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JOURNAL OF CHROMATOGRAPHY

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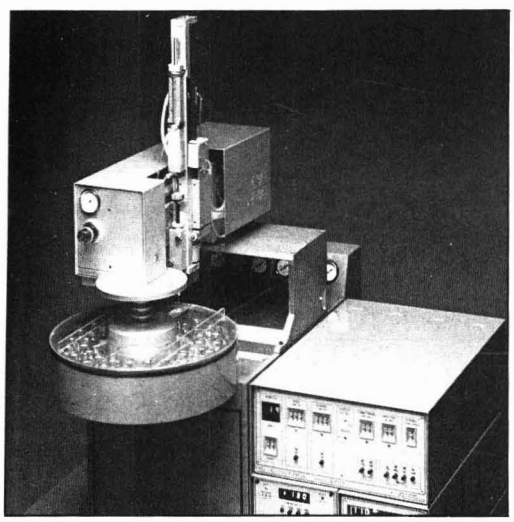

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GAS–LIQUID CHROMATOGRAPHIC STUDY OF THE THERMODYNAMICS OF 1-IODOACETYLENE–LEWIS DONOR MOLECULAR ASSOCIATIONS

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(First received December 10th, 1980; revised manuscript received January 19th, 1981)

SUMMARY

Gas–liquid chromatography was used to study molecular associations involving 1-iodo-1-dodecyne and 21 Lewis donors. The data sometimes show slight positive or negative deviations from the diachoric model of solutions but they agree fairly well with the latest developments of conventional theories. However, Purnell's classical simple relationship $K_{R(M)} = f(C_A)$ gives precise thermodynamic parameters of donor–acceptor complexations. The behaviour of 1-iodododecyne towards very different Lewis bases shows that the electrostatic contribution accounts for 13–80% of molecular interactions.

INTRODUCTION

Iodoalkynes are Lewis acids, so they form molecular associations with electron donors[1]. Interactions can be divided into electrostatic and covalent types. In particular, experimental data relating to charge transfer (CT) complexes between 1-iodo-1-dodecyne and various Lewis bases can be correlated using the so-called double scale equation proposed by Drago *et al.*[2]:

$$-\Delta H = E_A E_D + C_A C_D \quad (1)$$

where E_A and E_D, C_A and C_D are the susceptibility of the acid and base, respectively, to undergo electrostatic and covalent interactions.

UV spectroscopy cannot be used owing to the difficulty in observing the CT band lying in the vacuum ultraviolet region and the strong absorption of the Lewis bases. In IR spectroscopy, the stretching frequency ν_{CI} lies near 400 cm^{-1} and its low molar absorptivity makes the study difficult. However, spectroscopic evidence for a 1:1 complex between 1-iodo-1-dodecyne and pyridine was provided by the splitting of the ν_{6a} band of pyridine: at a base concentration of 0.425 mole dm^{-3} in carbon tetrachloride and a 1-iodo-1-dodecyne concentration varying from 0 to 1.585 mole dm^{-3}, an isosbestic point was observed (Fig. 1). The corresponding equilibrium constant by Liptay's method[3] is 1.07 ± 0.30 mole dm^{-3}.

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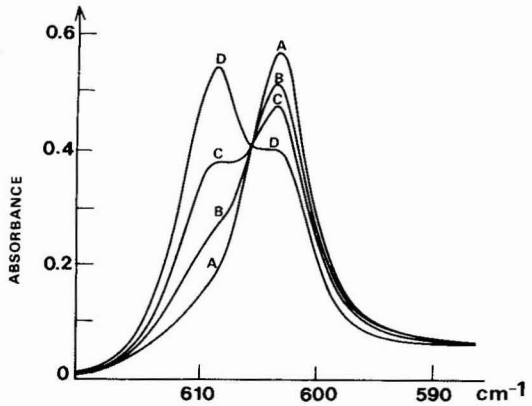


Fig. 1. ν_{6a} infrared band of pyridine in CCl_4. Pyridine concentration 0.425 mole dm^{-3}. 1-Iodo-1-dodecyne concentrations: 0, 0.277, 0.713 and 1.585 mole dm^{-3} for A, B, C and D, respectively.

