I.D. *ca.* 0.2 mm. The registration was effected with a conductivity detector (a.c. mode) with a potential gradient probe of I.D. 0.2 mm. The potential recorder was equipped for zero suppression (for this analysis, five times). At the arrow (off), the electric current is switched off. R = increasing electric resistance. $J = 0.064\ \mathrm{A \cdot cm^{-2}}$.

CONCLUSION

From these experiments, we recommend the use of narrow-bore tubes with an inside diameter smaller than the *ca.* 0.4 mm commonly used, especially for analytical purposes. Even for preparative isotachophoresis, a series of narrow-bore columns, mounted in parallel, is worth considering, instead of increasing the inside diameter. Undesirable temperature increases, especially if thermally labile components are of interest, can be prevented.

The use of soluble linear polymers, which decrease the convective disturbances, is also recommended. The concentration of these polymers depends on, amongst other factors, the molecular weight, the type of polymer, the diameter of the narrow-bore tube and the current density applied.

Owing to the construction of our injection system and the way our conductivity detector is constructed, we found a narrow-bore tube with an I.D. of *ca.* 0.2 mm to be almost optimal.

The advantages of decreasing the inside diameter of the narrow-bore tubes are:

(1) smaller amounts of sample are needed for full qualitative and quantitative separation;

(2) the qualitative information is no longer a function of the current density;

(3) the temperature differences between the various zones are smaller;

(4) the total time for analysis can be decreased, if required;

(5) terminators with a lower effective mobility can be applied.

Whether or not the diameter can be decreased further in the future depends on, amongs other factors, the electroendosmosis, the availability of current-stabilized power supplies that enable one to work at low electric currents ($I < 10\ \mu A$) and better injection systems.

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CHROM. 9595

WHISKER-WALLED OPEN-TUBULAR GLASS COLUMNS IN GAS CHROMATOGRAPHY

I. DEACTIVATION

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SUMMARY

A number of techniques for deactivating the surface of whisker-walled open-tubular glass columns have been investigated. Silanization and treatment with surface-active agents were the most successful techniques.

INTRODUCTION

Techniques for growing silica whiskers on the inner surface of glass open-tubular columns have been described previously[1,2]. The internal surface area can be increased considerably by these means and, when coated with stationary phase, acceptable values of the phase ratio, β, can be obtained[1,3].

The method of growing whiskers results in a highly active surface, which leads to excessive tailing of the eluted peaks, particularly when a non-polar stationary phase is used. Deactivation of the surface of the whiskers is therefore necessary before coating with the stationary phase if the potential performance of the column is to be realized. We report here an investigation that was carried out to assess various methods for the deactivation of the surface.

EXPERIMENTAL

Glass columns 10–15 m long and of I.D. *ca.* 0.03 cm were drawn and the inner surface was modified by growing silica whiskers as described previously[2]. After deactivation by the methods outlined below, the columns were connected with heat-shrinkable PTFE tubing (Raychem, Olifantsfontein, South Africa) to a variable splitter[4] in a Varian Aerograph Model VA 1800 gas chromatograph. The column outlet was connected in a similar manner to a flame-ionization detector, which was modified to allow the introduction of make-up carrier gas[4]. The splitter was constructed from glass-lined stainless-steel tubing (Scientific Glass Engineering, Mel-

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bourne, Australia), the inner surface of which was deactivated by silanization as described below. This procedure ensured that any tailing that was observed originated in the column and not in the inlet. The column was operated at 200° using nitrogen as the carrier gas.

The methods used to deactivate the surface of the whiskers were silanization, sorption of surface-active agents, surface carbonization and application of a non-extractable polymer layer.

Silanization

Three different methods of silanization were used. Firstly, a plug of dimethyldichlorosilane in toluene (5%, v/v) was propelled through the column using dry nitrogen as described by German and Horning[5] and Pierce[6], then washed with toluene and methanol and finally dried in a stream of dry nitrogen at 200°. The second method, which has been described by Novotný and Tešarìk[7], entailed bubbling nitrogen through a mixture of hexamethyldisilazane and trimethylchlorosilane (5:1) at 25° and filling the column with the resulting vapour at the same temperature. The column ends were then sealed and the column was heated at 200° for 48 h. The third method was similar to that of Novotný and Tešarìk except that the silanization vapour mixture was passed continuously through the column at 200° for 24 h. In each of the procedures the column was finally cleaned by flushing it with dry nitrogen at 200° for 6–12 h.

Sorption of surface-active agents

This technique has been discussed by Novotný and Zlatkis[8]. A plug of a 0.2–1.0% (w/v) of the surface-active agent in a suitable solvent is propelled through the column using dry nitrogen. The excess of surfactant is washed out with solvent, which is then flushed using dry nitrogen at 200°. Benzyltriphenylphosphonium chloride was chosen as the surface-active agent because of its ready availability and good thermal stability[9].

Surface carbonization

Three methods were used. The first has been described by Grob[10,11]. Nitrogen was bubbled through pure dichloromethane at 20° and the vapour used to fill the column at a slightly higher temperature. After sealing its ends, the column was heated at 550° for 30–45 min and then flushed with dry nitrogen. In the second method, which has been briefly referred to by Grob[10,11], pure acetylene was passed through the column, its ends were sealed and the acetylene was pyrolyzed at 550° for 30–45 min. The column was then flushed with dry nitrogen. In the third method, one end of the column was connected to a high-vacuum pump and the other end to a septum through which pure *n*-hexane was injected until approximately 10% of the column volume was filled with liquid *n*-hexane. The ends of the column were sealed and the column was heated at 550° for 30–45 min, the column subsequently being flushed with dry nitrogen.

Application of a non-extractable polymer layer

This method was evolved by Aue *et al.*[12] and applied to open-tubular columns by Cronin[13]. The column was filled with a 2% (w/v) solution of Carbowax 20M in di-

chloromethane and allowed to stand for 3–6 h, then the solution was flushed out using dry nitrogen at 250° for 24 h. The column ends were closed and the column was heated at 280° for 24 h. The column was then opened, flushed with dichloromethane and methanol and dried with nitrogen at 200°.

RESULTS

The success of a particular method of deactivation was determined by injecting into each of the deactivated columns a series of compounds containing different functional groups that could be expected to be encountered in a wide variety of practical separations. Samples of 0.2–0.5 μl of the liquid solutes were injected, and also 20–50-μl samples of methane gas. These compounds and their associated functional groups are listed in Table I. The tailing factor of each peak:

$$\text{TF}\ (\%) = \frac{a}{b} \times 100\%$$

where a and b were measured at 10% of the peak height, as shown in Fig. 1[14]. The values that were obtained for the different deactivated surfaces are given in Table II.

As it would be unrealistic to expect that a support surface could be produced that is completely unreactive to all solutes, *i.e.*, TF = 100%, the results obtained

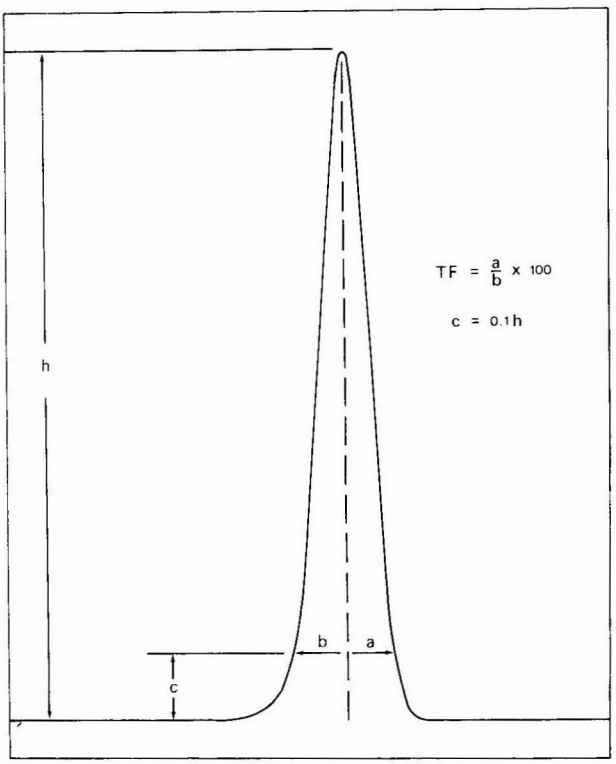

Fig. 1. Definition of the tailing factor, TF (%).

TABLE I

COMPOUNDS USED IN THE STUDY OF THE EFFICIENCY OF DEACTIVATION

Functional group	*Solute*
Aliphatic	Methane, *n*-heptane
Aromatic	Benzene
Ether	Diethyl ether
Ketone	Methyl isobutyl ketone
Alcohol	Methanol, cyclohexanol
Amine	Dimethylaniline, pyridine

were compared with those pertaining to a support surface that is generally considered to be the best attainable in practice, *i.e.*, Chromosorb W HP (Johns-Manville, Denver, Colo., U.S.A.)[15]. The tailing factors obtained by injecting the solutes listed in Table I onto this surface are therefore included in Table II for comparison purposes.

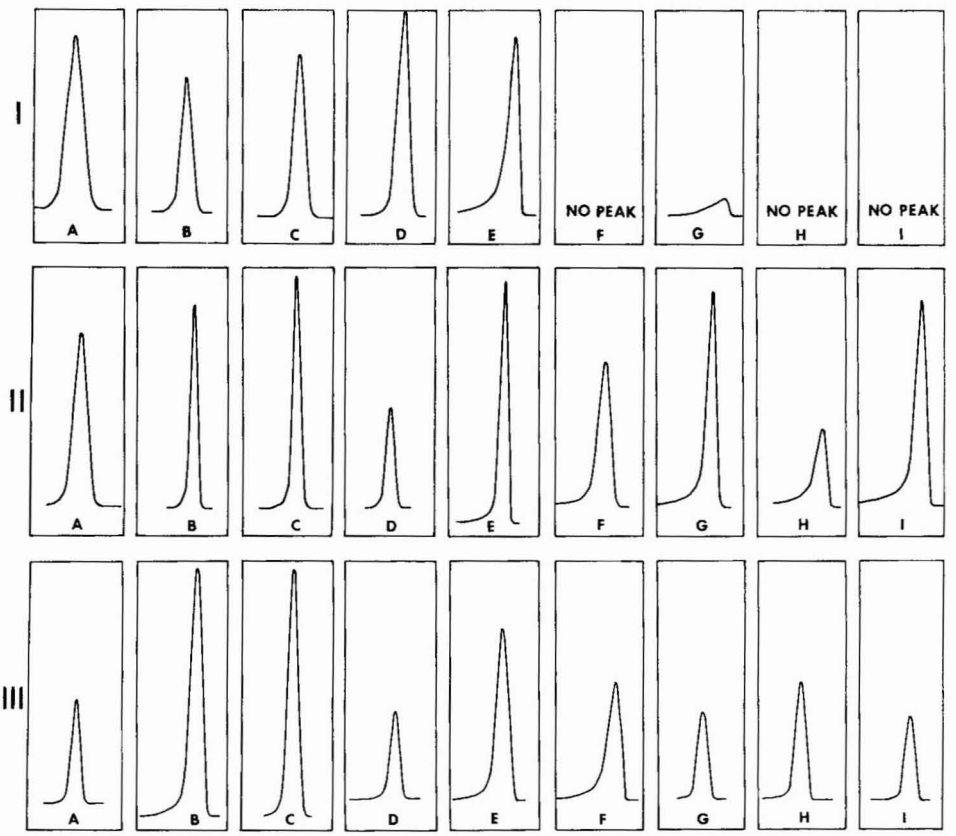

Fig. 2. Chromatograms of test solutes on various supports: I, untreated silica whiskers; II, continuous-flow silanization; III, Chromosorb W HP. Solutes: A, methane; B, *n*-hexane; C, benzene; D, diethyl ether; E, methyl isobutyl ketone; F, methanol; G, cyclohexanol; H, dimethylaniline; I, pyridine.

TABLE II

TAILING FACTORS DETERMINED FOR THE COMPOUNDS LISTED IN TABLE I ON VARIOUS SURFACES

Solute	*Tailing factor, TF(%), for different surfaces*									
	Whiskers	*Whiskers and BTPPC*	*Whiskers and Carbowax 20M*	*Whiskers and acetylene*	*Whiskers and dichloro-methane*	*Whiskers and n-hexane*	*Whiskers and DMDCS*	*Whiskers and HMDS/ TMCS*	*Whiskers and HMDS/ TMCS (continuous)*	*Chromosorb W HP*
Methane	96	94	94	95	80	98	99	64	92	90
n-Hexane	80	80	74	70	14	85	68	69	98	78
Benzene	78	85	70	65	<5	60	63	78	100	72
Diethyl ether	86	73	72	75	<5	80	NP*	11	100	71
Methyl isobutyl ketone	33	73	60	66	<5	70	8	<5	73	65
Methanol	NP*	50	54	NP*	NP*	60	NP*	17	69	44
Cyclohexanol	21	37	51	50	NP*	38	NP*	NP*	60	77
Dimethylaniline	NP*	57	34	NP*	NP*	14	NP*	NP*	30	69
Pyridine	NP*	35	22	NP*	NP*	10	NP*	NP*	40	63

* NP = no peak observed.

DISCUSSION

The best deactivation of the whisker surface was obtained using the third silanization method. Only for compounds containing an amine group did this surface compare badly with Chromosorb W HP. This problem could possibly be overcome by treating the column with sodium or potassium hydroxide, as is often done in the chromatographic separation of amines[16,17].

The other deactivation techniques were less effective but, with the exception of the carbonization of dichloromethane and acetylene, may be acceptable for many applications.

Examples of the chromatograms obtained using the best silanization deactivation technique are shown in Fig. 2, together with those obtained on undeactivated whiskers and also on Chromosorb W HP.

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CHROM. 9596

WHISKER-WALLED OPEN-TUBULAR GLASS COLUMNS IN GAS CHROMATOGRAPHY

II. CHROMATOGRAPHIC PERFORMANCE

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(First received December 5th, 1975; revised manuscript received July 27th, 1976)

SUMMARY

A variety of parameters relating to column efficiency (including the plate height, effective plate height, number of effective plates per unit time, phase ratio, sample capacity, performance index and percentage utilization of the theoretical efficiency) have been determined experimentally for whisker-walled open-tubular glass columns, and compared with values pertaining to other types of open-tubular columns.

INTRODUCTION

We have previously reported[1,2] a method by which silica whiskers can be grown on the inner surface of open-tubular glass columns and used as a support for the liquid stationary phase. Preliminary experiments indicated that these whisker coatings have considerable promise as a means of obtaining high performance. In this paper we report more extensively on this aspect.

EXPERIMENTAL

Columns were drawn as described previously[2] from borosilicate glass (Pyrex, J. A. Jobling & Co., Staffs, Great Britain) to an O.D. of 0.1 cm, an I.D. of 0.020–0.035 cm and a length of 45–50 m. Whisker coatings were grown[2] on the inner surfaces of the columns; a typical portion of the surface obtained is shown in Fig. 1. The whiskers are *ca.* 10 μm long and 1 μm in diameter with a density of *ca.* 25 whiskers per 100 μm^2. From these data, the surface area is estimated to be 8–9 times that of a smooth surface.

The surface of freshly grown whiskers is extremely active and can be de-activated, before coating with stationary phase, in a variety of ways, as reported elsewhere[3]. One method of de-activation, which was adequate for the present purpose, employs a 0.2% (w/v) solution of benzyltriphenylphosphonium chloride (BTPPC) in chloroform. Excess of solution was removed by washing with pure chloroform.

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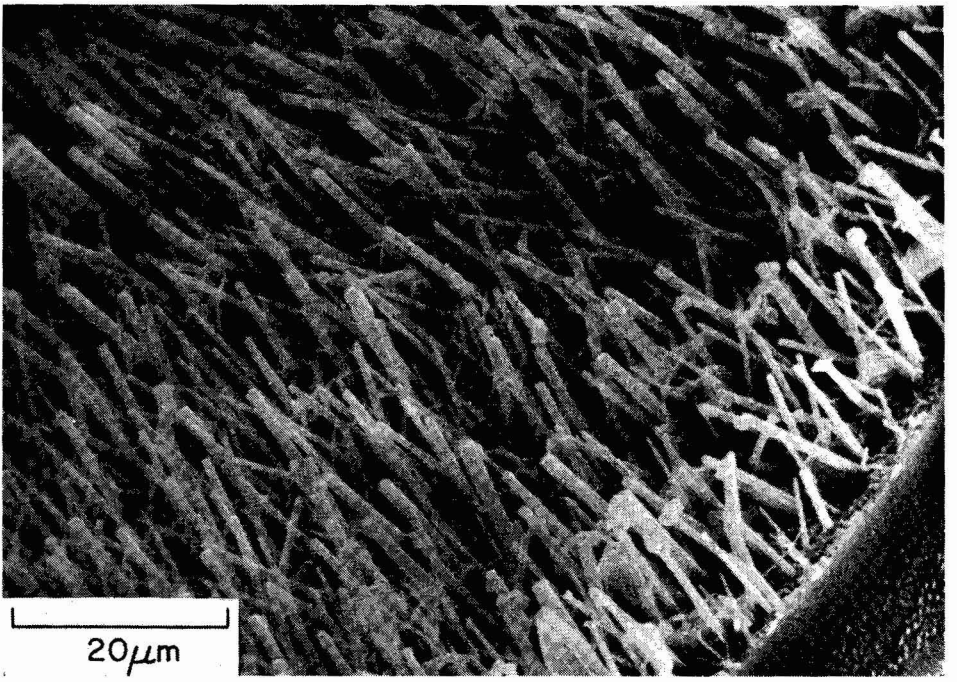


Fig. 1. A typical portion of the surface of a whisker-walled open-tubular column.

Stationary phase was coated on to the whiskers using the dynamic coating method[4]. Squalane was chosen as the stationary phase solely because data that are needed for estimating the phase ratio (β) of the column are accurately known. A short plug (*ca.* 10% of the column volume) of a solution of squalane in chloroform (2.5–5%, w/v) was forced through the column using dry nitrogen as a propellant, at a linear velocity of 3 to 4 cm·sec^{-1}. Excess of solvent was then removed by continuing the nitrogen flow for 24 h at room temperature. The column was then conditioned by increasing the temperature slowly to 130° and maintaining it there for a further 24 h. The internal diameter of the column was estimated by filling a short length, *e.g.*, 35 cm, with distilled water and weighing it. As a check, a short length of the column was viewed end-on using a scanning electron microscope. The value of the internal diameter obtained in this way was within 4% of that obtained by weighing.

Columns were installed in a Varian Aerograph Model VA 1800 gas chromatograph that was modified by adding a splitter similar to that described by Ettre[5] and connections for make-up gas[5]. The outlet of the column was fed to a flame-ionization detector. Heat-shrinkable PTFE tubing (Raychem, Olifantsfontein, South Africa) was used to make the connections between the columns and the remainder of the system. The column temperature was maintained at 81°. *n*-Heptane was used as a test sample and methane as an "air peak".

CHROMATOGRAPHIC PERFORMANCE

A single, overall parameter describing column performance is still lacking and

a number of alternative parameters have been used. Numerical values of these parameters that were obtained in this study are summarized in Table I. Values of the mass distribution coefficient, k, were determined from the measured retention times of the solute and methane peaks[5]. The phase ratio, β, was estimated from the equation[5]

$$K = k \cdot \beta$$

where the concentration distribution coefficient, K, is 152 for the present system[6]; S in Table I refers to the percentage (w/v) of the stationary phase in the coating solution.

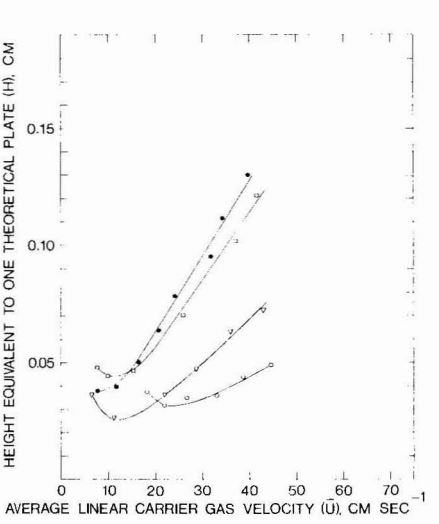
TABLE I

SUMMARY OF CHROMATOGRAPHIC PERFORMANCE OF FOUR WHISKER-WALLED OPEN-TUBULAR GLASS COLUMNS

Parameter	*Column*			
	1	*2*	*3*	*4*
Length (m)	55.4	52.9	44.8	44.8
Radius (cm)	0.016	0.016	0.012	0.012
Stationary phase	Squalane	Squalane	Squalane	Squalane
Temperature (°C)	81	81	81	81
S (%)	2.5	5	5	5
Phase ratio, β	125	90	80	80
B_0 (cm^2) (experimental)	$198 \cdot 10^{-7}$	$198 \cdot 10^{-7}$	$245 \cdot 10^{-7}$	—
B_0 (cm^2) (calculated)	$320 \cdot 10^{-7}$	$320 \cdot 10^{-7}$	$180 \cdot 10^{-7}$	$180 \cdot 10^{-7}$
Carrier gas	N_2	N_2	N_2	He
Sample	*n*-Heptane	*n*-Heptane	*n*-Heptane	*n*-Heptane
k	1.2	1.7	1.85	1.85
H_{min} (cm)	0.04	0.045	0.025	0.030
h_{min} (cm)	0.13	0.11	0.06	0.075
$\bar{u}_{opt}$ (cm·sec^{-1})	7.5	10	12.5	22.5
$N \cdot m^{-1}$ (max)	2630	2270	3880	3130
$N_{eff} \cdot m^{-1}$ (max)	780	900	1635	1320
$N_{eff} \cdot sec^{-1}$ (max)	100	57	90	130
$\bar{u}^*_{opt}$ (cm·sec^{-1})	21.5	26	22.5	30
B (cm^2·sec^{-1})	0.143	0.207	0.197	0.2580
C (sec)	$32 \cdot 10^{-4}$	$29 \cdot 10^{-4}$	$17.5 \cdot 10^{-4}$	$10.0 \cdot 10^{-4}$
C_L (sec)	$6.5 \cdot 10^{-4}$	$8.0 \cdot 10^{-4}$	$5.0 \cdot 10^{-4}$	$5.0 \cdot 10^{-4}$
C_G (sec)	$25.5 \cdot 10^{-4}$	$20.5 \cdot 10^{-4}$	$12.5 \cdot 10^{-4}$	$5.0 \cdot 10^{-4}$
PI (poise)	0.4	0.40	0.65	—
UTE (%) ($a = 0.1$)	62	55	71	58
Sample capacity (μg per component)	1.5–2.5	1.5–2.5	0.5–1.5	0.5–1.5
TZ (I = 600–700)	29.0	28.5	36.5	—

Values of the plate height, H, were obtained in the usual way from the chromatogram and are plotted against the mean carrier gas flow velocity, $\bar{u}$, in Fig. 2. The minimum values of the plate height, H_{min}, at the optimum mean carrier gas flow velocity, $\bar{u}_{opt}$, are listed in Table I. Values of the plate number per metre of column length, $N \cdot m^{-1}$, were calculated from $N \cdot m^{-1} = 100/H$ (H measured in centimetres). The effective plate height was calculated using the equation[7,8]

$$h = \frac{(1 + k)^2}{k^2} \cdot H$$



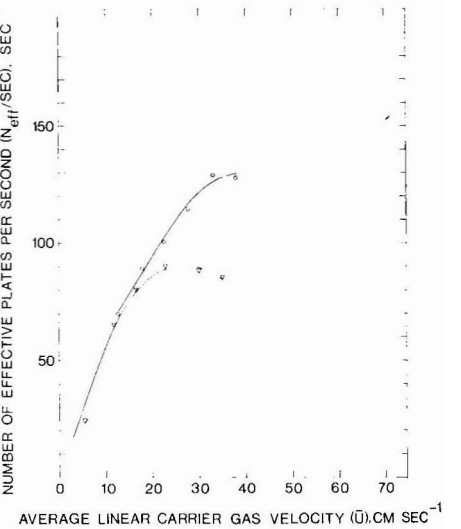


Fig. 2. Plate height (H) *versus* mean carrier gas flow velocity. Solute: *n*-heptane. Stationary phase: squalane. ●, $r = 0.016$ cm, nitrogen as carrier gas, $k = 1.2$, $\beta = 120$, column length = 55.4 m; □, $r = 0.016$ cm, nitrogen as carrier gas, $k = 1.7$, $\beta = 90$, column length = 52.9 m; ▽, $r = 0.012$ cm, nitrogen as carrier gas, $k = 1.85$, $\beta = 80$, column length = 44.8 m, ○, $r = 0.012$ cm, helium as carrier gas, $k = 1.85$, $\beta = 80$, column length = 44.8 m.

Fig. 3. Number of effective plates per second ($N_{\text{eff}} \cdot \text{sec}^{-1}$) *versus* mean carrier gas flow velocity ($\bar{u}$). Column length, 44.8 m; radius, 0.012 cm; solute, *n*-heptane ($k = 1.85$); $\beta = 80$. Carrier gas: ○, helium; ▽, nitrogen.

Values of the effective plate number per metre, $N_{\text{eff}} \cdot \text{m}^{-1}$, follow from the above two equations.

The maximum number of effective plates per second, $N_{\text{eff}} \cdot \text{sec}^{-1}$ (max), refers to the highest value obtained when the effective plate number per second is plotted against flow velocity, as shown in Fig. 3. $N_{\text{eff}} \cdot \text{sec}^{-1}$ is determined by dividing N_{eff} by the time required to elute the *n*-heptane; $\bar{u}^*_{\text{opt}}$ is the linear gas velocity pertaining to $N_{\text{eff}} \cdot \text{sec}^{-1}$ (max). B, C, C_{L} and C_{G} refer to the Golay equation[5]:

$$H = \frac{B}{\bar{u}} + C\bar{u}$$

where $C = C_{\text{L}} + C_{\text{G}}$, B = longitudinal gaseous diffusion, C = total mass transfer, C_{L} = resistance to mass transfer in the liquid phase and C_{G} = resistance to mass transfer in the gas phase. B has been calculated[6] from

$$B = \frac{H_{\text{min}}\, \bar{u}_{\text{opt}}}{2}$$

and the data in Fig. 1. C was determined from the slopes of the plate height *versus* flow velocity graphs at high linear velocities. C_{L} was estimated by the method of Perrett and Purnell[9], *viz.*

$$C_{\text{L}} = \frac{\Delta H}{\Delta u}$$

where $\Delta H = H_{He} - H_{N_2}$, *i.e.*, the difference of the plate height for a single component (*e.g.*, *n*-heptane) using helium and nitrogen as carrier gases at a flow velocity ratio that equals the ratio of the diffusion coefficients of the solute in the two carrier gases. C_G follows from the difference between C and C_L.

The performance index[10], PI, was calculated from the equation

$$PI = \left(\frac{W_h}{t_r - t_m}\right)^4 \left(\frac{t_r - t_m}{t_r}\right)^4 \left(\frac{t_m}{t_r - 0.9375\, t_m}\right) t_r \Delta P \text{ (poise)}$$

where W_h = peak width at half height (seconds), t_r = retention time of retarded peak (seconds), t_m = retention time of unretarded peak (seconds) and ΔP = pressure drop across the column (dyne·cm^{-2}).

The percentage utilization of the theoretical efficiency (UTE, %)[6]

$$\text{UTE} = \frac{H_{\text{min (theoretical)}}}{H_{\text{min (measured)}}} \cdot 100$$

was calculated using the experimental data for H_{min} (*cf.*, Table I) and the corresponding values of H_{min} (theoretical) obtained from the extended Golay equation[11]:

$$H_{min} \text{ (theoretical)} \approx 2(BC_G)^{\frac{1}{2}} \quad \text{(ref. 6)}$$

$$= r\left(\frac{1 + 6k + 11k^2}{3(1 + k)^2} + \frac{8a}{3} + \frac{16\, ka}{3(1 + k)^2}\right)^{\frac{1}{2}} \quad \text{(ref. 6)}$$

where a is the ratio of the thickness of the whisker layer to the column radius, as determined from a scanning electron micrograph of the column[2].

The separation number, TZ, was determined from the relationship proposed by Kaiser[12]:

$$TZ = \frac{1.699\, t_R(z + 1) - t_R(z)}{W_b(z) + W_b(z + 1)} - 1$$

where $t_R(z)$ and $t_R(z + 1)$ are the retention times of two consecutive peaks in a homologous series and $W_b(z)$ and $W_b(z + 1)$ are the two corresponding peak widths.

The sample or load capacity refers to the maximum amount of solute that can be injected on to the column without appreciably (*e.g.*, 10%) affecting the band width, *i.e.*, the plate height.

The column permeability, B_0, was determined from the well-known equation[5]

$$\Delta P = \eta \frac{L\, \bar{u}}{B_0}$$

where ΔP = the pressure drop across the column, η = the viscosity of the carrier gas, L = column length and $\bar{u}$ = linear carrier gas velocity.

Comparison with other types of surface-modified open-tubular columns

For the purpose of comparison, it is convenient to classify open-tubular columns[6] into "classical" wall-coated open-tubular (WCOT) columns, in which the stationary phase is spread as a thin film on the inner surface of the column, and porous-layer open tubular (PLOT) columns, in which the inner surface is either chemically

TABLE II

PERFORMANCE PARAMETERS OF VARIOUS TYPES OF MODIFIED OPEN-TUBULAR COLUMNS

Type of column	*Workers*	*Reference*	*Modifying reagent/solid support*	*Column material*	*Column dimensions*		*h (cm)*	*β*	*Sample capacity*	*Most polar stationary phase used*	*F*
					Length (m)	*I.D. (cm)*					
Wall-treated open-tubular	Novotný and Tešarík	13–15	HCl (vapour)	Alkali-type glass	10–50	0.015–0.027	H^*=0.04–0.06	—	—	Triethanol-amine	1.5–2
			HF (gas)	Borosilicate glass	10–50	0.015–0.027	H^*=0.04–0.06	—	—	Triethanol-amine	1.1–1.3
	Alexander and co-worker	13, 16, 17	HCl (vapour)	Alkali-type glass	20	0.022	0.034 (0.28)	400	—	DEGA	1.5–2
	Novotný and Bartle	13, 18	HCl (vapour)	Alkali-type glass	50	0.5	0.05	—	—	SF-96	1.5–2
	Schomburg *et al.*	13, 19	HCl (vapour)	Alkali-type glass	18	0.025	0.065	—	—	DEGS	1.5–2
	Rijswick and Tešarík	13, 20	HF (vapour)	Borosilicate glass	15	0.018–0.022	0.0425	—	—	OV-101	1.1–1.3
	Grob	21, 22	CH_2Cl_2(vapour)	Borosilicate	50	0.026	~0.07	—	—	XE-60, PPG	—
	Schieke *et al.*	1, 2	HF (vapour)	Borosilicate	45	0.024	0.06 (0.06)	80	0.5–2 μg/component	Carbowax 20M	8–9
Porous-layer open-tubular	Horning and co-workers	23–26	Silanox	Glass	30–60	0.03	0.18	—	—	Silar-5CP PZ-176	—
	Nikelly and Blumer	27–29	Silanox	Glass/stainless steel	11	0.05	0.19 (0.25)	50	5 μg/component	FFAP, DEGS	—
	Bertsch *et al.*	30	Silanox	Nickel	100–200	0.05	0.14	—	2–5 μl of complex sample	Carbowax 20M	—
	Watanabe and Tonita	13, 31	NaCl crystals	Glass	5–20	0.056	0.032	—	—	OV-101	1.5–2
	Nota *et al.*	32	Graphite	Glass	14	0.05	H^*=0.07	—	—	Carbowax 20M	—
	Grant	6, 33	Diatomaceous earth and LiCl	Glass	20	0.5	~0.2	—	—	Dilauryl phthalate	5–10
	Cronin	34	Diatomaceous earth and glass powder	Glass	36	0.05	0.15 (0.4)	150	—	Carbowax 20M	—
	Ettre and Purnell	6	Diatomaceous earth	Stainless steel	17	0.05	0.1 (0.1)	76	~10^{-2} μl/component	DEGS	30
	Scientific Glass Engineering	35	Diatomaceous earth	Glass	50	0.05	0.16	—	—	Carbowax 20M	—

* H = theoretical plate height.

modified, *i.e.*, wall-treated (WTOT) columns, or a layer of support material is deposited on the inner surface [support-coated open-tubular (SCOT) columns]. Several of the performance parameters mentioned in the previous section are listed in Table II. The data were either obtained from the sources cited or were calculated from other available data. Where several values of a particular parameter were available, the best value is quoted.

It is important to note that performance parameters have been compared at similar values of k (*e.g.*, $k = 2$–5) and K. This latter point is important because from the equation[7,8]

$$h = \left(\frac{\beta}{K} + 1\right)^2 H$$

it is clear that a column with an intrinsically high β value may be made to appear spuriously effective by calculating H from the peak of a substance with a large K value. The unbracketed values of h listed in Table II refer to k values of 2–5; the K values vary over a large, and often unknown, range of values. Therefore, for the reasons just mentioned, an attempt has been made to correct the effective plate height to similar values of K (= 150). This correction can be performed only if the phase ratio is known and furthermore it is assumed that H is not unduly affected by changes in K.

Table II also includes two quantities that are not efficiency parameters, but which nevertheless are related to column performance. The first relates to the spreading characteristics of the stationary phase on the support. A support surface is only useful in practice to the extent to which a variety of liquid stationary phases can be spread on it as a thin film without forming droplets. For this to be possible, γ_c^s, the critical surface tension of the surface, of the support surface must be higher than that of the stationary phase. Values of γ_c^s for the various types of open-tubular columns are sparse; as an alternative, the most polar stationary phase that has been reported to have been used succesfully on a particular modified surface will be specified. This assumes that the surface tension of a stationary phase increases with its polarity[16].

The second quantity is the F value, which has been defined by Golay[11] as the ratio of the surface area of the modified support to that of an unmodified open-tubular column of similar internal diameter. High F values enable low values of the phase ratio, β, to be obtained and at the same time, depending on the geometry of the surface, to spread the stationary phase in a thin uniform film.

DISCUSSION

The data presented here suggest that in whisker-walled open-tubular (WWOT) columns band spreading is essentially determined by mobile phase effects. This is consistent with the fact that the plate height, H_{min}, decreases when either the column radius is decreased or the diffusivity of the solute in the mobile phase is increased, whereas a decrease in the phase ratio, β, does not significantly increase the plate height.

The effective plate height (at comparable K values) of WTOT columns is generally higher than that of SCOT columns; WWOT columns are exceptional in

this regard. This difference is basically ascribable to differences in the phase ratios.

All of the modified surfaces can succesfully accommodate polar stationary phases and the surfaces can be deactivated sufficiently well so that peak tailing does not cause any significant problems. SCOT columns have larger F values thán do WTOT columns, again with the exception of the WWOT columns.

The amount of sample that can be handled without significantly decreasing the column efficiency is, with the exception of WWOT columns, larger in SCOT columns that WTOT columns.

The data in Table II shows that WWOT columns combine the best performance charateristics of WTOT and SCOT columns. The fact that they are easily and cheaply constructed in long lengths and have been found to withstand relatively rough handling are further attractive features.

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CHROM. 9597

WHISKER-WALLED OPEN-TUBULAR GLASS COLUMNS IN GAS CHROMATOGRAPHY

III. APPLICATIONS

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SUMMARY

Examples are given of the use of whisker-walled open-tubular columns for separating various mixtures, *viz.*, hydrocarbons, steroids, essential oils, pesticides and fatty acids.

INTRODUCTION

Various aspects of whisker-walled open-tubular (WWOT) columns, including methods of construction and chromatographic performance, have been reported previously[1–3]. In this paper, examples are given of the separations that can be achieved using non-polar, slightly polar and polar stationary phases with a variety of types of mixtures that are important in practice.

EXPERIMENTAL

Columns were drawn from borosilicate glass and provided with a layer of silica whiskers as described previously[2]. The columns were 45–55 m long with I.D. 0.02–0.04 cm. The silica whiskers were deactivated using benzyltriphenylphosphonium chloride[4] when coated with a non-polar stationary phase. When a polar or slightly polar stationary phase was used, prior deactivation was not necessary. Stationary phase was coated on to the whiskers using the dynamic coating method[5]. The columns were then conditioned at room temperature for 24 h, during which time dry nitrogen was passed through them. Finally the temperature was increased at the rate of 1–2°/min to 10–30° above the working temperature of the column and maintained at this level for 24 h (*cf.*, Table I).

The effective plate height, h, and the effective number of plates per metre ($N_{eff} \cdot m^{-1}$) were determined in the normal way[3] by injecting a solute for which the mass

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TABLE I

SUMMARY OF THE CHARACTERISTICS AND PERFORMANCES OF THE DIFFERENT COLUMNS USED

Parameter	*1*	*2*	*3*	*4*	*5*
Stationary phase	Squalane	Squalane	OV-101	Dexsil 410	Carbowax 20 M
Column length (m)	44.8	55.4	54	55	48.6
Column radius (cm)	0.012	0.016	0.012	0.014	0.012
Concentration of coating mixture $\left(\frac{\text{weight of stationary phase}}{\text{volume of solvent}}, \%\right)$	5	5	2.5	2.5	5
Conditioning temperature (°C)	130	130	250	270	230
Plate height, H (cm)	0.04	0.04	0.045	0.065	0.055
Effective plate Height, h (cm)	0.09	0.13	0.10	0.15	0.10
$N_{eff} \cdot m^{-1}$	1100	770	950	660	1000
Mean carrier gas flow Velocity, $\bar{u}$ (cm·sec^{-1})	24	14	16	25	24
Mass distribution Coefficient, k	1.85	1.2	1.9	1.9	2.9
Column temperature (°C)	81	81	200	200	220

distribution coefficient, k, varies between 1 and 3, into the separating system at the operating temperature for the column. Data pertaining to the columns are set out in Table I.

A Varian Aerograph Model VA 1800 gas chromatograph was modified to accomodate the column[3]. A flame-ionization detector was used. The splitting ratio at the inlet was 75:1.

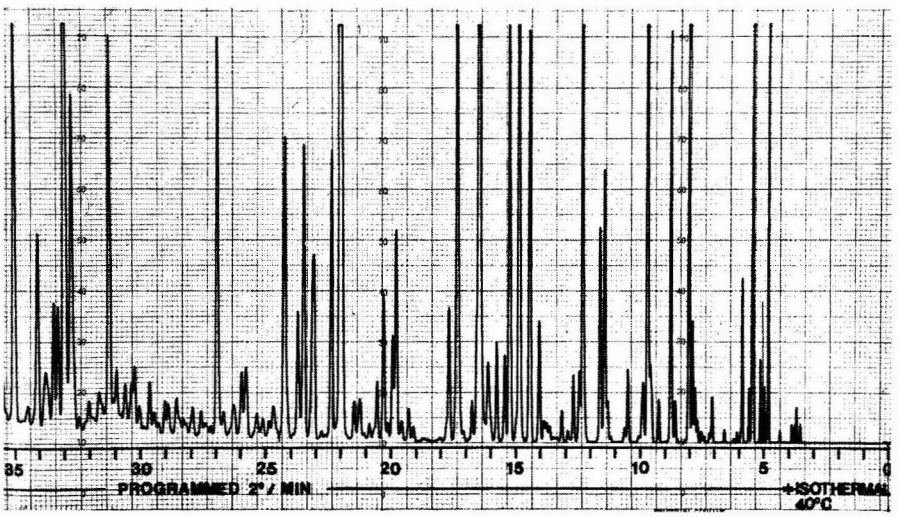

Fig. 1.

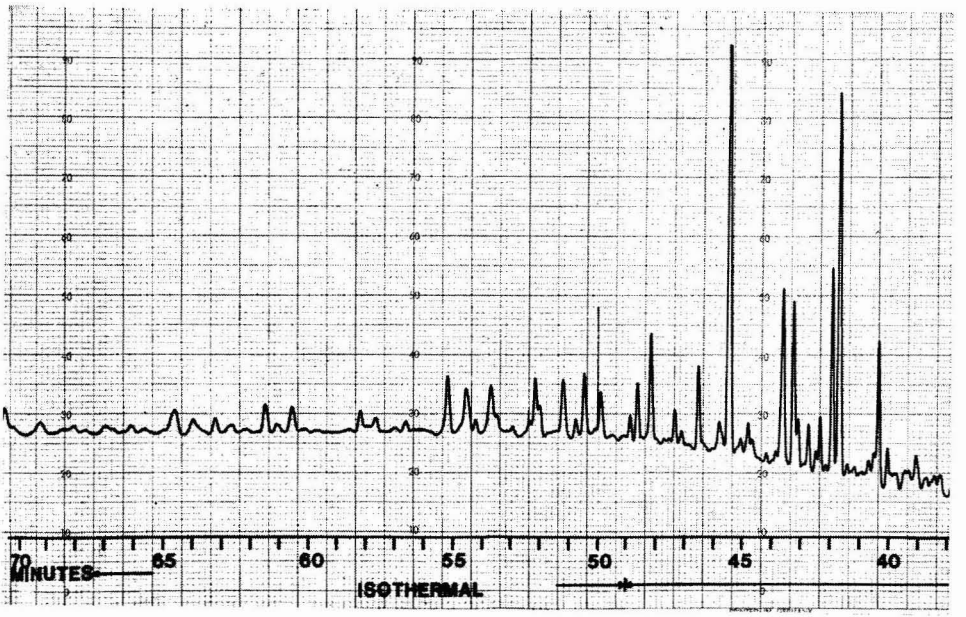

Fig. 1. Analysis of high-octane gasoline prepared synthetically from coal. Column dimensions: 44.8 m × 0.024 cm I.D.; stationary phase: squalane; carrier gas: helium; mean carrier gas flow velocity: 24 cm·sec^{-1}; sample volume injected: 2 μl; column temperature: programmed as shown.

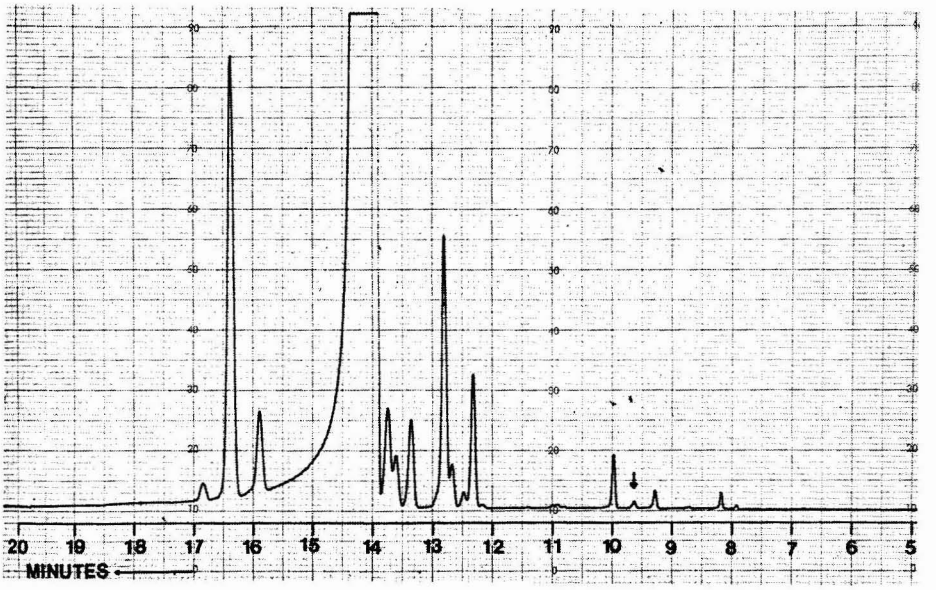

Fig. 2. Analysis of analytically pure *n*-heptane. The peak marked with an arrow corresponds to an amount less than 1 ppm. Column dimensions: 55.4 m × 0.032 cm I.D.; stationary phase: squalane; carrier gas: nitrogen; mean carrier gas flow velocity: 14 cm·sec^{-1}; sample volume injected: 2 μl; column temperature: 85°.

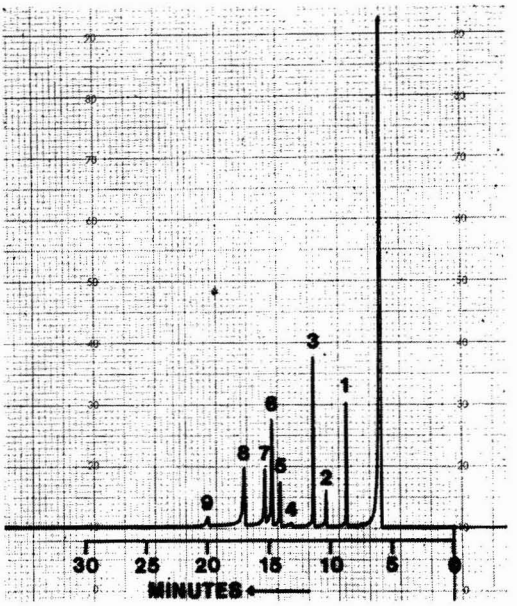

Fig. 3. Analysis of a synthetic mixture of chlorinated pesticides. Column dimensions: 54 m × 0.024 cm I.D.; stationary phase: OV-101; carrier gas: nitrogen; mean carrier gas flow velocity: 15 cm·sec^{-1}; sample volume injected: 1 μl; column temperature: 220°. Peaks: 1 = γ-BHC; 2 = heptachlor; 3 = aldrin; 4 = α-thiodane; 5 = p,p'-DDE; 6 = β-Thiodane; 7 = p,p'-TDE; 8 = p,p'-DDT; 9 = dieldrin.

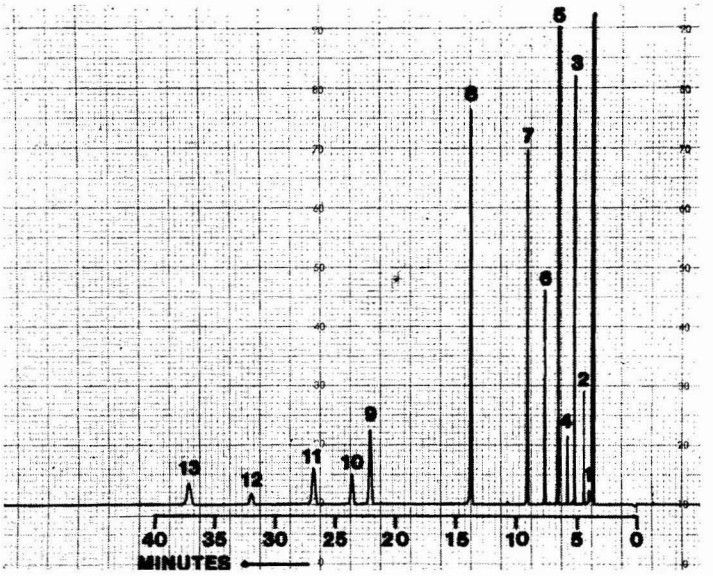

Fig. 4. Analysis of a synthetic mixture of C_6–C_{20} fatty acid methyl esters. Column dimensions: 48.6 m × 0.024 cm I.D.; stationary phase: Carbowax 20M; carrier gas: nitrogen; mean carrier gas flow velocity: 24 cm·sec^{-1}; sample volume injected: 1 μl; column temperature: 220°. Peaks: 1 = methyl caproate (n-C_6); 2 = methyl caprylate (n-C_8); 3 = methyl caprate (n-C_{10}); 4 = methyl undecanoate (n-C_{11}); 5 = methyl laurate (n-C_{12}); 6 = methyl tridecanoate (n-C_{13}); 7 = methyl myristate (n-C_{14}); 8 = methyl palmitate (n-C_{16}); 9 = methyl stearate (n-C_{18}); 10 = methyl oleate (n-C_{18}, 1 double bond); 11 = methyl linoleate (n-C_{18}, 2 double bonds); 12 = methyl linolelaidate (n-C_{18}, 3 double bonds); 13 = methyl arachidate (n-C_{20}).