We turned to gas–liquid chromatography, which has been used successfully by several workers[4–10] for the study of molecular associations; in our laboratory good agreement with the IR spectroscopic procedure was found as regards hydrogen-bonding associations between alkynes and proton acceptors[11].

A molecular interaction theory using a chromatographic procedure has recently been developed[12]. In this work an attempt has been made to check its ability to separate specific from non-specific interactions in donor–acceptor associations.

EXPERIMENTAL

Materials

We prepared 1-iodo-1-dodecyne (A) using Shostakovskii *et al.*'s method[13] from commercial 1-dodecyne and purified it by fractional distillation. Its low vapour pressure enabled us to use it as a liquid stationary phase below 80°C (P_0 = 0.1 mmHg).

The solvent squalane (S) was free from ethylenic hydrocarbons. Iodododecane, the reference liquid for Martire and Riedl's method[8] and the injected base solutes (D) were commercial products and were used without further purification. Table I gives the boiling points (T_b), the saturation vapour pressures (P_0), the molar volumes (V_D) and the second virial coefficients (B_{DD}) of the solutes. Saturation vapour pressures were calculated from literature data[14,15] and second virial coefficients from Berthelot's relationship from critical temperatures and pressures.

The densities of mixtures of iodododecyne and squalane and of pure liquids were evaluated using pyknometry (Table II). There is no excess volume of mixing:

$$V_M = V_S x_S + V_A x_A \qquad (2)$$

where x_i is the molar fraction of component i and V_i is the molar volume.

Apparatus and procedure

We used the injector and katharometer of a Girdel 3000 apparatus. Stainless-

TABLE I

PHYSICAL CONSTANTS OF BASES

Base	T_b^{760}	P_0 (25°C)	B_{DD} (cm^3)	V_D (cm^3)
Pyridine	115.5	20.7	1280.7	97.82
Propionitrile	97	46.7	1616	70.90
Dioxane	101	40.6	1468	85.71
Tetrahydrofuran	66	176	1138	80.63
Diethyl sulphide	92.1	58.4	1829	108.5
Diethyl selenide	110	31	–	111.4
Acetone	56.2	229.2	1024.9	74.0
2-Butanone	80	100	1292.1	90.1
3-Methyl-2-butanone	93	62.25	–	107.9
Chloroacetone	119	20	–	80.5
Butadione	88	79.7	–	87.0
Trifluoroacetone	22	–	–	–
Ethyl acetate	77	94.5	1396.2	98.3
Methyl acetate	57.5	216.2	1032.1	79.9
Ethyl thioacetate	116	23.6	–	106.6
Ethyl propionate	97	51.3	1817.2	115.7
Acetaldehyde	21	900.7	562.2	57.11
Diethyl ether	34.6	534.2	1046.0	104.75
Furan	31	614	–	73.11
Benzene	80	95.2	1378.8	89.4
Thiophene	84	87.7	–	79.47

steel columns (1 m × 2.2 mm I.D.) were placed in a liquid bath thermoregulated to within ±0.05°C. A mercury manometer measured the inlet pressure, about 300 mmHg above atmospheric pressure.

The flow-rate of the carrier gas (helium) (*ca.* 20 $cm^3\ min^{-1}$) was measured with a soap film flowmeter. The pressure drop and the flow-rate were approximately constant for the 22 columns used.

The lack of a mixed second virial coefficient for the solute D and the carrier gas, B_{DHe}, prevented us from calculating the correction for zero pressure drop to obtain K_R^0, partition coefficient at zero total pressure. Indeed, corrections are not important; at 25°C in pure squalane, K_R, the partition coefficient at the column

TABLE II

DENSITIES OF 1-IODODODECYNE–SQUALANE MIXTURES AT FIVE TEMPERATURES

x*	*Density* ($g\ cm^{-3}$)				
	15°C	*25°C*	*35°C*	*45°C*	*55°C*
0	0.8150	0.8093	0.8023	0.7960	0.7890
0.189	0.8574	0.8514	0.8440	0.8367	0.8295
0.503	0.9550	0.9480	0.9406	0.9329	0.9249
1	1.2660	1.2555	1.2450	1.2345	1.2242