EXAMPLES OF SEPARATIONS

Chromatograms of various separations are shown in Figs. 1–7.

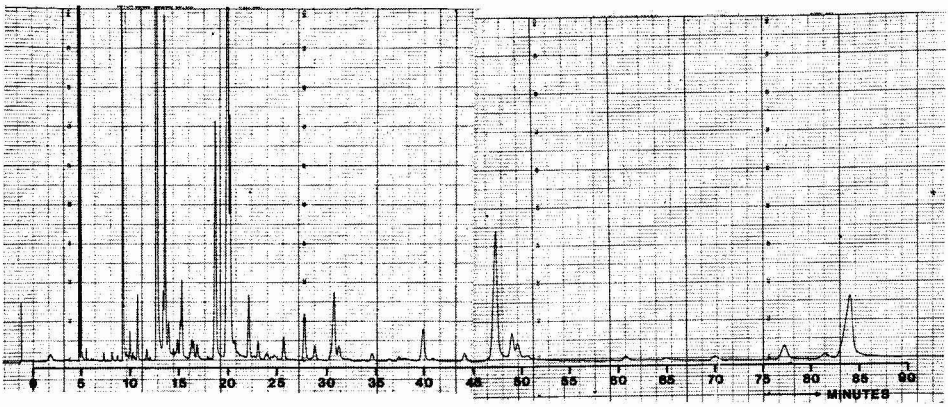

Fig. 5. Analysis of fatty acid methyl esters of a polished mackerel oil. Column dimensions: 48.6 m × 0.024 cm I.D.; stationary phase: Carbowax 20M; carrier gas: nitrogen; mean carrier gas flow velocity: 16 cm·sec^{-1}; sample volume injected: 1 μl; column temperature: 230°.

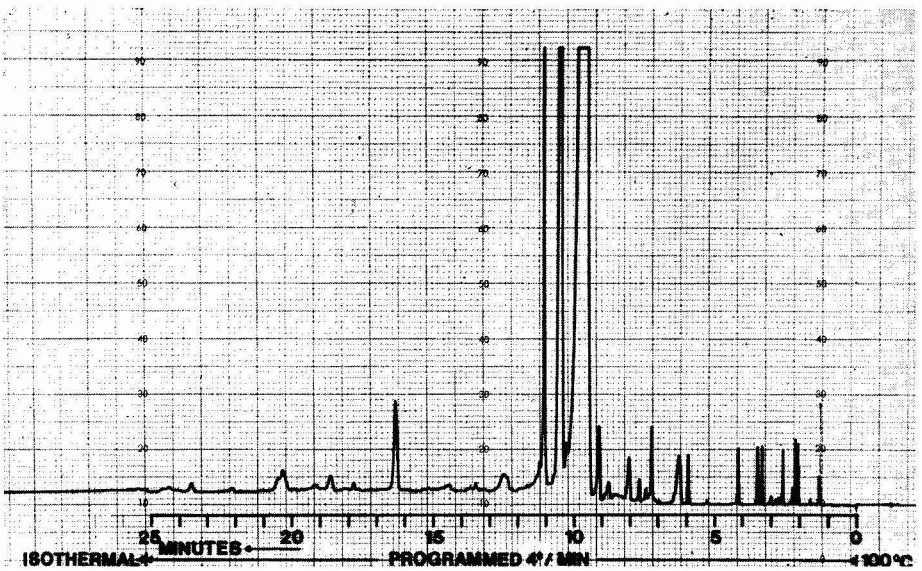

Fig. 6. Analysis of a peppermint oil sample. Column dimensions: 48.6 m × 0.024 cm I.D.; stationary phase: Carbowax 20M; carrier gas: nitrogen; mean carrier gas flow velocity: 24 cm·sec^{-1}; sample volume injected: 1 μl; column temperature: programmed as shown.

DISCUSSION

WWOT columns can be satisfactorily coated with non-polar and polar stationary phases and can be employed to separate many important types of mixtures.

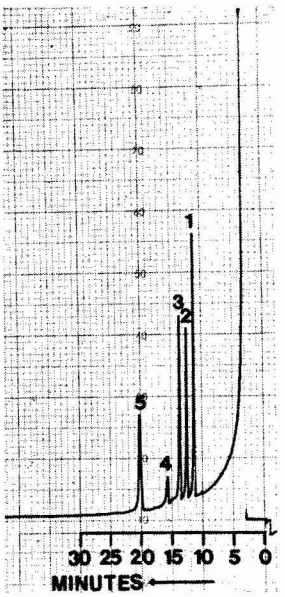

Fig. 7. Analysis of a synthetic mixture of trimethylsilyl (TMS) derivatives of 17-ketosteroids. Column dimensions: 48.6 m × 0.024 cm I.D.; stationary phase: Dexsil 410; carrier gas: nitrogen; mean carrier gas flow velocity: 25 cm·sec^{-1}; sample volume injected: 2 μl; column temperature: 220°. Peaks: TMS derivatives of 1, etiocholanolane; 2, androsterone; 3, dehydroepiandrosterone; 4, 11-ketoandrosterone; 5, 11-β-hydroxyetiocholanolane.

The resolution obtained is at least comparable to that of other types of open-tubular columns. The amount of solute mixture that can be handled by WWOT columns (1–5 μg of a single component) is similar to that with conventional porous-layer open-tubular columns[6].

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CHROM. 9709

COMBINED THIN-LAYER AND GAS–LIQUID CHROMATOGRAPHIC IDENTIFICATION OF TRICYCLIC ANTIDEPRESSANTS IN URINE

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SUMMARY

A combined thin-layer and gas–liquid chromatographic (TLC–GLC) procedure for determination of tricyclic antidepressant drugs in urine is described. GLC on 1.5% OV-17 was utilized to analyze basic urine extracts directly for the tricyclics and to confirm the identity of spots extracted from thin-layer chromatograms, and TLC was used to confirm the results of these GLC screens. Lower limits of sensitivity for the thin-layer spot extraction procedure were found to range from 2 μg/ml for amitriptyline and imipramine to 10 μg/ml for desipramine and nortriptyline. Turn-around-times for the GLC tricyclic screens were usually less than 1 h. The finding that nortriptyline and desipramine were chemically altered under various extraction conditions was used as a means of confirming the identity of these drugs, and the identity of these chemically altered derivatives was discussed with reference to their low resolution mass spectra.

INTRODUCTION

Tricyclic antidepressant drugs (amitriptyline, doxepin, imipramine, nortriptyline, and desipramine) are frequently used in the treatment of anxiety and depression, and it is not surprising that the incidence of self-poisonings with these drugs has increased dramatically in recent years[1,2]. Spiker *et al.*[1] reported recently that approximately 25% of all drug-overdosed patients seen in their emergency room were overdosed primarily with a tricyclic. The most dangerous complication in patients poisoned with these drugs is the cardiovascular toxicity[3–5]. Physostigmine has been reported[3,6] to rapidly reverse the cardiovascular action and antagonize the central nervous system effects of the tricyclic antidepressants, and as such, this drug has found frequent use in the treatment of patients overdosed with these drugs. However, because the administration of physostigmine itself is not without risk[7], it is important that identification of tricyclics be established prior to treatment with this drug. Unfortunately, most laboratory methods of analysis for tricyclics are either very time consuming or do not provide unequivocal identification of the tricyclics or both. Furthermore, no method has been reported which simultaneously analyzes for all five major tricyclics.

The rapid urine colorimetric test of Forrest *et al.*[8] is fairly specific, but it does not give positive reactions with doxepin, amitriptyline, or nortriptyline. UV spectrophotometric techniques[9–11] have found frequent use for the various individual tricyclics, but these techniques either employ tedious extraction procedures or require chemical transformation of the tricyclics prior to analysis. Thin-layer chromatography (TLC), although not very expedient, is undoubtedly the most widely used technique for identification of these drugs in urine, and several procedures have been reported[10,12,13]. Several gas chromatographic (GLC) procedures have also been reported[14–19], although again none of them have been used to analyze for all of the tricyclics simultaneously. The technique of combined GLC and mass fragmentography has been used to quantitate tricyclics in blood and urine[1,20–22], but this technique can hardly be considered practical for most routine laboratories.

This report describes a combined thin-layer and gas–liquid chromatographic (TLC–GLC) approach for the simultaneous identification of the tricyclics doxepin, amitriptyline, imipramine, nortriptyline, and desipramine in human urine. Herein is described not only a GLC method for the confirmation of the TLC results, but also a rapid GLC screen for tricyclics from urine directly.

MATERIALS AND METHODS

Apparatus

A Model 2100 (Varian Aerograph, Walnut Creek, Calif., U.S.A.) gas chromatograph equipped with dual flame ionization detectors was used in these studies. The instrument was fitted with U-shaped glass columns (1.83 m × 2 mm I.D.) packed with 1.5% OV-17 on 80–100 mesh Chromosorb G-HP (Applied Science Lab., State College, Pa. ,U.S.A.). The column was conditioned before use by heating to 300° overnight. Operating conditions were as follows: nitrogen as carrier gas was set at 30 ml/min, hydrogen at 30 ml/min, air at 300 ml/min, the injection port and detector temperatures at 280°, and the initial column oven temperature at 180°; the program rate was 10°/min up to 280° with 5 min isothermal at 280°.

Mass spectral analyses were performed on a Finnigan Model 3200 gas chromatograph–mass spectrometer with an electron energy of 60 eV and an emission current of 450 μA. The GLC conditions for this system were identical to those described above.

Reagents

All solvents were chromatoquality and were used without further purification unless otherwise indicated.

NH_4Cl–NH_4OH Buffer. To 100 ml of saturated NH_4Cl solution is added concentrated NH_4OH at 25° until pH 10 $\pm$ 0.1 is reached (approximately 80 ml).

Borate buffer. A solution of 100 ml of 25 mmoles/l Borax and 35.4 ml of 100 mmoles/l HCl is diluted to 200 ml with deionized water (pH 8.3 $\pm$ 0.1 at 25°).

Carbonate buffer. To 420 mg of $NaHCO_3$ is added 21 g Na_2CO_3 and 50 ml of deionized water. The pH is then adjusted to 11 $\pm$ 0.1 at 25° with 1 mole/l HCl or 1 mole/l NaOH.

Nalorphine standard. A 5 μg/μl aqueous standard was prepared by dissolving 5.0 mg nalorphine hydrochloride (Merck, Sharp and Dohme, West Point, Pa., U.S.A.) in 1 ml deionized water.

Cyheptamide standard. A 0.5 μg/μl methanolic cyheptamide standard was prepared by dissolving 50 mg of cyheptamide (Ayerst Labs., New York, N.Y., U.S.A.) in 100 ml of absolute methanol.

Thin-layer chromatography

All chromatography was performed on 10 × 20 cm glass chromatoplates coated with an absorbent layer of 250 μm silica gel 60 F_{254} (E. Merck, Darmstadt, G.F.R.). The developing solvent was prepared fresh daily and consisted of ethyl acetate, methanol, and concentrated ammonium hydroxide in the ratio of 170:20:10 ml, respectively.

Spray reagents

The spray reagents and their composition were as follows: (a) 0.4% ninhydrin, prepared by dissolving 0.4 g ninhydrin in 100 ml of acetone, (b) 0.5% sulfuric acid, and (c) neutral iodoplatinate, prepared by adding 1 ml of 10% platinum chloride to 25 ml of a 4% solution of potassium iodide.

Extraction procedure (modification of the method of Davidow et al.[23])

To 15 ml of urine in a 50-ml centrifuge tube were added 10 μl of the 5 μg/μl nalorphine standard and enough ammonium chloride–ammonium hydroxide buffer (one to five drops) to bring the pH of the urine to 8.5 $\pm$ 0.1. To this solution were added 15 ml of chloroform–isopropanol (9:1) solvent (AR grade), and the resultant mixture was shaken gently on a mechanical shaker for 5 min and centrifuged at 1500 rpm for 10 min. After aspirating the aqueous layer to waste, the organic layer was filtered through approximately 5 g of anhydrous sodium sulfate. Subsequently four drops of 0.5% methanolic sulfuric acid were added to this solution, and the extract was evaporated to dryness at 40° under a gentle stream of nitrogen.

Development and spraying procedure (modification of the method of Mulé[24])

The residues from the extracts were reconstituted with 25 μl of methanol and spotted in entirety onto the silica gel plates. After allowing the spots to dry, the plates were developed in rectangular developing tanks by allowing the solvent front to migrate a distance of 10 cm from the point of application of the extracts. The plates were removed, air dried for 5 min, then oven dried at 100° for 5 min. The following sprays were then applied in succession: (1) 0.4% ninhydrin lightly, followed by UV for 10 min and 100° for 5 min, (2) 0.5% sulfuric acid lightly, and (3) iodoplatinate heavily. The plates were allowed to dry before recording R_F values.

TLC spot extraction procedure (modification of the method of Reynolds[25])

After allowing the final spray to dry for at least 15 min, the appropriate spot was scraped into a small vial. To this solution were added 1 ml of the pH 8.3 borate buffer ,10 μl of the 0.5 μg/μl cyheptamide standard, and a "pinch" of Na_2SO_3. After swirling the mixture briefly and allowing it to stand for 5 min, 2 ml of chloroform–isopropanol (9:1) solvent (AT grade) were added and the contents of the vial shaken for 5 min on a mechanical shaker. The mixture was then centrifuged for 2 min at 2400 rpm, and the aqueous layer was aspirated to waste. After drying the organic layer over anhydrous Na_2SO_4 briefly, it was transferred to a clean vial and evaporated

to dryness at 50° under a gentle stream of nitrogen. The residue was reconstituted with 50 μl of methanol, and 5 μl of this solution were injected into the gas chromatograph.

GLC extraction procedure

To a 50-ml centrifuge tube were added 10 ml of urine and 20 μl of the 0.5 μg/μl cyheptamide standard. The pH of this mixture was adjusted to 9.0 $\pm$ 0.2 with carbonate buffer (around 1 ml of buffer is required for most urines), 10 ml of chloroform was added, and the resultant mixture shaken gently on a mechanical shaker for 5 min and centrifuged at 2000 rpm for 5 min. After removing the aqueous layer, 1 g of anhydrous Na_2SO_4 was added to the chloroform solution and it was swirled briefly. The chloroform solution was transferred to a dry tube and evaporated to dryness at 50° under nitrogen. The residue was reconstituted with 50 μl of methanol, and 5 μl of this solution was injected into the gas chromatograph.

RESULTS

R_F values for the tricyclic antidepressants and the more common drugs of abuse which were found to extract from urine at a basic pH (8.5) were determined, as shown in Table I. Assuming a maximum variation of $\pm$ 0.04 for these values, seven drugs were found to have chromatographic properties similar to those of the dimethylamine tricyclics (amitriptyline, imipramine, and doxepin), four to those of nortriptyline, and two to those of desipramine. Most of these drugs could be distin-

TABLE I

R_F VALUES OF VARIOUS DRUGS AND METABOLITES*

Drug	*R_F value*	*Drug*	*R_F value*
Amitriptyline metabolite**	0.10	Pentazocine metabolite**	0.58
Nortriptyline metabolite**	0.10	Nortriptyline	0.58
Doxepin metabolite**	0.12	Nicotine	0.62
Nicotine metabolite**	0.17	Doxepin metabolite	0.64
Morphine	0.17	Norpropoxyphene	0.66
Atropine	0.24	Meperidine	0.67
Desipramine metabolite**	0.25	Promethazine	0.68
Imipramine metabolite**	0.25	Promazine	0.70
Nalorphine	0.27	Diphenhydramine	0.70
Ephedrine	0.28	Imipramine	0.72
Phenylpropanolamine	0.28	Doxepin	0.72
Pentazocine metabolite**	0.30	Amitriptyline	0.74
Codeine	0.34	Pentazocine	0.74
Benztropine	0.40	Thioridazine	0.76
Quinine	0.46	Methaqualone	0.76
Desipramine	0.46	Chlorpromazine	0.78
Methamphetamine	0.48	Methadone	0.80
Amphetamine	0.52	Propoxyphene	0.80
Chlorpheniramine	0.54	Phencyclidine	0.83
Doxepin metabolite	0.54	Methadone metabolite	0.85
Hydroxyzine	0.56		

* R_F values relative to solvent front at 10 cm. See Materials and methods for details.

** Not confirmed with authentic sample.

guished from the tricyclics by their characteristic metabolite patterns and/or color reactions with the various spray reagents, but the possibility of multiple drug ingestion and the run-to-run variation in the TLC parameters prevents unambiguous identification of the tricyclics from these data alone. To confirm the identification of the tricyclics, the appropriate spots from the thin-layer plates after spraying were scraped off and extracted as described above. These extracts were concentrated and subjected to GLC analysis on an OV-17 column, and Table II lists the relative retention times (cyheptamide as standard) of authentic samples of drugs from Table I added to blank urines (also included in this list are drugs of abuse found in urine which might extract under the above conditions). With the exception of the drugs nortriptyline and desipramine (which are discussed in detail below), all drugs treated in this manner had the same relative retention times as corresponding authentic standards which had not been subjected to TLC prior to analysis by GLC; and it was concluded from these findings that the spot extraction procedure did not chemically alter these drugs. Allowing a variation of 0.04 in these relative retention times, only pentazocine and methaqualone had both TLC and GLC properties similar to doxepin, amitriptyline, and imipramine. Metabolite patterns and color reaction differences with the spray reagents make it possible to easily distinguish these drugs from the tricyclics, however.

In order to distinguish true negatives from those urines with low levels of drugs, the sensitivity of the spot extraction procedure was investigated. Known amounts of the appropriate drugs were added to blank urines and extracted at pH 9

TABLE II

RELATIVE RETENTION TIMES FOR VARIOUS DRUGS AND METABOLITES EXTRACTED FROM URINE

Drug	*RRT**	*Drug*	*RRT**
Nicotine	0.10	Imipramine	0.79
Barbital	0.19	Doxepin	0.81
Butalbital	0.30	Methaqualone	0.84
Butabarbital	0.31	Nortriptyline***	0.84, 1.19
Amobarbital	0.33	Nordiazepam	0.84
Meperidine	0.35	Desipramine***	0.85, 1.24
Pentobarbital	0.37	Pentazocine	0.85
Secobarbital	0.39	Promethazine	0.87
Diphenhydramine	0.44	Promazine	0.95
Phencyclidine	0.46	Norpropoxyphene	1.00
Meprobamate	0.51	Cyheptamide	1.00
Glutethimide	0.52	Oxazepam	1.05
Secondary methadone metabolite	0.54	Codeine	1.05
Caffeine	0.56	Chlorpromazine	1.10
Chlorpheniramine	0.61	Phenytoin	1.21
Primary methadone metabolite	0.62	Diazepam	1.22
Phenobarbital	0.66	Trifluoperazine	1.27
Methadone	0.70	Chlordiazepoxide	1.31
Propoxyphene**	0.46, 0.72	Flurazepam	1.37
Amitriptyline	0.76	Thioridazine	2.10

* Relative to cyheptamide as internal standard. See Materials and methods for GLC details.

** Thermally unstable, gives two peaks.

*** See Discussion for explanation of second peak.

as described above. These extracts were subjected to TLC, and the appropriate spots were removed, extracted, and subjected to GLC. Table III records the lowest concentrations of the drugs in urine at which the peak height of the drug was found to be equal to or greater than 10% of the peak height of the internal standard.

TABLE III

THIN-LAYER CHROMATOGRAPHY SPOT EXTRACTION EFFICIENCIES

Drug	*Lower limit of sensitivity (μg/ml)* *
Amitriptyline	2
Doxepin	5
Imipramine	2
Nortriptyline	10
Desipramine	10
Chlorpheniramine	2
Diphenhydramine	30
Meperidine	2
Methadone	2
Methadone metabolites	
Primary	20
Secondary	20
Nicotine	10
Norpropoxyphene	15
Pentazocine	5
Propoxyphene	5

* Based on initial concentration of pure drug in urine.

GLC analysis of extracted spots from thin-layer plates is a rather laborious process (4–8 h turn-around-time) and not one that is usually performed more than once a day. For a more rapid screen to rule out the presence of tricyclics, urine was extracted at pH 9 with chloroform and the extracts were analyzed directly by GLC without derivatization using cyheptamide as an internal standard. Since the GLC conditions were identical to those of the spot confirmation procedure, the data listed in Table II apply to both procedures. Screens were considered positive only when the peak height of the tricyclic drug was equal to or greater than 50% of the peak height of the internal standard. Under these conditions, normal endogenous metabolites frequently found in urine did not give chromatographic interference. Positive screens are routinely reported to the physicians and later confirmed by TLC.

DISCUSSION

By using data from both TLC and GLC, the tricyclic antidepressant drugs could be easily distinguished from the other common drugs of abuse. Methaqualone was the only drug found to have chromatographic properties on both systems similar to the tricyclics doxepin, imipramine, and amitriptyline. In this case, however, the characteristic orange-brown color of methaqualone when over-sprayed with Dragendorff's spray provided a means of differentiating between these tricyclics and metha-

qualone. And although it is probably of little clinical importance, the combined TLC–GLC analysis of patient urine specimens makes it possible to distinguish between these tricyclics themselves by using the R_F values and relative retention times of both the parent drugs and their desmethyl metabolites (0.54 doxepin metabolite, nortriptyline, and desipramine, respectively). *In vivo* metabolism of these drugs is rapid[19], and in all the overdose cases involving these tricyclics that we have studied to date, both the parent drugs and their desmethyl metabolites have been present. Thus, imipramine and doxepin, even though they have very similar R_F values on TLC and relative retention times on GLC, are easily distinguished by virtue of the large differences in the corresponding R_F values and relative retention times for the desmethyl metabolites.

The rapid GLC screen for tricyclics in urine gave results in a more clinically useful time frame. Typically results could be returned to the physician in less than 1 h from receipt of specimen. *A priori* one might expect many false positives with such a screen. However, this proved not to be the case. Not only were normal urines relatively free of endogenous metabolites with relative retention times similar to those of the tricyclics, but by defining positive screens as described above, metabolites that did have similar retention times were excluded. To date we have found 23 patients as positive for tricyclics by this screen, and subsequently all 23 were later confirmed by TLC. The sensitivity of this screen was of some concern, however, since it has been reported[19] that only 0.4% of doxepin and 0.15% of amitriptyline appeared in the urine of normal volunteers on therapeutic doses of these drugs in the first 24 h following their administration. Table IV shows, however, that as far as our overdose cases are concerned, sensitivity has not been a problem. Patient 4, who purportedly only took four 25-mg tablets of amitriptyline, still had a peak height ratio of amitriptyline to internal standard greater than 0.5. Several urine specimens from patients on therapeutic doses of doxepin, amitriptyline, and imipramine were analyzed and found to consistently have peak height ratios of tricyclic to internal standard of less than 0.5. Nevertheless, insufficient data exist as yet to be certain that only overdosed patients and not those on therapeutic doses have ratios greater than 0.5, and it is probable,

TABLE IV

CLINICAL AND LABORATORY FINDINGS OF PATIENTS DURING THE FIRST 24 H AFTER OVERDOSE

Patient	*Sex*	*Age*	*Drug taken*	*Amount taken by history*	*Other drugs*	*Patient status**	$\frac{h_{Tricyclic}}{h_{STD}}$ **
1	F	37	Doxepin	unknown	Codeine, salicylate	comatose	7.8
2	F	19	Amitriptyline	1500 mg		drowsy	6.0
3	F	34	Amitriptyline	600 mg	Alcohol, salicylate	semicomatose	2.2
4	M	14	Amitriptyline	100 mg	Diazepam, 60 mg	drowsy	0.6
5	M	56	Doxepin	>750 mg		awake	7.2
6	M	61	Imipramine	unknown	Phenytoin	confused	4.2
7	M	9	Doxepin	unknown		comatose	15.0
8	F	21	Amitriptyline	350 mg	Perphenazine	drowsy	2.0

* At time of initial examination in emergency room.

** Ratio of peak heights.

given the nature of drug metabolism and individual variations in excretion patterns, that some overlap exists. In an overdosed patients, however, it is the presence or absence rather than the amount of tricyclic that is currently used to determine whether or not to start physostigmine therapy. A large amount of urine was available on Patient 5, and ten consecutive extractions of his urine were chromatographed and found to give peak height ratios of doxepin to cyheptamide which ranged from 6.5 to 7.9. Thus it would appear that results from this screen have adequate reproducibility and are both sensitive and specific enough to be used as a guide to therapy in case of patients suspected of being poisoned with tricyclic antidepressant drugs.

As was mentioned earlier, nortriptyline and desipramine gave anomalous results upon GLC. That is ,when methanolic standards of these drugs were chromatographed directly, one peak was observed for each drug with a relative retention time of 0.85; whereas when these drugs were removed from thin-layer plates following chromatography and then analyzed by GLC, only peaks at 1.19 for nortriptyline and 1.24 for desipramine were observed (see Fig. 1). When the spots were scraped from the thin-layer plate without spraying and the material analyzed by GLC, however, both peaks were observed for each drug. Subsequently, it was found that when aqueous standards of these drugs were extracted between pH 8 and 9, only the peaks at 0.85 were observed; while for the same standards extracted at pH 10.5, small peaks at 0.85 and large peaks at 1.19 and 1.24 were observed when analyzed by GLC directly. Since these phenomena were reproducible for both aqueous standards and patient urines, it was concluded that a base catalyzed rearrangement and/or oxidation

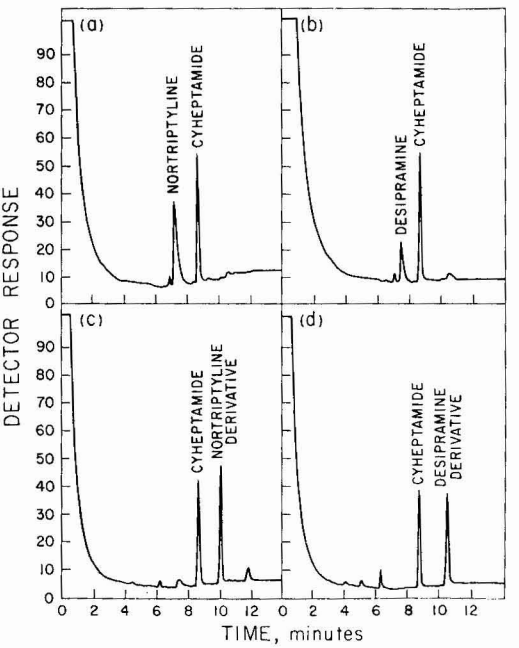
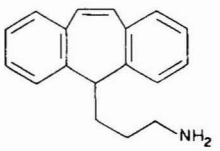
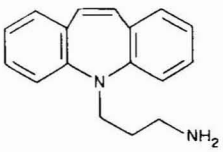


Fig. 1. Gas–liquid chromatograms of (a) equimolar methanolic solution of nortriptyline and cyheptamide; (b) equimolar methanolic solution of desipramine and cyheptamide; (c) spot extract from thin-layer chromatogram of (a); and (d) spot extract from thin-layer chromatogram of (b).

was occurring. Borga and Garle[16] reported the "*in vitro*" metabolism of nortriptyline and desipramine in acid via the 10-hydroxy intermediates to give 10,11-dehydrodesmethylnortriptyline (I) and 10,11-dehydrodesmethyldesipramine (II), respectively.



I



II

Under their chromatographic conditions (OV-1 column), these metabolites also had somewhat longer retention times than the parent drugs. Mass spectral analyses of the RRT 1.19 and 1.24 peaks, however, indicate that these derivatives are not the same as metabolites I and II seen by Borga and Garle. The published spectra for nortriptyline and desipramine[22] and the spectra for the derivatives shown in Fig. 2 all have intense

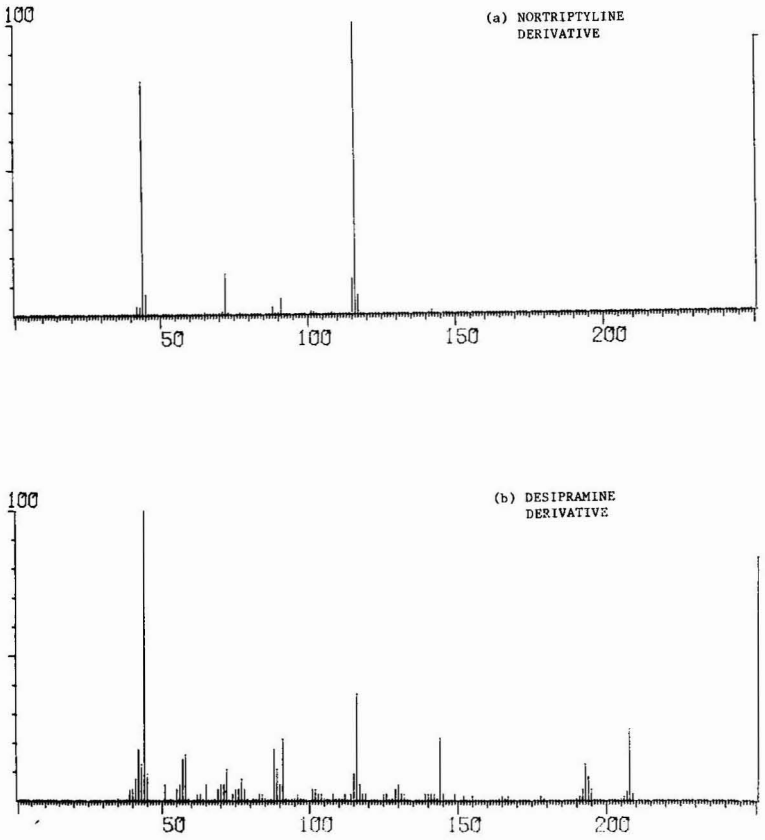


Fig. 2. Mass spectra of (a) compound with RRT of 1.19 in Fig. 1c (nortriptyline derivative) and (b) compound with RRT of 1.24 in Fig. 1d (desipramine derivative).

ions at *m/e* 44 characteristic of α-cleavage in secondary N-alkylmethylamines[26]. As is common in the mass spectra of alkylamines

$$\underset{m/e\ 44}{CH_2 = \overset{+}{N}H\text{–}CH_3}$$

no molecular ions were observed in either spectrum. The only ion of note besides the *m/e* 44 ion was that at *m/e* 116 (base peak for the nortriptyline derivative and second largest peak for the desipramine derivative). Although formation of this ion in each case is obviously energetically favorable, it is difficult to deduce their structures starting from the parent drugs and speculating probable metabolic pathways. High-resolution and chemical ionization mass spectral studies are now in progress to determine the structure(s) of this ion and to help establish the identities of these derivatives. The only conclusion possible at the present time is that the N-alkylmethylamine side chain of the parent drug in each case is still intact.

Even though the identities of these derivatives have not yet been established, the fact that their formation is reproducible has been found to be quite useful in identifying nortriptyline and desipramine. For instance, when a peak with a relative retention time of 0.84–0.85 is noted in the gas–liquid chromatogram of a urine extracted under normal conditions, the urine is re-extracted at pH 10.5 and analyzed again by GLC. If peaks at 1.18 or 1.24 appear, then positive identification of nortriptyline or desipramine is established.

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PARALLEL FLAME IONIZATION DETECTION–TOTAL ION CURRENT RECORDING IN CAPILLARY GAS CHROMATOGRAPHY–CHEMICAL-IONIZATION MASS SPECTROMETRY

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SUMMARY

The need for independent dual detection in the analysis of complex mixtures by gas chromatography–chemical-ionization mass spectrometry is illustrated. Monitoring of the gas chromatographic effluent by means of universal detection and unaltered uniform response by flame ionization detection is a desirable supplement to the customary, highly selective, monitoring of the total ion current, especially in consecutive analyses of the same sample, where the chemical-ionization reagent gas is varied. Since the gas chromatograms obtained by flame ionization detection are unaffected by changes in chemical-ionization conditions, they provide a safer common basis for interrelating spectral data from successive runs than do the total ion current records. For the special case of reconstructed gas chromatograms generated by computers from digitized total ion current values, direct correlations are conveniently achieved by calibrating both the flame ionization detection and total ion current records in spectrum index numbers through the use of an event marker, triggered by the data system.

INTRODUCTION

The combination of high-efficiency gas chromatography with chemical-ionization mass spectrometry (GC–CIMS) appears to offer a promising new approach for the determination of the structures of unknown compounds present in complex mixtures which resist the isolation of pure materials. The potential of this method was realised when it was found that the use of more than one chemical-ionization (CI) reagent could result in new, complementary, structural evidence and thus provide a means of selective probing of certain structural features**. Convenient variation of the GC–CIMS experiment on the same sample by changing the reagent gas became possible through dual-gas interfacing, gases other than the GC carrier gas being employed and freely selected[3–5]. By the use of such equipment, the reagent gas is not

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** For recent reviews of the reagent gases in current use see refs. 1 and 2.

admixed to the GC effluent before entering the ion source of the mass spectrometer, and thus the carrier gas and, consequently, the GC conditions can be kept unchanged throughout a series of runs. As a first benefit, directly comparable GC traces can be obtained simply by total ion current (TIC) monitoring, at least as far as the retention times are concerned. In many cases this will secure a sufficiently broad basis for an immediate correlation of the results although they are obtained under widely differing CI conditions.

Difficulties with this mode of single detection may, however, arise when exactly analogous data of poorly resolved components have to be compiled from the various runs and correlated throughout the series of experiments. Usually, this will involve selecting, from a set of spectra in a given run, the most representative scan of the component of interest, and locating the latter, as accurately as possible, within a GC peak pattern of the TIC chromatogram. This will determine the position of the spectrum on the time axis of a two-dimensional mass–time data field. The next step will aim at finding exactly corresponding positions within the TIC chromatograms of all other CI runs. This will ensure that only those spectra are correlated which correspond to identical, or at least very similar, retention indices[6] and thus reflect comparable concentrations of the constituent in question. This step may also be viewed as the retrieval of a time-coherent two-dimensional subset of data along the CI reactant axis from a three-dimensional mass–time–reactant array of mass spectral data.

In spite of the fact that the retention times remain largely unchanged during a series of CI experiments, locating of the most representative spectra of an unresolved component on a purely visual basis, *i.e.*, comparing analogous GC peak patterns in the TIC traces, is likely to be difficult for several reasons. Under varying CI conditions, the intensity profiles of unresolved GC peak dusters can vary considerably from run to run, rendering them sometimes past recognition. Marked changes of profile are even common when the sample is heterogeneous with respect to the classes of compounds of which it is composed. In such cases, the differences in the proton affinities between the substrate and reagent gas may become too small to allow efficient protonation of some constituents, *i.e.*, CI will discriminate heavily against them especially when mildly acidic reagent gases are employed. Unfortunately, these highly selective reactants are the very ones which have to be chosen for attempts at a differential analysis of the special structural features that are hard to detect by other mass spectroscopic methods[1,2].

Other problems of single CI–TIC recording, posed by instrumentation rather by than gas phase ion chemistry, relate to the sharp demarcation of the TIC of the sample and reactant, which is not always easily attained. Mildly acidic reagent gases, which are frequently quite polar, tend to form oligomeric cluster ions of the type $(H–X)_nH^+$ ($n = 2$–4), in relative abundances that are not only sensitive towards changes in source conditions but which may also extend into mass ranges commonly occupied by sample ions to be collected in TIC recording. Since the abundances of reactant ions generally exceed those of sample ions by some orders of magnitude, they must be rigorously excluded from TIC recording in order to attain acceptable sensitivity. When quadrupole mass spectrometers are used, this can be conveniently achieved by integrating mass-resolved ion currents over pre-set limited mass ranges and cutting off lower portions which contain reactant but no sample ions (*e.g.*,

when using methane or isobutane, the TIC is usually sampled above *m/e* 60). With polar reagents like methanol or small aliphatic amines, cluster ions will, however, extend even beyond *m/e* 100, thus interfering with the mass range to be taken as representative of the compound to be analyzed. With data systems, the digitized mass-resolved TIC can be recorded with deletion of sets of specified *m/e* values, thus permitting deliberate exclusion of such contributions provided that no overlaps occur. The use of labelled reagent gas (*e.g.*, $[^2H_4]$methanol instead of CH_3O^2H) may have to be considered if overlap occurs.

Besides the potentially large disparities in peak shapes of the unresolved components due to discriminatory CI response, small differences in the absolute positions along the time coordinate may add to the uncertainty in visual correlation of TIC traces of complex mixtures. These minor deviations in retention times naturally arise from the lack of exact reproducibility in practical work in the GC part of the experiment, in spite of the efforts to keep the parameters constant. Unfortunately, the feasibility of correlating spectral data via unaffected retention indices rather than retention times is similarly impaired by the pronounced selectivity of the CI response. With most gases of interest, other than methane, the internal standards for index measurements, usually homologous *n*-paraffins, are not ionized at all.

One simple way of overcoming these problems of correlation experimentally is by splitting the GC effluent and feeding a portion of it into a universal rather than a selective independent detector, run in parallel to the TIC-recording mass spectrometer. The flame ionization detector (FID), often used in electron-impact GC–MS (GC–EIMS) systems[7–9] for various other reasons, is the obvious device of choice. It exhibits a largely uniform response to widely differing organic substrates, and is sufficiently sensitive to produce signal amplitudes which are comparable to those obtained by TIC recording. FID gas chromatograms, now identical in both dimensions, *i.e.*, retention time and concentration amplitude, will be produced in successive runs irrespective of which reagent gas is used. The details of the design and performance of such a dual-recording GC–CIMS systems are given below.

EXPERIMENTAL

Fig. 1 shows a schematic diagram of the capillary GC–CIMS system used in our laboratory for GC–EIMS and multiple GC–CIMS analyses of mixtures, and which is equipped with provisions for rapid switching of the reagent gases. The main components of the system are a Carlo Erba Fractovap 2101 AC capillary gas chromatograph (a), a Finnigan 3300 mass spectrometer (i) and a Finnigan 6000 interactive data system (j). It also incorporates an effluent splitter (d) with *ca.* 1:1 splitting ratio, a conventional hydrogen FID (e), a coaxial dual-gas GC–CIMS interface (g, h)[5] and a pair of vaporizers (p–r, of which only one is depicted in the scheme) for liquids to be used as CI reagent gases. The splitting device (d), manufactured from glass and platinum capillaries according to Etzweiler and Neuner-Jehle[10], is placed in the GC oven and feeds a portion of the effluent to the FID (e) as the universal detector. By use of a *ca.* 1:1 splitting ratio, it was not necessary to add a make-up gas in order to prevent losses in resolution due to the dead volume of the connecting capillary lines.

The two vaporizers for liquid reactants can be operated independently through assemblies of regulating and simple shut-off valves (n and o, respectively). The heated

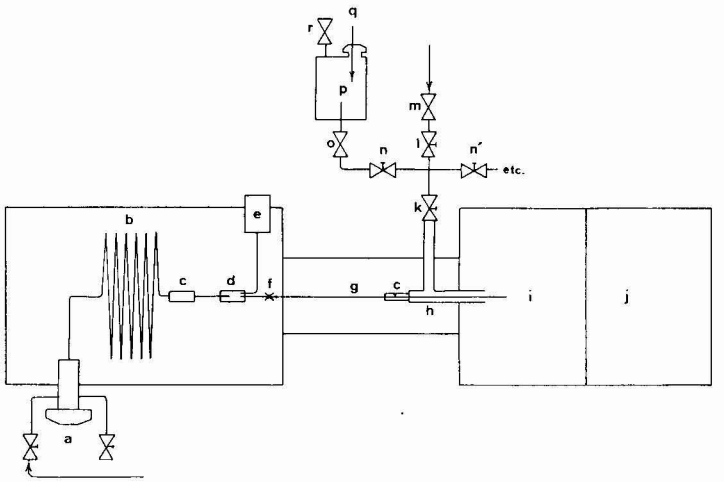

Fig. 1. Schematic diagram of the dual-recording capillary GC–CIMS–computer system equipped with an effluent splitter, vaporizers for liquid reactants and coaxial dual-gas interface. Components: (a) Fractovap 2101 AC gas chromatograph with Grob-type injector, (Carlo Erba, Milan, Italy); (b) Emulphor-ON 870 glass capillary column (50 m × 0.35 mm) (H. and G. Jaeggi, Trogen, Switzerland); (c) polyimide ferrule, Vespel SP-1 (Dupont, Genève, Switzerland); (d) splitting device, glass and platinum capillaries[10]; (e) hydrogen FID (Carlo Erba); (f) platinum restriction; (g, h) coaxial dual-gas interface; (g) interface capillary, AR-glass (0.9 mm O.D., 0.3 mm I.D., pre-treated according to ref. 11; (h) stainless-steel reactant gas line (3.2 mm O.D., 1.2 mm I.D.) (*cf.* ref. 5); (i) Finnigan 3300 mass spectrometer; (j) Finnigan 6000 interactive data system; (k, l, n) SS-4BMG bellows metering valves, (Nupro; Kontron, Zürich, Switzerland); (m, o, r) 4172G2Y bellows shut-off valves (Hoke; Mathemie, Therwil, Switzerland); (p) stainless-steel vessel, 200 ml; (q) silicon rubber septum.

200-ml vessels (p) are charged with liquids (*ca.* 5 ml) by means of injection by syringe via silicon-rubber septa (q), or discharged by applying a reduced pressure to the vent valves (r). The vessels may also be used as expansion volumes for gases like NH_3, for which constant flow is hard to maintain when supplying them directly from a pressure cylinder through the main reagent gas line (k–m). For measurements, 1-μl samples of a 0.1 % solution were injected without stream-splitting while maintaining the column temperatures at *ca.* 50°. The column temperature was programmed after an initial period (3 min) from 50 to 200° with a gradient of 2°/min. Helium was supplied as carrier gas at a pressure of 1.6 bar. The interface temperature was maintained at 260°.

For CI operation, reagent gases of the highest available purity were employed: methane and isobutane (L'Air Liquide, Genève, Switzerland, types N 55 and CH 35, respectively); ammonia (Fluka, Buchs, Switzerland; purissimum); CH_3O^2H (Ciba-Geigy, Basel, Switzerland; > 99.5 atom % deuterium). Optimized CI conditions were secured by operating the source at pressures of 0.7–1 mbar (readings from an uncalibrated thermocouple gauge) with reagent: carrier-gas ratios of *ca.* 10:1. The filament current, electron energy and ion-source temperature were maintained at 200 μA, 150 eV and 140–150°, respectively.

Analog recording of the total ion current gas chromatograms (TIC–GC) required adjusting the electronically integrated mass ranges to the respective reagent

gas, *e.g.*, *m/e* 60–350 for methane and isobutane, or *m/e* 103–350 for CH_3O^2H, in order to exclude reactant-ion contributions. Alternatively, TIC traces were generated as reconstructed gas chromatograms (TIC–RGC) by use of the dedicated interactive data system (j), acquiring and storing CI mass spectra (for computing the digital TIC values) continuously during the analyses over specified mass ranges at selected scan rates. For example, for a CI(NH_3) run, a mass range of *m/e* 72–350 was chosen with integration times of 7 msec/a.m.u. In plotting these TIC–RGC records, spectrum index numbers rather than time equivalents were used for the calibration of the time axis. In order to allow direct correlations, this calibration scale was simultaneously transferred as a spectrum number (SN) trace on to the analog FID chromatogram (Perkin-Elmer Model 56 recorder equipped with an event marker, triggered by the SN generator of the data system), a pulse signal being supplied at suitable intervals, *e.g.*, at every fifth spectrum acquired. The complete spectra were inspected after the components had been located by means of the spectrum numbers from the TIC–RGC plots, or, more conveniently, by use of a cathode-ray tube (CRT) display (Hewlett-Packard, Type 1311 A) which allows the simultaneous observation of TIC–RGC and mass traces. The sequential inspection of a series of spectra, taken across the GC peak, permits a rapid search for the most representative scan for unresolved components.

RESULTS AND DISCUSSION

The performance of the system was tested by subjecting a synthetic mixture of approximately equal amounts of aliphatic and aromatic compounds, comprising 70 components, to a series of GC–CIMS experiments with different reagent gases. Besides saturated and unsaturated hydrocarbons, the mixture contained alcohols, ethers, halides, amines, esters, aldehydes and ketones. Only little deterioration of the GC resolution was observed for either mode of recording when compared to single detection by means of FID (GC alone) or TIC (GC–MS).

Some results obtained with this mixture are reproduced to illustrate the difficulties in correlating different CI runs as discussed above. Thus, Figs. 2–4 show the markedly differing CI–TIC gas chromatograms of runs with CH_4, $(CH_3)_3CH$ and CH_3O^2H, respectively, *i.e.*, with increasingly milder, and hence more selective, reactants (upper curves). The figures also display the uniform FID traces (lower curves) which were recorded in order to facilitate identification of the GC fractions in spite of the differing signal amplitudes. The assignments of the various components (*cf.* Table I; the numbers refer to the GC fractions) were, in the case of overlapping, arrived at on this basis, and were verified by analysis of the spectra recorded at appropriate instants during these runs.

Methane (Fig. 2), a strongly acidic and thus a rather universal protonating agent (CH_5^+ ions!), ionized and permitted detection of all of the components. Hence a record was produced which more closely resembles the FID chromatogram than those of the other reactant gases employed. In contrast, isobutane (Fig. 3) displayed, as expected on thermodynamic grounds, an essentially complete suppression of aliphatic hydrocarbons (components 1, 3, 10, 20, 26, 33 and 44), and moderate to severe discrimination towards aromatic hydrocarbons (*e.g.*, components 2, 4, 5, 8, 9, etc.). The CI–TIC trace of the analogous CH_3O^2H run (Fig. 4) showed the expected enhancement of this discriminatory effect, as well as its extention to additional com-

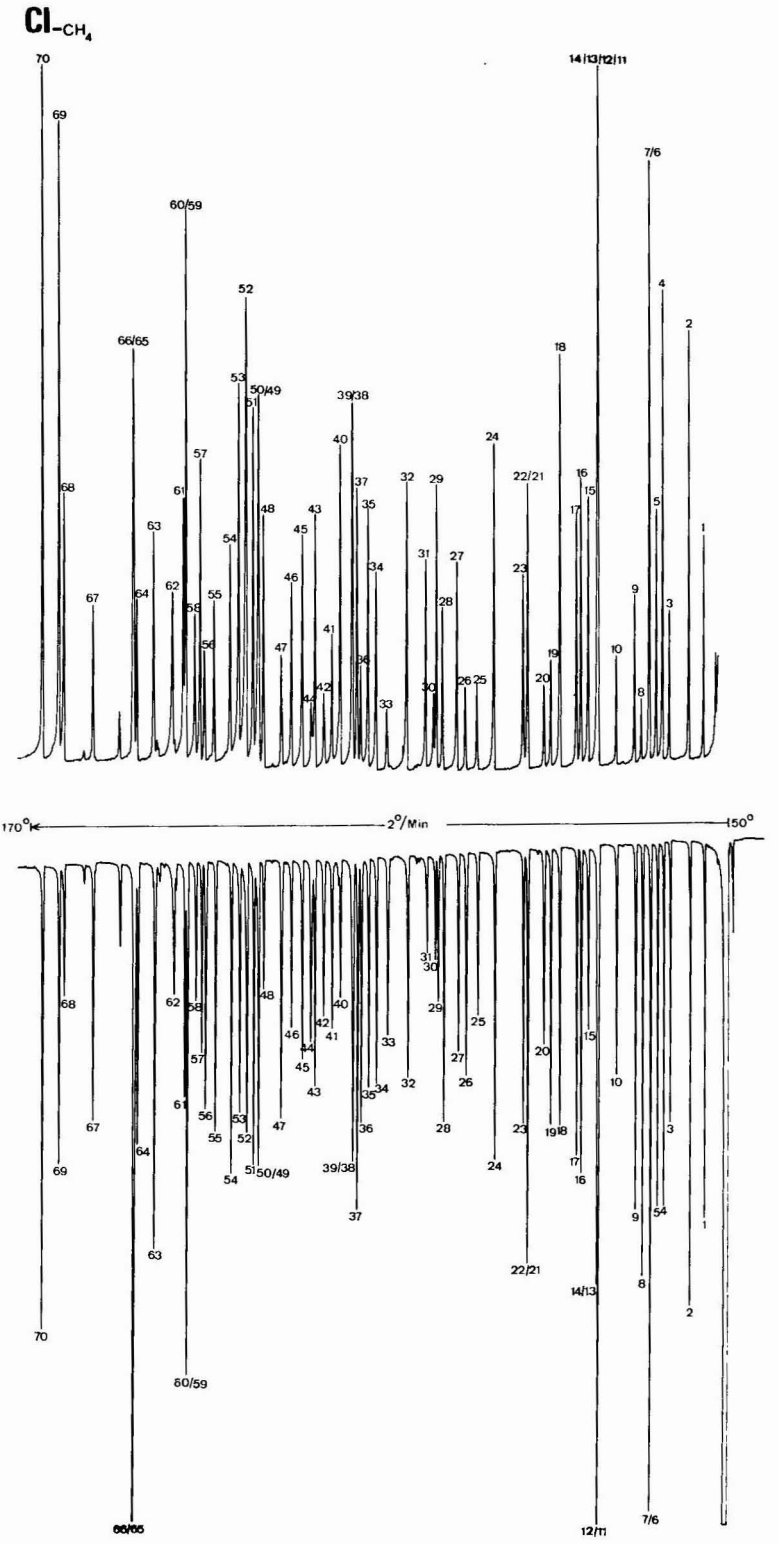

Fig. 2. Comparison of total-ion-current (upper trace, methane reactant) and flame-ionization detection (lower trace) for a synthetic mixture (*cf.* Table I). Glass capillary column (50 m × 0.35 mm) coated with Emulphor-ON 870. Mass range of analog TIC integration, *m/e* 60–350.

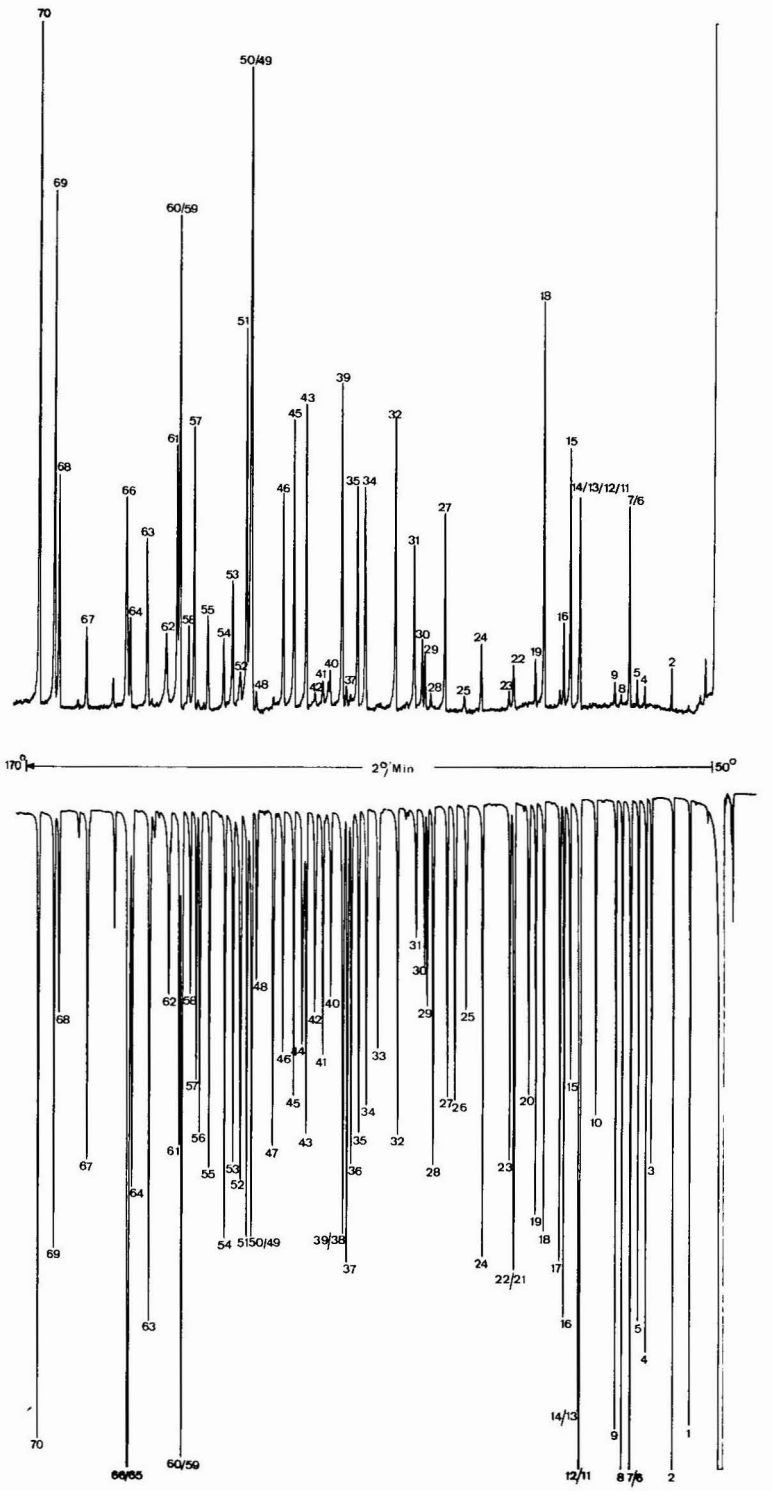

Fig. 3. Comparison of total-ion-current (upper trace, isobutane reactant) and flame-ionization detection (lower trace) for a synthetic mixture (*cf.* Table I). Details as in Fig. 1.

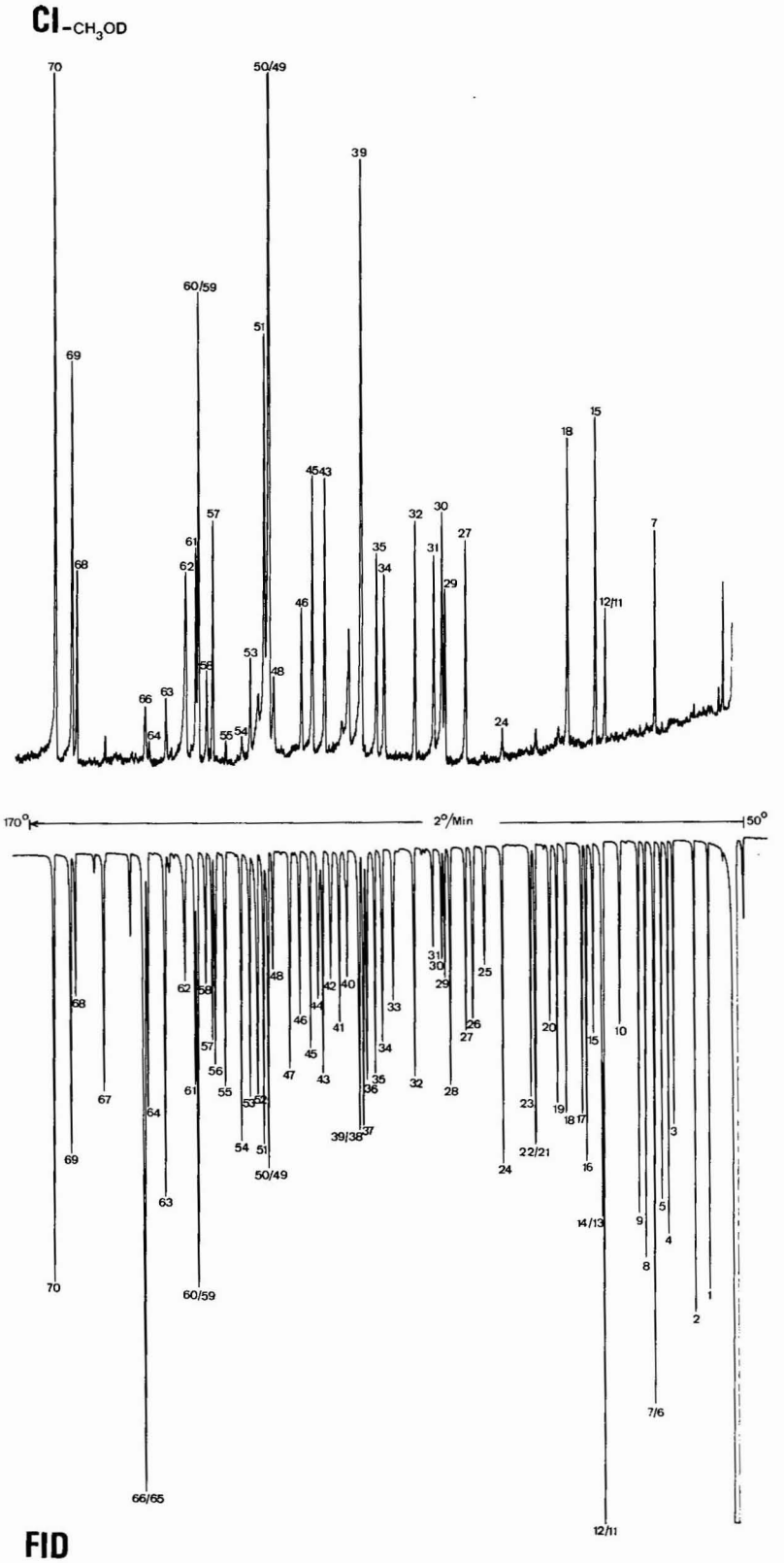

Fig. 4. Comparison of total-ion-current (upper trace, CH_3O^2H reactant) and flame-ionization detection (lower trace) for a synthetic mixture (*cf.* Table I). Column as in Fig. 1. Mass range of analog TIC integration, *m/e* 103–350.

TABLE I

IDENTITIES OF GC FRACTIONS 1–70 (INCREASING RETENTION VALUES) OF A SYNTHETIC MIXTURE

The fraction numbers correspond to those in Figs. 2–5.

No.	*Compound*	*No.*	*Compound*
1	*n*-Decane	36	*n*-Heptylbenzene
2	Ethylbenzene	37	Phenylcyclohexane
3	*n*-Undecane	38	2,6-Dichlorostyrene
4	Chlorobenzene	39	Diethyl succinate
5	*n*-Propylbenzene	40	1,5-Dibromopentane
6	4-Ethyltoluene	41	Naphthalene
7	1,8-Cineole	42	2-(*n*-Heptyl)-1-nonene*
8	*tert.*-Butylbenzene	43	2-Methylacetophenone
9	*sec.*-Butylbenzene	44	*n*-Hexadecane
10	*n*-Dodecane	45	4-Methylacetophenone
11	2-Methyl-3-phenyl-1-propene	46	4-Isopropylbenzaldehyde
12	Anisole	47	*n*-Octylbenzene
13	*n*-Butylbenzene	48	1,6-Dibromohexane
14	Bromobenzene	49	Nerol
15	6-Methyl-5-hepten-2-one	50	2-Bromobenzaldehyde
16	*tert.*-Pentylbenzene	51	2,6-Dimethylaniline
17	*sec.*-Pentylbenzene	52	2-Chlorophenol
18	Cyclohexyl methyl ketone	53	Benzyl alcohol
19	1-Phenyl-2-butene	54	1-Methylnaphthalene
20	*n*-Tridecane	55	1-Phenyl-1-heptyne
21	1,4-Diisopropylbenzene	56	*n*-Nonylbenzene
22	1,4-Dichlorobenzene	57	2-Chloroaniline
23	*n*-Pentylbenzene	58	Diethyl adipate
24	Coumarone	59	2-Ethylnaphthalene
25	2-(*n*-Hexyl)-1-octene*	60	2-Methoxybenzaldehyde
26	*n*-Tetradecane	61	2,6-Dichloroaniline
27	N,N-Dimethylaniline	62	2-Bromophenol
28	*n*-Hexylbenzene	63	1,3-Dimethylnaphthalene
29	1,4-Dibromobutane	64	1,4-Dimethylnaphthalene
30	4-Bromochlorobenzene	65	*n*-Decylbenzene
31	Diethyl malonate	66	1,2-Dimethylnaphthalene
32	Methyl benzoate	67	1,8-Dimethylnaphthalene
33	*n*-Pentadecane	68	4-Chloroaniline
34	(+)-Pulegone	69	3-Chloroaniline
35	Ethyl benzoate	70	2-Aminoacetophenone

* Samples were obtained from Dr. G. Schomburg, MPI für Kohlenforschung, D-4330 Mülheim/Ruhr, G.F.R.

ponents (*e.g.*, 13, 14 and 16). This increased selectivity for molecules with functional groups can be of distinct advantage in certain applications, *e.g.*, when hydrocarbon background or hydrocarbon component suppression is desirable, as may be the case in specific probing with this reactant for acidic protons by hydrogen–deuterium exchange[12]. With CH_3O^2H (or CH_3OH), ionization became so selective that less than half of the 70 components remained, for practical purposes, sufficiently detectable. The attempts to recognize and identify components only on the basis of unchanged retention times in other runs were ambiguous, if not futile, without careful reference to the three parallel FID traces. This may be illustrated by components 48–54, which

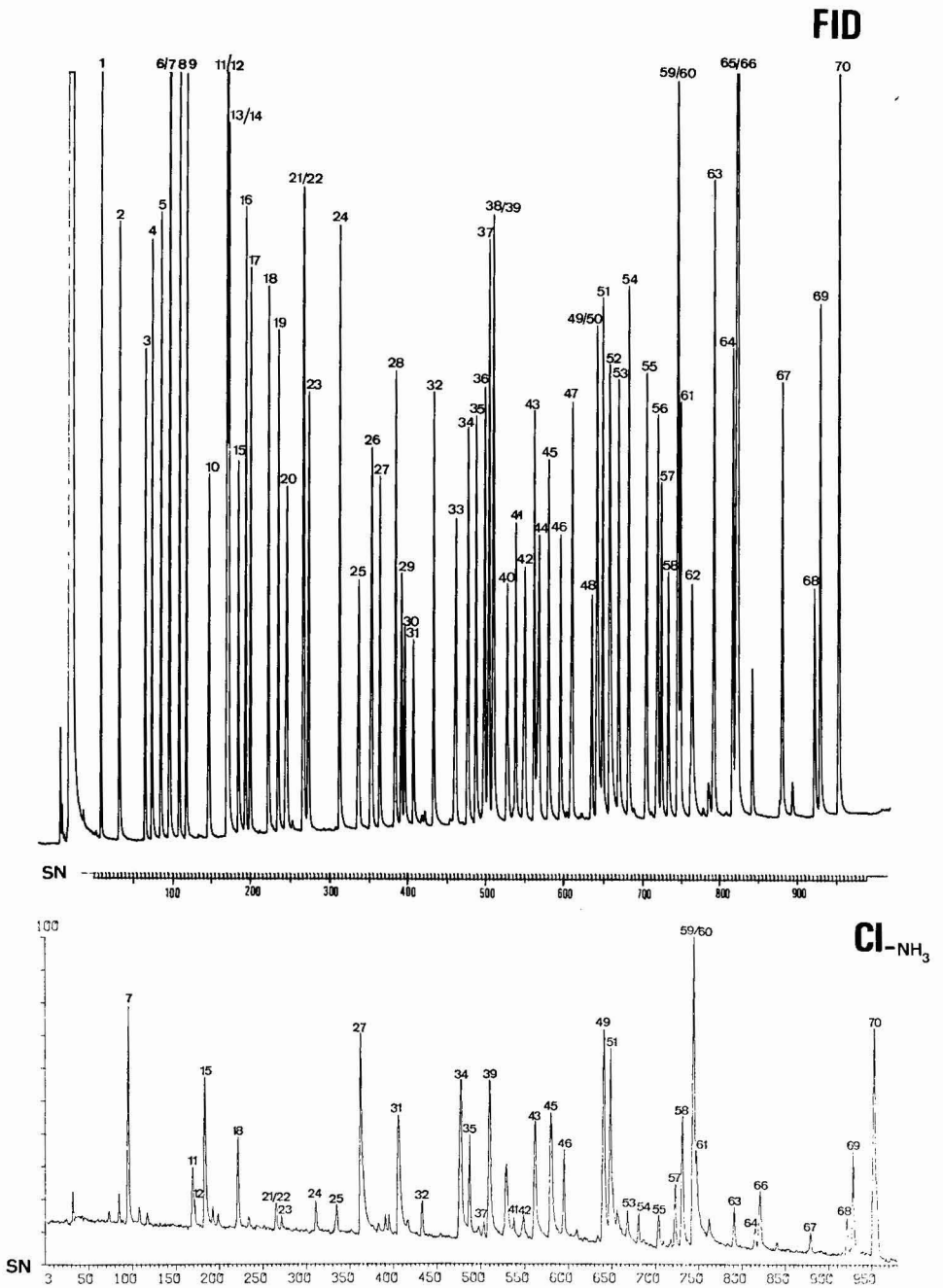

Fig. 5. Comparison of computer-reconstructed total-ion-current (lower trace, NH_3 reactant) and flame-ionization detection (upper trace) for a synthetic mixture (*cf.* Table I). Column as in Fig. 1. Mass range of digital TIC integration, *m/e* 72–350.

represent a tightly adjacent group exhibiting large discrepancies in the CI responses to CH_4 compared with those to isobutane and CH_3O^2H. In such a case it is necessary to identify the components by means of their spectra rather than their retention behaviour, particularly when overlapping of the components is complete (*e.g.*, 49 and 50). With the exception of isomers, this will also permit to detect which of the overlapping components are lost if they fail to respond when a new reagent gas is employed (*e.g.*, 6, 13 and 14, when substituting methanol for isobutane). In reality, the concentrations of the components are likely to differ over much larger ranges than in the present test mixture, and this may constitute another source of ambiguity in correlation work of this type.

The near-synchronous analog recording of the FID signal and the electronically integrated TIC (time lag *ca.* 1 sec with this configuration) yields traces having essentially superimposable time scales, which permit a careful timing of representative mass scans and their exact localization in the complex patterns of the chromatograms without tedious interpolation. However, in most cases, spectra will be recorded continuously in large numbers during the CI experiments rather than in single scans, and will be processed by means of a data system (*cf.* the Experimental section). This technique is illustrated by the TIC–RGC plot of the CI(NH_3) run of the same mixture (Fig. 5, lower curve). Although the TIC–RGC records differ considerably from analog FID records in their format, the calibration of both traces in identical spectrum numbers (SN) secures a quick and straightforward reference between the unlike traces. Correlations of data from different runs, by comparison of indexed spectra, thus become a matter of quick extraction of the spectra from corresponding sets or files. In spite of some loss in the apparent GC resolution of the TIC–RGC trace, owing to the pronounced incremental nature of the TIC sampling in this mode (only one sample per complete scan cycle; *i.e.*, 2 sec), this type of search for the most representative and exactly analogous spectra is preferred for practical work.

The great wealth of complementary data of independent, yet interrelatable, GC–CIMS experiments can be safely and reasonably managed only in this manner for more than a very few components of a mixture of some complexity. Only in this fashion can this wealth be exploited in an approach that aims to determing the structural features, one by one, for many compounds, rather than many features for one compound at a time.

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ANALYSIS OF THE PROTEINS IN SWEAT AND URINE BY AGAROSE-GEL ISOTACHOPHORESIS

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SUMMARY

A sensitive and practical method is described for the analysis of the proteins contained in human sweat and urine which does not require pre-concentration of the sample. Technical details are provided of the agarose-gel isotachophoresis and the proteinograms of normal and pathological urine samples, as well as proteinograms of human sweat. The method can also be applied as an electro-concentration system in a field-strength gradient. By means of this electro-concentration system, and in combination with immunodiffusion against monospecific antisera, a detection limit of albumin of 50 ng/ml has been obtained.

INTRODUCTION

Unconcentrated human sweat and urine contain trace amounts of proteins which are difficult to analyse by means of electrophoresis, where the diffusion forces constantly counteract the separation power of the method. Even in disc electrophoresis[1,2] the sharpness of the fraction is partially disrupted by diffusion effects. For this reason an analytical method such as free isotachophoresis, which combines a concentration power with an antidiffusion force created by ionophoresis in the potential gradient, is of great interest[3,4].

This paper describes technical details of the agarose-gel isotachophoresis of diluted biological fluids without pre-concentration. The selectivity and the reproducibility of the protein pattern are discussed.

MATERIALS

Samples

For feasibility studies, solutions (1 mg/ml) of human albumin and human transferrin (Behringwerke, Marburg, G.F.R.) were analysed. "Sauna" sweat was collected from healthy volunteers at the Zeepreventorium, Den Haan. The individual

samples were stored at 4°. Urine samples (Department of Nephrology, St. Jans-hospitaal, Brugge, Belgium) exhibiting a pattern of glomerular, tubular and mixed proteinuria were selected by routine agarose-gel electrophoresis. When spacer molecules were required, ampholines (pH 6–8) (LKB, Stockholm, Sweden) were added to the samples before isotachophoresis[5,6].

Antisera

Commercial rabbit antisera (Behringwerke) against human albumin, total human serum, human α-lipoprotein, human β-lipoprotein, human transferrin and human Ig were used. An antiserum against sweat lipoproteins was raised in the rabbit at our laboratory.

METHODS

Isotachophoresis

Isotachophoresis was carried out with tap-water cooling on an LKB Type 2117 electrophoresis system. Glass plates (1.5 × 11 cm or 3 × 11 cm) were covered with agarose gel containing the leading electrolyte. For clinical purposes (analysis of sweat and urine), 0.018 *M* orthophosphoric acid adjusted with tris(hydroxymethyl)amino-methane (Tris) to pH 5.5 was used as the leading electrolyte at the anode. After gelification, one third of the leading gel was cut off and replaced by the cathodic agarose gel containing a terminating electrolyte solution of 0.04 *M* glycine adjusted by use of Tris to pH 8.6. In some experiments 0.014 *M* orthophosphoric acid (adjusted with Tris to pH 7) and 0.028 *M* glycine (adjusted with Tris to pH 8.6) were used as leader and terminator. De-ionized and twice distilled water was used in all of the solutions. Connection of the gel with the corresponding electrolyte vessels was by means of paper wicks.

The protein samples, with or without spacer molecules, were applied to a filter strip (3 × 1.5 cm) and placed on top of the terminator gel. A constant current of 5 mA was applied under a voltage of 300 V. After 2 h, at the end of the run, the voltage rose to 400 V depending on the concentration, pH and mobility of the ions. Coomassie brilliant blue staining and destaining was carried out before drying of the gel[7]. For Sudan Black B and PAS (periodic acid Schiff) staining, the gels were first fixed in ethanol–acetic acid–water (10:1:9) and stained after drying.

Immunological analysis

For gel immunoisotachophoresis, samples were separated in the presence of 2 μl of ampholine. Antiserum was applied in a slot cut out along the migration path. For immunological analysis of the leader–terminator boundary, antiserum was applied in holes punched in the gel at 5 mm from the boundary. After immuno-diffusion for 24 h, the gels were washed, dried and stained as described earlier[8].

RESULTS

Isotachophoretic protein patterns

Selectivity and reproducibility of the method. 500 μl of a solution of albumin transferrin containing 10 μg of each protein and 2 μl of ampholine was analysed at

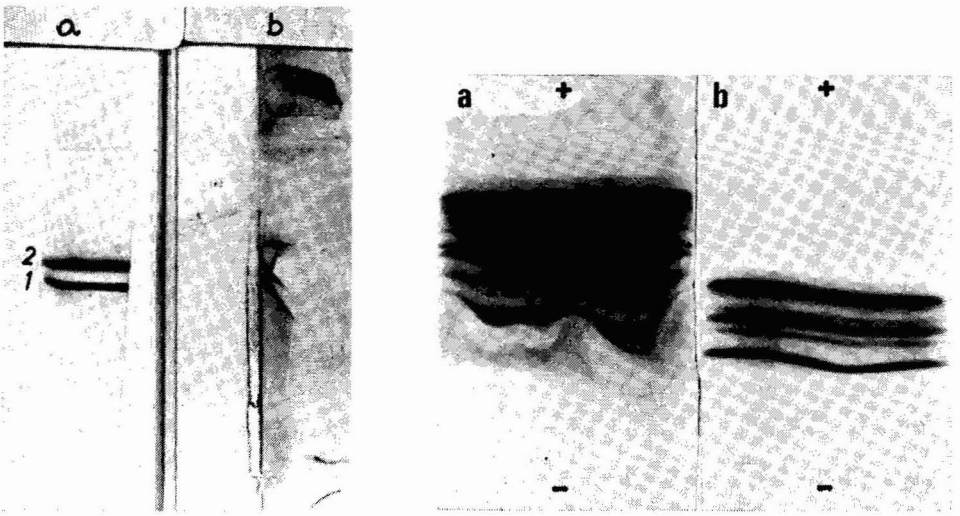

Fig. 1. Isotachophoresis (a) and immunoisotachophoresis (b) in agarose gel of solutions of albumin (1) and transferrin (2).

Fig. 2. Isotachophoresis of a proteinuria sample (250 μl) without pre-concentration. (a) Leading buffer, Tris–0.018 *M* H_3PO_4 (pH 5.5); terminating buffer, Tris–0.04 *M* glycine (pH 8.6). (b) Same sample as in a: leading buffer, Tris–0.014 *M* H_3PO_4 (pH 7); terminating buffer, Tris–0.028 *M* glycine (pH 8.8).

several pH values and electrolyte concentrations. Optimal separation for both proteins was obtained at a leader-electrolyte concentration of 0.014 *M* orthophosphoric acid adjusted by use of Tris to pH 7 and at a terminator concentration of 0.028 *M* glycine adjusted by Tris to pH 8.6 (Fig. 1a). The purity of the protein fractions was demonstrated by immunoisotachophoresis and two crossed well-separated immunoprecipitation lines were obtained (Fig. 1b).

The selectivity of the method is dependent on the composition of the leading electrolyte as demonstrated by a comparison of the protein pattern of a nephrotic urine sample at pH 5.5 and pH 7 (Fig. 2). Previous studies have shown that the protein patterns of human urine and human sweat stored at 4° in the presence of thymol are reproducible over a period of 6 months[5,8,9].

Proteinogram of human sweat. The protein pattern of unconcentrated human sweat is shown in Fig. 3a. The pattern shows three main protein bands next to several smaller fractions. The major band corresponds to albumin, as was demonstrated by immunological techniques. The second band corresponds to the inter-α band detected by electrophoresis of human sweat after a 200-fold concentration[9–11]. The third band contains transferrin and fast-migrating γ-globulins, as confirmed by immunoisotachophoresis with specific antisera. Moreover, as shown in Fig. 3b, three lipophilic fractions appeared after staining with Sudan Black B. The presence of α- as well as of β-lipoproteins was established, but the major component is a lipid protein fraction having plasma-albumin mobility. The concentration of this native fraction is season dependent[9]. After staining for polysaccharides by Schiff's method, two glycoprotein lines were demonstrated as shown in Fig. 3c.

Proteinogram of urine. The proteinograms of healthy fasting patients at rest

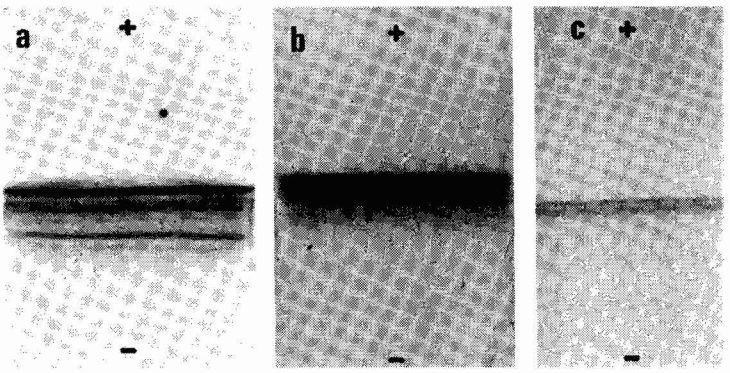

Fig. 3. Isotachophoresis of normal human sweat (500 μl). With Coomassie brilliant blue (a), Sudan Black B (b) and periodic acid Schiff staining (c).

showed only trace amounts of albumin. Samples from patients with orthostatic albuminuria showed, in addition to the presence of albumin, some α- and β-proteins, which were not visible after classical zone electrophoresis without pre-concentration[4]. Urine samples from patients with kidney disease were analysed for classification according to the type of proteinuria.

A glomerular proteinuria is shown in Fig. 4a with albumin as the major and transferrin as a minor fraction. A pattern of tubular proteinuria (Fig. 4b) had nine well-defined protein fractions from which prealbumin, albumin, transferrin and γ-globulins were identified by immunoisotachophoresis. Several samples of proteinuria belonged to an intermediate type characterized by a high number of protein fractions having relatively high albumin concentrations (Fig. 4c).

Protein concentration at the leader–terminator boundary

Isotachophoresis without spacer molecules concentrates all of the proteins present in the sample into one band at the leader–terminator boundary. For testing the sensitivity of the method, a very dilute sample of albumin was analysed. By means of immunological analysis after isotachophoresis, we were able to detect the presence

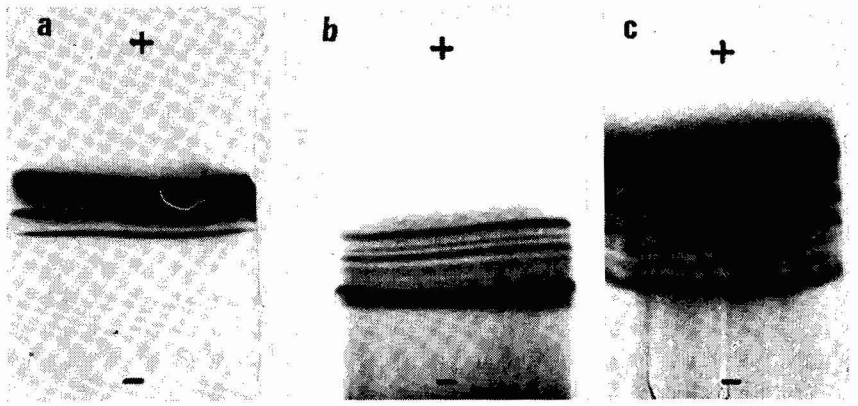

Fig. 4. The proteinuria patterns: glomerular (a), tubular (b) and mixed (c).

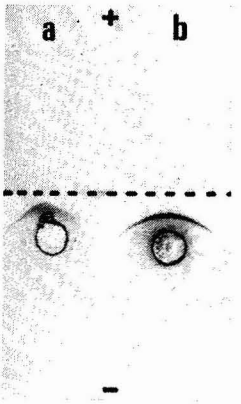

Fig. 5. The immunological analysis of the leader–terminator boundary of human sweat (500 μl) using 5 μl (a) and 10 μl (b) of rabbit anti-sweat lipoprotein.

of 24 ng of albumin which had been previously diluted with 500 μl of water. This concentration procedure was also applied to the detection of proteins and lipoproteins in human sweat (Fig. 5). We were able to demonstrate the presence of α- and β-lipoproteins in sweat collected on filter paper[5]. The same method can also be applied to the detection of trace amounts of albumin on fatty hair and in tears and to the detection of immunoglobulins in nasal secretions, all without previous concentration.

DISCUSSION

By means of agarose-gel isotachophoresis, proteins present in very low concentrations in different biological fluids can be separated. The fractionation will be a function of the experimental conditions of pH and ionic strength, of the nature of the spacer used and of the mobility of the leading and terminating ions[12].

The isotachophoretic urine pattern shows all of the proteins possessing a mobility intermediate between those of the leading and terminating ions. The observed patterns correlate well with those obtained by classical electrophoretic techniques after concentration. As the mobility of glycine is nearly the same as that of the γ-globulins, the described conditions cannot be used for differentiation of γ-globulins. ε-Aminocaproic acid as terminating ion, having a mobility lower than that of glycine, would give better results.

The high concentration power of agarose-gel isotachophoresis is an important advance in the analysis of proteins in urine and sweat. Owing to the high sensitivity of the technique, new ways are made available for further study of the pathology of the kidney, and this technique could be of interest in the analysis of urine from newborn babies.

The applicability of the described method to biological fluids containing very low protein concentrations is shown by the proteinogram of 500 μl of human sweat. Since normal electrophoretic techniques require a 200-fold pre-concentration of the sample before any result can be observed, most published studies describe results obtained on pooled samples of human sweat which were pre-concentrated before analysis. The introduction of the isotachophoretic agarose technique enables the

analysis of protein and glycoprotein fractions in individual samples. The presence of a lipoprotein which does not precipitate anti-α and anti-β plasma lipoproteins in human sweat is also of importance in the study of the function of sweat glands in the metabolism of human skin. Immunoisotachophoresis and the immunological analysis of the proteins concentrated at the leader–terminator boundary are helpful tools in the identification of dilute protein solutions.

ACKNOWLEDGEMENT

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THE VAN URK–SALKOWSKI REAGENT — A SENSITIVE AND SPECIFIC CHROMOGENIC REAGENT FOR SILICA GEL THIN-LAYER CHROMATOGRAPHIC DETECTION AND IDENTIFICATION OF INDOLE DERIVATIVES

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SUMMARY

The chromogenic reagent described has been tested with seventy-nine indole derivatives and found to be very sensitive and indole-specific. The lower limit of detection on silica gel thin-layer plates was between 25 and 50 ng for most indoles. Phenols and hydroxy-, and amino-benzoic acids, hydroxy-, and methoxy-cinnamic acids did not yield chromophores with the exception of *p*-amino-benzoic acid and *p*-hydroxy-cinnamic acid which gave yellow and pink chromophores at concentrations greater than 1 and 2 μg. Although many of the C-3 substituted indoles such as indole-3-acetic acid and tryptamine had colors in the reddish-violet-blue color region, most exhibited sufficient color differentiation to allow their identification by thin-layer chromatography. The procedure was simple and required only 10 min from the time of spraying the thin-layer plate until full color development was reached. The colors had a wide spectral range from yellow of the indole-3-glyoxylamide chromophore to blue of the melatonin chromophore, and were extremely stable.

INTRODUCTION

Silica gel thin-layer chromatography (TLC) has become a powerful technique in the purification, separation and possible identification of natural and synthetic indole derivatives[1–4]. The advantages over paper chromatography are short developing times, inertness of the silica gel layer towards corrosive spray reagents and minimal zone spreading of the chromatographing compounds, resulting in a 10–20-fold decrease of the detection limits[5].

The indole compounds have been visualized on TLC plates by one of the following chromogenic reagents: (a) Salkowski reagent[6–13] (strong mineral acid plus oxidant; (b) Ehrlich reagent[5,14–34] (*p*-dimethylaminobenzaldehyde–HCl with or without oxidant); (c) van Urk reagent[35–42] (*p*-dimethylaminobenzaldehyde–H_2SO_4 and oxidant); (d) Renz and Loew reagent[43–49] (*p*-dimethylaminocinnamaldehyde–HCl);

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(e) Adamkiewicz reagent[50–57] (formaldehyde–HCl); (f) Maickel and Miller reagent[58–61] (*o*-phthalaldehyde–HCl).

The last two reagents give strongly yellow fluorescing indole condensation products, which makes them the most sensitive reagents available. Their use is limited, because extracts (especially from plant material) contain many non-indolylic yellow fluorescing substances, and the visible yellow-orange colors are not diagnostic for indole derivatives.

The Renz and Loew[43–47] reagent is claimed to be more sensitive for indoles on TLC[48,49,62] than the Ehrlich[14–21] or van Urk[35–37] reagents. A comparative study of the three reagents on TLC with a number of biologically important indoles, such as indole-3-acetic acid (Iaa), tryptophan (Trp) and indole-3-acetyl esters, has shown[63,64] that the *p*-dimethylaminocinnamaldehyde (*p*-DMAC) reagent is 3–8 times less sensitive for most of the indoles. In addition the *p*-DMAC reagent develops a yellow to red background within 12 h which makes the subsequent identification of the colored indole condensation products difficult.

The Ehrlich and van Urk reagents are, to date, the most specific chromogenic reagents for indole derivatives, but color development is slow (3–8 h) and the colors are not stable, due to the mineral acid retained on the silica gel layer. We have reported a modified van Urk spray reagent procedure[25] which resulted in considerable color stability, but color development was slow (5–8 h). Color development with the Salkowski[6–13] reagent is rapid (15–30 min), but the colors change quickly to non-diagnostic brown tones. The sensitivity is about 10-fold less than for the Ehrlich and van Urk reagents, and has poor specificity for indoles except for Iaa and some Iaa derivatives.

A spray reagent has now been developed that has a high sensitivity and specificity for indole compounds, gives rapid color development and color stability of the indole condensation products.

EXPERIMENTAL

Materials

Sources of indoles. The (indole-3-acetyl)-*myo*-inositols, di-O-, and tri-O-(indole-3-acetyl)-*myo*-inositols, (indole-3-acetyl)-*myo*-inositolglycosides, 2-O-, 4-O-, and 6-O-(indole-3-acetyl)-D-glucopyranoses, N-(*p*-coumaryl)-tryptamine and N-ferulyltryptamine were isolated from sweet corn kernels of *Zea mays*[25,29–31]. The 1-O-(indole-3-acetyl)-β-D-glucopyranoside was a gift from Dr. D. Keglević (Institute "Ruder Bošković", Bijenička 54, Zagreb, Yugoslavia).

Other indoles were obtained from the following sources: 5-benzyloxy-6-methoxyindole, 6-benzyloxy-5-methoxyindole, N-acetyl-indole, indole-3-acetonitrile and 5-methoxytryptophol from Regis (Morton Grove, Ill., U.S.A.); 1-methylindole from Eastman-Kodak (Rochester, N.Y., U.S.A.); ethylindole-3-acetate, indole-3-propionic acid, indole-3-butyric acid and indole-3-acetyl-L-aspartic acid from Calbiochem (La Jolla, Calif., U.S.A.); tryptophol, gramine, tryptamine·HCl and bufotenine from Sigma (St. Louis, Mo., U.S.A.). All other indoles were obtained from Aldrich (Milwaukee, Wisc., U.S.A.).

Indole standards. The indoles were dissolved in either absolute ethanol, 50% ethanol, 2-propanol or chloroform to give 1- or 2-μg/μl solutions. From these stock

solutions serial dilutions containing 10, 25, 100, 200 and 500 ng/μl were prepared.

Solvents and reagents. Methanol, ethanol, 2-propanol, 2-butanone, ethylacetate, and chloroform were reagent grade and further purified with activated charcoal and fractional distillation. The purity was monitored by UV. Water was distilled, deionized, and redistilled in an all-glass distillation apparatus. The reagents were made as follows. (A) van Urk[37] reagent: 1 g *p*-dimethylaminobenzaldehyde (Aldrich) decolorized with activated carbon and recrystallized from ethanol–water (m.p. 74.5°), was dissolved in 50 ml conc. HCl (specific gravity 1.190) and 50 ml absolute ethanol was added; this reagent is stable for several months at room temperature when stored in a brown glass bottle. (B) Salkowski[6] reagent (as modified by Tang and Bonner[9]): 2.03 g $FeCl_3 \cdot 6\ H_2O$ were dissolved in 500 ml water and 300 ml conc. H_2SO_4 (specific gravity 1.840); this reagent is stable indefinitely.

Spray reagent. The new TLC spray reagent used, was made up of reagent A and B (1:3). The spray reagent may be kept at room temperature for several weeks.

Silica gel TLC plates. Precoated silica gel G TLC glass plates with or without fluorescent indicator and a layer thickness of 0.25 mm were used throughout this study (E. Merck, Elmsford, N.Y., U.S.A.).

TLC solvent systems. The indoles listed in Table I were chromatographed in one of the following solvent systems: (1), butanone–ethyl acetate–ethanol–water (3:5:1:1); (2) propanol–water (8:2); (3), propanol–water–28 % ammonium hydroxide (8:1:1); (4), chloroform–methanol–water (84:14:1).

Methods

Visualization of indoles. After one-, or two-dimensional TLC of indole standards or partially purified extracts containing indolylic compounds in the appropriate solvent system the plate was dried at 45° until all traces of solvent had evaporated (5–10 min). The dry plate could then be examined under UV for fluorescing and/or quenching spots. Spraying of the TLC plate was done in a fume hood, using a glass atomizer such as a Desaga® standard glass atomizer (100-ml capacity; Brinkmann, Westbury, N.Y., U.S.A.) connected to an air line. The plate was sprayed evenly in an upright position until the silica gel layer became transparent. If the plate was accidentally oversprayed the excess reagent on the silica gel layer could be removed with a paper towel.

The plate was heated in a 100° oven for 5 min, then removed from the oven and allowed to cool to room temperature. Heating up to 10 min had no adverse effect on the indole condensation products, but heating for more than 10 min caused a greying of the silica gel background. The plate was immersed in distilled water (2–3 l per 20 × 20-cm plate), agitated periodically for 1 min. The plate was washed two more times as before. Thorough washing of the TLC plate was necessary to assure the complete removal of acids since it was found that incomplete washing of the plate resulted in yellowing of the silica gel within a few weeks.

The plate was removed from the last water wash and blotted with a dry paper towel. At this time the colors of the indole condensation products were evaluated (Table I; wet-plate color reading). The plate was then dried at 45° (20–30 min). The colors of the indole condensation products were evaluated once more (Table I; dry-plate color reading). The colors of the indole condensation products are extremely stable and fade resistent. We have kept TLC plates at room temperature in the dark

TABLE I

COLOR REACTIONS AND LOWER LIMITS OF DETECTION OF INDOLE AND INDOLE DERIVATIV CHROMATOGRAPHED ON SILICA GEL TLC AND SPRAYED WITH THE VAN URK–SALKOWS REAGENT

Color region, color name and page number from the Horticultural Colour Chart[66]. For each compound the co name for wet-plate color reading is followed by the name for dry-plate color reading, if the color name does not ha a page number, the color could not be matched with one of the 200 color plates, and a descriptive color name w chosen. Limit of detection: see *Methods*.