* x = Molar fraction of iodododecyne.

pressure for benzene, is 525.3 and a value of 524.7 was derived from the correction using the equation[16]

$$\text{Ln } V_N = \ln K_R^0 V_1 + \frac{2\,B_{DHe} - V_D'}{RT} \cdot P_0 J_3^4 \qquad (3)$$

where V_N is the net retention volume, V_1 the volume of liquid phase in the column, V_D^∞ the partial molar volume of solute D at infinite dilution in the liquid phase, P_0 the column outlet pressure, R the gas constant, T (°K) the column temperature and

$$J_3^4 = \frac{3\left(\frac{P_i}{P_0}\right)^3 - 1}{4\left(\frac{P_i}{P_0}\right)^4 - 1}$$

where P_i is the column inlet pressure.

The support was Chromosorb P, acid washed, DMCS treated (60–80 mesh). The stationary phase was dissolved in dichloroethane and the solvent evaporated in a rotary dryer under a weak vacuum. The total mass of packing was determined by weighing and weight-percent of solvent mixture by ashing.

The contributions of solid and liquid interfaces to the partition coefficient were obtained from 10, 20 and 40% loadings (10, 20 or 40 g of liquid and 100 g of solid support). The loadings, molar and volume fractions and concentrations for each column are listed in Table III.

Measurements were made with triple injections of *ca.* 0.1 μl of liquids using a minimal attenuation katharometer. Infinite dilution of solute D in the stationary phase was derived from the variation in sample size and extrapolation to zero. Peaks were generally symmetrical, sometimes including weak tailing (Langmuir-type distribution). We computed the retention times using Conder and Young's procedure[17]; no significant variation of K_R *versus* $1/V_1$ was observed, except for the pure squalane column at 10% loading; solid–liquid interface adsorption was not taken into account[18].

TABLE III
COLUMN CHARACTERISTICS

x_A	C_A (*mole dm*$^{-3}$)	φ_A	L = 10%	L = 20%	L = 40%
0	0	0	×*	×	×
0.1195	0.245	0.057		×	×
0.1894	0.405	0.0943		×	×
0.265	0.5947	0.1384	×	×	×
0.328	0.766	0.1784		×	×
0.503	1.338	0.3114	×	×	×
0.690	2.143	0.4988		×	×
0.8995	3.436	0.7995		×	×
1	4.2967	1	×	×	×

* × = Existing column.

METHODS FOR CALCULATING EQUILIBRIUM CONSTANTS

K_R and γ_D' were computed by the classical relationships

$$K_R = \frac{V_N}{V_1}$$

and

$$\gamma_D' = \frac{nRT}{P_0 V_N} \cdot \exp\left(- \frac{B_{DD} - V_D}{RT} \cdot P_0 \right) \tag{4}$$

We used four methods for calculating the equilibrium constant, the first proposed by Purnell[4], the latest by Harbison *et al.*[12], another method given by Eon *et al.*[9] improving Purnell's, and a simpler method by Martire and Riedl[8] using a reference compound.

Purnell's classical method[4]

The formation of a 1:1 complex between a volatile solute D and an involatile additive A in solution in an inert solvent S produces a change in the partition coefficient, as shown by Purnell[4]:

$$K_{R(M)} = K_{R(S)} (1 + K_c C_A) \tag{5}$$

where $K_{R(M)}$ is the partition coefficient for solute D between the mixed liquid phase (M = S + A) and the gas phase, $K_{R(S)}$ is the same coefficient in the pure solvent and K_c is the formation constant of complex AD in the mixed solvent.

Method of Harbison et al.[12]

In studying solute–alkane and solute–binary mixed alkane systems, Laub *et al.*[20] used Prigogine's treatment as modified by Janini and Martire[21] to calculate partition and activity coefficients. Harbison *et al.*[12] extended this study to ternary systems between aliphatic, acyclic and aromatic solutes and squalane–dinonyl phthalate solvent. First, for ternary systems without molecular association, they obtained

$$\ln K_{R(M)} = \ln K_{R(S)} + \left[\left(\frac{V_D}{V_A} - \frac{V_D}{V_S} \right) + \left(\chi_S^D - \chi_A^D + \frac{V_D}{V_A} \cdot \chi_S^A \right) \right] \varphi_A - \frac{V_D}{V_A} \cdot \chi_S^A \varphi_A^2 \tag{6}$$

where χ_j^i is the Flory-type interaction parameter between i and j and φ_A is the volume fraction of additive/acceptor A.

Eqn. 6 can be written as

$$\ln K_{R(M)} = U + V\varphi_A + W\varphi_A^2$$

For alkanes, U, V and W are found by a polynominal regression[22], so χ_S^A, independent of solute D, is known. Then, when only 1:1 complexes are present in ternary systems, a rigorous treatment gives the following equation:

$$\ln K_{R(M)} = \ln K_{R(S)} + \left[\left(\frac{V_D}{V_A} - \frac{V_D}{V_S}\right) + \chi_S^D - \chi_A'^D\right]\varphi_A + \frac{V_D}{V_A}\cdot\chi_S^A\,\varphi_A\varphi_S + \ln\left(1 + \frac{K_c}{V_A}\cdot\varphi_A\right) \quad (7)$$

$\chi_A'^D$ is the non-complexing component of interaction between D and A. Eqn. 7 can be rearranged to give

$$\ln K_{R(M)} - \chi_S^A\cdot\frac{V_D}{V_S}\varphi_A\varphi_S = r + s\varphi_A + \ln(1 + t\varphi_A) \quad (8)$$

where $r = \ln K_{R(S)}$, $s = (V_D/V_A - V_D/V_S) + \chi_S^D - \chi_A'^D$ and $t = (K_c/C_A)$. For each φ_A, the left-hand side of eqn. 8 is known; r, s and t can be calculated by the weighted non-linear least-squares method[22] and χ_S^D, $\chi_A'^D$ and K_c thus obtained.

Martire and Riedl's method[8]

From the equilibrium D + A ⇆ AD between a volatile solute D and A giving rise to the thermodynamic constant K:

$$K = \frac{[AD]}{[A]\,[D]}$$

Martire and Riedl derived

$$K' + 1 = \frac{V_{gA}^D\,V_{gR}^{al}}{V_{gR}^D\,V_{gA}^{al}} \quad (9)$$

where $K' = K[A] = K\gamma_A C_A$, V_{gj}^i is the specific retention volume of an injected solute i in liquid phase j, al represents alkane and R is the reference stationary liquid phase. γ_A was obtained from

$$\gamma_A = \frac{V_{gA}^{al}\,M_A}{V_{gR}^{al}\,M_R} \quad (10)$$

where M_i is the molecular weight of i.

Iodododecane was chosen as the reference stationary phase owing to its similarity to 1-iodododecyne as regards molecular size and polarizability. According to eqn. 9, only four specific retention volumes are needed in order to obtain K.

Eon et al.'s method[9]

Eqn. 5 takes into account neither the size differences between molecules nor the activity coefficients of A, S and D. Eon *et al.*[23] established the relationship

$$K_{R(M)}[V_S + (V_A - V_S)\,x_A] = V_S K_{R(S)}[1 - (\psi + K_x^*)\,x_A] \quad (11)$$

with

$$\psi = \frac{\gamma^{\infty a}_{D(S)}}{\gamma^{\infty a}_{D(A)}} \approx \frac{V_A}{V_S}\left[\frac{\exp\left(\frac{V_D}{V_A}\right)}{\exp\left(\frac{V_D}{V_S}\right)} - 1\right]$$

where $\gamma^{\infty a}_{D(S)}$ and $\gamma^{\infty a}_{D(A)}$, from Flory–Huggins theory, are athermal activity coefficients at infinite dilution for D in S and A, respectively; the standard state is pure liquid D. K^*_x is the thermodynamic constant:

$$K^*_x = \frac{x_{AD}}{x_A x_D} \cdot \frac{\gamma^*_{AD}}{\gamma^*_A \gamma^*_D}$$

where γ^*_i is the activity of species i with a reference state of infinite dilution.