Substitution	*Name of compound*	*Color region*	*Color name (page no.)*	*Limit o detecti (ng)*
None	Indole	reddish violet	Royal Purple (174)	25
			grey with reddish cast	25
N-1	1-Methylindole	reddish violet	Violet Purple (161)	25
		orange	Mars Orange (104)	50
	1-Acetylindole	reddish violet	Royal Purple (174)	25
			reddish grey	25
	1-Indoleacetic acid	reddish violet	Doge Purple (96)	25
		reddish violet	Lilac Purple (115)	25
C-2	2-Methylindole	violet red	Peony Purple (95)	25
		greenish yellow	Naples Yellow (121)	50
	2-Phenylindole	bluish violet	Dauphin's Violet (117)	25
		yellowish green	Fern Green (186)	50
	Ethyl indole-2-carboxylate	red	Victoria Violet (97)	25
		bluish violet	Dauphin's Violet (117)	25
C-3	3-Methylindole	green	Ivy Green (200)	25
			greyish-blue	25
	Indole-3-methanol	violet red	Peony Purple (95)	50
		reddish violet	Pansy Violet (116)	50
	Indole-3-ethanol	blue	Princes Blue (98)	25
		greenish blue	Capri Blue (52)	25
	3-Acetylindole	reddish violet	Mauve (80)	1000
			light grey	1500
	Indole-3-carboxaldehyde	reddish violet	Mauve (80)	300
		orange red	Orient Pink (124)	600
	Indole-3-acetic acid	bluish violet	Aconite Blue (180)	25
		violet blue	Sea Blue (119)	25
	Indole-3-propionic acid	violet blue	Sea Blue (119)	25
		violet blue	Sea Blue (119)	25
	Indole-3-butyric acid	violet blue	Sea Blue (119)	25
		green	Carnation Green (194)	25
	Indole-3-pyruvic acid	yellowish green	Fern Green (186)	100
		yellowish green	Sage Green (198)	100
	Indole-3-lactic acid	blue	Princes Blue (98)	25
		violet blue	Sea Blue (119)	25
	Indole-3-acetamide	violet blue	Sea Blue (119)	50
		violet blue	Sea Blue (119)	50
	Indole-3-glyoxylamide	reddish violet	Petunia Purple (32)	25
		yellow	Straw Yellow (67)	50
	Indole-3-acetic acid hydrazide	blue	Oriental Blue (47)	50
		violet blue	Sea Blue (119)	50
	Indole-3-acrylic acid	red	Oxblood Red (191)	25
			light siena	25
	Indole-3-acetone	yellowish green	Fern Green (186)	50
		yellowish green	Fern Green (186)	50

ABLE I (*continued*)

ubstitution	*Name of compound*	*Color region*	*Color name (page no.)*	*Limit of detection (ng)*
	Indole-3-acetonitrile	bluish violet	Dauphin's Violet (117)	25
		violet blue	Sea Blue (119)	25
	Gramine	reddish violet	Pansy Violet (116)	50
		bluish violet	Methyl Violet (39)	50
	Tryptamine	blue	Princes Blue (98)	25
		blue	Oriental Blue (47)	25
	Ethyl indole-3-acetate	blue	Princes Blue (98)	25
		violet blue	Sea Blue (119)	25
	1-O-(indole-3-acetyl)-β-D-glucopyranose	bluish violet	Aconite Blue (180)	50
		violet blue	Sea Blue (119)	50
	2-O-(indole-3-acetyl)-D-glucopyranose	bluish violet	Aconite Blue (180)	50
		violet blue	Sea Blue (119)	50
	4-O-(indole-3-acetyl)-D-glucopyranose	bluish violet	Aconite Blue (180)	50
		violet blue	Sea Blue (119)	50
	6-O-(indole-3-acetyl)-D-glucopyranose	bluish violet	Aconite Blue (180)	50
		violet blue	Sea Blue (119)	50
	2-O-(indole-3-acetyl)-*myo*-inositol	bluish violet	Aconite Blue (180)	50
		violet blue	Sea Blue (119)	50
	1-DL-1(4)-O-(indole-3-acetyl)-*myo*-inositol	bluish violet	Aconite Blue (180)	50
		violet blue	Sea Blue (119)	50
	1-DL-5-O-β-L-arabinopyranosyl-1-O-(indole-3-acetyl)-*myo*-inositol	bluish violet	Aconite Blue (180)	75
		violet blue	Sea Blue (119)	75
	5-O-β-L-arabinopyranosyl-2-O-(indole-3-acetyl)-*myo*-inositol	bluish violet	Aconite Blue (180)	75
		violet blue	Sea Blue (119)	75
	1-DL-5-O-β-L-galactopyranosyl-1-O-(indole-3-acetyl)-*myo*-inositol	bluish violet	Aconite Blue (180)	75
		violet blue	Sea Blue (119)	75
	5-O-β-L-galactopyranosyl-2-O-(indole-3-acetyl)-*myo*-inositol	bluish violet	Aconite Blue (180)	75
		violet blue	Sea Blue (119)	75
	Di-O-(indole-3-acetyl)-*myo*-inositol	bluish violet	Aconite Blue (180)	40
		violet blue	Sea Blue (119)	40
	Tri-O-(indole-3-acetyl)-*myo*-inositol	bluish violet	Aconite Blue (180)	30
		violet blue	Sea Blue (119)	30
	DL-Tryptophan	violet blue	Sea Blue (119)	25
		blue	Oriental Blue (47)	25
	N-Acetyltryptophan	blue	Princes Blue (98)	25
		violet blue	Sea Blue (119)	25
	Glycyl-L-tryptophan	violet blue	Sea Blue (119)	25
		blue	Cerulein Blue (46)	25
	L-Tryptophyltyrosine	violet blue	Sea Blue (119)	25
		blue	Butterfly Blue (85)	25
	Indole-3-acetyl-L-aspartic acid	reddish violet	Cobalt Violet (87)	50
		bluish violet	Moorish Blue (163)	50
	5-Methylindole	reddish violet	Dahlia Purple (178)	25
			light umbra	25
	5-Fluoroindole	reddish violet	Dahlia Purple (178)	25
		reddish orange	Garnet Brown (192)	50
	5-Nitroindole	reddish violet	Plum Purple (179)	25
		reddish violet	Magnolia Purple (114)	25
	5-Hydroxyindole	reddish violet	Plum Purple (179)	25
			greyish-brown	50

(Continued on p. 272)

TABLE I (*continued*)

Substitution	*Name of compound*	*Color region*	*Color name (page no.)*	*Limit of detection (ng)*
C-7	7-Methylindole	violet	Royal Purple (174)	25
		reddish violet	Rose Purple (140)	50
	7-Nitroindole	reddish violet	Maroon (185)	25
		violet red	Erythrite Red (190)	25
N-1, C-2	1-Methyl-indole-2-carboxylic acid	bluish violet	Moorish Blue (163)	25
		violet blue	Bluebird Blue (118)	25
C-2, C-3	Carbazole	violet blue	Sea Blue (119)	25
		greenish blue	Porcelain Blue (49)	25
	Isatin (2,3-Indolinedione)	yellow*	Light Chrome Yellow (144)	300
		yellow	Amber Yellow (132)	600
	Tetrabyrine		siena	300
		violet red	Erythrite Red (190)	300
C-2, C-5	Ethyl 5-ethylindole-2-carboxylate	bluish violet	Moorish Blue (163)	25
		violet blue	Bluebird Blue (118)	25
	5-Hydroxy-indole-2-carboxylic acid	violet	Royal Purple (174)	25
		bluish violet	Dauphin's Violet (117)	25
	2,5-Dimethylindole	reddish violet	Peony Purple (95)	25
			siena	25
	5-Ethylindole-2-carboxylic acid	violet blue	Lobelia Blue (41)	25
		bluish violet	Dauphin's Violet (117)	25
C-3, C-5	5-Hydroxy-indole-3-acetic acid	violet blue	Sea Blue (119)	25
		greenish blue	Porcelain Blue (49)	25
	5-Methyl-indole-3-acetic acid	violet blue	Sea Blue (119)	50
		greenish blue	Enamel Blue (48)	100
	5-Hydroxytryptophan	violet blue	Sea Blue (119)	25
		blue	Ethyl Blue (142)	25
	5-Methoxytryptophol	bluish violet	Moorish Blue (163)	25
		bluish green	Capri Blue (52)	25
	5-Hydroxytryptamine	violet blue	Bluebird Blue (118)	25
		bluish green	Capri Blue (52)	25
	5-Fluorogramine	violet	Cobalt Violet (81)	200
		bluish violet-red	Sea Lavender Violet (153)	200
	5-Ethylgramine	bluish violet	Dauphin's Violet (117)	100
		violet blue	Sea Blue (119)	150
	5-Methoxytryptamine	blue	Princes Blue (98)	25
		bluish green	Langite Green (53)	25
	N-Acetyl-5-methoxy-tryptamine	blue	Princes Blue (98)	25
	(Melatonine)	blue	Ethyl Blue (142)	25
	N,N-Dimethyl-5-hydroxy-tryptamine	violet blue	Cornflower Blue (164)	25
	(Bufotenine)	bluish green	Capri Blue (52)	25
	5-Fluoro-α-methyltryptamine	violet blue	Sea Blue (119)	25
		violet blue	Sea Blue (119)	25
	N-Acetyl-5-hydroxytryptamine	blue	Pomas Blue (98)	25
	(N-acetylserotonin)	bluish green	Capri Blue (52)	25
	5-Benzyloxy-6-methoxyindole	bluish violet	Aconite Violet (180)	25
		green	Leek Green (197)	25
	6-Benzyloxy-5-methoxyindole	bluish violet	Aconite Violet (180)	25
		green	Carnation Green (194)	25
C-3, C-6	6-Fluoro-α-methyltryptamine	violet blue	Sea Blue (119)	25
		violet blue	Sea Blue (119)	25
C-1, C-2, C-3	1-Phenylcarbazole	bluish violet	Moorish Blue (163)	25
		blue	Cerulein Blue (46)	25

TABLE I (*continued*)

Substitution	*Name of compound*	*Color region*	*Color name (page no.)*	*Limit of detection (ng)*
C-2, C-3, C-5	5-Chloro-2-methylindole-3-acetic acid	reddish violet	Rose Purple (140)	75
		reddish violet	Pastel Mauve (127)	100
	5-Methoxy-2-methylindole-3-acetic acid	reddish violet	Pansy Violet (116)	25
		bluish violet	Pastel Lavender (129)	25
	Ethyl 2-ethoxy-5-hydroxy-indole-3-carboxylate		light umbra	50
		yellowish green	Pod Green (120)	25
	2,3,5-Trimethylindole		light brown rose	100
		reddish orange	Orient Pink (124)	100
C-2, C-3, C-6	Reserpine	green	Cyprus Green (59)	300
		yellowish green	Pod Green (120)	300
	Rescinnamine	bluish green	Verdigris (88)	600
		yellowish green	Pod Green (120)	600

* Yellow before spraying.

for more than two years with little or no fading of the original dry-plate colors. However the silica gel background tended to become slightly grey-yellow if the plates were stored for more than six months. This could be eliminated by covering the dry TLC plate with permanent, invisible mending tape (Highland-Brand No. 6200; 3M Co., St. Paul, Minn., U.S.A.) or similar tape. TLC plates covered with this tape have been kept for more than one year with no detectable color changes of the indole condensation products, and have retained a white silica gel background.

Purity and identity of indole standards. In order to evaluate the color reaction of each indole standard listed in Table I the indoles were chromatographed at 1-, 5-, 10- and 20-μg concentrations in one of the four solvent systems to assure a minimum migration of 6 cm of each indole from the origin (solvent front, 10 cm). After the plates were sprayed and processed as described above, they were examined for possible secondary chromogenic spots. In addition each indole standard was analyzed by gas–liquid chromatography and the identity confirmed by mass spectrometry (GLC–MS)[65].

Colors of the indole condensation products. Each indole standard with the exception of the indole derivatives isolated from mature sweet corn karyopses (see *Sources of indoles*) was spotted at 1-, 5-, 10- and 20-μg concs. on a 5 $\times$ 10-cm TLC plate. The corresponding spot areas were 20, 30 and 60 mm^2. The plate was sprayed and processed as described above. After the third water wash of the plate and removal of excess water the color of the indole condensation product was matched by eye with one of the 200 plates of 64 full hues, 60 tints, 38 shades and 38 greyed hues of the Horticultural Colour Chart[66]. The 200 plates had been arranged according to color families, and the matching could be done routinely in less than 1 min (Table I; wet-plate color reading). After the plate had been dried the color was matched again (Table I; dry-plate color reading). In addition each color was rematched three more times after 6, 12, and 24 h to evaluate any color changes which might have occurred. The color evaluation of the indole condensation products of the indole derivatives isolated from mature sweet corn of *Zea mays* was done the same way except that the indoles were first chromatographed on a 20 $\times$ 20-cm TLC plate at 1-, 5-, and 10-μg concentrations in solvent 1.

Limits of detection. The indoles were chromatographed at concentrations ranging from 10–1500 ng in the appropriate solvent systems and a minimum spot migration of 6 cm. The limit was determined as the smallest amount of indole to give a 5–7-mm^2 detectable color spot.

RESULTS AND DISCUSSION

The colors of 79 indole condensation products with *p*-DMAB on silica gel TLC and their limits of detection are listed in Table I. Most of the indoles can readily be detected at the 25–50-ng level which makes this reagent a very sensitive and indole-specific chromogenic reagent. Certain phenols and aromatic acids are known to give positive color reactions with *p*-DMAB–HCl (Ehrlich reagent)[67]. A number of hydroxy-, and aminobenzoic acids, hydroxy-, and methoxycinnamic acids were examined and found that only *p*-aminobenzoic acid (yellow) and *p*-coumaric acid (pink) give a positive color reaction. However the limit of detection for these compounds is about 40–80 times (1–2 μg) higher than for most of the indoles tested.

The indoles in Table I are arranged according to their substitution(s) on the indole ring system. It has been reported that condensation of the indole derivative with *p*-DMAB occurs at the free C-2 position and results in a violet-blue color product if C-3 has a $-CH_2-R$ group[68]. A structural analysis of a number of indole condensation products by low- and high-resolution MS has shown that condensation of *p*-DMAB can also occur at C-3, N-1, and to a limited extent on C-5 and C-6 of the indole ring system (see Table I, 1-methylindole-2-carboxylic acid, carbazole and 1-phenylcarbazole)[69].

Although many of the C-3 substituted indoles such as Iaa and its esters, tryptamine and its derivatives have colors in the reddish-violet-blue color region, most exhibit sufficient color differentiation to allow their identification on TLC. We have chosen the Horticultural Colour Chart to illustrate this color differentiation (Table I, color region and color name). Frequently it is possible to identify tentatively endogenous indole derivatives from plant extracts by their colors and R_F values on TLC, and the subsequent identification by GLC–MS confirms the great diagnostic value of the chromogenic spray reagent.

Another advantage of the spray reagent for TLC is the relatively simple procedure. It takes about 10 min from the time of spraying until the wet-plate colors can be evaluated, and less than 1 h for the permanent color evaluation. The majority of the colors of the indole condensation products are extremely stable. TLC plates of some 40 indole standards at 1- and 5-μg concentrations and developed in solvent systems (1) and (4) have been kept for more than two years with virtually no fading or change of the original dry-plate colors.

Some indoles, such as indole, 1-methylindole, 1-acetylindole, 2-methylindole and indole-3-acrylic acid exhibit a characteristic color change as the plate dries (see Table I). It was found that this color shift and or fading is reversed to the original wet-plate color by rewetting the plate. It is thus possible to evaluate the wet-colors of those indoles which in the dry-plate state are no longer diagnostic. The colors of some of the indoles have been reversed as often as ten times in one day with no apparent loss of color or color intensity.

An important aspect of this reagent is the finding that it can also be used as

a colorimetric reagent with a sensitivity and specificity for many indoles equal to that on TLC. The use of this reagent for the colorimetric determination of indole derivatives will be published in a separate communication.

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DETERMINATION OF CHLORINATED PESTICIDES IN POTABLE WATER

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SUMMARY

A procedure is described for monitoring organochlorine pesticides in potable water. Material adsorbed from water on to Amberlite XAD-2 macroreticular resin was eluted with *n*-hexane and the concentrated eluate was analyzed without further treatment.

Electron capture (^{63}Ni) gas chromatography utilizing a column of 4% QF-1 and 2% SE-30 on 80–100 mesh HP Chromosorb W at 180° permitted identification of organochlorine pesticides and indicated the presence of phthalates. Confirmatory qualitative and quantitative analyses were done by specific ion-monitoring mass spectrometry. Levels determined in Ottawa drinking water were 17 ppt α-BHC, 1.3 ppt lindane, 0.7 ppt aldrin, and less than 0.05 ppt for each of heptachlor, heptachlor epoxide, dieldrin, endrin, chlordane, *o,p'*-DDT, *p,p'*-DDD, *p,p'*-DDT, and *p,p'*-DDE.

INTRODUCTION

The ubiquity of chlorinated pesticides and polychlorinated biphenyls (PCBs) and the consequential environmental hazards has created much interest. Although their occurrence in finished waters has been confirmed[1,2], the levels detected vary widely[1–5] and are dependent upon location, time of analysis, and method of analysis. Nevertheless, tentative standards for drinking water, based on toxicological considerations, have been established (Table I).

A variety of analytical procedures capable of measuring low levels of chlorinated organic compounds in water is documented. These include the use of activated

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TABLE I

COMPARISON OF DRINKING WATER STANDARDS

Pesticide	*Maximum permissible level (mg/l)* *		
	*Canada, NH & W. 1968** (ref. 6)*	*U.S. E.P.A.*	
		1972 (ref. 7)	*1975 (ref. 8)*
Aldrin	0.017	0.001	0.001
Chlordane	0.003	0.003	0.003
DDT	0.042	0.05	0.05
Dieldrin	0.017	0.001	0.001
Endrin	0.001	0.0005	0.0002
Heptachlor	0.018	0.0001	0.0001
Heptachlor epoxide	0.018	0.0001	0.0001
Lindane	0.056	0.005	0.004

* Equivalent to parts per million.

** Tentative standards for raw as well as treated water.

carbon[9,10], solvent extraction[11,12], reversed-phase liquid–liquid partition[3], reverse osmosis[4], polyurethane foams[13], support-coated silicones[5], and volatile stripping[14,15], and are discussed elsewhere[1,4]. Recently the use of porous polymer resins has found considerable success in the analysis of aqueous samples[16,17]. A low-polarity styrene–divinyl benzene copolymer (Amberlite XAD-2; Rohm & Haas, Philadelphia, Pa., U.S.A.) with a high sorptive capacity has been widely used[17]. The method[17] has been demonstrated to be accurate and reliable for the analysis of a large number of compounds, including chlorinated pesticides, occurring in water.

The present work was initiated in order to evaluate the use of macroreticular resins in the development of analytical methodology for Canadian drinking water. Organochlorine pesticides have been detected in numerous natural[1,3,5] and finished[2–5] water supplies, thus presenting a possible health hazard to the population. With this in mind, these investigations were focussed on the determination of chlorinated pesticide levels in Ottawa drinking water.

EXPERIMENTAL

Preliminary studies were conducted to determine a suitable method for the isolation of organochlorine pesticides from tap water. Liquid–liquid extraction[18] using *n*-hexane and cyclohexane, and extraction using 30 g XAD-2 and XAD-4 columns followed by elution with *n*-hexane (see below) was applied to four 100-l tap water samples. Comparison of the concentrated extracts by means of electron-capture gas chromatography (GC–ECD) indicated that cyclohexane and XAD-2 afford optimum pesticide recovery and minimum background. Recent advances with macroreticular resins[17] prompted the use of XAD-2 for further work.

All glassware was triply rinsed with distilled water, acetone, and hexane in turn and was oven-dried at 130° after each solvent wash. Residue-free (distilled in glass, Caledon Labs., Georgetown, Canada) solvents were used throughout. PTFE tubing, stopcocks, and liners were used where indicated due to the inert nature and versatility of the material.

Preparation of XAD-2 column

The column employed consisted of a 50 cm × 1.8 cm I.D. glass tube equipped with a removable PTFE stopcock and a 500-ml reservoir with a 24/40 $ joint. Amberlite XAD-2 resin (30 g, Rohm and Haas) was slurried in distilled water, and the fines were decanted; this procedure was repeated twice. A silanized glass wool plug was placed in the bottom of the column, and the aqueous slurry of XAD-2 was poured into the column and allowed to settle and drain until a 2-cm layer of water covered the resin. Another silanized glass wool plug was secured atop the resin and a modified clean-up procedure[19] as follows was applied. The column was eluted with distilled water (250 ml), *n*-hexane (250 ml), acetone (250 ml), and again with *n*-hexane (250 ml). Concentration of the final *n*-hexane eluate to 2 ml using a rotary evaporator was followed by GC–ECD (see below) of a 1-μl sample. Column elution with 250-ml portions of *n*-hexane was continued until a clean GC tracing of the concentrates was obtained.

Tap water extraction

The column reservoir was connected to a laboratory water tap by means of glass and PTFE tubing. Provision was made for overflow of water from the reservoir. The influent water, sampled during January, 1975, had a pH in the range 8.4–8.6 and was adjusted to a temperature of 20 ± 2° by control of cold and warm water taps.

A measured volume (100, 200, or 300 l) of tap water was passed through the column at a flow-rate of 15–20 ml/min. The column was allowed to drain until the water level reached the upper glass wool plug and was then eluted with 250 ml *n*-hexane at a rate of 15 ml/min. The *n*-hexane eluate, collected in a 500-ml, 24/40 $ jointed round bottom glass flask, was reduced to 5 ml by means of a Büchi rotary evaporator. A 50-ml, 24/40 $ jointed glass flask connected at the bottom to a 3-ml, calibrated, tapered tube[2] was used to further reduce the combined concentrate and three *n*-hexane rinses to a volume of 0.3 ml. The tightly glass-stoppered flask was stored at 4° until 1 h prior to analysis.

Gas chromatographic analysis

A Hewlett-Packard Model 5710 A gas chromatograph, equipped with a ^{63}Ni electron capture detector and a coiled glass column, 1.8 m × 3.5 mm I.D., packed with a mixture of 4% QF-1 and 2% SE-30 on 80–100 mesh HP Chromosorb W was used throughout the procedure.

Columns packed with 3% OV-17 on 80–100 mesh HP Chromosorb W, and a mixture of 3% QF-1 and 2% SE-30 on 80–100 mesh HP Chromosorb W, gave slightly inferior separation and were utilized as a confirmatory procedure. High-purity argon–methane (95:5) was used as the carrier gas at a flow-rate of 55 ml/min. The injector, oven, and detector temperatures were kept at 200°, 180°, and 300°, respectively. One microliter of standard and extract solutions were injected on to the columns.

Standard solutions were prepared by dissolving selected organochlorine pesticides and phthalates in *n*-hexane. All standards were analyzed individually, on an additive basis, and as a composite mixture in order to determine possible effects on retention time and for peak identification (Fig. 1, Table II). A negligible shift in relative retention time was noted upon comparison of results obtained with the composite mixture and the individual components.

TABLE II

GC–ECD IDENTIFICATION OF SOME ORGANOCHLORINE PESTICIDES IN OTTAWA TAP WATER

XAD-2 extract peak No.	*Retention time relative to aldrin*		*Standard compounds*
	XAD-2 extract	*Standards mixture**	
1	0.13	0.11	chlordane
2	0.18	0.18	chlordane
3	0.23**		
4	0.28	0.31	chlordane
5	0.35	0.35	α-BHC
6	0.40**		
7	0.43	0.44	lindane
8	0.57**		
9	0.64	0.61	chlordane
10	0.74	0.72	dieldrin
11	0.84	0.80	heptachlor
12	0.94**		
13	1.00	1.00	aldrin
14	1.02	1.02	chlordane
15	1.22	1.22	chlordane
16	1.33	1.34	heptachlor epoxide
17	1.41**		
18	1.63	1.62	chlordane
19	1.77	1.77	chlordane
20	1.93	1.89	chlordane
21	2.04	2.05	endrin + *p,p'*-DDE
22	2.21**		
23	2.68**		
24	2.87	2.92	*o,p'*-DDT
25	3.16**		
26	3.51**		
27	3.75	3.77	*p,p'*-DDD
28	4.14**		
29	6.40**		
30	7.78**		
31	9.09**		
32	10.50	10.50	di(2-ethylhexyl) phthalate

* Only peaks which closely match those in XAD-2 extracts are listed.

** Comparison with Arochlor 1254 suggests that these may represent PCBs.

Since the interference of PCBs on pesticide analyses has been noted[20], a standard solution containing 0.3 ng/μl of each component and an excess (5.0 ng/μl) of Arochlor 1254 was analyzed. Retention time and peak height of the other components were unaffected by the preponderance of Arochlor 1254. Initially the Armour–Burke PCB separation procedure[21] was successfully applied to a tap water extract and a composite standards mixture. However, use of this technique was discontinued since it was found that analyses of untreated samples, using the reported conditions, gave better overall results.

Comparison of results obtained from analyses of standards, tap water extracts, and tap water extracts spiked with small quantities of aldrin confirmed the occurence of organochlorine pesticides and phthalates in Ottawa tap water (Table II, Figs. 1 and 2).

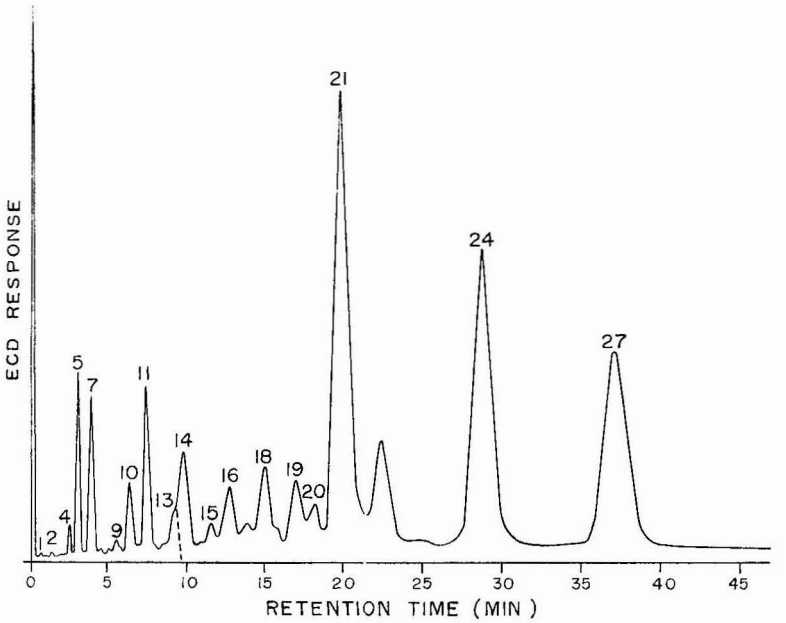

Fig. 1. Electron capture gas chromatogram of standard pesticide mixture (0.3 ng of each component). For peak numbering, see Table II. The operating parameters for the gas chromatograph are described in the text.

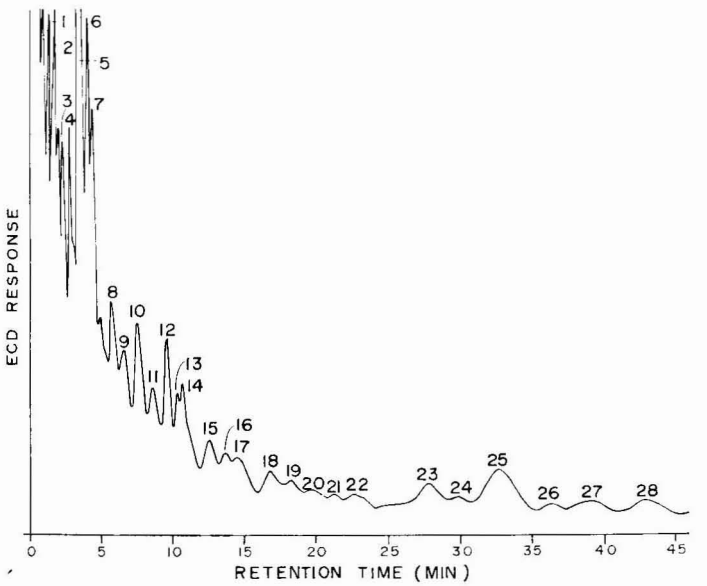

Fig. 2. Electron capture gas chromatogram of XAD-2 extract (from 200 l of Ottawa tap water). For peak numbering, see Table II. The operating parameters for the gas chromatograph are described in the text.

Gas chromatographic–mass spectrometric analysis

Qualitative and quantitative analyses of standards and tap water extracts were performed on a Varian Model 1440 gas chromatograph interfaced to a Varian MAT 311A high-resolution mass spectrometer with Watson-Biemann separator.

The GC conditions were: columns, 4% QF-1 and 2% SE-30 on 80–100 mesh HP Chromosorb W in a coiled glass column, 1.8 m × 3.5 mm I.D.; column temperature isothermal, 180–240°, depending on samples; injector temperature, 275°; interface temperature, 275°; carrier gas, helium at a flow-rate of 30 ml/min.

The mass spectrometric (MS) conditions were: source temperature, 250°; emmission, 3 mA; accelerating voltage, 3 kV; S.E.M. voltage, 2 kV; electron energy, 70 eV; resolution, $M/\Delta M = 5000$ (10% valley).

The instrument was operated in the specific ion-monitoring mode with perfluorokerosene as the reference compound. Masses to be monitored were set on the peak matching unit and were checked regularly against the reference peak. Comparison of peak heights with those of standards analyzed under identical conditions allowed calculation of quantitative data (Table IV).

Recovery studies

Although studies on the retention of organics in water by macroreticular resins vary in methodology[16,17,22,23], they generally indicate high retention efficiency. A procedure was developed to confirm the recovery efficiency of the present analytical scheme.

Two XAD-2 columns, identical to the type used for tap water extraction, were connected in tandem by means of a short piece of PTFE tubing and 24/40 $\bar{\S}$ jointed

TABLE III

PER CENT RECOVERY EFFICIENCY FOR XAD-2 TAP WATER EXTRACTION DETERMINED BY GG–MS

Compound	*1 µl standard solution**		*1 µl XAD-2 eluate*** *peak height (cm)*	*Per cent recovery*
	Weight injected (ng)	*Peak height (cm)*		
α-BHC	13	16.3	12.2	75
Lindane	1.0	1.3	1.0	77
Heptachlor	1.0	11.4	8.5	75
Heptachlor epoxide	1.0	3.7	2.7	73
o,p'-DDD	0.5	24.0	17.0	71
o,p'-DDT	0.5	5.3	4.9	92
p,p'-DDT	0.5	1.8	1.8	100
p,p'-DDE	0.5	12.8	9.4	73
Aldrin	1.0	5.1	4.1	80
Chlordane	0.5	14.5	13.4	92
Dieldrin	1.0	10.3	9.0	87
Endrin	1.0	10.9	9.2	84
Dibutyl phthalate	30	22.5	23.5	104
Di(2-ethylhexyl) phthalate	100	16.3	21.0	129

* 0.3 ml of this solution on an XAD-2 column was washed with 100 l of XAD-2 treated tap water and the components were eluted with 250 ml *n*-hexane which was concentrated to give 0.3 ml of XAD-2 eluate (see text).

glass connecting tube. The apparatus was connected to a laboratory water tap as previously indicated and 100 l of tap water were passed through at a flow-rate of 15–20 ml/min. Both columns were allowed to drain and were eluted separately with 250 ml *n*-hexane. The eluates were concentrated to 0.3 ml in the usual manner. GC–MS spectrometric analysis of the eluate from the upper column indicated the presence of organochlorine pesticides and phthalates, while no such materials were detected in the lower column eluate blank.

Per cent recovery determinations with this apparatus were preceded by reapplication of the column clean-up procedure and flushing with tap water (500 ml) to remove residual *n*-hexane. A 0.3-ml aliquot of standards in *n*-hexane solution (Table III) was added to the lower column reservoir, which contained 450 ml of tap water treated by passage through the upper column. The water level was drained to within 2 cm of the glass wool plug, 400 ml of treated water were added to the reservoir, the apparatus was assembled, and 100 l of tap water were passed through the system. The columns were drained, eluted with 250 ml *n*-hexane, and the eluates were concentrated to 0.3 ml.

Comparison of the lower column eluate and the solution containing standards in *n*-hexane by means of GS–MS allowed calculation of per cent recovery data (Table III).

TABLE IV

GC–MS ANALYSIS OF OTTAWA TAP WATER XAD-2 EXTRACT

Compound	*Ion mass monitored**	*Column temperature (°C)*	*Retention time (min)*	*Concentration detected in Ottawa tap water*** (ppt)*
α-BHC	218.9116 180.9379	180	2.0	17
Lindane	218.9116	180	2.4	1.3
Aldrin	66.0469 363.8728	180	4.7	0.70
Heptachlor	271.8102	180	3.8	⩽0.013
Heptachlor epoxide	271.8102	180	5.9	⩽0.044
Dieldrin	379.8678 271.8102 344.8988	180	6.7	⩽0.0095
Endrin	344.8988	200	5.3	⩽0.013
o,p'-DDD	235.0081	200	4.7	⩽0.0012
o,p'-DDT	235.0081	200	6.0	⩽0.0001
p,p'-DDD + *p,p'*-DDT***	235.0081	200	7.4	⩽0.0005
p,p'-DDE	317.9351	200	4.5	⩽0.016
Chlordane	372.8260	200	3.5	0.0053
Dibutyl phthalate	149.0239	200	2.5	29
Di(2-ethylhexyl) phthalate	149.0239	240	3.5	78

* Data obtained from ref. 27.

** Quantitative data obtained by comparison of GC–MS analyses of standards and tap water extracts were adjusted by per cent recovery values (Table III).

*** Both *p,p'*-DDD and *p,p'*-DDT had identical retention times and both gave strong peaks at *m/e* 235. They can be distinguished by their different molecular ions, but since none could be detected distinction was unnecessary.

RESULTS AND DISCUSSION

The use of XAD-2 resin for isolation and recovery of a variety of organic compounds from water and for development as a standardized analytical procedure has been explored[17]. The present study has confirmed the utility of the method for determining selected organochlorine pesticides in drinking water.

Measured volumes of tap water were extracted by passage through 30 g XAD-2 columns. The modified XAD-2 column clean-up usually resulted in clean blanks after only one series of *n*-hexane, acetone, *n*-hexane washes. This rapid and efficient method obviated the use of long sequential solvent extraction methods[17]. Use of the appropriate GC column (4% QF-1 and 2% SE-30 on HP Chromosorb W) allowed direct injection and analysis of the concentrated *n*-hexane eluate from the XAD-2 column. Under the specified conditions, good resolution and retention time reproducibility as well as suppressed PCB response and interference were achieved.

Major GC peaks with retention times greater than 0.1 (relative to aldrin) were identified (Table II). Confirmatory analysis and quantitation was achieved by means of specific ion-monitoring GC–MS (Table IV). Material from at least 200 l of tap water was required for quantitative analysis. The sensitivity of the method is achieved, in part, by efficient extraction (see Table III) and effective concentration of the sample extract from 300–0.3 ml, *i.e.*, a factor of 10^6. Analytical sensitivity is largely limited by operational parameters as well as nature of the sample. Use of a Varian Model 1440 gas chromatograph interfaced to a Varian MAT 311A high-resolution mass spectrometer operated in the specific ion-monitoring mode gave sensitivity in the order of nanograms for most of the chlorinated pesticides. The overall limit of detection under the experimental conditions is approximately 0.01 ppt* for the compounds studied. This limit could be extended by the application of aids such as time-averaging computer.

A procedure utilizing XAD-2 treated tap water was designed for the determination of per cent recovery for the analytical scheme. The aim of the design was to duplicate the experimental conditions as closely as possible, without introduction of unknown quantities of the materials under study. Determination of blanks for the clean-up column and the analytical column (see above) indicated, as anticipated, the presence of materials in the former column while no material was detected in the latter column. Per cent recoveries, ranging from 71–100% for organochlorine pesticides were found (Table III). These values are consistent with those found by other workers[16,17]. Values exceeding 100% for the phthalates are probably due to contamination resulting from the ubiquity of phthalates.

The possible presence of taste- and odour-producing or toxic organic contaminants, even in low concentration, in drinking water is of concern to the consumer. Concentrations of organochlorine pesticides detected in Ottawa tap water (Table IV) are expressed in terms of 17 ppt or less. These correspond to values found elsewhere[3] and are well below published maximum permissible levels for drinking water (Table I). They also correspond to levels determined by *n*-hexane liquid–liquid extraction[24] and are somewhat lower than those found in Ottawa River water[25]. The results (Table IV) show very low levels of DDT and its analogues, compounds whose

* Throughout this article, the American trillion (10^{-12}) is meant.

occurrence in the environment has resulted in many investigations and controversies. Also, the preponderance of α-BHC as compared to the isomeric but more toxic γ-BHC (lindane) and the other identified pesticides should be noted. Similar results have been observed elsewhere in surface water[26].

The use of XAD-2 macroreticular resin in the determination of low levels of organochlorine pesticides in water is a useful technique. Its potential for development into a standard procedure is clearly indicated.

Qualitative and quantitative analysis of other organic components in treated water is being pursued. Application of temperature programming (70–180°) to the analytical method provides a means to resolve the more volatile substances in the extracts. Recent studies indicate the occurrence of many such substances in Ottawa drinking water in low concentration.

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CHROM. 9704

GAS CHROMATOGRAPHIC DETERMINATION OF PROPOXYPHENE AND NORPROPOXYPHENE IN PLASMA

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SUMMARY

Propoxyphene and its major metabolite norpropoxyphene have been determined simultaneously by using gas chromatography. In order to avoid the on-column decomposition of the propoxyphene, derivatives were formed with the aid of lithium aluminium hydride, which cleaves the propionic acid ester of propoxyphene and reduces the norpropoxyphene amide. Promethazine was used as an internal standard.

Propoxyphene and norpropoxyphene levels in plasma were determined in samples from six male volunteers receiving a single oral dose of 150 mg of propoxyphene hydrochloride in a sustained-release form (Abalgin Retard®).

INTRODUCTION

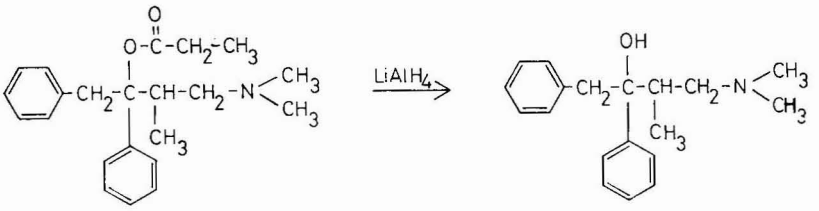
In recent years, several papers have been published on the determination of propoxyphene in plasma and in most instances[1–10] gas chromatography was employed. Only a few of the authors[1–3] mentioned the on-column decomposition of the drug. Sparacino *et al.*[1] reported that the nature of the solid support and of the silanization agent are decisive for this breakdown. However, we have experienced that even columns which do not decompose propoxyphene immediately do so progressively after a few months' routine work, and silanization does not improve the results.

In order to overcome these problems, we decided to prepare a derivative of propoxyphene. The cleavage of the propionic acid ester leads to the formation of a chromatographically stable tertiary alcohol (Fig. 1), and the reagent used, lithium aluminium hydride, also reacts with norpropoxyphene amide, yielding 4-(methylpropylamino)-1,2-diphenyl-3-methyl-2-butanol (Fig. 2). The retention times of the reacted compounds are shorter than those of the unreacted drugs (Table I).

EXPERIMENTAL AND RESULTS

Chemicals and reagents

Propoxyphene and norpropoxyphene. An aqueous stock solution containing 2.5 μg/ml of *d*-propoxyphene hydrochloride and 5 μg/ml of N-desmethylpropoxyphene maleate (norpropoxyphene) is stable for several months when stored at 4°.



d - Propoxyphen

Fig. 1. Reaction of propoxyphene with lithium aluminium hydride.

Internal standard. An aqueous solution of promethazine hydrochloride (Dott. Bonapace et Cie, Milan, Italy) (25 μg/ml) must be prepared freshly every day.

Derivatization agent. Lithium aluminium hydride (Riedel de Haën, Seelze-Hannover, G.F.R.) (100 mg) was shaken with 10 ml of dried diethyl ether for 15 min. When the excess of the hydride has settled, the solution is ready for use.

Other chemicals. Redistilled *n*-butyl chloride, dried distilled diethyl ether, distilled water, carbon tetrachloride (Uvasol, Merck, Darmstadt, G.F.R.), 1 *M* carbonate buffer [pH 9.8 (ref. 11)], 0.2 *N* hydrochloric acid, 4 *N* sodium hydroxide solution and a 2% solution of trimethylamine in acetone were used.

Glassware

Centrifuge tubes of 15-ml volume with screw-caps were silanized as described

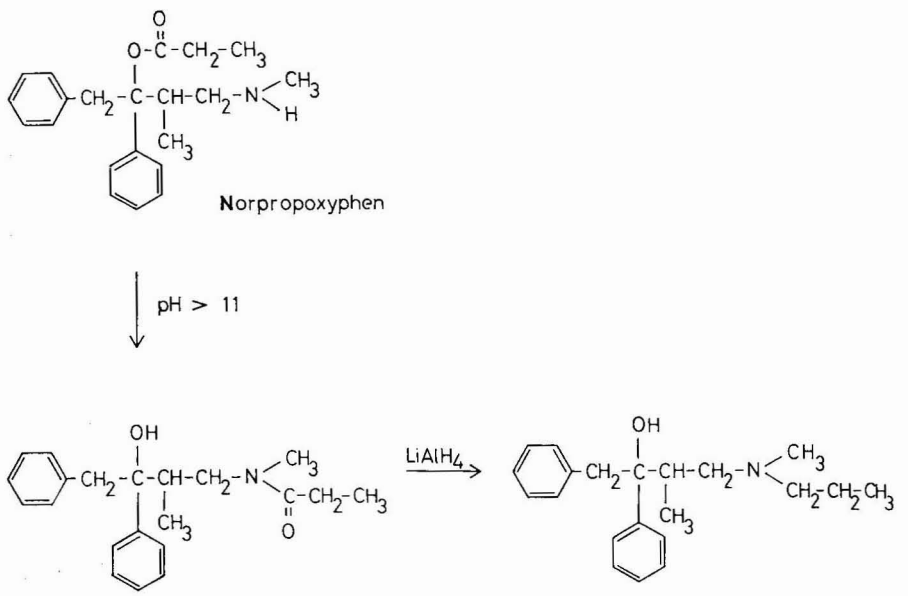


Fig. 2. Conversion of norpropoxyphene into an amide and subsequent reduction with lithium aluminium hydride.

by Walle and Ehrsson[12] and washed before use with the 2% solution of trimethylamine in acetone in order to prevent adhesion of the amines to the glass.

Gas–liquid chromatography (GLC)

The GLC analysis was performed on a Varian Aerograph Model 2100 chromatograph equipped with a flame ionization detector. The U-shaped glass columns (1.8 m × 2 mm I.D.) were silanized. The packing material consisted of 3% OV-17 on Chromosorb W AW DMCS, 80–100 mesh. The temperature of the oven was 180°, injection port 200° and detector 250°. The carrier gas was nitrogen at a flow-rate of 40 ml/min. The hydrogen flow-rate was 40 ml/min and the oxygen flow-rate 200 ml/min. The detector sensitivity was $8\text{–}64 \cdot 10^{-12}$ A/mV at full scale.

Sample handling

Blood samples were drawn into heparinized tubes, centrifuged and the plasma was decanted and stored at −24°. This storage does not interfere with the analytical results[4,10].

Extraction procedure

The extraction procedure was a modification of the method described by Verebely and Inturrisi[6]. To 5.00 ml of plasma were added 0.200 ml of the solution of the internal standard, 1.00 ml of carbonate buffer and 5.00 ml of *n*-butyl chloride. The plasma was extracted by vertical rotation (32 rpm) for 15 min and centrifuged immediately for 5 min at 1700 *g*.

After freezing (−24°), the upper phase (*n*-butyl chloride) was decanted into another tube containing 2.00 ml of 0.2 *N* hydrochloric acid. The residue was washed with 1.0 ml of *n*-butyl chloride, and the combined *n*-butyl chloride phases were shaken automatically for 5 min with the acidic phase. After centrifugation for 5 min, the *n*-butyl chloride phase was discarded and the acidic phase washed by shaking it for 5 min with 5.0 ml of diethyl ether. The diethyl ether was discarded after centrifugation and the residue washed with 1.0 ml of *n*-butyl chloride.

TABLE I

RETENTION TIMES OF PROPOXYPHENE, NORPROPOXYPHENE AND PROMETHAZINE

Compound	*Retention time (min)*	
	Untreated	*$LiAlH_4$ treated*
Propoxyphene	12.1	8.0
Norpropoxyphene	82.0	13.0
Promethazine	20.3	20.3

The aqueous phase was made alkaline by the addition of 0.300 ml of 4 *N* sodium hydroxide solution and 15 min later was extracted with 1.00 ml of *n*-butyl chloride by shaking for 5 min. After centrifugation for 5 min, the aqueous phase was frozen and the *n*-butyl chloride decanted. Lithium aluminium hydride in diethyl ether (0.500 ml) was added and the samples were left for 15 min in a water-bath (35–40°).

Excess of lithium aluminium hydride was destroyed by the addition of 0.200 ml of water and, after centrifugation, the water was frozen. The organic phase was decanted and evaporated to dryness in vacuum. The tubes were washed with three drops of *n*-butyl chloride and the *n*-butyl chloride was evaporated. The residue was dissolved in 10 μl of carbon tetrachloride and 1 μl of the solution was injected into the gas chromatograph.

Quantitation

Peak heights were measured and the ratios of propoxyphene and norpropoxyphene to the internal standard calculated. Four standards at two concentrations were made every day in order to construct calibration graphs. All values were calculated as propoxyphene hydrochloride equivalents expressed in nanograms.

Linearity and precision

Linearity of the peak-height ratio was established by the assay of 18 plasma samples containing known amounts of propoxyphene and norpropoxyphene, six samples for each of the three levels used. The results are shown in Table II.

TABLE II

LINEARITY AND PRECISION

Parameter	*Propoxyphene*			*Norpropoxyphene*		
Plasma concentration (ng/ml)	20	55	90	40	110	180
Peak-height ratio (mean for $n = 6$)	0.052	0.168	0.294	0.064	0.183	0.303
Standard deviation of peak-height ratio	0.0063	0.0136	0.0301	0.0059	0.0190	0.0266
(% of mean)	(12)	(8)	(10)	(9)	(10)	(9)
Correlation coefficient ($n = 18$)	0.984			0.984		

It can be seen from the correlation coefficients that the linearity is excellent, while the precision, as expressed by the standard deviation, must be considered satisfactory in view of the very low plasma concentrations.

Later experience showed that the slight positive intercepts on the ordinate by the calibration lines resulting from this experiment (5 ng for propoxyphene and 2 ng for norpropoxyphene) were fortuitous, so in routine work all standard graphs are made to pass through the origin.

Recovery

Internal standard was added to plasma samples ($n = 6$), taken through the whole procedure and propoxyphene alcohol dissolved in *n*-butyl chloride (corresponding to 50 ng/ml of propoxyphene in plasma) was added just before evaporation. The ratio of the peak heights was calculated to be 100%. The internal standard and propoxyphene (50 ng/ml in plasma) were added to other plasma samples ($n = 6$) and taken through the procedure. The peak-height ratio thus obtained was divided by the 100% ratio, and showed a net recovery of 53%.

DISCUSSION

Experimental conditions

pH. Like other workers, we found it essential to maintain a pH of 9.8 during the extraction from plasma. A higher pH will cause the rearrangement of norpropoxyphene to an amide that cannot be re-extracted from *n*-butyl chloride with hydrochloric acid. On the other hand, when carrying out the final part of the extraction procedure, it is important that the pH is increased to above 11 (refs. 6, 13 and 14) and that the samples are allowed to stand for 15 min before extraction in order to ensure a 100% yield of the amide (see Fig. 2).

Freezing of the samples. The samples are frozen in order to reduce the working time required. In the step just before the formation of the derivative, this procedure also ensures the essential low content of water in the *n*-butyl chloride phase (see below).

Washing of the acid phase with diethyl ether. This procedure will remove cholesterol, which cannot be eliminated with *n*-hexane as used by Verebely and Inturrisi[6]. The cholesterol has a retention time of about $2\frac{1}{2}$ h under the GLC conditions mentioned.

Derivatization of propoxyphene

Comparing the GLC retention times and the IR spectra of the hydrochlorides (Fig. 3), we found the derivative of propoxyphene to be identical with *d*-4-(dimethylamino)-1,2-diphenyl-3-methyl-2-butanol, synthesized by Ravensberg GmbH. To investigate whether this alcohol from propoxyphene is formed *in vivo*, we analyzed some samples without the formation of the derivative. Plasma samples were obtained from 10 male volunteers 3 h after administration of 150 mg of timed-release propoxyphene hydrochloride (Abalgin Retard®). The concentration of propoxyphene in the pooled plasma samples was 45 ng/ml, and we were not able to detect the alcohol (the detection limit is *ca.* 2 ng/ml).

Derivatization of norpropoxyphene

Norpropoxyphene has to be converted into the amide before reaction with lithium aluminium hydride, otherwise two reaction products will be formed. Under

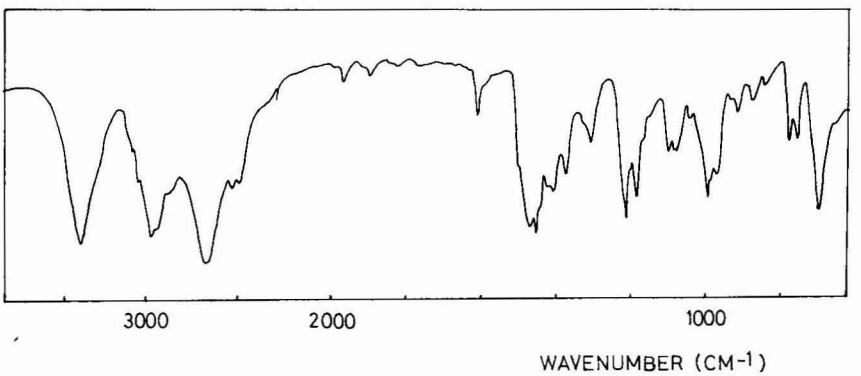


Fig. 3. IR spectrum of the derivative of propoxyphene identical with *d*-4-(dimethylamino)-1,2-diphenyl-3-methyl-2-butanol.

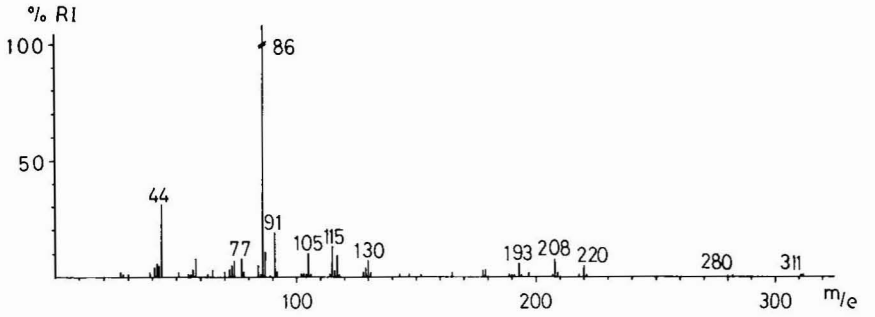


Fig. 4. Mass spectrum of the derivative of norpropoxyphene amide identical with 4-(methylpropylamino)-1,2-diphenyl-3-methyl-2-butanol.

the conditions described (pH above 11 and the samples allowed to stand for 15 min before extraction), only one product is formed. This derivative was identified by mass spectrometry as 4-(methylpropylamino)-1,2-diphenyl-3-methyl-2-butanol (see Fig. 4). When analyzing plasma without the formation of the derivative, no peak is seen in the chromatograms with a retention time corresponding to this compound.

Lithium aluminium hydride

As lithium aluminium hydride reacts violently with water, very low water contents in the *n*-butyl chloride phase and in the diethyl ether are essential. In destroying lithium aluminium hydride with water, highly alkaline reaction products are formed and thus all of the amines remain in the *n*-butyl chloride–diethyl ether phase. Promethazine does not react with lithium aluminium hydride.

The formation of the derivative can be performed at the relevant concentrations with the following standard deviations: propoxyphene 4% ($n = 14$) and norpropoxyphene 6% ($n = 15$).

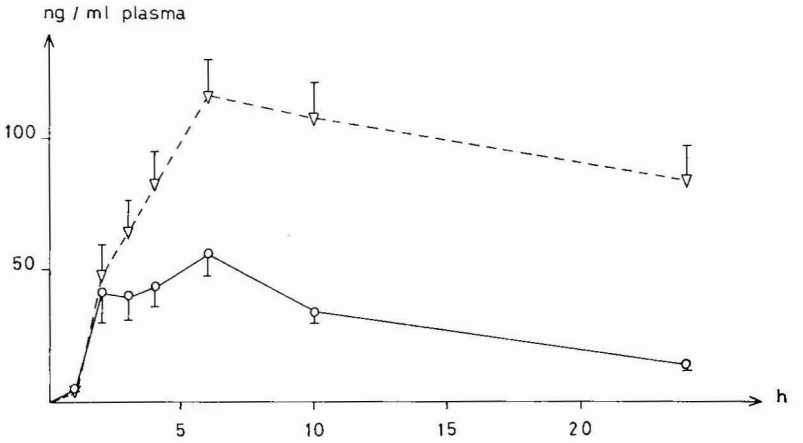


Fig. 5. Plasma levels of propoxyphene (———) and norpropoxyphene (— - - —) following an oral dose of 150 mg of propoxyphene hydrochloride in a sustained-release form (Abalgin Retard). The results represent the mean for six male volunteers, and the standard error of the mean is indicated.

Solvent

Carbon tetrachloride is a suitable final solvent as the flame-ionization detector gives a very small response to this compound. Unfortunately, the internal standard is not stable in this solvent. Consequently, the residue has to be dissolved immediately before the injection into the gas chromatograph.

APPLICATION

Six male volunteers received 150 mg of propoxyphene hydrochloride in a sustained-release form (Abalgin Retard). *In vitro*, an amount of 150 mg is released by 26–44%, 56–74% and not less than 80% after 1, 4 and 8 h, respectively[15].

Blood samples were drawn at 0, 1, 2, 3, 4, 6, 10 and 24 h. Apart from an overnight fast, no specific dietary measures were taken except that the consumption of alcohol was not permitted. The results are given in Fig. 5 and examples of the chromatograms are given in Fig. 6.

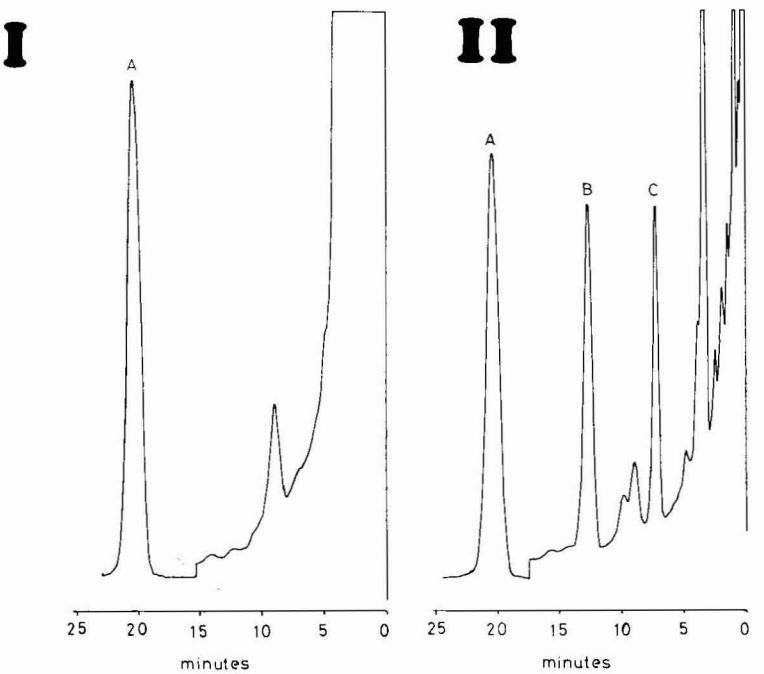


Fig. 6. Gas chromatograms of human plasma extracts. I, Blank containing internal standard (A); II, plasma sample drawn 6 h after administration (A represents the internal standard, B represents 97 ng/ml of norpropoxyphene in plasma and C represents 47 ng/ml of propoxyphene in plasma).

CONCLUSION

Gas chromatography is the method most widely used for the determination of propoxyphene in plasma (and urine), and so on-column decomposition is a problem of general importance. Sullivan *et al.*[8] and Wolen *et al.*[9] avoided the problem by the use of deuterium-labelled internal standards and chemical-ionization mass fragmento-

graphy in combination with gas chromatography. In this instance, the decomposition that may still occur causes no problems as the internal standard will decompose to exactly the same degree.

The method described in this paper avoids the decomposition through derivatization and is thus rendered practicable for the equipment possessed by most analytical laboratories.

The formation of the derivative is not time consuming, the reacted compounds have a shorter retention time than the unreacted drugs, they are chromatographically stable and they do not represent natural metabolites that occur in plasma in detectable amounts. Finally, the standard deviation of the method of about 10% is satisfactory, considering the low plasma concentrations.

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CHROM. 9614

DÉTECTION PAR SPECTROMÉTRIE INFRAROUGE EN CHROMATOGRAPHIE LIQUIDE À HAUTE PERFORMANCE

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(Reçu le 7 juillet 1976)

SUMMARY

Detection by infrared spectrometry in high-performance liquid chromatography

The cell of a Perkin-Elmer 521 spectrophotometer is adapted to the requirements of infrared detection. It is possible to choose a mobile phase which is suitable at the wavelength used. Two types of separations are realised with this detection system.

The first example is realised with isooctane as a mobile phase. The transparency of this classical solvent in infrared permits to work with 1748 cm^{-1} radiation corresponding to the absorption of the function $\diagdown C = O \diagup$ of esters.

The second example is made with a mixture of tetrachlorodiflouroethane–trichlorotrifluoroethane (20:80). This eluent can be used for many separations, its absorption in infrared is almost the same as that of carbon tetrachloride.

The experimental conditions for utilisation of the infrared detector are discussed.

INTRODUCTION

Depuis quelques années, d'énormes progrès ont été réalisés en chromatographie liquide. En effet, les supports de fine granulométrie (5 et 10 μm) ont permis d'atteindre des efficacités tout à fait remarquables et simultanément l'appareillage a suivi cette évolution.

Cependant, la détection reste un point en retrait par rapport aux efficacités de séparation maintenant réalisables. Les deux modes classiques sont insuffisants: la spectrophotométrie dans l'ultraviolet est limitée dans son application et la réfractométrie différentielle par sa sensibilité trop faible. Il faut donc s'intéresser à d'autres modes de détection.

Récemment, certains auteurs, dont les travaux ont entre autre été rappelés au XI Symposium de Chromatographie à Barcelone[1], ont essayé d'utiliser la détection électrochimique. Celle-ci peut permettre de résoudre un certain nombre de problèmes, qu'il s'agisse des méthodes coulométriques ou ampérométriques. Dans le cas de cette

dernière nous avons pu montrer que, par l'apport d'un électrolyte support, on peut adapter cette détection à tous les types de chromatographie[2]. Malheureusement, la pollution qui apparait aux électrodes restreint un peu cette méthode de détection et ce problème ne peut être considéré comme réellement résolu.

Parallèlement, nous nous sommes intéressés à un autre mode de détection faisant appel à la spectrométrie infrarouge. Ce mode de détection ne parait pas avoir encore donné lieu à publication. Toutefois, la spectrométrie infrarouge a déjà été utilisée en aval du détecteur pour réaliser le spectre des solutés après séparation afin de permettre leur identification[3,4]. Comme tous les composés organiques présentent au moins une bande de vibration infrarouge active, le champ d'application de cette technique parait assez étendu. Plusieurs problèmes sont à résoudre: (i) choix de l'appareillage et construction d'une cellule à circulation de liquide de faible volume, (ii) choix du solvant et (iii) choix des conditions opératoires et notamment de la fréquence de travail. Notre travail a abordé ces différents points.

PARTIE EXPÉRIMENTALE

Le montage chromatographique comporte une pompe (Minipump Dosapro) avec un système d'amortissement constitué de deux tubes Bourdon reliés par un capillaire. La détection est assurée à l'aide d'un spectrophotomètre à double faisceau et monochromateur à réseau (Perkin-Elmer Type 521) muni de cellules à circulation de liquide. L'injection est faite par seringue (0.5–5 μl) à travers une tête d'injection (Reeve-Angel).

Les colonnes utilisées ont été conçues pour des séparations en chromatographie de partage à l'aide de phases imprégnées. Les colonnes ont été remplies au laboratoire par voie humide[5] et imprégnées soit de β,β'-oxydipropionitrile (ODPN), soit de Carbowax 400[6].

RÉSULTATS ET DISCUSSION

Détection du signal

On cherchera à obtenir un signal proportionnel à la concentration du soluté traversant la cellule du détecteur. Dans le cas présent, ceci sera réalisé si, selon la loi de Lambert-Beer on détecte la variation d'absorbance de l'éluat en fonction de la concentration du soluté. Or l'appareil utilisé fournit un enregistrement linéaire en pourcentage de transmission. De plus les variations de transmittance produites par le soluté n'excèdent pas en général 10%. Aussi il est nécessaire d'utiliser un montage annexe à la fois pour amplifier le signal et pour le rendre linéaire en unités de densité optique et donc en concentration. Ce résultat est obtenu en utilisant l'amplificateur logarithmique d'un lecteur de concentration (Quanta de Type S 32). L'ensemble est schématisé (Fig. 1). L'enregistrement se fait alors sur un enregistreur extérieur à plusieurs sensibilités (Solea-Tacussel Type EPL 1).

Cellule

Deux types de cellules de détection ont été utilisés. (1) La Fig. 2 présente le montage le plus simple: une cellule à liquide scellée classique, de chemin optique 1 mm, a eu son volume interne réduit à 100 μl par l'utilisation d'un spacer en plomb

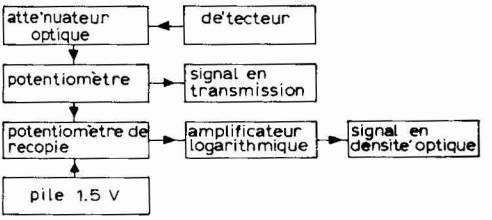


Fig. 1. Montage annexe pour la mesure des densités optiques.

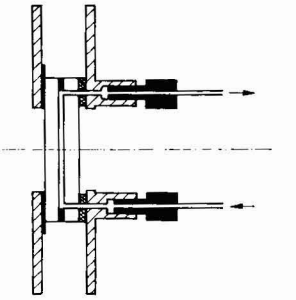


Fig. 2. Cellule à liquide classique.

muni d'une ouverture plus étroite. Les bouchons en PTFE de la cellule sont percés, suivant leurs axes, aux diamètres extérieurs des canalisations de jonctions, elles-mêmes en PTFE Introduites dans les bouchons, les extrémités de ces canalisations sont munies d'un méplat. L'étanchéité est assurée d'une manière convenable par compression de ces méplats contre le fond des ouvertures du porte-cellule. (2) La Fig. 3 présente un ensemble constitué d'une microcellule scellée à fenêtres KBr, de chemin optique 0.2 mm, de volume 5 μl (Perkin-Elmer) et d'un porte-cellule en acier inoxydable. Ce dernier est muni de deux capillaires de 0.5 mm de diamètre interne et diamétralement opposés qui permettent la circulation de l'effluent chromatographique à l'intérieur de la cellule. L'étanchéité est assurée par compression de joints en PTFE entre les faces externes des fenêtres de la cellule et les faces internes du porte cellule. Dans ces deux montages, la circulation du liquide est réalisée du bas de la cellule vers le haut afin de permettre un meilleur remplissage et d'éviter la formation de bulles.

Choix du solvant

On a déjà signalé que tous les produits organiques possèdent des vibrations actives dans l'infrarouge. Il est donc indispensable de choisir un solvant qui présente

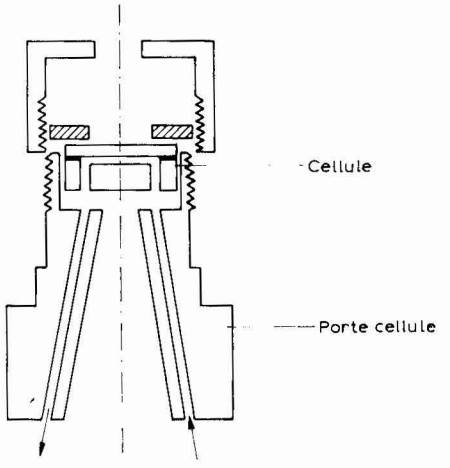


Fig. 3. Microcellule.

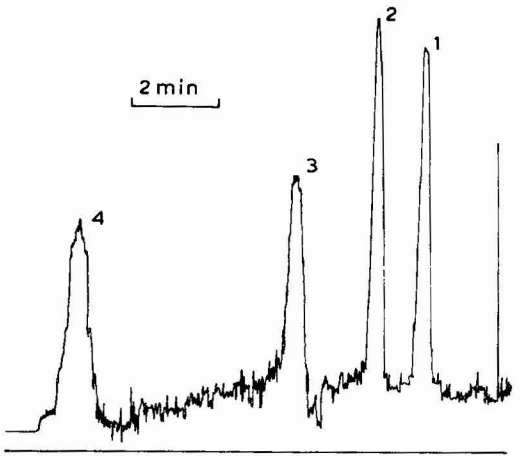


Fig. 4. Séparation d'esters en chromatographie de partage. Colonne, LiChrosorb SI-60 (5 μm) + ODPN; éluant, isooctane, 2 ml/min; s = 250 mV; $\bar{\nu}$ = 1748 cm^{-1}; fentes, 150 μm; cellule, 100 μl (l = 1 mm); injection, 1 μl. 1 = Dioctylphtalate; 2 = diéthyladipate; 3 = diéthylphtalate; 4 = diméthylphtalate.

une fenêtre de transmission au voisinage de la fréquence de détection des solutés. Le chromatogramme (Fig. 4) montre une séparation d'esters réalisée en utilisant l'isooctane comme solvant, la détection se faisant à 1748 cm^{-1} sur la vibration de valence

\
C=O de la bande fonctionnelle.
/

En fait, pour rendre ce mode de détection universel, il semble nécessaire de travailler entre 3100 et 2800 cm^{-1} dans la région des vibrations de valence des groupements alkyls. Or la plupart des solvants utilisés en chromatographie liquide présentent de tels groupements et absorbent donc dans ce domaine. Les solvants classiques utilisés en spectroscopie infrarouge, comme CS_2 et CCl_4, peuvent être de bons éluants pour la chromatographie liquide. Toutefois, leur force éluante, respectivement 0.15 et 0.18 dans l'échelle de Snyder[7], est trop importante pour remplacer les hydrocarbures pratiquement apolaires. De plus, ils possèdent des points d'ébullition assez bas et une toxicité élevée.

Un essai réalisé avec un mélange 20:80 de tétrachlorodifluoroéthane (CCl_2F–CCl_2F) et de trichlorotrifluoroéthane (CCl_2F–$CClF_2$), Foranes 112 et 113, (Ugine Kuhlmann), montre que la polarité de cette phase mobile est encore trop importante, donc cette phase ne peut remplacer systématiquement les hydrocarbures (Fig. 5). L'utilisation de solvants plus proches des fluoroalcanes devrait permettre de résoudre ce problème.

Fréquence de détection

La fréquence de détection du soluté détermine le choix du solvant mais l'ouverture des fentes du monochromateur est également un paramètre fondamental. D'une part, l'énergie reçue par le détecteur est proportionnelle à la surface de la fente d'entrée du monochromateur donc à la largeur géométrique de cette fente. D'autre part,

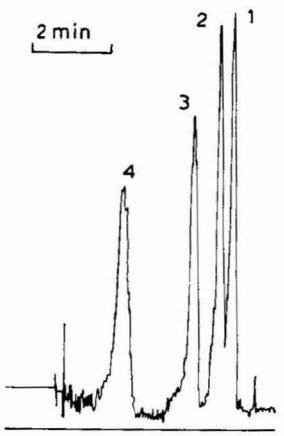


Fig. 5. Exemple d'utilisation d'un mélange de foranes. Memes conditions que Fig. 4, sauf: éluant, Forane 112–Forane 113 (20:80), 1.5 ml/min.

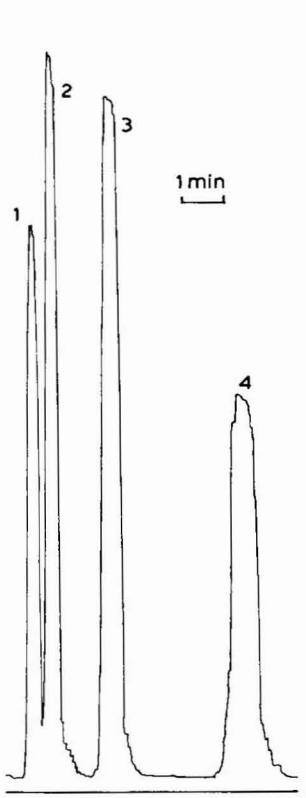
le pouvoir de résolution du spectrophotomètre dépend de la largeur de fente spectrale, qui est une fonction linéaire de la largeur de fente géométrique. Enfin, si on ouvre plus largement les fentes, on peut obtenir un signal donné, avec un gain d'amplification du détecteur plus faible. En réduisant ainsi le bruit de fond électronique, il est possible d'abaisser le seuil de détection.

Comme nous opérons à fréquence fixe, un pouvoir de résolution élevé présente peu d'intérêt et même pas du tout dans le cas d'un mélange de solutés absorbant à des fréquences différentes. Comme il est nécessaire d'avoir au niveau du détecteur infrarouge un maximum d'énergie disponible pour abaisser le seuil de détection et le temps de réponse de la chaîne de mesure, il semble primordial d'opérer avec une ouverture de fente importante. Malheureusement une telle ouverture a une grande influence sur les coefficients d'extinction moléculaire apparents. Robinson[8] a montré qu'une largeur de fente supérieure à 40% à la largeur de demi-bande infrarouge diminue le coefficient d'extinction molélulaire apparent de la bande et la loi de Lambert-Beer n'est plus vérifiée.

Les chromatogrammes (Figs. 6 et 7) ont été réalisés avec une ouverture de fente importante; ils présentent des pics chromatographiques élargis dont le sommet est écrêté. Lorsqu'il s'agit de détecter, un mélange de solutés dont les maxima d'absorption sont situés à des fréquences assez voisines, il convient de trouver une fréquence de travail moyenne qui permette un compromis: en ouvrant les fentes, on diminue la résolution, on obtient donc un signal pour des produits auparavant non détectés, mais on abaisse les coefficients d'extinction moléculaires respectifs de ces solutés donc la sensibilité de leur réponse.

En général, les seuils de détection obtenus pour différents composants d'un mélange seront plus élevés que ceux trouvés en analysant séparément, aux fréquences optimales, chaque constituant.

Nous avons vu précédemment qu'il est important que la réponse du détecteur (en unités de densité optique) varie linéairement en fonction de la concentration du soluté, c'est à dire que la loi de Lambert-Beer soit vérifiée. La Fig. 8 montre que cette



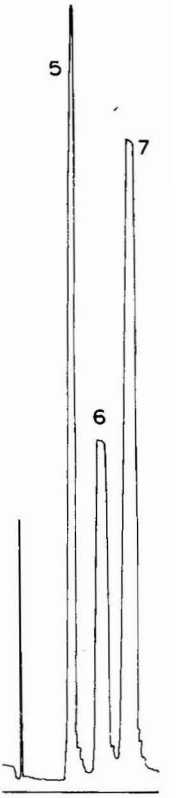


Fig. 6. Séparation de phtalates. Colonne, LiChrosorb (10 μm) + Carbowax 400; éluant, isooctane; s = 50 Mv; $\bar{\nu}$ = 1741 cm^{-1}; fentes, 973 μm; cellule, 0.2 mm; injection, 5 μl. 1 = Dioctylphtalate; 2 = dibutylphtalate; 3 = diéthylphtalate; 4 = diméthylphtalate.

Fig. 7. Séparation d'esters aliphatiques. Même conditions que Fig. 6. 5 = Butyrate d'éthyle; 6 = adipate d'éthyle; 7 = malonate d'éthyle.

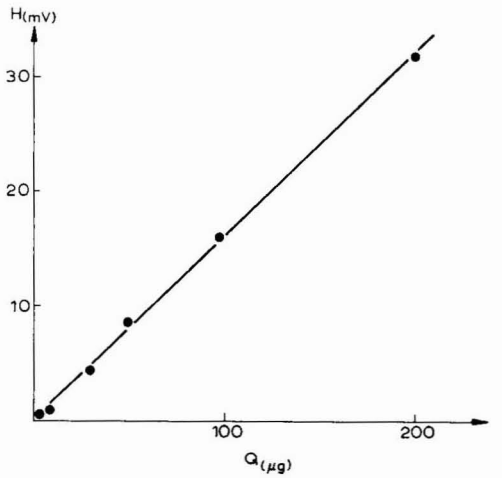


Fig. 8. Linéarity de réponse sur l'adipate d'éthyle. $\bar{\nu}$ = 1748 cm^{-1}; fente, 400 μm.

condition est vérifiée, dans le cas de l'adipate d'éthyle, pour des quantités injectées variant entre 5 et 200 μg. Il est certainement possible en réduisant le bruit de fond électronique, d'abaisser le seuil de détection ci-dessus. Cette réduction peut être obtenue par l'amélioration du montage électrique annexe, représenté sur la Fig. 1.

Signalons enfin le grand intérêt présenté par la détection infrarouge pour l'étude d'un mélange de composés aromatiques et aliphatiques qui ne peut être analysé complètement en ultraviolet.

CONCLUSION

Un spectrophotomètre infrarouge classique, muni de cellules à circulation de liquide, peut être avantageusement utilisé comme détecteur de chromatographie liquide. En réduisant la longueur de la canalisation colonne–cellule et le volume interne de la cellule, on peut encore espérer abaisser le seuil de détection de tous les solutés analysables.

RÉSUMÉ

Les auteurs proposent une cellule adaptée à une telle détection. Il est possible de choisir un éluant chromatographique compatible avec la longueur d'onde de travail. Deux séparations ont été réalisées avec une telle détection.

La première montre que l'utilisation d'un éluant tel que l'isooctane est envisageable par exemple pour l'étude de fonctions $\diagdown\!\!\diagup C{=}O$ à 1748 cm^{-1}.

La deuxième est un essai avec un solvant plus transparent dans le domaine infrarouge: tétrachlorodifluoréthane–trichlorofluoréthane (20:80).

De tels solvants peuvent permettre l'étude des groupes alkyls et couvrent donc un très large domaine.

Les conditions expérimentales dans un tel cas sont discutées.

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IMPROVED CHROMATOGRAPHIC IDENTIFICATION OF COLOURED AMINO ACID THIOHYDANTOINS

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SUMMARY

A new N-terminal reagent for peptides and proteins, 4-N,N-dimethylaminonaphthylazobenzene-4′-isothiocyanate, is described which gives purple thiohydantoin derivatives; chromatographic separation of 24 amino acid thiohydantoins is reported. Such standard purple derivatives can be used as markers in the separation of the red 4-N,N-dimethylaminoazobenzene-4′-thiohydantoins. Conversely, standard red thiohydantoins can be used as markers in the separation of unknown purple amino acid thiohydantoins.

In two-dimensional thin-layer chromatography, the precision of identifying the unknowns can be greatly improved by running markers with a colour different from the unknowns on the same side of the sheet.

INTRODUCTION*

The introduction of DABITC as a coloured N-terminal reagent[1] and the combined use of DABITC and phenyl isothiocyanate in the extended manual sequence determination of peptides and proteins[2] has demonstrated that DABITC possesses several advantages over conventional methods[3]. The yield of the acid-labile N-terminals, such as asparagine, glutamine and tryptophan, and the colour change among isothiocyanate, thiocarbamoyl and thiohydantoin derivatives, which provides a rapid identification of N-terminal amino acids, were the major advantages over the dansyl method[4,5].

Due to the small size of the polyamide sheets used for the identification of the amino acid thiohydantoins (3 × 3 cm) and the difficulty in running markers on the back of the sheet due to differential solvent migration on the front and back, we wished to improve the ease and reliability of identification of the thiohydantoins. The generation of some blue markers in the DABITC method which could be used

* Abbreviations: DABITC = 4-N,N-dimethylaminoazobenzene-4′-isothiocyanate; DABTC = 4-N,N-dimethylaminoazobenzene-4′-thiocarbamoyl; DABTH = 4-N,N-dimethylaminoazobenzene-4′-thiohydantoin; DANABITC = 4-N,N-dimethylaminonaphthylazobenzene-4′-isothiocyanate; DANABTC = 4-N,N-dimethylaminonaphthylazobenzene-4′-thiocarbamoyl; DANABTH = 4-N,N-dimethylaminonaphthylazobenzene-4′-thiohydantoin.

to distinguish immediately most of the DABTH-AAs (amino acids) suggested the idea of replacing the application of standard markers on the reverse side of the sheet by internal markers of contrasting colours.

Under the existing procedure, although identification between DABTH-Asn and DABTH-Ser and DABTH-Gln and DABTH-Thr could be achieved solely by judging their relative positions to the major blue by-product E, we wish to establish further criteria for the rapid and accurate confirmation of DABTH-Asn, DABTH-Ser, DABTH-Gln, and DABTH-Thr by locating some authentic purple markers around these four red DABTH-AAs.

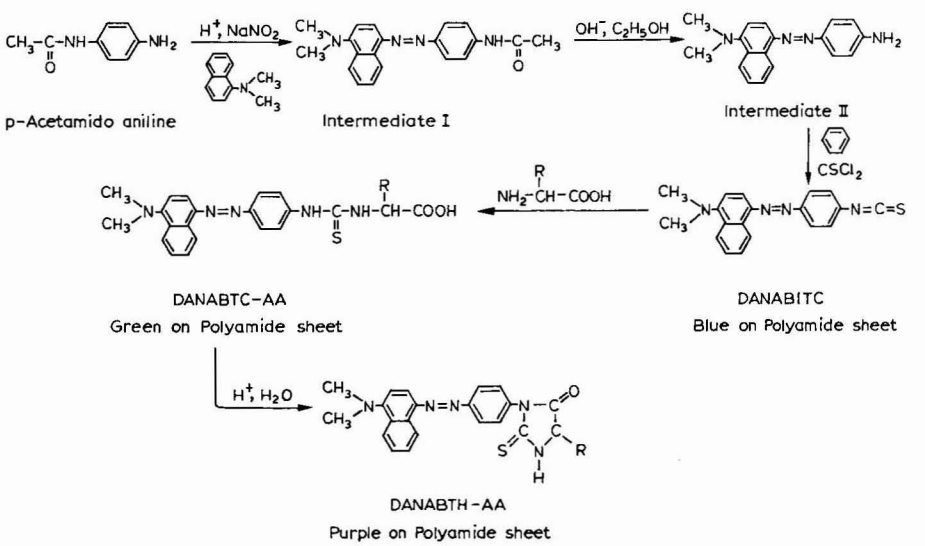
For this purpose, we synthesized another azo dye isothiocyanate, DANABITC, which gives rise to purple amino acid thiohydantoin derivatives. The purple-coloured DANABTH-Asp and DANABTH-Ser were selected as two markers to distinguish red DABTH-Asn and DABTH-Ser and DABTH-Gln and DABTH-Thr (see Fig. 1) in the DABITC method. Conversely, the red-coloured DABTH-Gly and DABTH-Hyp were used as the authentic markers to help the identifications of DANABTH-AAs (see Fig. 2).

MATERIALS AND METHODS

Peptides and proteins were the highest purity obtainable from normal commercial sources. Other chemicals used in the synthesis of DANABITC and for thin-layer chromatography (TLC) were A.R. grade. Chen Chin polyamide sheets were from Pierce (Rockford, Ill., U.S.A.).

Synthesis of DANABITC

The preparation of DANABITC (Scheme 1) was similar to the synthesis of



Scheme 1. Preparation of 4-N,N-dimethylaminonaphthylazobenzene-4′-isothiocyanate (DANABITC) and 4-N,N-dimethylaminonaphthylazobenzene-4′-thiohydantoin amino acids (DANABTH-AA). The colour appeared after exposure to HCl vapour.

DABITC[1], except for the following modifications. (a) The precipitation of intermediates I and II was found to be difficult. Two portions of ethyl acetate (500 ml) were used to extract both intermediate I and intermediate II from the aqueous layers. The extracts were evaporated to dryness in vacuum, giving the creamy intermediates I and II. The synthesis could be continued without purifying the crude intermediates. (b) The conversion of intermediate I to intermediate II was accomplished by refluxing with ethanol (100 ml) and 11 *N* NaOH (20 ml) for 1 h. (c) The final product, DANABITC, after having been eluted from a silica gel column by benzene, was dried (also a creamy product) and redissolved in the minimum volume of acetone. Deep red crystals precipitated when left in the refrigerator overnight (m.p. 125–126°). Its IR spectrum had a strong $-N=C=S$ peak at 2110 cm^{-1}.

Analysis. Calc. for $C_{19}H_{16}N_4S$: C, 68.67; H, 4.82; N, 16.87; S, 9.64. Found: C, 68.76; H, 4.96; N, 16.51; S, 9.44%.

Microsynthesis of DANABTH amino acids

A solution of 150 nmoles amino acid in 40 μl buffer (50 ml acetone + 50 ml distilled water + 0.2 ml triethylamine + 5 ml 0.2 *M* acetic acid; pH 9.65) was treated with 40 nmoles of DANABITC in 20 μl of acetone (2 nmoles/μl) and heated at 50° for 75 min. The mixture was dried in a vacuum over P_2O_5 and taken up in a mixture of 20 μl of distilled water and 40 μl of acetic acid saturated with HCl. The solution was allowed to stand at 50° for 45 min, and then dried again in the desiccator. The residue was redissolved in 40 μl of ethanol for TLC. 0.01–0.02 μl of each DANABTH-AA was applied on a 3.75 × 3.75 cm polyamide sheet.

Special buffer with higher pH value (0.4 ml triethylamine + 5 ml 0.2 *M* acetic acid + 50 ml acetone + 50 ml distilled water; pH 10.4) was used for the synthesis of DANABTH-Glu, DANABTH-Asp and DANABTH-Cys(O_3H), due to the higher pK_a values of glutamic acid, aspartic acid and cysteic acid.

N-Terminal determination of peptides and proteins by the DANABITC method—TLC separations

The separations were performed as described previously[1,2].

RESULTS AND DISCUSSION

Double-sided coated polyamide sheets are commercially available[6,7], and both sides can be used independently. They offer another advantage by applying known standards on the reverse side, followed by correlating the unknown spot to those standards. In our work[1] of separating DABTH-AAs on a small-size polyamide sheet (such as 2.5 × 2.5 cm), however, it was found that the speed of solvents was always different on the opposite sides when the sheet was not developed in a precisely vertical position. Hence, we prefer to apply standards on the same side of the polyamide sheet and then identify the unknowns by relating their positions to the applied markers. The appearance of the major blue by-product E fortuitously meets this requirement. By its help, the discrimination of threonine–serine, glutamine–asparagine and glutamic–aspartic pairs was clear. However, the distinction between asparagine and serine and between glutamine and threonine could be improved, even though they are well separated on two-dimensional TLC. This problem arises mainly

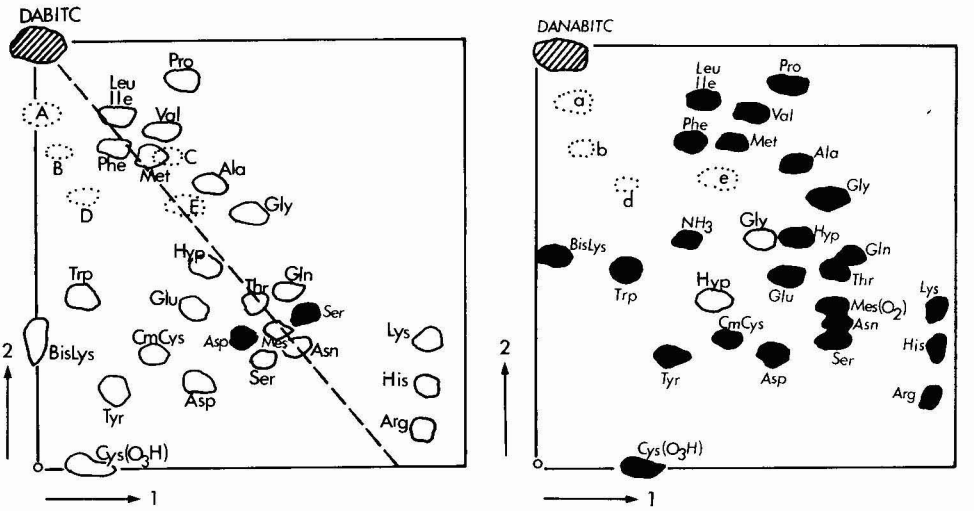


Fig. 1. Tactics of discriminating thiohydantoin derivatives of glutamine and threonine and asparagine and serine using the DABITC method. For details, see text and ref. 1. Solvents: (1) water–acetic acid (2:1); (2) toluene–*n*-hexane–acetic acid (2:1:1). The striped area was the unreacted DABITC. Abbreviations: BisLys = α-DANABTH-(ε-DANABTC)-lysine; CmCys = carboxymethylcysteine; Cys(O_3H) = cysteic acid; Mes = methionine sulphone; A, B, C, D and E = blue by-products in the DABITC method.

Fig. 2. Two-dimensional TLC separation of DANABTH amino acids and DANABTC-NH_2 (blackened areas) on a polyamide sheet using the DANABITC method. All the blackened areas were of purple colour except that BisLys was greenish purple and NH_3 (DANABTC-NH_2) was green. The greenish by-products (broken circles; a, b, d and e) and the red spots (unbroken circles; DABTH-Gly and DABTH-Hyp) were used as the reference markers. The striped area was the unreacted DANABITC. Solvents as in Fig. 1. Mes(O_2) = methionine sulphone; other abbreviations as in Fig. 1.

because the size of the polyamide sheet used was only 2.5 × 2.5 cm. Two criteria are proposed to help in resolving this problem:

(1) Extrapolation of the line joining the centre point of unreacted DABITC and spot E (Fig. 1) should go through the centre point of DABTH-Thr and DABTH-Asn and leave the DABTH-Gln and DABTH-Ser located outside this line. This is the easiest way, which has been successfully employed during the sequence determination of glucagon and insulin A chain.

(2) Application of the purple standards DANABTH-Ser and DANABTH-Asp, together with the unknowns at the origin when one of those four DABTH-AAs is suspected. Fig. 1 shows the relative positions of the red-coloured DABTH-Gln, DABTH-Asn, DABTH-Thr and DABTH-Ser to the two purple markers, DANABTH-Asp and DANABTH-Ser (blackened areas). The colour difference between markers and unknowns offers an advantage in that, even though they partly overlap, one can still tell their relative positions by the heterogeneous colour intensity. When this criterion is to be applied, the diameter of the origin spot should be as small as possible. The amount of authentic markers applied should be less than 20 picomoles for a 2.5 × 2.5 cm sheet.

The colour changes of DANABITC (blue), DANABTC derivatives (green) and DANABTH derivatives (purple), as indicated in scheme 1, were the major

TABLE I

COLOURS IN THE DABITC AND DANABITC METHODS

Azo dye derivative	*Colour*	*Azo dye derivative*	*Colour*
DANABITC	Blue	DABITC	Purple
DANABTC-AA	Green	DABTC-AA	Blue
DANABTH-AA	Purple	DABTH-AA	Red

differences between the DABITC and the DANABITC method. As anticipated, the replacement for the benzene ring in DABITC by the naphthalene ring in DANABITC shifted the absorptions to longer wavelengths. The colours of DANABITC, DABITC and their derivatives are shown in Table I for comparison.

Fig. 2 shows the two-dimensional TLC separation of 25 different DANABTH-AAs. We found that they had very similar chromatographic behaviour to DABTH-AAs. The only amino acid pairs which could not be satisfactorily separated were: isoleucine and leucine, glutamine and threonine; asparagine, methionine sulphone and serine. The generation of the green by-products a, b, d, e was the same as those blue by-products produced in the DABITC method[1]. The major green by-product e, in addition to the two selected red DABTH-Gly and DABTH-Hyp, fulfilled the requirement of markers to distinguish most of the DANABTH-AAs.

We have successfully applied this new reagent on the N-terminal amino acid determination of peptides, Gly-Ala, Gly-Leu, His-Gly, Trp-Gly, Ala-Gly, Leu-Tyr, hexapeptide (Leu-Trp-Met-Arg-Phe-Ala), bradikinin (arginine), insulin A chain (glycine), glucagon (histidine), insulin (phenylalanine and glycine), ribonuclease and lysozyme (both with α-DANABTH-(ε-DANABTC)-lysine as the major cleaved N-terminal and α-DANABTH-lysine as the minor products), and human serum albumin (aspartic acid) with only 2 nmoles as the starting materials. The purple colour of the DANABTH-AAs makes the spots readily detectable on the white background of a polyamide sheet.

We are also using DANABITC to complement the DABITC method for the assessment of peptide purity and for the N-terminal map of tryptic digests of proteins which gave preliminary information about the amino acids which follow lysine and arginine. It appears that DANABITC is a very promising N-terminal reagent; it offers an alternative spectrum of amino acid derivative colours to the DABITC procedure.

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CHROM. 9545

ERYTHROMYCIN SERIES

V. QUANTITATIVE ANALYSIS OF CLADINOSE AND METHYLCLADINOSIDE BY DENSITOMETRY OF THIN-LAYER CHROMATOGRAMS

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SUMMARY

A direct, quantitative, thin-layer chromatographic method is described for the determination of sugar cladinose and methylcladinoside in the presence of other acid-degradation products of the antibiotics erythromycin oxime and erythromycylamine. Cladinose and methylcladinoside are separated from compounds which cause interference on pre-coated silica gel F_{254} plates, and are measured directly on the thin-layer plate using a densitometer. Standard graphs obtained for cladinose and methylcladinoside show a linear relation between the square root of the peak area and the logarithm of the amount of substance present in the spot, as well as between the square of the area and the logarithm of the amount. This method is very successful in stability studies on the antibiotics erythromycin oxime and erythromycylamine in an acid medium. The technique seems to be particularly useful in instances in which the usual analytical methods either cannot be applied or can be applied only with difficulty.

INTRODUCTION

A number of publications have appeared on the quantitative determination of erythromycin and its derivatives[1–4]. Most of the methods employed, however, are non-specific and time-consuming, so they are not satisfactory for stability studies in the presence of degradation products and for the determination of very small quantities. Thin-layer chromatography (TLC)[5–7] is a practical method of solving these problems rapidly and satisfactorily. The substance to be analysed is separated from compounds which might cause interference by means of TLC, which permits a direct densitometric determination.

In our laboratory we have applied TLC to the identification of erythromycin oxime and erythromycylamine, of their derivatives and of their degradation products[8]. Continuing the work in this field, we studied the stability of the antibiotics erythromycin oxime and erythromycylamine[9] in an acid medium. Thus, reaction of these substances with 0.23% hydrogen chloride in methanol or in water at room temper-

ature removed the neutral sugar cladinose and provided products having low antibacterial activity. The structures of these antibiotics and their hydrolytic products are shown in Fig. 1.

In order to test the stability of the above antibiotics, the chromatography–densitometry method has been developed for rapid quantitation of the liberated sugar cladinose and methylcladinoside. Although a number of publications[10–16] give TLC methods for sugars, the quantitative determination of cladinose and methylcladinoside has not been described previously.

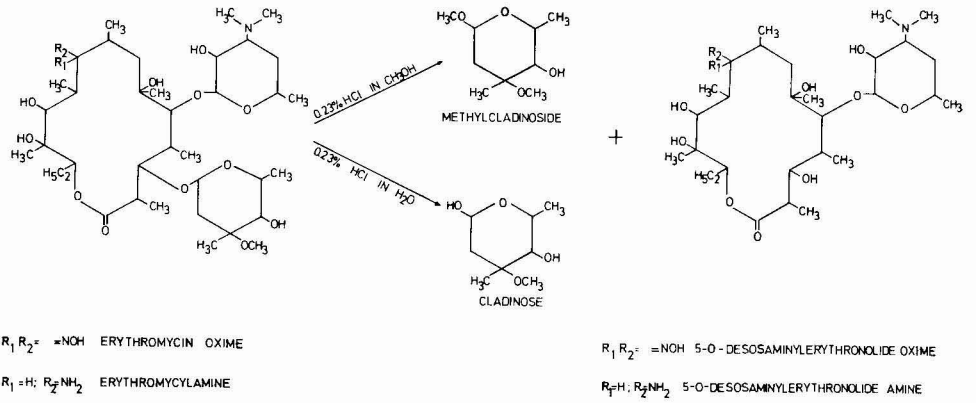

Fig. 1. Acid hydrolysis of erythromycin oxime and erythromycylamine at room temperature.

EXPERIMENTAL

Preparation of standards

The standards were prepared by the method for cladinose and methylcladinoside[17], and were kept in dark bottles at 17° as 1.60 and 1.45% solutions in chloroform, respectively.

Preparation of hydrolysates

Erythromycin oxime and erythromycylamine were hydrolysed in 0.23% HCl in methanol and water at room temperature for 48 h. Samples taken at intervals were neutralized with Na_2CO_3, the solvent was removed *in vacuo* and the residue was extracted with chloroform. The extract was dried and concentrated *in vacuo* to a solid. The sample solutions used for spotting were prepared in chloroform (10 mg/ml).

Chromatographic procedure

All of the solvents used were c.p. grade (E. Merck, Darmstadt, G.F.R.). Precoated silica gel F_{254} plates (20 × 20 cm, with a layer thickness of 0.25 mm) (Kemika, Zagreb, Yugoslavia) were used. The solutions were applied by means of 10-μl micropipettes and a semi-automatic Desaga micro-doser, in a 1-cm long lines. To set up the calibration graphs, amounts of standards of between 1 and 50 μg per spot were applied. In order to prevent the initial spots from spreading, the desired volume was added in 0.2-μl portions with drying between additions. After application of the

samples and standards, the plate was developed in chloroform–benzene (19:1) saturated with ammonia vapour, to a height of 18 cm, then heated in an oven at 110° for 10 min. The detection reagent was prepared by dissolving 3 g of phenol in 95 ml of absolute methanol and 5 ml of H_2SO_4. The plates were sprayed with this reagent and then heated for another 10 min at 110° to produce the dark brown spots.

Densitometry

Densitometry was carried out by use of a Photovolt 520 A densitometer, furnished with a drive unit, Model 42 B light source and Model 49 A integrator (Photovolt Corp,, New York, U.S.A.), through a blue filter of 465-nm wavelength. Plates were scanned parallel to the direction of development.

RESULTS AND DISCUSSION

Cladinose and methylcladinoside were separated satisfactorily from other components in hydrolysates. The R_F values for the antibiotics and their degradation products ranged between 0.08 and 0.70 (Table I).

TABLE I

R_F VALUES OBTAINED FROM TLC ON SILICA GEL WITH CHLOROFORM–BENZENE (19:1) IN A CHAMBER SATURATED WITH AMMONIA VAPOUR

Compound	R_F
Erythromycylamine	0.40
Erythromycin oxime	0.15
Cladinose	0.29
Methylcladinoside	0.70
5-O-Desosaminyl-erythronolide amine	0.35
5-O-Desosaminyl-erythronolide oxime	0.08

The spot areas of the analyses were compared with spot areas from standard solutions of the same compounds, the distribution of the substance within an individual spot being identical in the standard and the analysis sample. Standards were always applied near to the samples on the plates in order to avoid errors due to variations in the chromatographic conditions. In the range of 3–18 μg, the best linearity for cladinose and methylcladinoside was obtained when using the square root or the square of the area and the logarithm of the weight, as shown in Fig. 2. The expected variations in the areas observed during the scanning were determined by measuring the area of a cladinose standard. Three different amounts of cladinose, 20, 10 and 3 μg per spot, were each scanned 16 times. The coefficients of variation (1.4, 1.6 and 4.9 % respectively) demonstrate the good reproducibility of the scanning.

After the areas of the standard and sample spots had been measured, the amounts of compound in the unknown spots were calculated by arithmetical and graphical methods. Arithmetically the results were calculated according to eqn. 1

$$\log C = \log C_M + \log d \frac{A^2 - A_M{}^2}{A_V{}^2 - A_M{}^2} \quad (1)$$

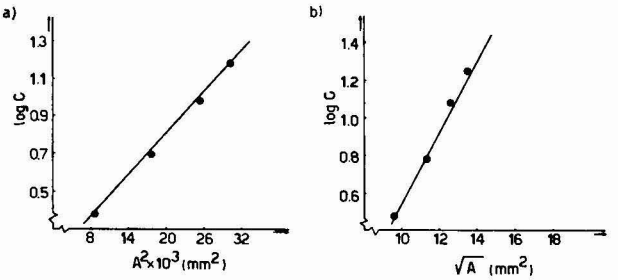

Fig. 2. Relation of the densitometric peak area (A) to the logarithm of the amount of sugar (log C) present on the chromatogram: (a) log C *versus* A^2; (b) log C *versus* $\sqrt{A}$.

TABLE II

QUANTITATIVE DETERMINATION OF CLADINOSE IN ACID HYDROLYSATES BY THE ARITHMETICAL METHOD

Amount of standard cladinose (μg)	*Dilution factor, d*	*Peak area of standard (mm²)*	*Peak area of cladinose in hydrolysates (mm²)*	*Amount of cladinose in hydrolysates* (μg)*
3	4	79	93	4.368
12		123	107	6.757
			108	6.990

* Calculated according to eqn. 1.

where C = the amount (μg) of the compound in the unknown spot, C_M = the amount (μg) of the smaller standard, d = the relation (dilution factor) between the two standards and A, A_M and A_V = the integral areas (mm²) of the unknown spot, of the smaller standard spot and of the larger standard spot, respectively. This equation is a modification of Nybom's equation[18], *i.e.*, instead of the area we used the square of the area. An example of the quantitative analysis by this method is given in Table II.

Beside the above arithmetical method we also applied a graphical method, where four or more standards were used. Straight lines were fitted to the calibration points by means of the least-squares method. The calibration graphs were individually calculated for each plate, and in this case we employed the linear relation between

TABLE III

QUANTITATIVE DETERMINATION OF CLADINOSE IN ACID HYDROLYSATES BY THE GRAPHICAL METHOD

Peak area of cladinose in hydrolysates, A (mm²)	$\sqrt{A} = x$	*Log* $C = y$*	*Amount of cladinose in hydrolysates, C (μg)*
93	9.64365	0.67632	4.746
107	10.34408	0.83671	6.866
108	10.39231	0.84776	7.043

* Calculated from the calibration graph: $y = 0.22899x - 1.53198$.

the logarithm of the amount of compound and the square root of the resulting spot area. An example of a quantitative analysis by this method is given in Table III.

Both the graphical and arithmetical methods of determination of cladinose and methylcladinoside gave similar results.

CONCLUSIONS

A quantitative analysis by direct densitometry of thin-layer chromatograms is described, which could be used for testing the stability of pharmaceutically active substances. In cases in which compounds causing interference are present and which necessitate the use of time-consuming separation techniques, this method is also suitable because of the accuracy of the results and the short period of time required. The results of the quantitative analysis presented here have successfully been employed for stability testing and defining the kinetic parameters of acid-catalysed hydrolysis of erythromycin oxime and erythromycylamine[19].

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AN *IN SITU* FLUORIMETRIC METHOD FOR THE DETECTION AND QUANTITATIVE ANALYSIS OF FENITROTHION, ITS BREAKDOWN PRODUCTS AND OTHER AMINE-GENERATING COMPOUNDS

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SUMMARY

A procedure is described for the *in situ* detection of primary amine-generating pesticides on thin layers of silica gel. The technique is based on the reaction of fluorescamine with primary amines to yield highly fluorescent derivatives. Nitro compounds are first reduced to their corresponding primary amines.

Pesticides studied were aminotriazole, fenitrothion, parathion and parathion-methyl. Possible breakdown products of fenitrothion were also included. Detection limits were in the low nanogram range.

The method is simple, rapid and very selective and can also distinguish between primary amines and nitro compounds. An example of a typical chromatographic separation is presented and quantitative aspects are discussed.

INTRODUCTION

Fenitrothion [O,O-dimethyl O-(3-methyl-4-nitrophenyl)phosphorothioate] has been determined by colorimetry and spectrophotometry[1–7], thin-layer chromatography[8–13] and gas chromatography[14–23]. Most of these techniques, however, cannot be used for the simultaneous determination of fenitrothion and its degradation products. The quantitative analysis of fenitrothion and its oxon and cresol derivatives can be performed by gas chromatography using an electron-capture detector[19] but the method lacks specificity. A sensitive and specific response is obtained for fenitrothion, fenitrooxon and aminofenitrothion when using a flame-photometric detector[19] but 3-methyl-4-nitrophenol and 4-amino-3-methylphenol are not detected and a separate column with an electron-capture detector has to be used.

It was intended in this study to develop an *in situ* fluorimetric method that would permit the simultaneous detection of fenitrothion and some of its major breakdown products and that could eventually be used for their quantitative evaluation in environmental samples.

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EXPERIMENTAL

Chemicals

Fluorescamine (Hoffmann-La Roche, Nutley, N.J., U.S.A.) was purchased from Fisher Scientific (Montreal, Canada). A solution was prepared containing 25 mg of the reagent in 100 ml of acetone. A solution of tin(II) chloride was prepared by dissolving 0.5 g of the compound in 5 ml of concentrated hydrochloric acid and diluting to 120 ml with a solution of 50 ml of water plus 65 ml of acetone. This solution was always freshly prepared.

Fenitrothion (Folithion) and fenitrooxon (Folithion oxygen analogue) were obtained from Chemagro (Kansas City, N.Y., U.S.A.). Parathion, parathion-methyl and aminotriazole were supplied by American Cyanamid Co. (Princeton, N.J., U.S.A.). 3-Methyl-4-nitrophenol and 4-amino-3-methylphenol were purchased from Pfaltz & Bauer (Flushing, N.Y., U.S.A.). Aminofenitrothion was synthesized according to Zitko and Cunningham[1]. Individual stock solutions were prepared at a concentration of 1 μg/μl in acetone, except for the two phenols, which were dissolved in ethanol. Dilutions were made in *n*-hexane or ethanol.

Layers of silica gel (20 $\times$ 20 cm) were prepared 250 μm thick using a mixture of 30 g of silica gel H (Brinkmann Instruments, Rexdale, Canada) and 80 ml of 0.1 *M* potassium dihydrogen orthophosphate solution. The plates were dried in air and were not activated before use.

Apparatus

A Turner Model III fluorimeter (G. K. Turner Assoc., Palo Alto, Calif., U.S.A.) equipped with a Camag TLC scanner was used for all quantitative fluorimetric measurements. A 7-60 excitation filter (360 nm) and a 2A secondary filter (>415 nm), both available from Corning Glass Works (Corning, N.Y., U.S.A.) were utilized. For measuring the fluorescence spectra, a Farrand spectrophotofluorimeter (UV–VIS Chromatogram Analyzer, Farrand Optical Co., New York, N.Y., U.S.A.), equipped with a xenon lamp and grating monochromators supplemented by a Corning 7-54 excitation filter (230–420 nm) and a 3-73 emission filter (>405 nm), was used.

Brinkmann spray guns using freon propellant cans and atomizer heads were utilized for the spraying of the chromatograms.

Methods

For chromatographic separation, the compounds were spotted 2 cm from the bottom of a chromatographic plate and the development was made to a height of 10 cm in an appropriate solvent system. Fenitrothion and its related compounds were separated in *n*-hexane–acetone (2:1), parathion-methyl and parathion were separated in *n*-hexane–acetone (4:1), while for aminotriazole the plate was developed in *n*-hexane–acetone (1:2).

For the detection of compounds bearing a primary amino group, the plate was first sprayed lightly with water–acetone (1:1) solution, then with the fluorescamine reagent followed by a second water–acetone spray until the plate was just moist. The chromatogram was dried in a stream of cold air and examined under long-wavelength UV light (360 nm).

The nitro compounds were reduced to their corresponding primary amines by the following procedure. The plate was sprayed to saturation with the tin(II) chloride solution, allowed to stand for 5 min and then dried in a stream of air. The acid on the plate was neutralized by spraying it lightly with 2 *M* sodium carbonate solution. Additional fluorescamine was sprayed and the chromatogram was examined under UV light to detect the nitro compounds.

For determining detection limits, measured aliquots of the compound being studied were spotted in a series of decreasing concentrations at the bottom of a chromatographic plate and, after migration of the spots in an appropriate solvent system, the fluorescence was formed according to the procedure described above. The spots were scanned on the Turner fluorimeter. The range selector (aperture) was set to its maximum position (30×) and the slit adjusted to 1 mm. A 10-mV recorder connected to the fluorimeter was used to record the fluorescence of the spots. As the baseline is free from noise, the lowest amount that gave a 1-cm deflection on the recorder under the experimental conditions was taken as the limit of detection.

RESULTS AND DISCUSSION

The procedure described here permits the simultaneous detection and determination of fenitrothion and some possible breakdown products on a thin-layer chromatogram. It is based on the reaction of fluorescamine with primary amines to give highly fluorescent derivatives[24,25]. It has already been shown to be reliable and sensitive for the analysis of amino acids[26–30], peptides[27,31], proteins[32], enzymes[33], polyamines[33–36] and other compounds of biological interest[37–39], both in solution and on thin-layer chromatograms. Lawrence and Frei[40] suggested the use of this reagent as a labelling compound for the detection of primary amine-generating pesticides. It was later used for the quantitative determination of Chloramben in vegetables[41].

Of the compounds investigated (Table I), only aminofenitrothion and 4-amino-3-methylphenol have a primary amino group that is capable of reacting with fluorescamine. Fenitrothion, fenitrooxon and 3-methyl-4-nitrophenol each possess a nitro group that can be reduced to the corresponding primary amine.

The *in situ* reaction of fluorescamine with the primary amines on thin layers of silica gel yielded derivatives that appeared as greenish yellow spots under long-wavelength UV light. A similar reaction and formation of fluorescence was observed with the nitro compounds after reduction to the corresponding amines. The excitation and emission maxima for each fluorescent derivative are given in Table II.

All of the compounds were detected by the fluorimetric procedure and the results in Table II indicate that the instrumental limits of detection are in the nanogram range. Under the experimental conditions used, 3-methyl-4-nitrophenol exhibits a weaker fluorescence than the other compounds at identical concentrations, but the fluorescence can be increased by spraying the plate lightly with a 10% solution of triethanolamine in ethanol. The phenol is also detected as a yellow spot immediately after spraying with the sodium carbonate solution. It is important to note that only a slight decrease in the fluorescence of the derivatives was observed after several days when the chromatogram had been treated with sodium carbonate solution, compared with almost complete disappearance of the fluorescence after 24 h for an untreated plate bearing the primary amines.

TABLE I

COMMON NAMES AND STRUCTURAL FORMULAE OF THE COMPOUNDS STUDIED

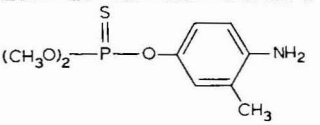
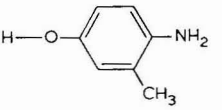
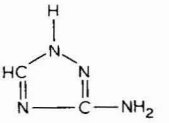
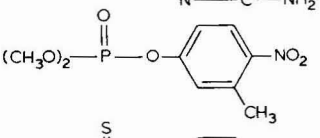
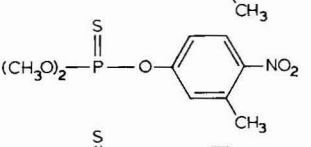
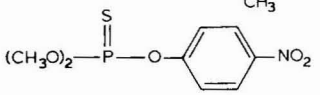
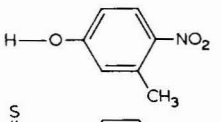
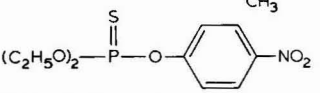
Compound	*Structure*
Aminofenitrothion	
4-Amino-3-methylphenol	
Aminotriazole	
Fenitrooxon	
Fenitrothion	
Parathion-methyl	
3-Methyl-4-nitrophenol	
Parathion	

TABLE II

FLUORESCENCE PROPERTIES OF THE FLUORESCAMINE DERIVATIVES ON THIN LAYERS OF SILICA GEL

Compound	*Excitation maximum (nm)*	*Emission maximum (nm)*	*Limit of detection (μg)*	*Upper limit of linear range (μg)*
Aminofenitrothion	382	500	0.01	0.5
4-Amino-3-methylphenol	382	492	0.02	0.5
Aminotriazole	382	488	0.01	0.5
Fenitrooxon	383	490	0.01	1.0
Fenitrothion	383	490	0.01	1.0
Parathion-methyl	390	510	0.01	1.0
3-Methyl-4-nitrophenol	385	490	0.08 (0.02*)	1.0
Parathion	390	510	0.01	1.0

* Limit of detection after spraying with 10% triethanolamine in ethanol.

TABLE III

REPRODUCIBILITY OF THE TECHNIQUE

*Plate**	*Relative standard deviation (%)*	
	Fenitrothion	*Aminofenitrothion*
1	4.76	6.40
2	13.90	4.73
3	8.55	6.51
4	8.82	9.30
5	10.07	9.33
Average	9.22	7.25

* Five spots per plate.

Quantitative analysis is possible as calibration graphs are linear over a definite range for each compound. However, the slopes of the graphs may vary from one plate to another, and it is therefore recommended that standards be included in each series of analyses.

The reproducibility of the technique was evaluated for aminofenitrothion and fenitrothion using 0.4-μg spots. The relative standard deviations for each compound are given in Table III. The diffusion of the spots and greater background irregularities

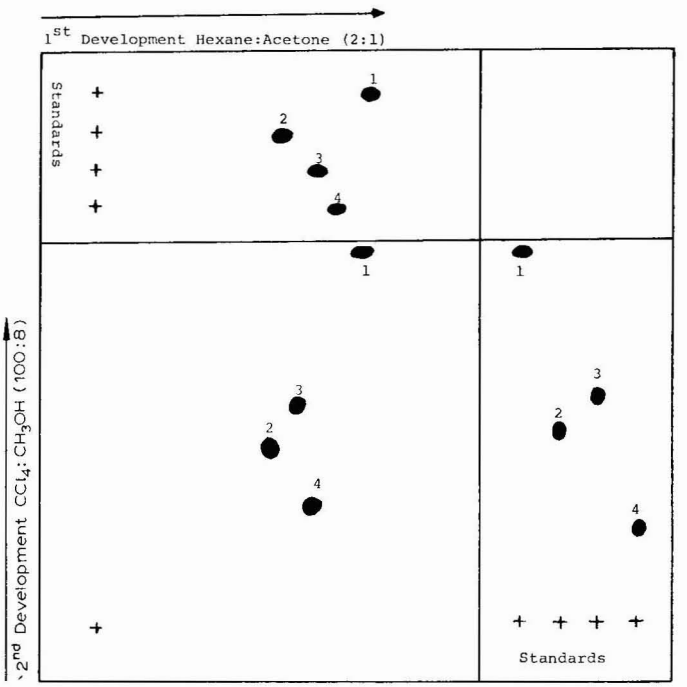


Fig. 1. Two-dimensional thin-layer chromatographic separation of fenitrothion and possible breakdown products. Spots: 1 = fenitrothion; 2 = fenitrooxon; 3 = aminofenitrothion; 4 = 3-methyl-4-nitrophenol.

on the chromatogram, caused by spraying with the inorganic reagents, explain the lower precision for fenitrothion compared with that for aminofenitrothion.

The amount of the sodium carbonate soluiton sprayed and the spraying technique used are important factors in obtaining reproducible results in the analysis of the nitro compounds because of the additional step involved. An excess of the reagent will cause flaking of the adsorbent and excessive diffusion of the spots. Good results were obtained when the plate was first sprayed lightly with the sodium carbonate solution, dried in a stream of air and re-sprayed in the same way.

Potassium dihydrogen orthophosphate was added to the layer in order to prevent the tailing of aminofenitrothion spots that was observed with certain developing solvents. Its use is not essential for rendering the spots visible by the fluorometric technique and untreated silica gel H can be used for most separations.

An example of a typical chromatographic separation of fenitrothion and some possible breakdown products is given in Fig. 1. The positions of the spots can be varied by changing the developing system. This figure clearly illustrates the feasibility of quantitatively analyzing more than one species on a single chromatogram. In fact, the technique is at present being used successfully in our laboratories for the determination of fenitrothion and some breakdown products in natural water samples.

CONCLUSION

The procedure described has been used successfully for the simultaneous detection and quantitation of breakdown products of fenitrothion in samples of natural water. Provided that the compounds to be measured are separated from the co-extractives, the *in situ* technique offers a simple and convenient method of analysis. It is possible to run more than one sample concurrently. When the separation of the compounds from the interfering material by one-dimensional thin-layer chromatography is not satisfactory, a two-dimensional separation of the extract can be carried out.

The technique is not limited to the compounds listed here, and could be used for the detection of other primary amino or nitro derivatives of fenitrothion or for the detection of several breakdown products of parathion and parathion-methyl. As the fluorogenic reagent is selective towards primary amines, the two-step procedure permits discrimination between amino and nitro compounds.

ACKNOWLEDGEMENT

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ELECTROPHORETIC INVESTIGATIONS OF THE COMPLEXING OF CADMIUM AND ZINC WITH EDTA

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SUMMARY

The formation of EDTA complexes of ^{109}Cd and ^{65}Zn in KNO_3 solutions has been investigated by high-voltage paper electrophoresis. The concentration of EDTA was varied from 1 μM to 10 mM, the concentrations of inactive cadmium in the systems were 0, 0.1 mM and 1 mM, the concentrations of inactive zinc were 0, 1 μM, 10 μM and 0.1 mM, and the pH of the system was 8.1. The behaviour of cadmium and zinc was examined by measuring the dependence of their electrophoretic mobilities on the EDTA concentration and on the age of the system up to 5 days. From the results obtained, it is evident that in 0.1 M KNO_3 both ions have similar cationic mobilities. At very low and at very high concentrations of EDTA, cadmium and zinc give well-defined cationic and anionic zones, respectively. In the intermediate range of EDTA concentration, *i.e.*, in the transition region, ^{109}Cd gives well-defined zones with continuous change in electrophoretic mobilities, but ^{65}Zn gives very pronounced multi-zonal tailing. This indicates that EDTA reacts rapidly with cadmium, but rather slowly with zinc. From the results, the variation in effective stability constants of the cadmium–EDTA and zinc–EDTA complexes with the age of the system was calculated, and the values are compared with the effective stability constants obtained for both ions in sea water and 0.55 M NaCl systems at pH 8.1. The stability constants have also been calculated from the electrophoretic data using methods devised by other authors; these methods are discussed on the basis of the results obtained.

INTRODUCTION

Electrophoresis has been used in the study of metal-ion complexes in aqueous solutions by Shvedov and Stapanov[1] and by Jokl and his co-workers[2–5], Jercan and Popa[6], Pučar[7], Musani-Marazović and Pučar[8–10]. This technique yields information on the physico-chemical behaviour of metal complexes, the number of stable species and the sign of the electrical charge on a complexed ion. From electrophoretic data, it is also possible to calculate the effective stability constants (conditional equilibrium constants) of a metal ion–ligand complex, even in such multicomponent systems as sea water.

The aim of this work was to compare the behaviour of Cd– and Zn–EDTA

complexes in a conventional supporting electrolyte KNO_3 at pH 8.1 and ionic strength 0.1 with previously published results[8-10] on their behaviour in sea water.

From the variation in electrophoretic mobility with concentration of EDTA, effective stability constants could be calculated, and the values for both ions in sea water and in 0.55 *M* NaCl could be compared.

MATERIALS AND METHODS

The electrophoretic experiments were performed as described previously[11], except that the temperature of the gasoline was maintained constant by means of an ultra-thermostat[12,13].

Cadmium complexes

The supporting electrolytes used (each of ionic strength 0.1) were

(a) KNO_3 + 1 m*M* barbitone,
(b) KNO_3 + 0.1 m*M* $Cd(NO_3)_2$ + 1 m*M* barbitone, and
(c) KNO_3 + 1 m*M* $Cd(NO_3)_2$ + 1 m*M* barbitone,

with or without EDTA; the ionic strength was kept constant by varying the KNO_3 concentration. The concentration of EDTA was varied from 1 μM to 10 m*M*, and the pH was adjusted to 8.1. For the filter paper used (Munktel 302), the correction factor due to the effect of the paper structure was 2.01; the free length and width of the paper strips was 66 × 1.5 cm. The electrophoretic parameters were 2200 V and 11 mA per strip (current density per 1 cm width of strip, 7.3 mA), and the specific electric effect on the strips was 0.24 VA cm^{-2}. The duration of an experimental run was 40 min, the temperature of the strip was 25 ± 0.1°, and the temperature of the gasoline was 19 ± 0.1°.

The solutions of ^{109}Cd were prepared from an original solution of carrier-free ^{109}Cd in 0.5 *M* HCl (NEN Chemicals GmbH) by evaporation to dryness and adding the appropriate supporting electrolyte to the residue. The specific activities of these solutions were 1 μC/20 μl (20 μl was the volume of solution applied to the starting point of each electrophoretic strip). The concentration of carrier-free ^{109}Cd added to the filter-paper strip at the point of application was *ca.* 35 n*M*.

Zinc complexes

The supporting electrolytes used (each of ionic strength 0.1) were

(a) KNO_3 + 1 m*M* barbitone,
(b) KNO_3 + 1 μM $Zn(NO_3)_2$ + 1 m*M* barbitone,
(c) KNO_3 + 10 μM $Zn(NO_3)_2$ + 1 m*M* barbitone, and
(d) KNO_3 + 0.1 m*M* $Zn(NO_3)_2$ + 1 m*M* barbitone,

with or without EDTA. The concentration of EDTA was varied from 1 μM to 1 m*M*. The electrophoretic parameters were 1100 V and 5.7 mA per strip (current density per 1 cm width of strip 3.6 mA) and the specific electric effect on the strips was 0.06 VA cm^{-2}. The duration of an experiment was 36 min, the temperature of the gasoline was 23.3 ± 0.1°, and the other conditions were as for the cadmium complexes.

The solutions of ^{65}Zn were prepared from an original solution of carrier-free ^{65}Zn in 1 *M* HCl (NEN Chemicals GmbH) by evaporation to dryness and adding the

supporting electrolyte to the residue. The specific activities of these solutions were 1 μC/20 μl, and the concentration of carrier-free ^{65}Zn was *ca.* 20 n*M*.

The behaviour of ^{109}Cd and ^{65}Zn was studied by measuring the change in electrophoretic mobility of carrier-free ^{109}Cd and ^{65}Zn with the concentration of EDTA and the age of the system (from 0 to 5 days).

The electrophoretic mobilities (in mm $V^{-1}h^{-1}$ cm) were corrected as described earlier[11]; these mobility values can easily be transformed into the usual dimensions ($cm^2\ V^{-1}\ sec^{-1}$) by multiplying by 0.278×10^{-4} (see ref. 11).

The variation in effective stability constants of the cadmium–EDTA and zinc–EDTA complexes and the number of ligands (n) per atom of cadmium or zinc with the age of the system were calculated from the electrophoretic data according to the method developed by Pučar[7], using the C.I.I. Model 90.40 computer at the "Rudjer Bošković" Institute.

RESULTS

The electrophoretic behaviour of cadmium and zinc was dependent on the concentration of EDTA in all the systems investigated. Both ions behaved as cations at lower concentrations of EDTA, and as anions at higher concentrations of EDTA; in the "transition region", cadmium and zinc changed their electrophoretic mobilities from cationic to anionic in nature. The age of the system was significant only with ^{65}Zn.

Cadmium complexes

Fig. 1a shows the electrophoretic mobilities of ^{109}Cd in KNO_3–EDTA systems as a function of the log. of the concentration of EDTA; the age of the system had no significant effect. In Figs. 1b and 1c, the electrophoretic mobilities of ^{109}Cd are presented as a function of the log. of the concentration of EDTA in the systems with 0.1 m*M* and 1 m*M* inactive $Cd(NO_3)_2$ added, respectively.

Comparison of Figs. 1b and 1c shows that the transition region of complexing was shifted to higher concentrations of EDTA when $Cd(NO_3)_2$ solution was added to the system. In the system with 0.1 m*M* $Cd(NO_3)_2$ added, the transition region lay between 20 μ*M* and 0.2 m*M* EDTA (Fig. 1b). For the system with 1 m*M* $Cd(NO_3)_2$ added (Fig. 1c), the transition region was shifted between 0.17 and 1.8 m*M* EDTA.

Zinc complexes

Fig. 2a shows the behaviour of ^{65}Zn in KNO_3–EDTA systems. In the transition region, cationic and anionic "tailing" occurs, giving ill-defined electrophoretic zones. At EDTA concentrations greater than 146 μ*M*, there was no change in the anionic electrophoretic mobility of ^{65}Zn.

Figs. 2b, 2c and 2d show the electrophoretic mobilities of ^{65}Zn as a function of the log. of the concentration of EDTA when 1 μ*M*, 10 μ*M* and 0.1 m*M* $Zn(NO_3)_2$ were added to the system.

When the concentration of zinc in the system was 1 μ*M* (Fig. 2b), ^{65}Zn behaved as a cation up to an EDTA concentration of 10 μ*M*, and in the transition region between 46.5 μ*M* and 0.333 m*M* EDTA, ^{65}Zn showed pronounced "tailing" in both cationic and anionic directions.

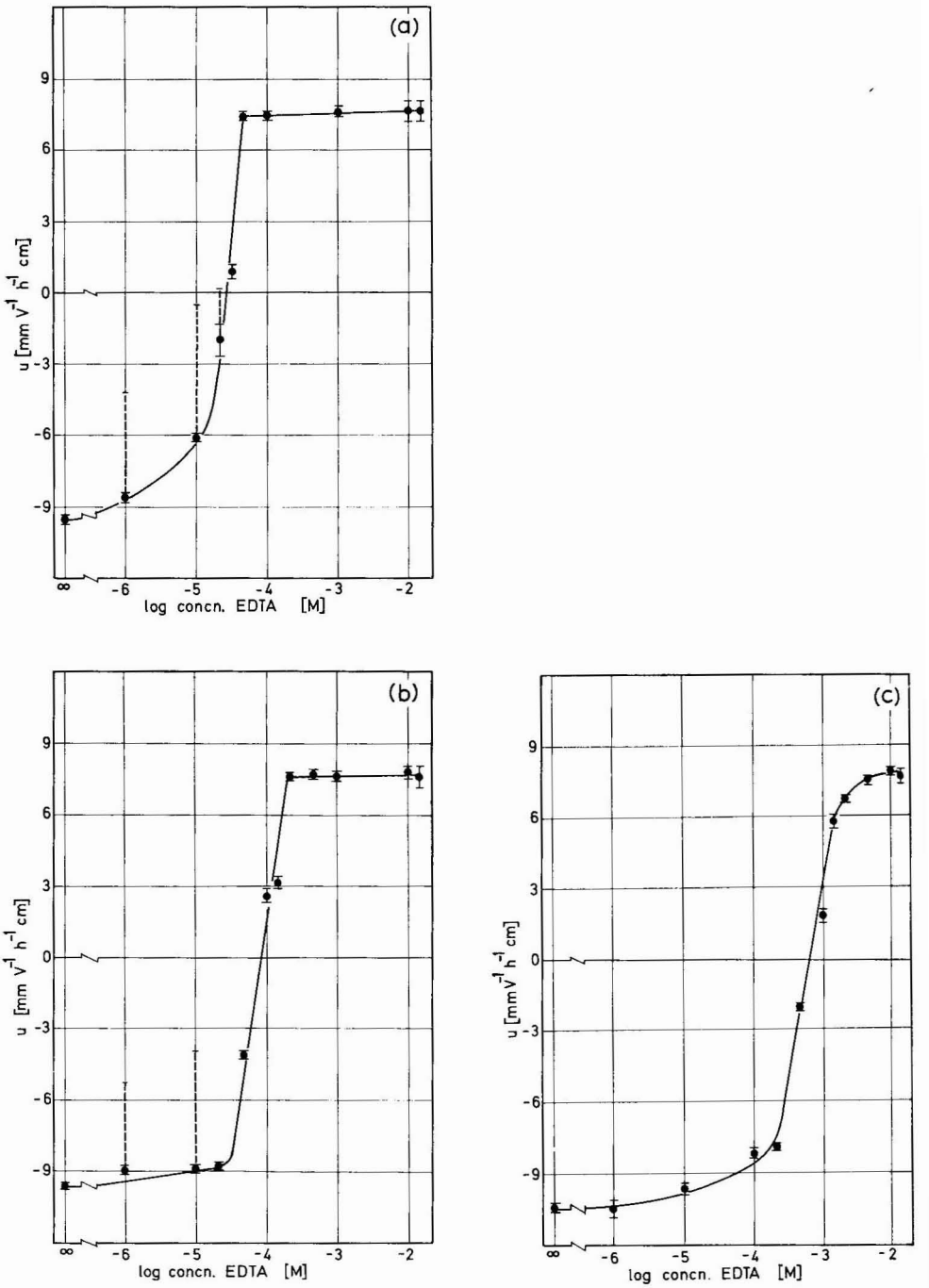

Fig. 1. Dependence of electrophoretic mobility of ^{109}Cd on age and EDTA concentration in KNO_3 medium (pH 8.1; ionic strength, 0.1). (a) KNO_3 + 1 m*M* barbitone; (b) KNO_3 + 0.1 m*M* $Cd(NO_3)_2$ + 1 m*M* barbitone; (c) KNO_3 + 1 m*M* $Cd(NO_3)_2$ + 1 m*M* barbitone. The curves do not change over the period 0–5 days.

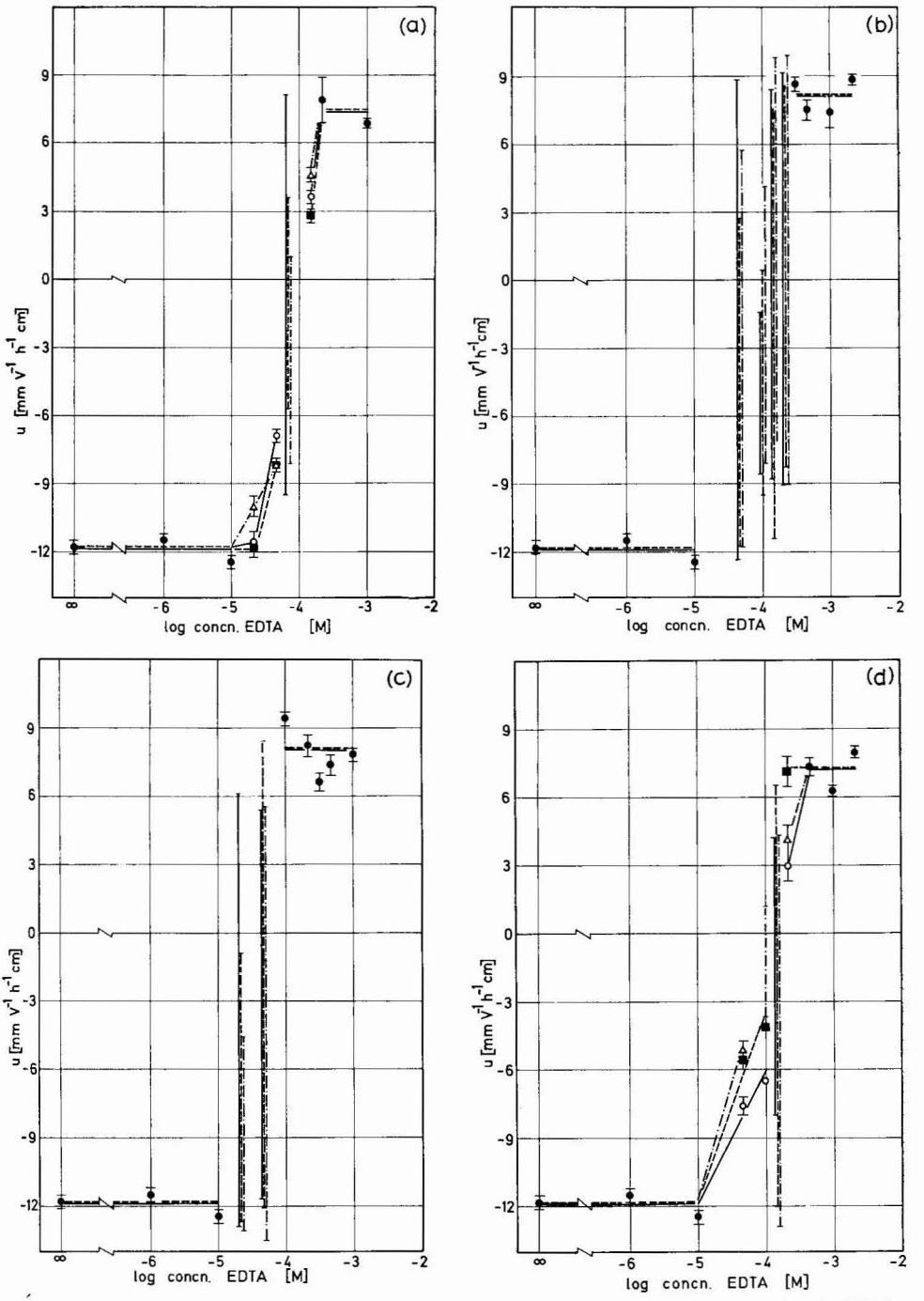

Fig. 2. Dependence of electrophoretic mobility of ^{65}Zn on age and EDTA concentration in KNO_3 medium (pH 8.1; ionic strength, 0.1). (a) KNO_3 + 1 m*M* barbitone; (b) KNO_3 + 1 μM $Zn(NO_3)_2$ + 1 m*M* barbitone; (c) KNO_3 + 10 μM $Zn(NO_3)_2$ + 1 m*M* barbitone; (d) KNO_3 + 0.1 m*M* $Zn(NO_3)_2$ + 1 m*M* barbitone. ○—○, 0th day; ■——■, 1st day; △-·-·-△, 5th day; ●- - -●, 0–5th day.

Fig. 2c shows the behaviour of ^{65}Zn in systems with 10 μM $Zn(NO_3)_2$; ^{65}Zn migrates cationically at EDTA concentrations up to 10 μM, and at EDTA concentrations higher than 0.1 mM, it migrates anionically.

With 0.1 mM $Zn(NO_3)_2$ added (Fig. 2d), the transition region lies between 10 μM and 0.215 mM EDTA. Above 0.465 mM EDTA, ^{65}Zn shows a well-defined anionic zone independent of the age of the system.

Evaluation of the experimental data for fast complexing[7]

When the concentration of a ligand A in a system is gradually increased, and the concentration of the metal species and the pH are kept constant, complexing of metal with ligand occurs and the charge on the metal species changes from $z+$ to $(z+ + nz-)$. Processes with a fast rate of complexing are characterised by the fact that, in the transition region of ligand concentration, the mobility of the metal species ($\bar{u}$) changes continuously from the maximum cationic mobility of the metal ion in the given media ($u-$) to the maximum anionic mobility of the metal–ligand complex ($u+$), and it is impossible to separate the complexed and uncomplexed forms by electrophoresis.

For calculating the effective stability constant, K, the equation

$$\frac{[MA_n^{z+ + nz-}]}{[M^{z+}]\,[A^{z-}]^n} = K \tag{1}$$

could be written as follows

$$\frac{C_X}{(C_M - C_X)\,(C_A - nC_X)^n} = K \tag{2}$$

where

$$C_X = C_M \cdot \frac{\bar{u} - u-}{u+ - u-} \tag{3}$$

In eqns. 2 and 3, C_M, C_A and C_X represent, respectively, the total concentrations of metal, ligand and complexed metal in the system, and $\bar{u}$, $u-$ and $u+$ represent the electrophoretic mobilities of the zone of the metal in the transition region of ligand concentration, the uncomplexed metal (M^{z+}) and the electrophoretic mobility of the complexed metal $MA_n^{z+ + nz-}$.

The electrophoretic mobility of the metal species can be presented in diagrams as a function of the total ligand concentration. In most cases, graphs of $\bar{u}$ against log C_A will give a straight line in the transition region, which is suitable for the statistical evaluation of experimental results. Other functions, such as $\bar{u} = f(C_A)$ can also be used if necessary. Eqns. 2 and 3 have been combined, and a computer program was devised for evaluating the results. For a given system, C_M, $u-$ and $u+$ are constants and C_A, $\bar{u}$ and n are variables, where $\bar{u} = f(C_A)$. The value of log K should satisfy the relationship $\log K_{n=\text{constant}} = f(\bar{u}) = \text{constant}$.

With the ^{109}Cd systems, the values for u were taken directly from the transition

region of the experimental curves; for ^{65}Zn systems, the values for $\bar{u}$ were taken from the straight line connecting the cationic and anionic parts of the complexing curves, *e.g.*, in Fig. 2c, the points $C_A = 10\ \mu M$, $u- = 11.8$, and $C_A = 0.1$ mM, $u+ = 8.1$ were joined.

Figs. 3 and 4 show some examples of results obtained by computing log K against electrophoretic mobilities for values of n between 0.33 and 3; these results refer to the experiments presented in Figs. 1a, 1b, 1c (Fig. 3) and 2d (Fig. 4). Since the value K depends on the number (n) of ligands chelated to one metal ion, the correct values of K and n are represented by the curve that gives constant values of K in diagrams of log K *vs.* electrophoretic mobility. The "straight" lines obtained show deviations at their ends because experimental curves expressed in the form $\bar{u}$ = f(log EDTA concentration) do not fit completely to straight lines, and only the middle parts of the curves can be taken into account.

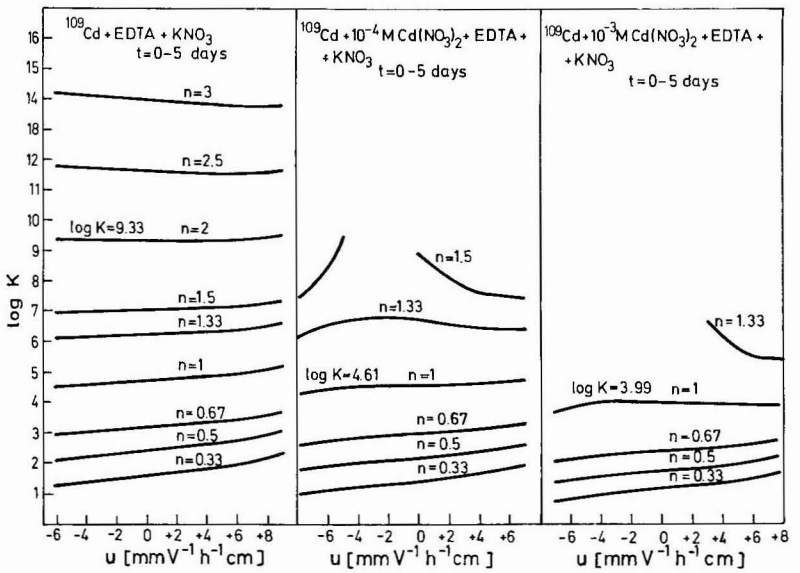

Fig. 3. Dependence of effective stability constants of the Cd–EDTA complexes in KNO_3 medium on the electrophoretic mobility of ^{109}Cd and the number of ligands (n).

In order to compare the effective stability constants of metal–EDTA complexes in different systems, and determined by other methods, the values log $K_{n=1}$ are introduced, although this value should not be assumed to be the same as the actual log K value. The values log $K_{n=1}$ are convenient for comparison because the absolute stability constants are usually calculated for n = 1.

DISCUSSION

The radionuclides ^{109}Cd and ^{65}Zn behave in 0.1 M potassium nitrate as cations with almost identical electrophoretic mobilities. From our previous results obtained

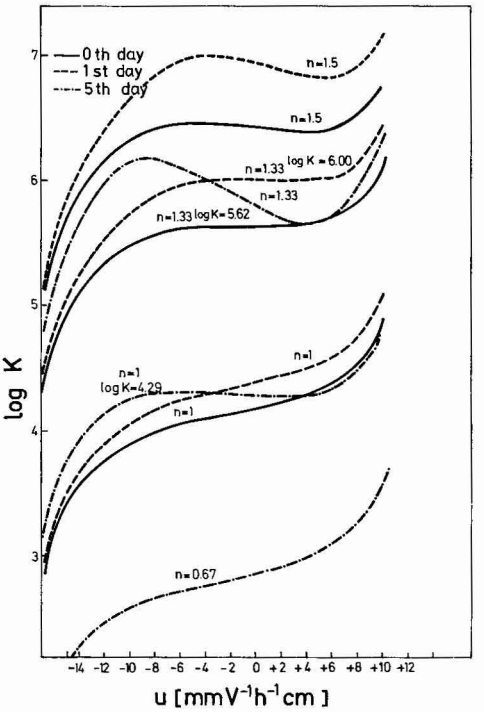

Fig. 4. Dependence of effective stability constants of the Zn–EDTA complexes in KNO_3 medium (Zn + 0.1 m*M* $Zn(NO_3)_2$ + EDTA + KNO_3) on the electrophoretic mobility of ^{65}Zn, the number of ligands (n) and the age of the system.

in chloride medium (sea water and 0.55 *M* sodium chloride), it is evident that ^{109}Cd has a low cationic mobility, showing formation of chloro- and/or dichloro-complexes[10]. In contrast, ^{65}Zn has a cationic mobility corresponding to that of uncomplexed divalent cations[9]. From a study of the hydrolysis of zinc in aqueous solutions, in which the tyndallometric method was used as well as high-voltage paper electrophoresis[14], we conclude that, in the systems investigated, ^{65}Zn behaves as a divalent cation with no hydrolytic species present. According to earlier results[9,14], in sea water and in sodium chloride solutions, ^{65}Zn behaves as divalent cation. In these experiments, ^{65}Zn was introduced in solutions in ionic form, and only the behaviour of ionic zinc was studied. For this reason, no conclusions could be formed as to the state of the zinc present in sea water. This was confirmed by the results of Piro *et al.*[15], who found that, in natural sea water at pH 8, 10–20% of the zinc was in ionic form (including labile complexes), 30–50% was in particulate form and 40–50% was in complexed form; these workers also established that, even after 1 year, radioactive ionic zinc does not enter into equilibrium with naturally complexed stable zinc, although it is partly transformed into particulate form. According to Florence and Batley[16], measurement of the recovery of ionic zinc added to the solution bears no relationship to the recovery of the metal original present in the water sample.

Our present results, as well as those published earlier[9,10], show that, in the systems without EDTA or at very low or very high concentrations of EDTA, both

radionuclides behave in the same way, giving well-defined cationic and anionic zones, respectively. In the intermediate range of EDTA concentrations, ^{109}Cd and ^{65}Zn differ slightly in behaviour. ^{109}Cd gives well-defined zones with continuous change in electrophoretic mobilities, whereas ^{65}Zn gives very pronounced "tailing", consisting of poorly-defined multi-zones instead of defined zones. This indicates that the interaction of cadmium with EDTA is fast, whereas the reaction-rate for zinc is fairly slow. The effect of age of the system shows that the rate of formation of cadmium–EDTA complexes is higher than that of the zinc complexes, because there is no change in the electrophoretic mobility of cadmium with the age of the system in the intermediate range of EDTA concentration.

When the rate of interaction is slow, complexed and uncomplexed species present in solution exhibit defined zones with constant electrophoretic mobilities; in the transition region of ligand concentration, only the intensity of the zones changes[7].

From the data in Figs. 2b and 2c, the effect of the age of the system corresponding to 0 and 1 day could not be evaluated in the form of stability constants and number of ligands, although the effects of aging were evidenced in the transition region by the different forms of tailing.

By increasing the metal-ion concentration in the system, the concentration of EDTA needed for total complexing of the metal increases; at lower concentrations of metal, relatively more EDTA is needed than at higher metal contents, where complexing with EDTA is almost stoichiometric. This is in agreement with literature data[17,18]. For comparison, the results already published and those obtained as described here are shown in Tables I and II.

The fact that values for the effective stability constants assuming n = 1 (log $K_{n=1}$) are almost the same shows that cadmium and zinc behave similarly in the EDTA-containing medium. At low metal-ion concentration in the system, the number of ligands, n, exceeds 1, thus increasing the value of the effective stability constant. This could be attributed to increasing competition between ligand ions for a metal

TABLE I

EFFECTIVE STABILITY CONSTANTS (K) AND NUMBERS OF LIGANDS (n) OF ^{109}Cd–EDTA COMPLEXES IN VARIOUS MEDIA

Electrolyte	*Ionic strength*	*Age of system (days)*	*n*	*Log K*	*Log K** $_{n=1}$	*Temp. (°C)*	*Ref.*
Sea water + EDTA	0.74	0	2.5	10.92	(4.34)	21	10
		1	2.5	10.93	(4.35)	21	
		7	1.33	5.54	(4.16)	21	
Sea water + 0.1 m*M* $Cd(NO_3)_2$ + EDTA	0.74	0–7	1.33	6.30	(4.44)	21	10
KNO_3 + EDTA	0.1	0–5	2	9.33	(4.50)	25	**
KNO_3 + 0.1 m*M* $Cd(NO_3)_2$ + EDTA	0.1	0–5	1	4.61	(4.61)	25	**
KNO_3 + 1 m*M* $Cd(NO_3)_2$ + EDTA	0.1	0–5	1	3.99	(3.99)	25	**
0.55 *M* NaCl + EDTA	0.55	0–10	2.5	8.52	(3.35)	21	10

* The effective stability constant when the number of ligands per atom of cadmium is 1.
** This paper.

TABLE II

EFFECTIVE STABILITY CONSTANTS (K) AND NUMBERS OF LIGANDS (n) OF ^{65}Zn-EDTA COMPLEXES IN VARIOUS MEDIA

Electrolyte	*Ionic strength*	*Age of system (days)*	*n*	*Log K*	*Log $K^*_{n=1}$*	*Temp. (°C)*	*Ref.*
Sea water + EDTA	0.74	0–10	1.5	4.25	(4.25)	21	8**
KNO_3 + EDTA	0.1	0	1.5	6.32	(4.32)	25	***
		1	1.5	6.18	(4.14)	25	
		5	2	8.34	(4.20)	25	
KNO_3 + 1 μM $Zn(NO_3)_2$ + EDTA	0.1	0–5	1.33	5.65	(4.26)	25	***
KNO_3 + 10 μM $Zn(NO_3)_2$ + EDTA	0.1	0–5	1.5	6.94	(4.60)	25	***
KNO_3 + 0.1 mM $Zn(NO_3)_2$ + EDTA	0.1	0	1.33	5.62	(4.10)	25	***
		1	1.33	6.00	(4.32)	25	
		5	1	4.29	(4.29)	25	
0.55 M NaCl + EDTA	0.55	0–10	1.5	5.52	(3.68)	21	8**

* The effective stability constant when the number of ligands per atom of zinc is 1.
** For experimental results, see ref. 9.
*** This paper.

ion. Generally, the values of log $K_{n=1}$ show that, at low concentrations of metal ions (micro-constituents), the stability of the EDTA complexes decreases; thus, special attention should be paid to the treatment of complex-formation at low metal-ion concentration.

With increasing cadmium nitrate concentration beyond 1 mM or zinc nitrate concentration beyond 0.1 mM, an apparent decrease in stability constant (due to competition of EDTA with impurities introduced by the reagents) is observed; this was markedly confirmed for 0.55 M sodium chloride[8]. In this respect, sea water, a relatively concentrated solution (ionic strength 0.74), had a relatively low content of impurities that interacted with EDTA; this is not so with high-purity chemicals.

Our calculated values for the stability constants of the cadmium- and zinc-EDTA complexes (see Tables I and II) differ from the data of Schwarzenbach and his co-workers[19,20]. For this reason, we have re-calculated, from our experimental data, the thermodynamic stability constants of these complexes according to the method developed by Shvedov and Stepanov[1], *viz.*

$$\log K = \log a + \log b + \log f \tag{4}$$

in which a (the activity of the free ligand $EDTA^{4-}$) is defined by

$$a = \frac{C_{EDTA}\, K_1\, K_2\, K_3\, K_4}{(H^+)^4 + (H^+)^3\, K_1 + (H^+)^2\, K_1\, K_2 + (H^+)\, K_1\, K_2\, K_3} \cdot f_{EDTA^{4-}} \tag{5}$$

where K_1, K_2, K_3 and K_4 are the acid dissociation constants of EDTA, C_{EDTA} is the total concentration of EDTA in the system and (H^+) is the hydrogen-ion activity.

The value of b is given by

$$b = \frac{u_{MEDTA^{2-}} - u_c}{u_c - u_{M^{2+}}} \tag{6}$$

where $u_{MEDTA^{2-}}$, $u_{M^{2+}}$ and u_c are the electrophoretic mobilities of the metal–EDTA complex, the free metal ion and the metal species in the transition region of ligand concentration, respectively, and the value of f by

$$f = \frac{f_{M^{2+}}}{f_{MEDTA^{2-}}} \tag{7}$$

$f_{M^{2+}}$ and $f_{MEDTA^{2-}}$ are the activity coefficients of the free and complexed metal ion, respectively.

From Table III, it is evident that the values of the stability constants calculated according to Pučar[7] (effective stability constants) and according to Shvedov and Stepanov[1] (thermodynamic stability constants) differ only for the pH factor, α, (the pH being 8.1), because $\log K_{eff} = \log K_{aps} - \log \alpha$ (see ref. 21). For a divalent-cation complex ($MEDTA^{2-}$), K_{aps} is approximately equal to K_{therm} because eqn. 7 reduces to 1. The factor α depends on the pH and the acid dissociation constant.

At the EDTA concentration where 50% of the metal is complexed, the effective stability constant of the metal–EDTA complex is approximately equal to the negative value of this EDTA concentration [$\log K_{n=1} \approx -\log C_A$ (compare columns 3 and 6 in Table III)].

Jokl and Majer[5] also calculated the absolute stability constants of cadmium– and zinc–EDTA complexes by using paper electrophoresis. From experimental data, Jokl *et al.*[4] calculated the curve of u (electrophoretic mobility) against $\log A$ (free-ligand concentration), for which the value of A depended greatly on pH. According to their conclusions, $\log K \approx -\log A$ at the point u (see ref. 19) (the mean value of the electrophoretic mobilities of two metal species, *e.g.*, free metal and metal complex).

TABLE III

STABILITY CONSTANTS OF Cd- AND Zn–EDTA COMPLEXES IN POTASSIUM NITRATE SOLUTIONS OF pH 8.1 AND IONIC STRENGTH 0.1

Species	*Electrolyte*	*Log* $K^{*}_{n=1}$	*Log* $K_{n=1} + \log \alpha^{**}$	*Log* K $(= -\log a)^{***}$	*Log* C_{EDTA}[§]
^{109}Cd	KNO_3 + EDTA	4.50	6.67	6.77	−4.60
^{109}Cd	KNO_3 + 0.1 m*M* $Cd(NO_3)_2$ + EDTA	4.61	6.78	6.28	−4.12
^{109}Cd	KNO_3 + 1 m*M* $Cd(NO_3)_2$ + EDTA	3.99	6.16	5.45	−3.28
^{65}Zn	KNO_3 + EDTA	4.20	6.37	6.27	−4.10
^{65}Zn	KNO_3 + 1 μ*M* $Zn(NO_3)_2$ + EDTA	4.26	6.43	6.40	−4.23
^{65}Zn	KNO_3 + 10 μ*M* $Zn(NO_3)_2$ + EDTA	4.60	6.77	6.67	−4.50
^{65}Zn	KNO_3 + 0.1 m*M* $Zn(NO_3)_2$ + EDTA	4.29	6.46	6.30	−4.13

* Effective stability constants calculated according to Pučar[7] (see Tables I and II).

** Absolute stability constants calculated according to Schwarzenbach and Flaschka[21] (at pH 8.1, $\log \alpha = 2.17$).

*** Thermodynamic stability constants calculated according to Shvedov and Stepanov[1]: $\log K = -\log a$ because $\log b = 0$ (when 50% of the metal is complexed) and $\log f = 0$ (the activity coefficients of M^{2+} and $MEDTA^{2-}$ are equal). See eqns. 4, 6 and 7.

§ Concentration of EDTA when 50% of the metal is complexed (taken from the diagrams).

This means that, for experiments at pH 8.1, it is impossible to obtain values for the absolute stability constants of the cadmium- and zinc–EDTA complexes as cited in literature by using the equations of Shvedov and Stepanov[1] or those of Jokl *et al.*[4]. To obtain the value log $K_{Cd-EDTA} = 16.5$ at pH 8.1, the concentration of EDTA must be approximately 0.01 pM; at this EDTA concentration, metal ions at concentrations between 10 nM and 1 mM can not be complexed with EDTA to the extent of 50%. This means that electrophoretic experiments should be done at pH values less than 4.

It should be noted that, according to the literature[22], zinc forms a simple $Zn\text{–}EDTA^{2-}$ complex in the pH range 4–11; if this is true, it should be irrelevant at which of these pH values the experiments are performed in order to calculate the stability constants of the zinc–EDTA complex.

Shvedov and Stepanov[1] calculated the stability constants of lanthanide–EDTA complexes; the origin of the main part of the numerical value of the thermodynamic stability constants obtained was that part of eqn. 4 corresponding to the activity of the free ligand $EDTA^{4-}$ (*i.e.*, log a) at a pH between 2 and 3.

We would like to point out the difference between the experimental conditions used in our method and in the other methods already discussed. While in other methods optimal conditions for the appearance of a certain metal–ligand complex were used, we use the conditions present in natural aquatic systems, *e.g.*, sea water. In this sense, our calculation of the effective stability constants takes into account the actual conditions in the investigated systems, *viz.*, pH, ionic strength, actual ionic species present, possible formation of metal–ligand complexes in proportions other than 1:1, possible mixed-ligand complexes, intermediary and concurrent reactions, and instances in which rather low concentrations of metal ions to be investigated (10 nM) are present in concentrated mixed-electrolyte solutions.

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Note

Electronic system for chart recorder absorbance output of the multicuvette system for liquid chromatography

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We have previously described a computer-interfaced multicuvette system for liquid chromatography in which quantitation is performed in specially built 40-section PTFE and glass multicuvettes that are transported by a motor drive across a monochromatic light beam[1]. A photomultiplier converts the transmitted light to a time varying signal which is then processed by the computer to extract the maximum transmission from each section, calculate absorbance and subtract a base value. After further data reduction a graph is formatted for an electrostatic printer/plotter. Such a system gives the convenience of computer reduction of chromatographic data at the cost of a minicomputer, a printer/plotter and interfacing.

The present note describes an electronic system that makes it possible to use the multicuvette system without these costly components providing a usable recording of absorbance values on an ordinary potentiometric recorder without sacrificing operating speed. The system is not only a substitute for the computer, but provides back-up capability when the computer or other components are not operating, since it can operate at about the same speed, about one chromatogram per min, preventing the pile-up of chromatograms in a routine operation.

APPARATUS

The heart of the system shown in Fig. 1 is the peak detector (Model 4084/25; Burr-Brown, Tucson, Ariz., U.S.A.) whose output follows the rise of the signal as the analyzing light beam is gradually uncovered by the cuvette window of the moving multicuvette. A diode within this module prevents the output from falling as the back edge of the window begins to eclipse the light beam. This maximum value is then transferred to a sample/hold module (Burr-Brown Model 4032/15) whose output then represents the transmittance of the cuvette. A logarithmic amplifier (Burr-Brown Model 4116) converts transmittance to absorbance. These circuits are gated by the position of the window being used so that the peak detector is reset as the leading edge is detected and the peak signal is transferred to the sample/hold when the trailing edge is detected. The window detector consists of an infrared (IR) light source

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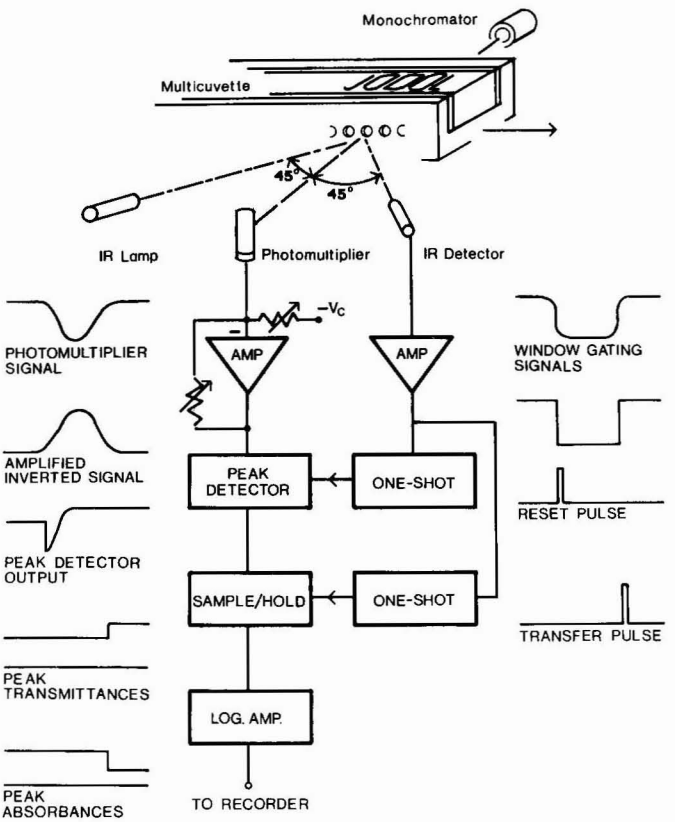

Fig. 1. Composite diagram showing at top the geometry of the window position detector and light absorption measuring system, at left the main signal units of amplifier, peak detector, sample/hold and logarithmic amplifier with typical waveforms, and at the right the window position gating circuit and its waveforms.

(Model L150; Skan-a-matic, Elbridge, N.Y., U.S.A.) mounted at 45° to the analyzing light beam and an IR detector (Skan-a-matic Model P171 IRC) at 45° on the other side of the monochromatic light beam, as shown at the top of Fig. 1. The IR light is at a wavelength of 900 nm so that it is clearly above the visible region of the spectrum used in spectrophotometry. The IR beam is reflected from the aluminium sides of the multicuvette between the windows so that almost no reflection occurs when a window is in the analyzing position. The detector output is shaped by an amplifier (Skan-a-matic Model T330D) and pulses are generated at both leading and trailing edges by two one-shots (Motorola MC74121P).

The analyzing light signal (+1 to −1 V) is inverted and scaled (typically 0 to +5 V) by an operational amplifier with adjustable gain and offset. Power supplies of +5, +15 and −15 V are provided. A switch allows selection of push-button control during the process of gain and offset setting. Almost any available chart recorder may be used. The operating speed of the transport is limited by the response of the recorder. With a variety of recorders we have operated at speeds of 1–5 min for a 120 fraction

readout, typically operating at 2 min with a Honeywell Electronik 194 recorder at a chart speed of 10 sec/in. At this speed the recorder has 1 sec to respond to the absorbance signal, for which the photomultiplier has been exposed for perhaps 0.1 sec.

The cost of the system is about US$ 500 for electronic components and modules, US$ 120 for the IR window detecting system, and in addition building a mount for the IR detector and lamp is required.

RESULTS AND DISCUSSION

The performance of the electronic system is illustrated by the simultaneous recording of the chromatograms of four common urinary corticosteroids (Fig. 2) using both the computer output on an electrostatic printer/plotter (top of Fig. 2) and the electronic system described here and recording on a chart recorder (bottom part). The corticosteroids run are *allo*-tetrahydrocortisone (a-THE), tetrahydrocortisone (THE), *allo*-tetrahydrocortisol (a-THF) and tetrahydrocortisol (THF). Gradient elution chromatography with a gradient of acetone in chloroform was used with quantitation by the blue tetrazolium reaction as described elsewhere[2]. Numerical

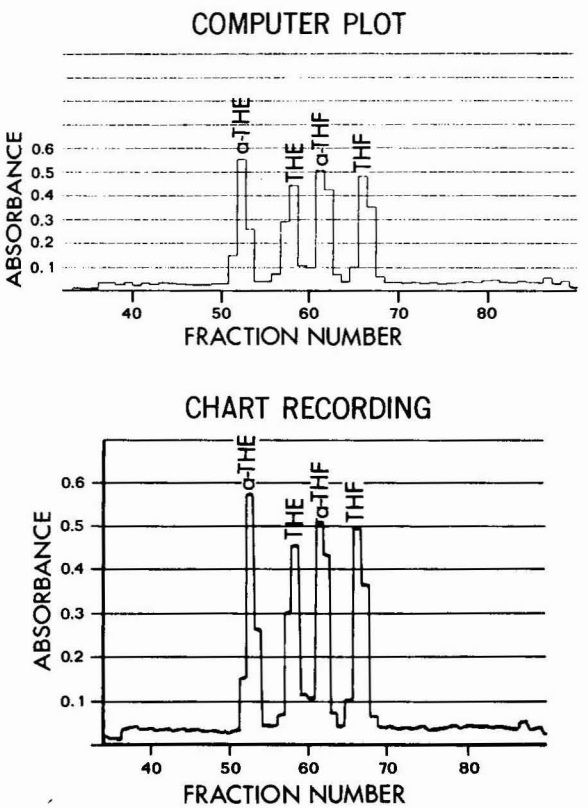

Fig. 2. Top: section of a printer/plotter output of chromatogram of corticosteroids (see text) from the computer based system. Bottom: chart recorder output from the electronic system for the identical cuvettes.

values taken from the chart outputs have agreed within 2% with the computer output.

This system may also be applied to a dual-beam spectrophotometer by placing the logarithmic amplifier at the input end together with a second logarithmic amplifier for the reference beam and an operational amplifier for subtracting the two absorbances to provide an inverted and offset signal for the peak detector.

The window detector circuitry is also useful during computer operation since threshold detection logic will sometimes fail to detect a fraction whose absorbance is greater than 2.

Additional details may be obtained from the authors.

ACKNOWLEDGEMENTS

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Note

Chromatographie sur couche mince des principaux cétoses

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(Reçu le 28 juillet 1976)

Depuis l'important travail de Strecker et Montreuil[1], qui ont séparé les principaux cétoses par chromatographie sur papier, cette question à notre connaissance n'a pas été traitée dans son ensemble, bien qu'il s'agisse d'une série de sucres relativement homogène. Nous proposons donc quelques solvants destinés à séparer, sur couche mince de gel de silice, tous les cétoses de C_3 à C_6, auxquels nous avons adjoint six des huit cétoses connus en C_7 et deux cycloses. Cette méthode a pour avantage l'usage de plaques de chromatographie sur couche mince toutes préparées du commerce, qui ne nécessitent ni imprégnation, ni traitement préalable.

PARTIE EXPÉRIMENTALE

Cétoses de référence (Tableau I)

Un certain nombre de cétoses n'étant pas disponible commercialement, il a été procédé à des préparations au laboratoire.

Techniques de preparation des cétoses

Cétoses préparés par épimérisation (Tableau II). Le D-*thréo*-pentulose, le D-*érythro*-pentulose, le D-psicose et le D-glucoheptulose ont été préparés par le procédé classique d'épimérisation des oses en milieu alcalin. Les trois premiers cétoses ont été obtenus par action de la pyridine sur respectivement le D-xylose, le D-arabinose et le D-fructose. Le D-glucoheptulose a été préparé par épimérisation du D-glucoheptose dans l'eau de chaux.

Cétoses préparés par oxydation biochimique (Tableau III). Un autre groupe de cétoses a été préparé à partir des polyols correspondants par action de l'*Acétobacter suboxydans* (Souche IP 53.162), cultivé à 30° en présence d'extrait de levure à pH 6.4.

Cétoses préparés par aldolisation. Un autre groupe de cétoses a été préparé par aldolisation en milieu alcalin selon Schoffer[6], par action équimoléculaire du dihydroxy-1,3 propanone-2 sur un aldotétrose dans l'eau de chaux à 20° pendant 20 min. Ainsi, avec le D-érythrose (origine: Fluka), on obtient un mélange en proportions variables de D-altro-, D-gluco- et D-alloheptulose; de même avec le D-thréose on obtient un mélange de D-ido- et D-galactoheptulose. (Le D-thréose avait été préparé par action du tétracétate de plomb sur le galactose[7].)

Méthodes particulières. (a) Le DL-*épi*-inosose a été préparé par oxydation par

* Filiale de Péchiney-Ugine-Kuhlmann S.A.

TABLEAU I

PRODUITS ÉTUDIÉS

Cétose	*Composé*	*Origine*
C_3	Dihydroxy-1,3 propanone-2 (dihydroxyacétone)	Aldrich (Milwaukee, Wisc., États Unis)
C_4	L-Glycéro-tétrulose* (erythrulose)	
C_5	D-*thréo*-Pentulose* (D-xylulose)	
	D-*érythro*-Pentulose* (D-ribulose)	
C_6	D-Tagatose	E. Merck (Darmstadt, Allemagne Fédérale)
	D-Fructose	Prolabo (Paris, France)
	L-Sorbose	Roquette (Lestrem, France)
	D-Psicose* (D-allulose)	
C_7	D-Idoheptulose*	
	D-Mannoheptulose	E. Merck
	D-Altroheptulose* (sédoheptulose)	
	L-Galactoheptulose* (perséulose)	
	D-Glucoheptulose*	
	D-Alloheptulose*	
Cycloses	D,L-*epi*-Inosose 2*	
	scyllo-Inosose*	

* Préparé au laboratoire.

TABLEAU II

CÉTOSES OBTENUS PAR ÉPIMÉRISATION

Cétose obtenu	*Ose de départ*	*Origine*
D-*thréo*-Pentulose[2]	D-xylose	Baker (Phillipsburgh, N.J., États Unis)
D-*érythro*-Pentulose	D-arabinose	Fluka (Buchs, Suisse)
D-Psicose[3]	D-fructose	Prolabo
D-Glucoheptulose	D-mannoheptulose	E. Merck

TABLEAU III

CÉTOSES OBTENUS PAR OXYDATION BIOCHIMIQUE

Cétose obtenu	*Polyol*	*Origine*
L-Glycérotétrulose	*m*-erythritol	E. Merck
L-Galactoheptulose[4]	perséitol	Sigma (St. Louis, Mo., États Unis)
scyllo-Inosose[5]	*m*-inositol	E. Merck

l'acide nitrique du *m*-inositol[8]. (b) L'altroheptulose a été préparé selon Strecker[9] par extraction du *Sedum acre** et destruction des aldoses à l'eau de brome en présence de carbonate de calcium, les aldoses s'oxydant en acides aldoniques qui sont éliminés sur résines échangeuses d'ions. La solution finale ne contenait que de l'altroheptulose et du fructose.

Technique chromatographique

Plaques chromatographiques. Nous avons utilisé les plaques finies pour CCM 20 cm × 20 cm (E. Merck, Art. 5721) de gel de silice 60, épaisseur de 0.25 mm sur support de verre.

* Famille: Crassulacées.

TABLEAU IV

SOLVANTS D'ÉLUTION

No.	*Solvant*	*Proportion*	*Réference*
1	Acétone–eau	90:10	10
2	Isopropanol–acétone–eau	40:50:10	
3	Propanol–acétone–eau	40:50:10	
4	Butanol–acétone–eau	40:50:10	11
5	Isopropanol–acétate d'éthyle–eau	40:50:10	
6	Isopropanol–acétate d'éthyle–eau	50:40:10	
7	Isopropanol–acétate d'éthyle–eau	60:30:10	
8	Isopropanol–acétate d'éthyle–eau	70:20:10	
9	Isopropanol–acétate d'éthyle–eau	83:11:6	12
10	Butanol–acétone–méthanol–eau	33:36:18:9	13
11	Acétate d'éthyle–butanol–méthanol–eau	80:15:15:10	
12	Ethanol–isobutanol–eau	60:30:10	14

TABLEAU V

COLORATIONS OBTENUES AVEC LE NAPHTHALÈNE DIOL-1,3

Les aldoses présentent une coloration bleu à bleu violet avec ce révélateur.

Cétose	*Couleur*
Dihydroxypropanone	rose violacé
Glycérotétrulose	gris vert
Cétopentoses	ocre jaune virant au bleu ensuite
Cétohexoses	rouge vif
Cétoheptoses	rouge violacé
Cycloses	vert pâle

Dépôts. Le dépôt est punctiforme de l'ordre de 1 μl contenant environ 2–5 μg de cétose à 1.5 cm du bord inférieur de la plaque. La plaque est déposée dans la cuve contenant le solvant préparé 1 h avant utilisation. La migration ascendante est effectuée sur environ 15 cm. Le séchage se fait à l'air chaud.

Solvants d'élution. Nous avons retenu 12 solvants de préparation directe par simple mélange (Tableau IV).

Détection des taches. La révélation* est effectuée par pulvérisation d'une solution de naphtalène diol-1,3 (naphtorésorcinol) à 0.2% dans l'éthanol contenant 5% d'acide sulfurique concentré, suivie d'un chauffage à 105–110° pendant quelques minutes. Les cétoses donnent les colorations variées et caractéristiques mentionnées dans le Tableau V.

RÉSULTATS ET DISCUSSION

Les $R_F \times 100$ obtenus par la méthode décrite ci-dessus sont donnés à titre indicatif, car ils peuvent varier légèrement selon les conditions opératoires. Le R_{Fru} est le rapport $\times 100$ entre le R_F du corps et le R_F du D-fructose (Tableau VI).

On peut faire la constatation suivante: grossièrement la migration tend à être inversement proportionnelle au poids moléculaire. En effet, le coefficient de partage

* Méthode classique décrite entre autre dans la réf. 15.

TABLEAU VI

VALEURS DES R_F ET R_{Fru}

a = $R_F \times 100$; b = $R_{Fru} \times 100$. Solvants d'élution 1–12, voir le Tableau IV.

	1		*2*		*3*		*4*		*5*		*6*		*7*		*8*		*9*		*10*		*11*		*12*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Dihydroxy-propanone	81	231	82	186	80	210	71	254	71	237	71	215	74	195	68	189	70	233	76	190	64	305	69	157
L-Glycéro-tétrulose	75	214	78	177	74	195	67	239	63	210	65	197	70	184	64	178	66	220	71	178	54	257	66	150
D-*thréo*-Pentulose	70	200	76	173	72	189	63	225	61	203	63	191	69	182	64	178	66	220	69	173	46	219	66	150
D-*érythro*-Pentulose	60	171	64	145	60	136	50	179	49	163	51	155	55	145	52	144	50	167	59	148	38	181	56	127
D-Tagatose	50	143	60	136	54	123	43	154	41	137	45	136	51	134	48	133	47	157	54	135	28	133	56	127
D-Psicose	49	140	55	125	50	114	39	139	41	137	43	130	48	126	45	125	41	137	49	123	29	138	50	114
L-Sorbose	42	120	51	116	46	121	34	121	35	117	38	115	44	116	42	117	37	123	48	120	24	114	50	114
D-Fructose	35	100	44	100	38	100	28	100	30	100	33	100	38	100	36	100	30	100	40	100	21	100	44	100
D-Altroheptulose	42	120	54	123	47	124	35	125	34	113	37	112	43	113	42	117	40	133	48	120	21	100	50	114
D-Manno-heptulose	32	91	43	98	39	103	27	96	29	97	32	97	38	100	38	106	33	110	41	103	18	86	47	107
D-Idoheptulose	33	94	41	93	36	95	28	100	30	100	31	94	35	92	35	97	27	90	35	88	19	90	41	93
D-Alloheptulose	24	69	37	84	32	84	21	75	25	83	28	85	33	87	33	92	27	90	33	83	14	67	39	89
D-Gluco-heptulose	22	63	33	75	29	76	20	71	23	77	26	79	31	82	32	89	24	80	32	80	14	67	39	89
L-Galacto-heptulose	17	49	25	57	22	58	15	54	19	63	19	58	23	61	23	64	16	53	23	58	12	57	29	66
D,L-*épi*-Inosose 2	10	29	9	20	9	24	6	21	6	20	6	18	7	18	6	17	4	13	10	25	6	29	9	20
scyllo-Inosose	4	11	4	9	4	11	2	7	3	10	3	9	4	11	3	8	2	7	5	13	3	14	5	11

dans la phase mobile diminue proportionnellement au nombre de groupements OH du cétose. Cependant, cette remarque est bien moins rigoureusement suivie pour les cétoses que pour les alditols[16]. Les cétoses, surtout à partir des cétopentoses, paraissent migrer sous forme cyclisée alors que les alditols le font sous forme linéaire. Ainsi s'expliquent les fréquents chevauchements entre les cétohexoses et cétoheptoses par exemple. C'est alors que la différence de couleur produite par le révélateur est utile pour l'identification et permet de ne pas confondre cétohexose et cétoheptose, même s'ils migrent dans la même zone.

Choix d'un solvant à utiliser

Il s'effectuera en fonction des séparations à effectuer. On utilisera les solvants suivants: No. 2 ou 3 pour la séparation des cétoheptoses, No. 2, 3, 4, 7, 8, 9 pour la séparation des cétohexoses, No. 1, 4 et 11 pour la séparation des cétopentoses, du glycéro-tétrulose et de la dihydroxypropanone. On note que les deux cycloses et les deux cétopentoses sont convenablement séparés dans tous les systèmes. On peut ainsi séparer les différents cétoses, soit en solution pure, soit à partir d'un hydrolysat de polyosides.

CONCLUSION

Après avoir préparé au Laboratoire un certain nombre de cétoses non aisément disponibles, les auteurs ont choisi plusieurs systèmes d'élution et de révélation pour la chromatographie sur couche mince des principaux cétoses. La technique peut s'appliquer non seulement aux solutions pures, mais aussi aux hydrolysats de polyosides contenant des cétoses.

REMERCIEMENTS

Nous remercions MM. les Professeurs J.-E. Courtois, J. Montreuil et J. Storck de l'intérêt qu'ils ont bien voulu montrer pour ce travail et de leurs conseils.

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CHROM. 9665

Note

Gas chromatographic study of binary diffusion of nitrobenzene and aniline in hydrogen*

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(First received July 6th, 1976; revised manuscript received August 24th, 1976)

Various gas chromatographic methods[1] have been employed for the measurement of the binary diffusion coefficients of gases and vapours of volatile compounds. The arrested elution method suggested by Knox and McLaren[2] bypasses most of the experimental and theoretical difficulties encountered in the continuous elution method developed by Giddings and Seager[3–5]. Recently, Cloete *et al.*[6] studied the binary diffusion of C_1–C_5 saturated hydrocarbons, sulphur hexafluoride, neon, nitrogen and helium in helium, argon and nitrogen at 0.85 atm and 294–483° by using the peak-arrest method.

In the present study, the arrested elution method was used for the determination of binary diffusion coefficients for nitrobenzene–hydrogen and aniline–hydrogen systems. The data are required for process design calculations for the catalytic vapour-phase hydrogenation of nitrobenzene to aniline.

EXPERIMENTAL

The chromatography was carried out on an AIMIL-NCL dual-column gas chromatograph with a flame-ionization detector (FID). Stainless-steel columns of length 220 cm and internal cross-sectional area 0.148 cm^2, without a packing, were used. The dead volumes in the injector and column connections were reduced by introducing solid glass rods into them. The experimental arrangement was similar to that used by Knox and McLaren[2], except that hydrogen was used both as the carrier gas and as a component of the gas–vapour binary system. Separate connections to the FID were made for the carrier gas and for the fuel (hydrogen).

The hydrogen was obtained from IOL (Poona, India) and was purified by passing over palladium–alumina (0.5%, w/w, of palladium) at 100° and silica gel in order to remove trace amounts of oxygen and moisture. The compounds under study, nitrobenzene and aniline, were obtained from BDH (Poole, Great Britain) and were of AnalaR grade.

In all experiments, a sharp band of nitrobenzene or aniline vapour was introduced into the column at a particular temperature by injecting 1 μl of liquid sample and eluted at a controlled and measurable volocity. When the band was about half

* NCL Communication No. 2032.

way down the column, the flow of carrier gas was arrested by diverting it by using a two-way stopcock for a time t, during which spreading could occur only by diffusion. The band was finally eluted from the column and its concentration profile and standard deviation were determined by using the detector.

The pressure drop across the column and the adsorption of nitrobenzene and aniline on the inner wall of the column were found to be negligible. The carrier gas velocity (v) was obtained by determining the retention time (t_R) of the compound under study without arresting the flow of carrier gas and from the column length (L) by using the equation $v = L/t_R$. The values of v and t_R were checked by measuring the volumetric gas flow-rates and the column volume directly.

The standard deviation (σ_t) of the eluted peak was obtained from the peak width (w) at the base-line by using the equation $\sigma_t = w/4$.

RESULTS AND DISCUSSION

Experimental results were obtained for the nitrobenzene–hydrogen and aniline–hydrogen systems at a pressure of 725 mm Hg and at temperatures varying from 210° to 295°. The standard deviations of the eluted peaks for arrest times ranging from 0 to 300 sec at different temperatures for both systems are given in Table I.

According to the equation[2]

$$\frac{\mathrm{d}\,\sigma_t^2}{\mathrm{d}t} = \frac{2\,D_g}{v^2} \tag{1}$$

for diffusional spreading in an empty tube, where D_g is the interdiffusion coefficient, a plot of variance (σ_t^2) against arrest time (t) should give a straight line of slope $2\,D_g/v^2$, from which D_g can be calculated. As v is raised to the second power in eqn. 1, its measurement should be very accurate for this method to be precise.

TABLE I

STANDARD DEVIATIONS (sec) OF ELUTED PEAKS FOR DIFFERENT ARREST TIMES

Values given are averages of more than three experimental values in each instance.

Arrest time, t (sec)	*Diffusing system*					
	Nitrobenzene–hydrogen			*Aniline–hydrogen*		
	215°	*250°*	*295°*	*210°*	*250°*	*290°*
0	3.50	3.27	3.09	3.62	3.56	4.28
30	—	3.35	3.23	3.72	3.63	4.50
60	3.68	—	3.28	3.84	3.75	4.62
90	3.81	3.56	3.37	3.87	3.90	—
120	4.00	3.79	3.43	4.02	4.00	4.96
150	3.94	3.94	3.40	4.12	4.20	5.12
180	3.94	3.08	3.59	4.13	4.20	5.19
210	4.03	4.06	3.62	4.19	4.36	5.31
240	4.13	4.00	3.56	4.37	4.40	5.50
270	4.18	4.22	3.70	4.50	4.41	5.56
300	4.30	4.37	3.79	—	4.56	5.77
v (cm/sec)	8.80	8.40	11.60	8.30	8.30	6.50

The plots of σ_t^2 *versus* t for both systems were linear and are shown in Figs. 1 and 2. The values of the binary diffusion coefficients D_g obtained from the slopes of the plots and those predicted from the theoretical equation based on the kinetic theory and the Lennard–Jones expression[7] for the intermolecular forces and from the expression developed by Fuller *et al.*[8] for both systems are presented in Table II. It can be seen that the experimental values of D_g for both systems are smaller than the values predicted from both expressions, the deviation of the predicted from the experimental value being 3.4–9.4% (average 5.6%) for the Lennard–Jones method and 2.3–8.6% (average 4.6%) for the Fuller *et al.* method. The latter method predicted the values of D_g more accurately than the former.

The arrested flow method is of high precision because the variance produced by the injector, column connections, detector and elution along the column are the same for all arrest times and accordingly cancel out. A fairly short column can be used for the pulse-broadening experiments. The main disadvantage of this method is the requirement of repeating the experiments at various arrest times. The continuous

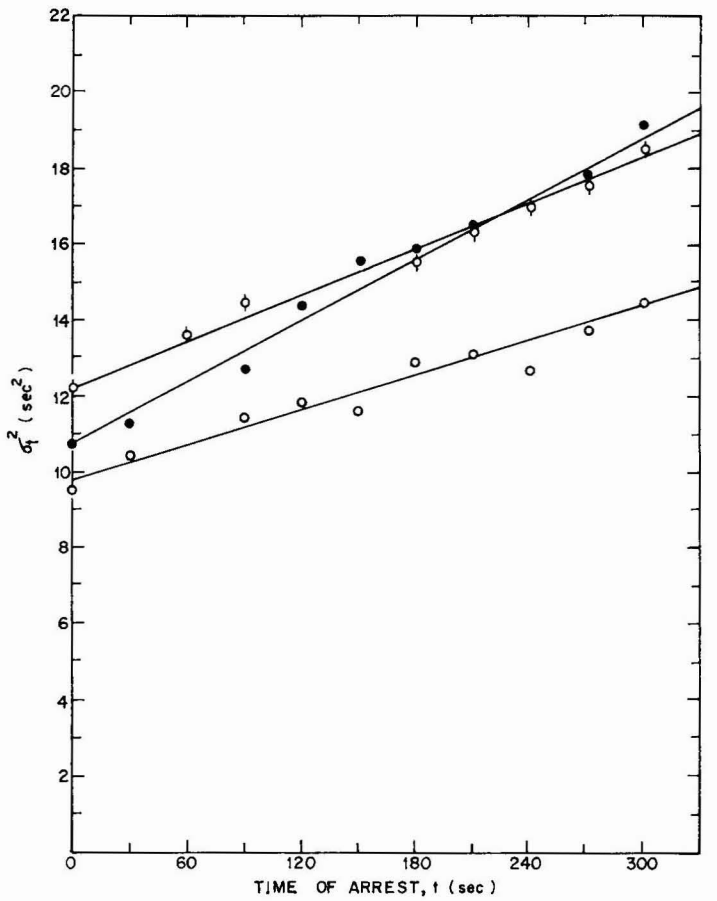

Fig. 1. Plots of σ_t^2 *versus* arrest time at different temperatures for the nitrobenzene–hydrogen system. ◌, 215° (v = 8.8 cm/sec); ●, 250° (v = 8.4 cm/sec); ○, 295° (v = 11.6 cm/sec).

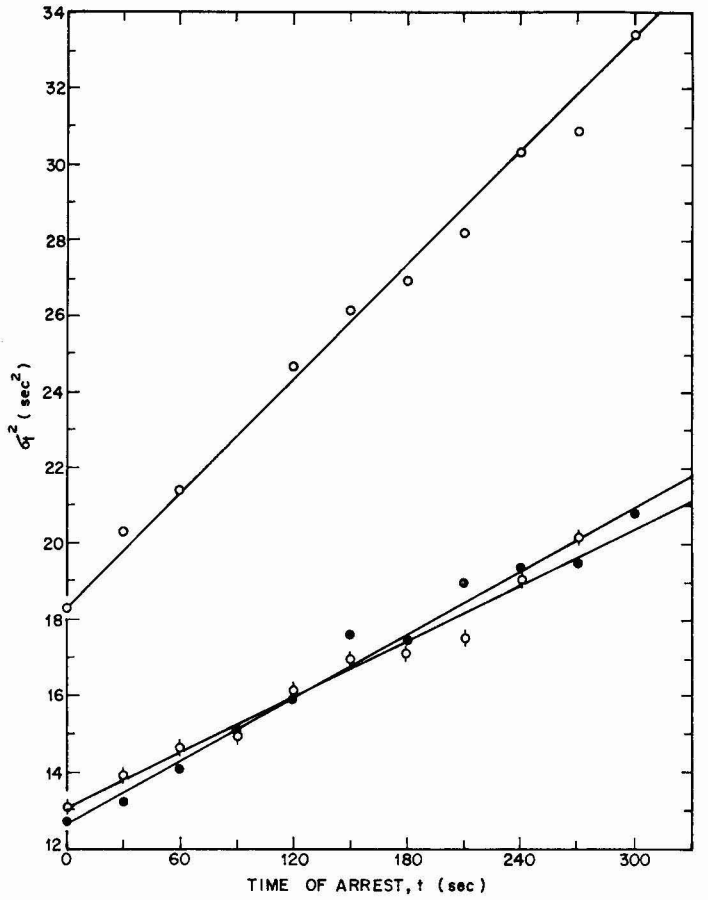

Fig. 2. Plots of σ_t^2 *versus* arrest time at different temperatures for the aniline–hydrogen system. ◌, 210° (v = 8.3 cm/sec); ●, 250° (v = 8.3 cm/sec); ○, 290° (v = 6.5 cm/sec).

TABLE II

RESULTS OF BINARY DIFFUSION OF NITROBENZENE AND ANILINE IN HYDROGEN
Pressure: 1 atm.

System	*Temperature (°C)*	*D_g (cm²/sec)*			*Deviation (%)***	
		*Experimental**	*Predicted by Lennard–Jones exp.*	*Predicted by Fuller et al. exp.*	*Lennard–Jones method*	*Fuller et al. method*
Nitrobenzene–hydrogen	215	0.750	0.820	0.814	9.4	8.6
	250	0.899	0.929	0.919	3.4	2.3
	295	1.014	1.068	1.062	5.4	4.8
Aniline–hydrogen	210	0.805	0.840	0.830	4.3	3.1
	250	0.926	0.973	0.954	5.0	3.0
	290	1.027	1.089	1.085	6.0	5.6

* Experimental value reduced to 1 atm pressure.
** Deviation of the predicted from the experimental value.

elution methods require the experiments to be carried out in a long column at very low flow-rates. Precise and accurate results can also be obtained[3,9–11] by the method by making corrections for the end-effects and the variance produced in the instrument dead volume and by controlling the experimental conditions precisely.

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Note

High-pressure liquid chromatography of anthocyanidins

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The separation and identification of anthocyanidins has been achieved in the past by either paper chromatography[1], two-dimensional cellulose thin-layer chromatography[2], or column chromatography[3]. More recently, a gas chromatographic procedure has been reported[4] which separated the anthocyanidins as their corresponding silylated quinoline derivatives after reaction with trimethylchlorosilane and hexamethyldisilazane. As these methods demand hours to perform, we would like to report a simpler procedure using a modified version of the reversed-phase high-pressure liquid chromatographic system developed by Wulf and Nagel[5] for the separation of flavonoids and phenolic acids. The examination by this method of plant materials containing anthocyanins will require a prior acid hydrolysis, to set free the anthocyanidins for analysis.

EXPERIMENTAL

Chromatograms were run on a Waters liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.) using a 30 cm × 4 mm I.D. μBondapak/C_{18} column (Waters Assoc.) and a Schoeffel SF770 UV–VIS detector set at 530 nm. A flow-rate of 2 ml/min was maintained employing a mixture of water–acetic acid–methanol (71:10:19) by volume as eluent.

Authentic specimens of delphinidin, cyanidin, pelargonidin and malvidin were obtained from commercial sources. Petunidin chloride was isolated from muscadine grapes by column chromatography of a crude hydrolysate on Polyclar AT[3]. Peonidin chloride was kindly supplied by Prof. S. Sakamura of Hokkaido University, Sapporo, Japan. These compounds were used to study the chromatographic separation, by injecting 25 μl of an aqueous solution containing approximately 1 mg/ml of each component. The exact concentration of each anthocyanidin was purposely varied according to the purity of the sample, in order to obtain chromatograms showing six well-resolved peaks of equal height.

For the analysis of plant materials, a sample (about 0.5 g) was extracted at room temperature for 1 h, with 20 ml of methanol made 0.01 *N* with concentrated HCl, the extract evaporated *in vacuo* and the residue hydrolyzed for 30 min at 100°

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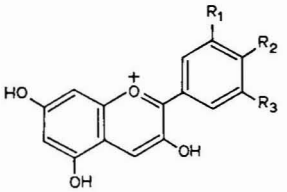
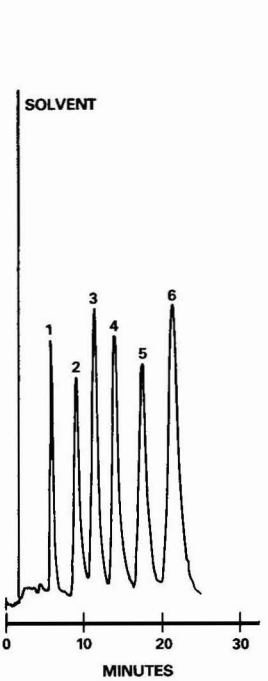
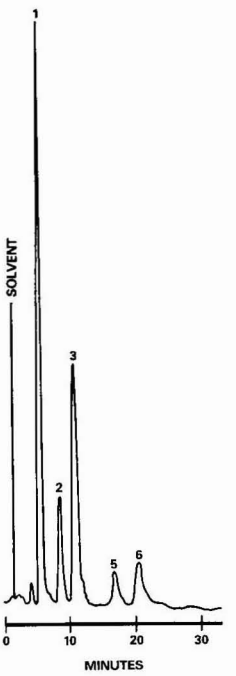
TABLE I

STRUCTURES AND RETENTION TIMES OF THE SIX MOST COMMON ANTHOCYANIDINS AS REFERRED TO IN THE TEXT



Peak No.	*Compound*	R_1	R_2	R_3	t_R *(min)*
1	Delphinidin	OH	OH	OH	5.7
2	Cyanidin	OH	OH	H	8.7
3	Petunidin	OCH_3	OH	OH	11.1
4	Pelargonidin	H	OH	H	13.6
5	Peonidin	OCH_3	OH	H	17.0
6	Malvidin	OCH_3	OH	OCH_3	20.8





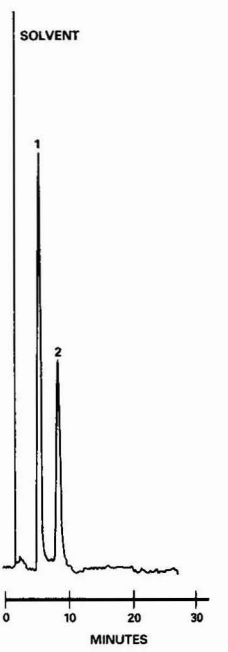


Fig. 1. Separation of the six reference anthocyanidins on μBondapak/C_{18} with water–acetic acid–methanol (71:10:19) by volume as eluent. The peak numbers are explained in Table I.

Fig. 2. Separation of *Vitis rontundifolia* anthocyanidins. The peak numbers are explained in Table I.

Fig. 3. Separation of *Hibiscus sabdariffa* anthocyanidins. The peak numbers are explained in Table I.

with 5 ml of 2 *N* HCl[6]. The hydrolysate was added to the top of a short (2 × 0.3 cm I.D.) Polyclar AT column, the column washed with two bed volumes of 0.01 *N* HCl, and the anthocyanidins eluted with 25 ml of 0.01 *N* HCl in methanol. The eluent was then concentrated to 1 ml on a rotary evaporator (< 30°) before injection on the chromatograph.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of a standard mixture of the six most common anthocyanidins. The structures and retention times of these anthocyanidins are given in Table I. The components were eluted in less than 30 min, started with the most hydrophilic one (delphinidin), and continued in order of decreasing hydrophilicity, as expected in a reversed-phase system.

Figs. 2 and 3 represent results from the injection of hydrolysates of extracts from muscadine grape skins (*Vitis rotundifolia*) and dried calyces of roselle (*Hibiscus sabdariffa*). The muscadine result corroborates previous determinations[7] which list delphinidin and petunidin as the major components and report the absence of pelargonidin. The roselle sample, in agreement with previous studies[8], showed only the presence of delphinidin and cyanidin.

ACKNOWLEDGEMENT

The authors are indebted to Professor S. Sakamura, Hokkaido University, Sapporo, Japan, for kindly providing a sample of peonidin chloride.

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Note

Analysis of urinary tract antibacterial agents in pharmaceutical dosage form by high-performance liquid chromatography

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A high-performance liquid chromatographic (HPLC) method of analysis for nalidixic acid (I) in urine has been reported[1]. A similar system was reported for the analysis of sulfanilamide antibiotics[2]. After suitable alteration of the system used for the analysis of I, the analysis of 1-ethyl-1,4-dihydro-4-oxo[1,3]dioxolo[4,5-*g*]cinnoline-3-carboxylic acid (cinoxacin) (II), a newly synthesized antibacterial agent[3], became possible. Sulfanilic acid or sulfamerazine was incorporated as an internal standard. This report describes the analysis of II in capsule and ampoule formulations and of I, II and oxolinic acid (III) as aluminum hydroxide gel suspensions.

I II III

EXPERIMENTAL

Materials

Standard solutions of I (purchased from Calbiochem, Los Angeles, Calif., U.S.A.), II (synthesized in the Eli Lilly Research Labs.) and III (purchased from Warner Lambert, Morris Plains, N.J., U.S.A.) were prepared by dissolving the material in 0.1 *M* borate buffer and a few drops of 1 *M* sodium hydroxide, if necessary, to yield a final concentration of 1 mg/ml. All reagents were obtained from commercial sources. Sulfanilic acid (2 mg/ml) or sulfamerazine (1 mg/ml) in the above buffer were routinely used as internal standards.

Liquid chromatography

Liquid chromatography was performed on a Varian Model 4100 liquid chromatograph equipped with a UV (254 nm) photometric detector and a Model A-20 strip chart recorder. The signal from the photometric detector was fed to an IBM 1800 computer for peak integration. Solutions were chromatographed at room tem-

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perature on a 1 m × 2.1 mm I.D. stainless-steel column packed with a strong anion-exchange resin (Zipax® SAX; E. I. DuPont, Wilmington, Del., U.SA.). The mobile phase was composed of 0.01 *M* sodium tetraborate, pH 9.2, and 0.003 *M* sodium sulfate. A flow-rate of 0.8 ml/min was maintained. A photometer range of 0.16 a.u.f.s. was adequate.

Sample preparation

Capsule contents were dissolved in 0.1 *M* borate buffer with the aid of 2 ml 1 *M* sodium hydroxide to give a final concentration of 1 mg/ml of II. Aliquots of ampoule contents were diluted to a similar concentration. Weighed samples of aluminum hydroxide gel suspensions were dissolved in 1 *M* sodium hydroxide to a final concentration as above. The diluted aluminum hydroxide gel suspensions of I and III were allowed to stand for a few hours to achieve complete dissolution. A separate determination of the density of the gel provided the necessary information for calculation of drug per volume.

The standard and samples were all further diluted for chromatography. Five-milliliter aliquots were transferred to 100-ml volumetric flasks containing 5 ml of the appropriate internal standard solution and the flasks were diluted to the mark with water. Injections of 10 μl were made. Routinely, sulfanilic acid was employed as the internal standard for II and III and sulfamerazine for I.

RESULTS AND DISCUSSION

The mobile phase described for HPLC analysis of I[1] was tested as the eluting solvent for II. Compound II was eluted at the solvent front. Retention was obtained by lowering the sodium sulfate concentration. In addition II could be resolved from possible synthetic impurities and from potential metabolites (Fig. 1).

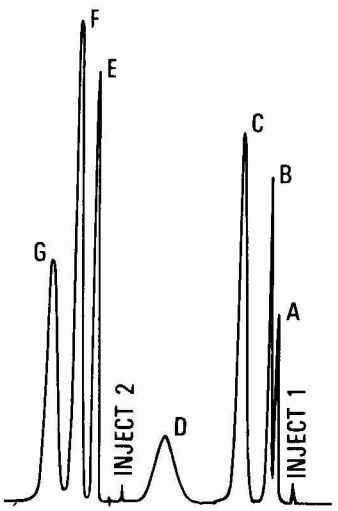

Fig. 1. HPLC recorder tracing. Injection 1: (A) decarboxylated II, (B) II, (C) sulfanilic acid, (D) 6-methoxy-7-hydroxy analogue of II; injection 2: (E) oxolinic acid, (F) nalidixic acid, (G) sulfamerazine.

TABLE I

RELATIVE RETENTION TIMES OF THE COMPOUNDS TESTED

Compound	t_R
Oxolinic acid	0.52
Nalidixic acid	0.86
Sulfamerazine	1.34
Decarboxylated II	0.34
II	0.45
Sulfanilic acid	1.00 (7.6 min)
6-Methoxy-7-hydroxy analogue of II	2.38

Sulfanilamide antibiotics had been successfully chromatographed in a similar solvent system also[3]. These were tested for potential use as an internal standard in the system under discussion. Sulfamerazine was satisfactorily resolved from the compounds under consideration. In the interest of keeping analysis time to a minimum, sulfanilic acid was chosen as the internal standard for II and III. Sulfamerazine was used for compound I.

The relative retention times of the compounds tested are compiled in Table I. Decarboxylated II is a possible synthetic impurity and possible degradation product. The 6-methoxy-7-hydroxy analogue of II is a possible metabolite comparable to one proposed for III[4]. Another compound tested, the 6,7-dihydroxy analogue of II was retained by the column in excess of 90 min. Further studies on the analysis of metabolites of II will be reported at a later date[5].

The response of the analytical system was linear in a range from 10–200% of

TABLE II

ANALYSES OF DIFFERENT DOSAGE FORMS

Dosage form	*HPLC*	*Microbial*	*Theory*
Capsule	50.3	50.2	50 mg/capsule
	51.4	49.5	
	51.1	50.1	
Capsule	254.0	256 9	250 mg/capsule
	258.7	256.9	
	257.1	256.9	
	248.0	245.8	
Ampoule	502	500	500 mg/ampoule
	494	489	
	500	504.6	
Aluminum hydroxide gel suspension	277	—	250 + 10% mg/5 ml
	263	—	
	267	—	
	274	—	
Aluminum hydroxide gel suspension of nalidixic acid	295	—	250 + 20% mg/5 ml
Aluminum hydroxide gel suspension of oxolinic acid	267	—	250 + 10% mg/5 ml

the described final sample concentration of 0.05 mg/ml. The extrapolated line passed through the origin and had a slope of 10 Area Ratio Units/mg·ml. The area ratios measured for multiple injections of the same solution were within 0.35% of the average value. Five replicate weighings of standard material gave an R.S.D. of $\pm$ 0.6%. Twenty replicate capsule samples gave values with $\pm$ 2.0% of the average and were 0.9% higher than label claim. Standard added to placebo gave 100% recovery. Some typical analytical results are shown in Table II, with microbiological assay results shown for comparison. Note that the suspensions could not be assayed microbiologically.

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Note

The separation of butylated hydroxyanisole isomers on Sephadex LH-20

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Butylated hydroxyanisole (BHA) is an antioxidant used to prolong the shelf life of many fats and fatty foods. Commercial BHA consists of two isomers (3-*tert.*-butyl-4-hydroxyanisole and 2-*tert.*-butyl-4-hydroxyanisole), and it may contain 5–37% of the 2-isomer[1,2].

Current research studies of various food additives require pure samples of each BHA isomer in sufficient quantity for biological testing. The two isomers differ in their reactivity due to the difference in degree of steric hindrance of the phenolic group by the *tert.*-butyl group. It has been reported[3] that Sephadex LH-20 retards hydroxyl compounds in chloroform; therefore, the chromatographic separation of BHA isomers was attempted using LH-20 with a chloroform–cyclohexane eluent.

EXPERIMENTAL

Thirty grams of Sephadex LH-20 gel (Pharmacia, Piscataway, N.J., U.S.A.) were swollen in chloroform–cyclohexane (1:1, v/v) overnight. The chloroform contained 0.75% alcohol as a stabilizer. The gel was transferred to a Sephadex SR-25 column and allowed to settle under gravity. The resulting column was 16.5 cm long, had a calculated volume of 84 ml, and was stabilized with the appropriate flow adapters. After continued use, the gel had a tendency to compress approximately 1 cm. The column was incorporated into a total chromatography system, which is outlined in Fig. 1.

BHA and BHT (2,6-di-*tert.*-butyl-4-methylphenol) were obtained from ICN Pharmaceuticals (Cleveland, Ohio, U.S.A.). Pure samples (98–100%) of the isomeric forms of BHA were kindly supplied by Eastman (Kingsport, Tenn., U.S.A.). The isomeric standards were used to calibrate the chromatographic column, thin-layer chromatography (TLC) system, and ultraviolet (UV) and infrared (IR) spectrophotometers.

Samples (10–200 mg) were dissolved in 0.5–1 ml of eluting solvent and transferred onto the column with 1-ml washings of eluting solvent. The effluent was monitored for UV absorption at 280 nm, and fractions of 5 ml were collected at a flow-rate of 30 ml/h. Fractions were pooled according to their UV absorption, and the solvent was removed under reduced pressure. The composition of the peaks was determined by TLC on silica gel[1] and IR internal reflection spectrometry.

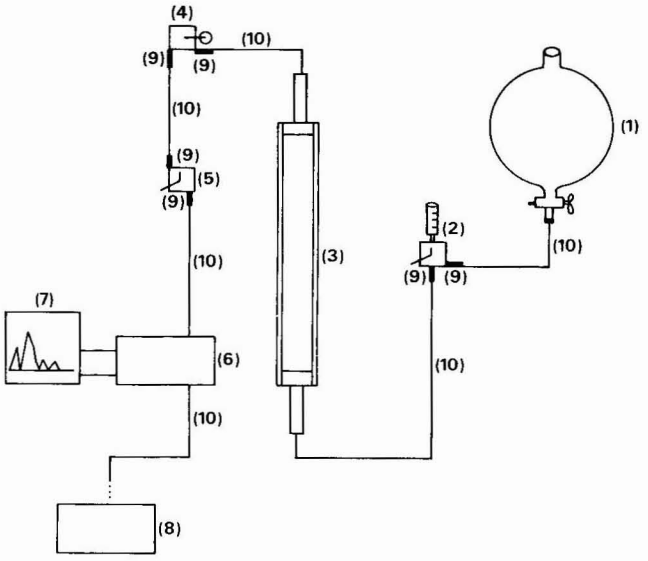

Fig. 1. Total chromatography system. 1 = Reservoir, 500 ml; 2 = 3-way valve with glass syringe; 3 = Sephadex SR-25 column; 4 = metering-type valve; 5 = on/off-type valve; 6 = UV monitor; 7 = recorder; 8 = fraction collector; 9 = 18-gauge stainless-steel needles ground to blunt point; 10 = PTFE tubing (I.D. 1.2 mm, O.D. 1.8 mm).

RESULTS AND DISCUSSION

Table I summarizes the elution peak volumes and the elution fractions containing the BHA isomers and BHT. BHT was included because it is a food antioxidant used in conjunction with BHA and contains a hydroxyl group sterically hindered by two *tert.*-butyl groups. Its early elution suggests hydrogen bonding between hydroxyl groups of the compounds, and Sephadex LH-20 is responsible for the adsorption observed in this solvent mixture.

Fig. 2 illustrates a typical separation of 200 mg of BHA. The separation of the two isomers is excellent. Three unknown peaks were also observed, but no attempt has been made to identify these trace impurities. The isomer peaks showed single spots in TLC[1], and their IR spectra compared identically to the spectra of known compounds and to published spectra[4].

TABLE I

SEPARATION OF BHA AND BHT ON SEPHADEX LH-20 WITH CHLOROFORM–CYCLOHEXANE (1:1) AS THE SOLVENT SYSTEM

Column height, 16.5 cm; flow-rate, 30 ml/h.

Compound	*Approx. elution peak (ml)*	*Elution fraction (ml)*
BHT	41– 45	36– 50
3-BHA	376–380	346–430
2-BHA	476–480	441–530

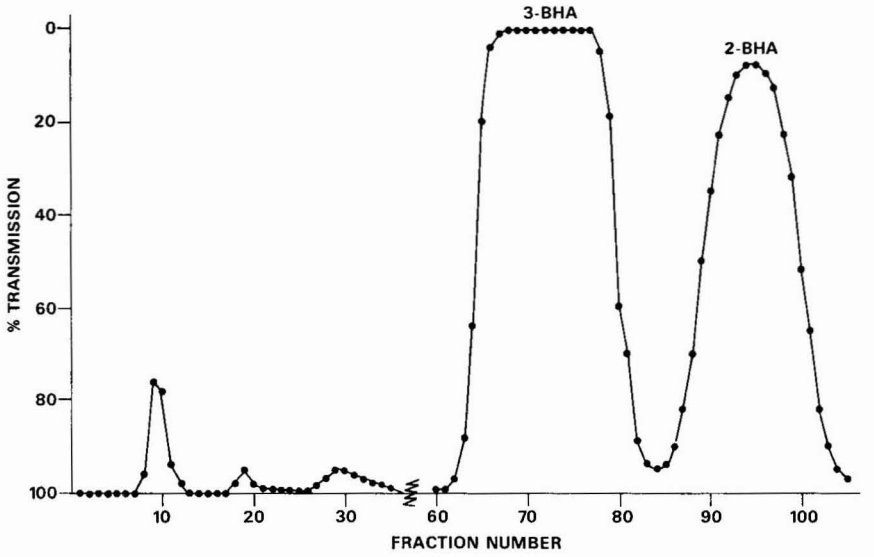

Fig. 2. Separation of BHA isomers on Sephadex LH-20 in cyclohexane–chloroform (1:1, v/v). Sample, 200 mg. Flow-rate, 30 ml/h. Ratio of 3-BHA to 2-BHA, 12:1.

The system described is applicable for the separation and purification of BHA isomers. Its advantage is that BHA remains stable, whereas in preparative TLC, the compounds discolor on silica gel after brief exposure to air.

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Note

Detection and quantitative analysis of sanguinarine in edible oils*

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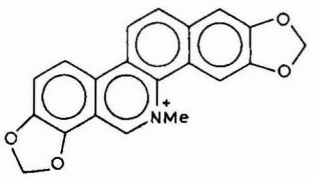
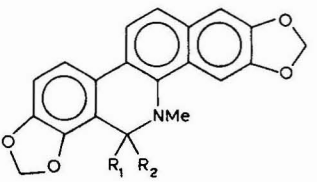
Since about 1886 widespread epidemics of dropsy have been recorded[1] in India, the last one occurring in Bombay as recently as 1966. An associated high-tension glaucoma was observed among some of the victims[2]. It has been established[3,4] that epidemic dropsy is caused by the adulteration of cooking oils with the seed oil of *Argemone mexicana* L., which grows prolifically across vast areas of India and other tropical countries. The two major alkaloids of this seed oil are[5] sanguinarine chloride (I) and dihydrosanguinarine (IIa), which together may be present in a concentration of 10 mg/ml. Sanguinarine is present in a large number of species of the Papaveraceae and Fumareaceae families[6] and it has been found[7] that sanguinarine appears in the milk and liver of cattle and sheep that graze upon pasture containing various species of poppies. It has been postulated[8] than an endemic glaucoma of various parts of the world may be caused by the innocent ingestion of very small amounts of sanguinarine over a period of time.

In order to test this hypothesis, and in order to screen cooking oils, especially in India, for the presence of *A. mexicana* seed oil it is important that a reliable method is available to detect and measure small amounts of sanguinarine. A method of detection involving paper chromatography (PC) has been described[6,7,9] but it is not completely reliable and the lower limit of detection is not known.



(I)



(IIa) $R_1 = R_2 = H$
(IIb) $R_1 = H$; $R_2 = OH$
(IIc) $R_1 + R_2 = O$
(IId) $R_1 = H$; $R_2 = CH_2COMe$

* This work was inspired by the enthusiasm of Dr. S. A. E. Hakim, who had for some years, devoted his attention to the possible relationship between the ingestion of sauguinarine and the incidence of glaucoma and cancer. Sohrab Hakim died of cancer in May 1976, and this paper is dedicated to his memory.

Hence, the aims of the present work were: (a) to improve on the method of detection of sanguinarine in certain foodstuffs and in biological samples such as urine, blood and liver, and to establish the lower limit of sensitivity and (b) to develop a method for the quantitative determination of small amounts of sanguinarine. Both procedures preferably should be capable of use in a rapid, routine method of screening by semi-skilled personnel and utilising the minimum of sophisticated equipment.

A thin-layer chromatographic (TLC) method for the detection of sanguinarine was found to be more sensitive, quicker, and more convenient than the PC and electrophoresis methods described by Hakim[6–9]. Silica plates were used, rather than alumina, since quaternary salts such as I can be transformed into pseudobases (*e.g.*, IIb) by the latter absorbant. Minor alkaloids present in the seed oil of *A. mexicana* (and identified here for the first time, see Experimental) do not interfere with the detection procedure. By using standard solutions of sanguinarine chloride in acidified ethanol, it was established that a spot applied to the TLC plate containing 10^{-9} g or more of sanguinarine could be detected.

The initial work on the quantitative determination of sanguinarine in the oils involved direct solvent extraction of I but this was thwarted by the peculiar solubility behaviour of the alkaloid and also by the readiness with which it undergoes a disproportionation reaction to give a mixture of dihydrosanguinarine (IIa) and oxysanguinarine (IIc). However, reduction of I with sodium borohydride gives IIa quantitatively and the latter, being a base, is easily extracted into dilute mineral acids (along with the other basic material present). Several methods were considered for the separation of IIa from this basic fraction, and for the quantitative measurement. PC and electrophoresis methods were quickly discarded, owing to slow development of the chromatograms and poor resolution. Gas–liquid chromatographic methods were also found to be unsatisfactory.

The TLC method developed for the detection of sanguinarine proved to be the best method. The spot of dihydrosanguinarine (IIa) present in the mixture of bases derived from the reduction procedure was well separated from other spots, and it could be easily and rapidly oxidised by air to sanguinarine by irradiating the plate with UV light. The sanguinarine, which appears as an orange spot under UV light, was quantitatively removed with a specially designed[10] micro "vacuum cleaner" and transferred to a standard volumetric flask. The concentration of sanguinarine in the spot was then derived from a measurement of the extinction coefficient at 330 nm of a solution in acidified ethanol. In this way reliable results were obtained with samples containing as little as 10^{-5} g sanguinarine in the spot applied to the TLC plate.

EXPERIMENTAL

TLC plates were prepared in the usual way by coating 20 × 20 cm glass plates with silica containing no indicator. Absorbances were measured using a Pye Unicam SP500 spectrophotometer in the null balance mode, or with the Perkin-Elmer 402. Developing solvents were distilled, and allowed to reach equilibrium in the developing chamber for 24 h before use.

Minor alkaloids of the seed oil of Argemone mexicana L.

Seed oil (200 g) was extracted by digestion with 95% ethanol at room temper-

ature over five days. The layers were separated and the ethanol evaporated under reduced pressure. The residue was dissolved in chloroform (200 ml) and extracted with 2 *M* hydrochloric acid (5 × 100 ml). The combined acid solutions were basified with ammonia and extracted with chloroform (5 × 100 ml). The organic solution was washed with water (2 × 100 ml), dried ($MgSO_4$), and evaporated under reduced pressure to leave a residue (600 mg; 0.3%) which was chromatographed on thick (1 mm) silica gel plates using benzene–methanol (6:1). The upper 80% of the orange sanguinarine band was removed and discarded. The remainder of the plate was extracted with ethanol, concentrated, and subjected to TLC on silica gel, using (A) benzene–methanol (6:1); (B) benzene–methanol (3:2), and (C) benzene–acetone–methanol (7:2:1).

Authentic samples of chelerythrine, berberine and protopine were also run on the same plates and all of the spots were visualised under UV light. Three spots were thus identified as chelerythrine (R_F values in solvents A, B, and C: 0.76, 0.72, and 0.67, respectively), protopine (R_F values in solvents A, B, and C: 0.35, 0.27, and 0.25, respectively), and berberine, which remained on the origin. Using the micro "vacuum cleaner"[10] the spots were removed, eluted and the UV spectra recorded to confirm identity. A further spot was tentatively identified as norsanguinarine [R_F value in benzene–chloroform (3:1): 0.2] but insufficient material precluded measurement of the UV spectrum. Two other minor alkaloids remained unidentified.

Detection of sanguinarine

A spot of the neat oil (*A. mexicana* L. or edible oil) was applied to a thick (750 μm) layer of silica gel G on a 20 × 20 cm plate in the usual way. The spot was developed in the first direction with benzene, and then at right angles with benzene–methanol (6:1). Sanguinarine appears, under UV light, as an orange spot (R_F values: 0.00 and 0.7–0.8 in the first and second direction, respectively). (Although the R_F value in the first solvent is zero, it was found that better resolution of the spots occurred in the second solvent if this procedure was adopted.)

Quantitative determination of sanguinarine

The sample of oil (*A. mexicana* seed oil or cooking oil) (5–30 g, as appropriate) was dissolved in petroleum ether (b.p. 40–60°) and a solution of sodium borohydride (500 mg) in ethanol (10 ml) was added with stirring. After stirring for 15 min the turbid liquid was treated with 2 *M* hydrochloric acid. The resultant lower acid layer was collected and the upper organic phase was extracted twice with 2 *M* HCl. The combined acid layers were basified ($NaHCO_3$ solution) and extracted into chloroform. The dried chloroform extract was evaporated, the residue was dissolved in ethanol, and the volume was adjusted to 1.00 ml in a standard flask.

Samples (2 μl) of this ethanol solution were spotted onto a 20 × 20 cm plate coated with silica gel G (250 μm) in the usual way. The spot runs were then scored and the chromatogram was developed over 10 cm with benzene–methanol (6:1). The plate was dried (air blast for 5 min), then irradiated with long-wavelength UV light for 15–20 min to effect oxidation of dihydrosanguinarine to sanguinarine. The latter appeared as an orange spot at R_F 0.78. This was removed from the plate with the micro vacuum cleaner[10], dissolved in ethanol containing one drop conc. HCl and the solution made up to 5.00 ml in a volumetric flask. The absorbance of this solution at 330 nm was measured and the concentration of sanguinarine computed from a calibration

curve of concentration of pure sanguinarine chloride *vs.* extinction at 330 nm. Usually ten samples of the test solution were processed on one plate and the average concentration was calculated.

RESULTS

The Beer–Lambert law was verified using solutions of sanguinarine chloride in acidified ethanol over the concentration range 0–4.0 mg per 100 ml. Measurements of absorbance were made at 330 nm.

Within a plate, using ten spots of sanguinarine chloride solution, the reproducibility of the R_F value was $\pm 3\%$ of the mean value provided that the plates were pre-saturated with solvent and the spot runs were scored. By developing plates spotted with the same sample solution in different chambers, the R_F value was $\pm 4\%$ above a mean value. The R_F value was found to be very sensitive to methanol concentration and to the method used for drying the plates after development.

A comparison of the concentration of sanguinarine chloride in ethanol solution after plating, eluting and absorbance measurement with the concentration of the original solution measured directly showed that an efficiency of $92 \pm 5\%$ was achieved in the analytical procedure.

The analysis was also tested using (a) *A. mexicana* seed oil containing a known concentration of dihydrosanguinarine and (b) mixtures of cooking oils to which known amounts of *A. mexicana* seed oil had been added.

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Note

Determination of dihydroxydianthrones by densitometry after thin-layer chromatographic separation*

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Medicinal plants such as senna, frangula, cascara and rheum have laxative activity resulting from their component dihydroxydianthrone glycosides. After oral intake of the crude drugs, the anthraglycosides are metabolized in the gastrointestinal tract by bacterial enzymes, and the free aglycones should cause the laxative effect[1]. According to Fairbairn and Moss[2], the sugars should have a transport function, enabling the aglycones to reach the site of action in the large intestine.

In order to study the metabolism of the senna glycosides in the body, where sennidines are formed, a specific method for the determination of dihydroxydianthrones is required. We have tried to determine 1,8-dihydroxydianthrones by densitometry on thin-layer chromatograms. This method has the advantage that in a mixture of aglycones, the dianthrones can be separated from the other anthracene compounds. Because of the specificity of the detection reaction, the method is more suitable for plant extracts than are the methods of Auterhoff and Kinsky[3] and Lawrence and Frei[4], in which interferences from natural compounds can affect the determination. The method is easier to perform than the method of Lemli[5] on paper chromatograms. We have applied the method to the determination of the dianthrone glycosides in senna plants.

EXPERIMENTAL AND RESULTS

Sennidine A was obtained by hydrolyzing a solution of 25 mg of sennoside A in 25 ml of 0.5% sodium hydrogen carbonate solution with 25 ml of 10 *N* sulphuric acid in a glycerine bath at 120° for 15 min. After cooling, the solution was extracted with 100 ml of diethyl ether and a 2-ml volume of the ethereal solution was evaporated to dryness (≡ 0.5 mg of sennoside A). Before use, the residue was dissolved in 2 ml of methanol.

The sennidine solution was spotted with a Hamilton microsyringe on precoated silica gel G (Merck, Darmstadt, G.F.R.) plates and developed twice over a distance of 5 cm with *n*-hexane–benzene–acetic acid (40:40:20). The plates were air

* 28th Communication on studies in the field of drugs containing anthracene derivatives.

dried, sprayed with a 30% solution of dimethylformamide in acetone and heated at 120° for 15 min. The plates were then cooled for 15 min. The sprayed plates were examined in the reflection mode using a Zeiss chromatogram spectrophotometer at a wavelength of 530 nm.

Calibration graphs with peak areas obtained using various amounts of the standard solution were plotted. Sennidine A showed a linear relationship between concentration and peak area over the range 0.10–1.5 μg of sennidine (Fig. 1). The colour of the spots was stable for at least 1 h.

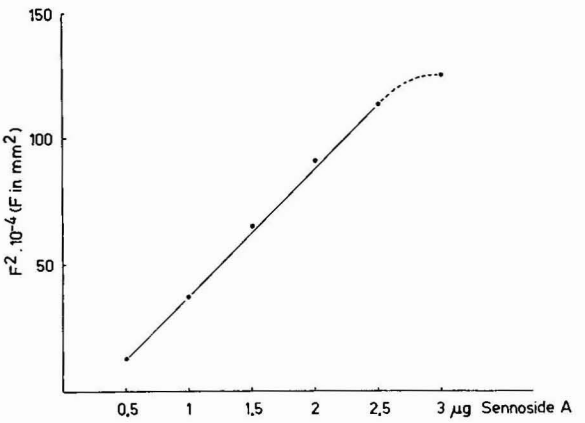

Fig. 1. Relationship between the amount of sennidine A and the corresponding peak area (F), calculated as sennoside A.

Hydrolysis of a senna leaf extract

Senna leaf (150 mg) was extracted with 30 ml of water and the extract was heated under a reflux condenser for 15 min. A 20-ml volume of the centrifuged liquid was transferred into a separating funnel and the aglycones were extracted with chloroform, after the addition of 0.1 ml of 2 N hydrochloric acid. After the addition of 100 mg of sodium hydrogen carbonate, the aqueous layer was shaken and centrifuged, then 10 ml of the liquid was hydrolyzed with 10 ml of 10 N sulphuric acid under a reflux condenser in a boiling water-bath for 15 min and, after cooling, was extracted with 50 ml of diethyl ether. A 20-ml volume of this solution was evaporated and, before use, the residue was dissolved in 2 ml methanol. For chromatography, 8-μl aliquots of this solution were spotted four times between the 4- and 6-μl spots of the standard sennidine A solution. A typical densitometric trace of a hydrolyzed senna leaf extract is shown in Fig. 2.

The order of separation on the plates was sennidine A + B (R_F 0.61) and sennidine C + D (R_F 0.41). Rhein (R_F 0.84) did not develop a colour after spraying with dimethylformamide. Densitometric analysis of senna leaf gave a concentration of 1.77% of sennoside A and B ($n = 6$, $S_{rel.} = 4.18\%$) and 0.52% of sennoside C and D, calculated as sennoside A. These results are in agreement with the results found by Lemli[5]. Spectrophotometric analysis of the total concentration of glycosides on the same senna leaf, following the method of the European Pharmacopea, gave 2.97%, calculated as sennoside B.

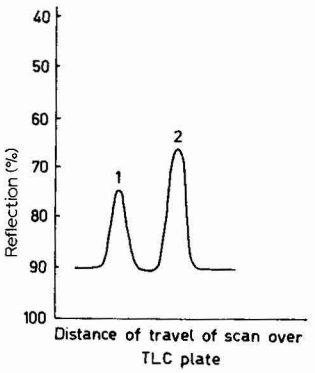

Fig. 2. A densitometric trace of the thin-layer chromatographic separation of sennidine C and D (1) and sennidine A and B (2) in a hydrolyzed extract from senna leaf.

DISCUSSION

The method described offers an approach to the analysis of sennidines and other 1,8-dihydroxydianthrones in mixtures of anthracene derivatives. The reaction of dimethylformamide is specific for 1,8-dihydroxydianthrones (*i.e.*, other dihydroxydianthrones and anthraquinones do not develop a colour with the detection solution). The method can be used to determine the composition of the dianthrone glycosides in laxative plants. This method will be applied in metabolic studies of the sennosides in the organism.

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Note

Thin-layer chromatographic separation of phosphonic acid derivatives

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We report here a convenient and sensitive thin-layer chromatographic (TLC) separation of several phosphonate compounds of current biological interest, including aminoethylphosphonic acid (ciliatine), a component of certain marine invertebrates[1]; aminomethylphosphonic acid, a plant growth regulator[2]; phosphonoacetic acid, a potent antiviral agent[3]; and methylphosphonic acid, a herbicide breakdown product found in plant tissues[4]. Ethylphosphonic acid was also included in the analysis. This TLC procedure is based on a minor modification of a solvent system developed by Libby[5], and of a phosphorus-specific visualization reagent used by Dittmer and Lester[6]. It offers a rapid procedure with a minimum number of operations, and good sample mobility, resulting in complete and reproducible resolution of all the compounds tested.

This procedure is a significant improvement over other TLC methods which are characterized by marginal resolution of phosphonic acid derivatives and/or less convenient chromatographic and visualization techniques[7–14]. Although not all the compounds tested would be expected to occur together in nature, the R_F values obtained in this study should be useful reference points on which to base TLC separations of these phosphonic acid derivatives from other materials.

EXPERIMENTAL

Materials and reagents

A plastic TLC sheet, 20 × 20 cm, precoated with a 100-μm cellulose MN-300 layer (Polygram Cell 300; Brinkmann, Westbury, N.Y., U.S.A.) is scribed to provide 2-cm wide vertical lanes. Heat activation is unnecessary. The sheet is developed in a 27.5 × 26 × 7 cm glass tank which has been lined on all sides with filter paper and pre-equilibrated for 15 min with 200 ml of developing solvent consisting of isobutanol–tetrahydrofuran–water–acetone–*p*-toluenesulfonic acid (80:60:50:10:3, v/v/v/v/w) and sealed with Saran Wrap under the lid.

The molybdenum blue visualization reagent is used to locate the phosphorus-containing solute zones and is prepared, with minor variations, by the procedure of Dittmer and Lester[6]. First two solutions are prepared. Solution I: 1 l of 25 *N* H_2SO_4 and 40.11 g of MoO_3 (Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A.) are mixed and boiled gently until the MoO_3 is dissolved (3–4 h); the light yellow

solution is allowed to cool at room temperature overnight, during which time the color changes to light blue. Solution II: a mixture of 1.78 g of powdered molybdenum (Matheson, Coleman and Bell) and 500 ml of solution I is boiled gently for 15 min, cooled and decanted from any remaining residue. The reagent is prepared by adding equal volumes of solutions I and II to 4.5 volumes of water to form a dark green solution. Solutions I and II are stable for several months when stored in the dark, but fresh visualization reagent must be prepared weekly.

All solvents and chemicals are commercially available reagent grade materials.

Procedure

A TLC sheet, with samples spotted 2.5 cm from the bottom, is developed in the system described above until the solvent front has migrated 16 cm (approximately 2 h, 45 min). The sheet is air dried for 1 h and sprayed with the molybdenum blue reagent until it is uniformly moist. Color development is complete within 30–60 min. Phosphorus-containing spots are dark blue on a light blue background. The dark background that develops after several hours can be lightened by spraying with water. If slightly more water is added to the spray reagent to produce a yellow-green solution, the time required for full color development will be increased considerably, but the background color of the sheet will be much lighter. Convenient, permanent records are obtained by outlining the spots with a felt-tip pen on the back of the sheet and photocopying the sheet from the back side.

RESULTS AND DISCUSSION

This TLC system gave complete and reproducible resolution of ethylphosphonic acid, methylphosphonic acid, phosphonoacetic acid, aminoethylphosphonic acid, and aminomethylphosphonic acid, as shown in Table I and Fig. 1.

TABLE I

R_F VALUES OF PHOSPHONIC ACID DERIVATIVES

Compounds were spotted in 0.10 *M* aqueous stock solutions. Means and standard deviations (S.D.) are based on 15 determinations.

Compound	*Formula*	*μg spotted*	*μl spotted*	$R_F \pm$ *S.D.*
Ethylphosphonic acid	$CH_3CH_2PO_3H_2$	22.0	2.0	0.78 ± 0.02
Methylphosphonic acid	$CH_3PO_3H_2$	38.0	4.0	0.71 ± 0.02
Phosphonoacetic acid	$HO_2CCH_2PO_3H_2$	56.0	4.0	0.62 ± 0.03
Aminoethylphosphonic acid	$NH_2(CH_2)_2PO_3H_2$	25.0	2.0	0.34 ± 0.03
Aminomethylphosphonic acid	$NH_2CH_2PO_3H_2$	22.2	2.0	0.21 ± 0.02

The molybdenum blue reagent was a convenient and sensitive means of visualizing the aforementioned phosphonic acid derivatives. Distinct blue spots were obtained with 0.2–0.4 μmoles of material, but spots were detectable with as little as 0.02 μmoles of phosphonate. On an equimolar basis, methylphosphonic acid and phosphonoacetic acid produced spots of approximately half the visual intensity of those produced by the other compounds.

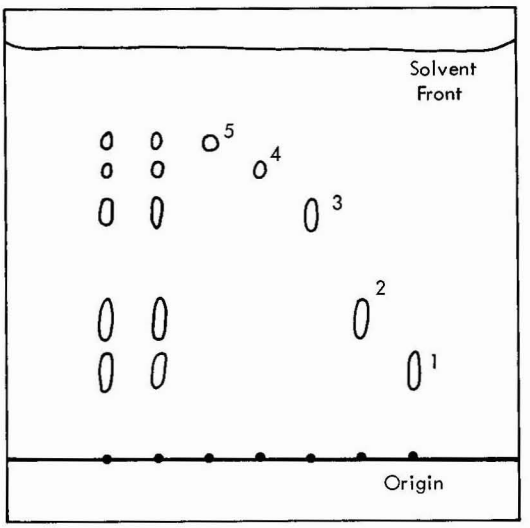

Fig. 1. TLC separation of phosphonic acid derivatives. 1 = Aminomethylphosphonic acid; 2 = aminoethylphosphonic acid; 3 = phosphonoacetic acid; 4 = methylphosphonic acid; 5 = ethylphosphonic acid.

The *p*-toluenesulfonic acid remaining on the plate after development and drying prevented the characteristic amino-specific color reaction of ninhydrin with the aminoalkylphosphonic acids. After spraying a plate with ninhydrin and heating at 100° for 30 min, all sample spots remained colorless on a light brown background.

Separation of the five phosphonic acid derivatives was complete and reproducible with this TLC system, and we obtained rapid sensitive visualization of the phosphonate spots with the molybdenum blue reagent. This system has been used successfully in our laboratory to detect impurities (approximately 20%) in 25-μg samples of aminomethylphosphonic acid preparations.

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journal of
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APPARATUS

N-968

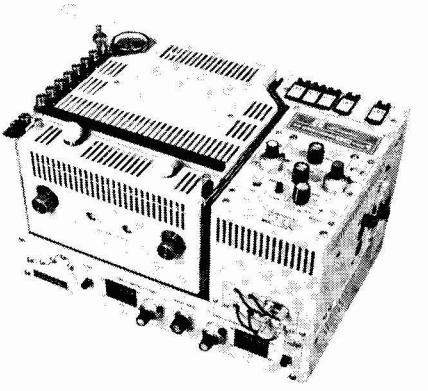
NEW PACKARD GAS CHROMATOGRAPH

The new Packard-Becker gas chromatograph (Series 428) is a dual column/dual detector system that can be arranged in many system configurations. Injection ports are optimised for each column type (metal, glass, capillary) and are interchangeable. Detectors are housed in a separate oven; any dual detector configuration is possible. The "analytical module" contains the injectors, columns and detectors. Complete column access is achieved by the lift mechanism which raises the analytical module. The flow system is kept completely modular by means of small plug-in units.

N-979

SHIMADZU MINI-GAS CHROMATOGRAPH

The Shimadzu GC-mini 1 gas chromatograph has been designed for glass capillary columns but can also be used with normal packed columns. It is equippped with a high-performance FID and features a dual column flow system, all-glass flow line, on-column injection system, solid-state temperature programmer.



N-977

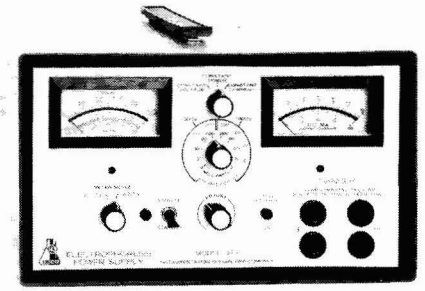
KONTES CHROMATOGRAPHY CATALOGUE

Now available from Kontes is their new 48-page chromatography catalogue. Featured sections include sample preparation, column chromatography, TLC systems, gas chromatography and accessories. The TLC section includes a list of the complete line of precoated plates.

N-972

ELECTROPHORESIS POWER SUPPLY

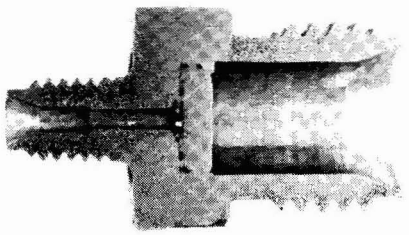
ISCO has introduced a new, pulseless, constant-wattage power supply for electrophoresis. The Model 493 delivers up to 200 watts at 1000 V. Two outlets allow simultaneous operation of dual electrophoretic systems. Five wattage ranges are provided; the unit will also provide constant voltage or constant current.



N-976

COLUMN TERMINATOR SNUBBER

Analabs' minimum-dead-volume snubber is a stainless-steel reducing union 1/4 in. to 1/16 in. incorporating a sintered stainless-steel filter with a two-μm opening. The snubber dampens pressure surges and thus helps protect downstream equipment.



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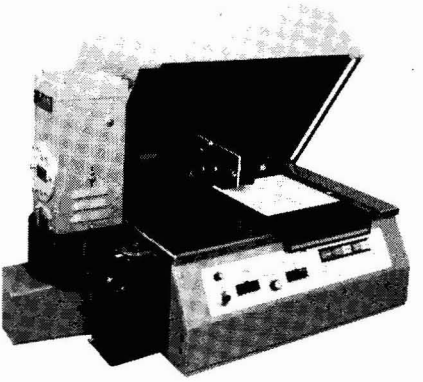
N-974

SPEED DRIVE FOR CHROMATOGRAM SCANNER

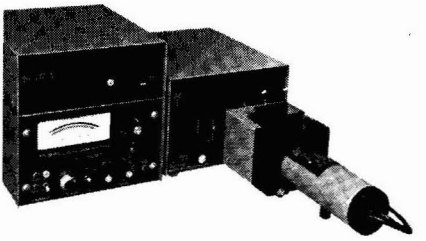
The Schoeffel Instrument Corp. has introduced a reversible variable scanning speed drive for the SD 3000 Chromatogram Scanner, to match the scanning stage speed to the chromatogram. Speed ranges include 8, 4, 2, 1, 0.5 and 0.25 inches/min. Selection of speeds is accomplished by a simple switch.



N-973

VARIABLE-WAVELENGTH FLUORESCENCE DETECTOR

The Schoeffel Instrument Corp. announce their new continuously variable-wavelength fluorescence detector for liquid chromatography. The FS 970 features a 5 μl capacity cuvette of 2π Steradian design, and a broad band deuterium (or tungsten) lamp with a high throughput monochromator.



N-978

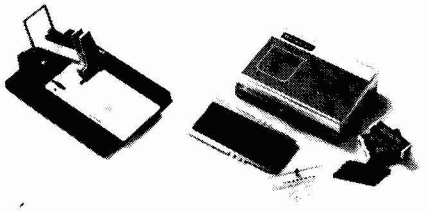
PYE UNICAM HIGH-PERFORMANCE LIQUID CHROMATOGRAPH

The new LC3 liquid chromatograph from Pye Unicam features the LC3 UV detector operating in the range 190–380 nm. The modular chromatograph is suited to quantitative determinations on a routine basis, and includes solvent reservoir facilities, a positive displacement pump and column mounting rack. Continuous operation of flow rates of up to 10 ml/min and 160 bars pressure can be achieved. A full range of accessories is available including the LC–EC detector.

N-975

MULTIPLE-SAMPLE APPLICATOR KIT

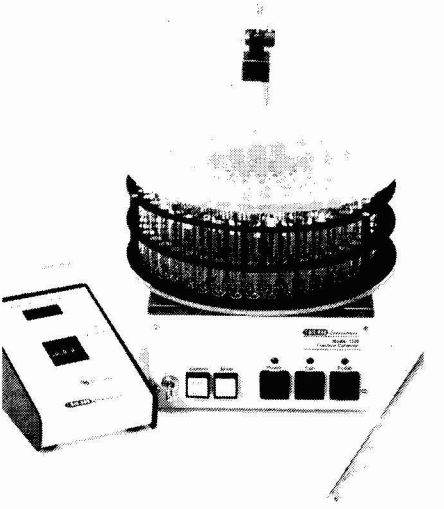
With the Beckman Multiple-Sample Applicator Kit (in box at right), the Agarose Applicator (left) can be converted to cellulose acetate capability by changing carrier plates and applicator tips. The application of samples is carried out by placing the buffered membrane on the carrier plate and then applying eight samples simultaneously. All parts of the kit are available separately on a replacement basis.



N-970

FRACTION COLLECTOR

The Bio-Rad Model 1320 fraction collector is one of their new "chromatographic compatibles". Four lift-off turntables are available: Standard test tube, preparative, scintillation vial, and spot collection. Indexing features include automatic digital counting and timed indexing from 1 to 999 seconds or 1 to 999 minutes. Counting can be 1 to 999 drops with the optional drop counter. The fraction collector is cold room compatible and is available in 220 V (50 Hz) or 110 V (60 Hz).



NEW JOURNAL

Journal of Chemical Research, The Chemical Society, Société Chimique de France and Gesellschaft Deutscher Chemiker. The journal will appear in two parts: Part S (Synopsis), containing synopses of all papers accepted for publication after a normal refereeing procedure (Part S will appear as a conventionally printed and bound journal), and Part M (Microfiche and Miniprint). Part M contains the complete articles for which synopses appear in Part S, in two forms: on microfiche or in miniprint format. The journal will be published on a monthly basis from January 1977 on. All sections will be published simultaneously. Total subscription price: £ 65.00 for 1977. Further information can be obtained from The Chemical Society, Burlington House, London W1V 0BN, Great Britain.

NEW BOOKS

The identification of molecular spectra, by R.W.B. Pearse and A.G. Gaydon, Chapman & Hall, London, 4th ed., 1976, *ca.* 424 pp., price £ 20.00, ISBN 0-412-14350-X.

Dictionary of organic compounds, 12th Suppl., edited by J.B. Thomson, Eyre & Spottiswoode/ E. & F.N. Spon, London, 1976, 272 pp., price £ 22.50, ISBN 0-413-60810-7.

Mass spectroscopy, (Wykeham Science Series, 44), by J.R. Majer, Taylor & Francis, London, 1976, *ca.* 180 pp., price *ca.* £ 3.00, ISBN 0-85109-550-X.

Principles of Mossbauer spectroscopy, by T.C. Gibb, Chapman & Hall, London, 1976, 260 pp., price *ca.* £ 9.00, ISBN 0-412-13960-X.

Molecular spectroscopy, Vol. 4, edited by R.F. Barrow, D.A. Long and J. Sheridan, Chemical Society, London, 1976, VIII + 279 pp., price £ 17.25, ISBN 0-85186-536-4.

Photochemistry of heterocyclic compounds, edited by O. Buchardt, Wiley-Interscience, New York, London, 1976, VII + 622 pp., price US$ 57.00, £ 33.00, ISBN 0-471-11510-X.

Terpenoids and steroids, Vol. 6, edited by K.H. Overton, Chemical Society, London, 1976, X + 363 pp., price £ 20.50, ISBN 0-85186-306-X.

The determination of sulphur-containing groups, Vol. 2, by M.R.F. Ashworth, Academic Press, New York, London, 1976, XI + 288 pp., price US$ 21.50, £ 9.80, ISBN 0-12-065002-9.

Separation and purification methods, Vol. 4, edited by E.S. Perry,, C.J. van Oss and E. Grushka, Marcel Dekker, New York, Basel, 1976, XII + 413 pp., price US$ 32.50.

Dyes and their intermediates, by E.N. Abrahart, Edward Arnold, London, 1977, *ca.* 272 pp., price *ca.* £ 12.00, ISBN 0-7131-2580-2.

Equilibrium in solids: Surface and colloid chemistry, by G. Scratchard, Harvard Univ. Press, Cambridge, Mass., London, 1976, XXXV + 306 pp., price £ 20.40, ISBN 0-674-26025-2.

Handbook of analysis or organic solvents, by V. Sedivec and J. Flek, Ellis Horwood, Chichester, 1976, 455 pp., price £ 18.00, US$ 34.20, ISBN 0-85312-030-7.

Modern fluorescence spectroscopy, Vol. 2, edited by E.L. Wehry, Plenum Press, New York, London, 1976, XX + 459 pp., price US$ 35.40, ISBN 0-306-33904-8.

Air pollution from pesticides and agricultural processes, edited by R.E. Lee, Jr., CRC Press, Cleveland, Ohio, 1976, *ca.* 300 pp., price *ca.* US$ 37.00, ISBN 0-87819-151-8.

Carbamate insecticides: Chemistry and biochemistry, by R.J. Kuhr and H.W. Dorough, CRC Press, Cleveland, Ohio, 1976, 312 pp., price US$ 49.95, ISBN 0-87819-052-X.

Guidelines for analytical toxicology programs, edited by I. Sunshine, P. Bondo and J. Thoma, CRC Press, Cleveland, Ohio, 1976, *ca.* 300 pp., price *ca.* US$ 49.95, ISBN 0-87819-150-X.

Handbook of chemistry and physics, edited by R.C. Weast, CRC Press, Cleveland, Ohio, 57th ed., 1976, 2365 pp., price US$ 29.95, ISBN 0-87819-456-8.

1977 Annual book of ASTM standards, ASTM, Philadelphia, Pa., 1977, 48 parts, over 39,000 pp., total price US$ 1170.00.

Calibration in air monitoring, (STP 598), ASTM, Philadelphia, Pa., 1976, 356 pp., price US$ 33.00.

HPTLC – High performance thin-layer chromatography, (Journal of Chromatography Library, Vol. 9), edited by A. Zlatkis and R.E. Kaiser, Elsevier, Amsterdam, Oxford, New York, and Institute of Chromatography, Bad Dürkheim, 1976, 240 pp., price Dfl. 110.00, US $ 43.95, ISBN 0-444-41525-4.

Modern methods of chemical analysis, by R.L. Pecsok, L.D. Shields, T. Cairns and I.G. McWilliam, Wiley, New York, Santa Barbara, London, Sydney, Toronto, 2nd ed., 1976, XVIII + 573 pp., price £ 13.50, US $ 21.50, ISBN 0-471-67662-4.

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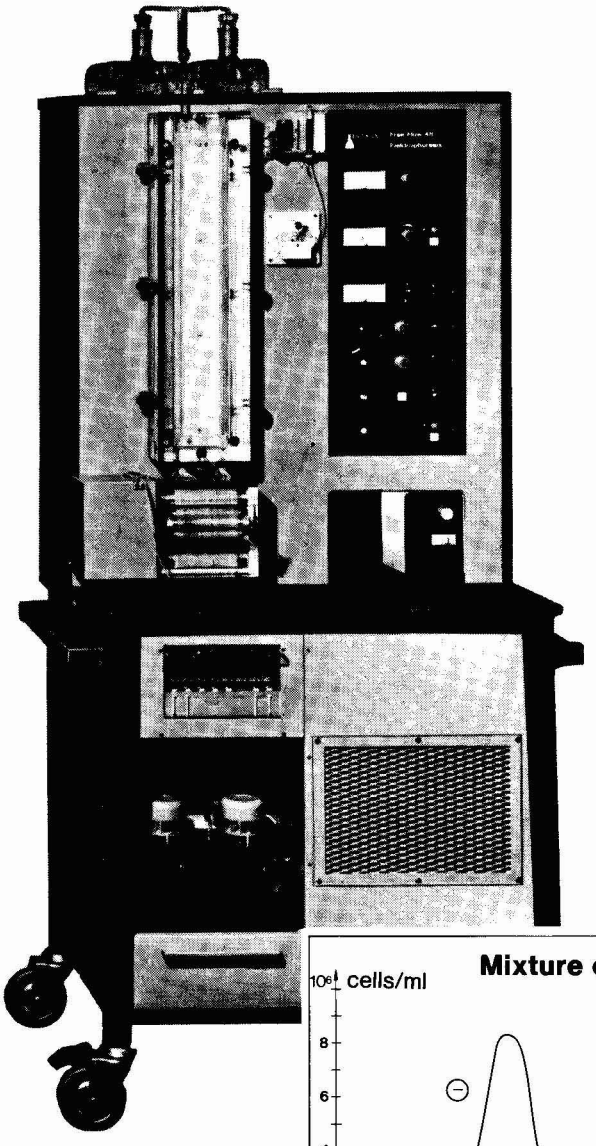

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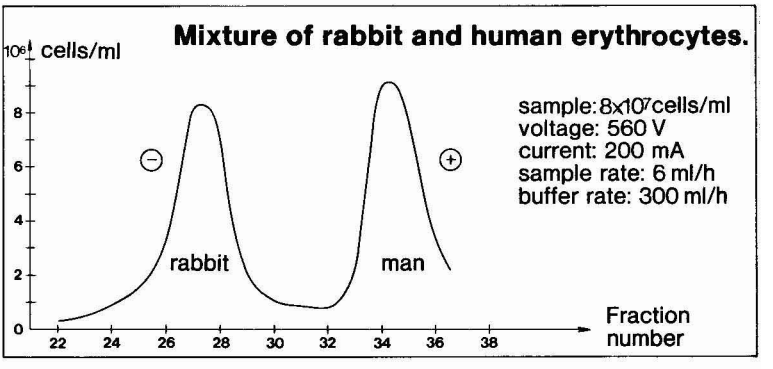

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