Eqns. 2, 5 and 11 can be combined to give[24,25]

$$K^*_x = \frac{K_C}{v_S} + \frac{V_A}{V_S}\left[1 - \frac{\exp\left(\frac{V_D}{V_A}\right)}{\exp\left(\frac{V_D}{V_S}\right)}\right] \tag{12}$$

RESULTS AND DISCUSSION

Equilibrium constants and enthalpy variations

The value of $K_{R(S)}$ in pure squalane, $K_{R(A)}$ in pure iodododecyne and γ^{∞}_D at 25°C are given in Table IV. The $K_{R(M)}$ values were too numerous to be listed. As shown by a good correlation coefficient, varying from 0.97 to 0.998 for nine points, a linear relationship occurs between $K_{R(M)}$ and C_A for all bases. Nevertheless, it is worth noting that such a correlation coefficient may sometimes be found even if the data obey the following polynominal equation:

$$K_{R(M)} = U + VC_A + WC_A^2$$

where U, V, W are numerical parameters. For instance, for diethyl sulphide, in spite of the linear correlation coefficient of 0.995, the experimental data are better fitted by a parabolic regression. The inclusion of an extra power in C_A effects a reduction Δ in the sum of squares about the regression relative to the s^2 estimate. A suitable statistical test[26] for establishing whether or not this improvement is significant was then applied:

$$T = (n - m - 1) \cdot \frac{\Delta}{(\Sigma W_i D_i^2)_{m+1}}$$

TABLE IV

PARTITION AND ACTIVITY COEFFICIENTS IN PURE LIQUIDS AT 25°C

Solute	$K_{R(S)}$*	$K_{R(A)}$*	$\gamma^{\infty}_{D(S)}$	$\gamma^{\infty}_{D(A)}$
Propionitrile	108.7	573.1	6.045	2.990
Dioxane	631.4	3191.1	1.418	0.597
Tetrahydrofuran	320.2	1250.7	0.641	0.361
Diethyl sulphide	754	3054	0.760	0.435
Diethyl selenide	1550	7103	0.740	0.362
Acetone	49.9	255	3.057	1.377
2-Butanone	177.3	772.2	2.022	0.997
3-Methyl-2-butanone	365.4	1472	1.43	0.872
Chloroacetone	393.1	1528	4.32	2.613
Butadione	160.8	524.6	2.051	1.910
Trifluoroacetone	10.1	16.4	–	–
Ethyl acetate	218.6	750.4	1.726	1.128
Methyl acetate	81.1	281.7	2.044	1.326
Ethyl thioacetate	1450	4517	1.026	0.749
Ethyl propionate	690.1	2120.1	1.007	0.734
Acetaldehyde	17.75	60.7	1.966	1.500
Diethyl ether	90.7	207.6	0.746	0.734
Furan	70.15	121.4	0.826	1.071
Benzene	525.3	983.5	0.699	0.839
Thiophene	541.4	1114.7	0.700	0.817
Pentane	114.4	105.6	0.624	1.521
Hexane	383.3	324.4	0.645	1.562
Heptane	1119.2	1024	0.699	1.633

* Confidence limits between 1 and 2%.

where n is the number of points and $\Delta = (\Sigma W_i D_i^2)_m - (\Sigma W_i D_i^2)_{m+1}$, where $(\Sigma W_i D_i^2)_m$ and $(\Sigma W_i D_i^2)_{m+1}$ are the weighted sum of the square of the residuals for m and $(m + 1)$ parameters, respectively.

For diethyl sulphide, the hypothesis $W = 0$ may be rejected at the 0.05 confidence level because $T = 33.78 > F_{1,4,0.05} = 7.71$ (seven points). Benzene, thiophene, diethyl ether, diethyl selenide and dioxane showed a similar behaviour; for acetaldehyde, tetrahydrofuran and propionitrile $T \approx F$, but for ketones and esters the rejection of the hypothesis outlined above is more significant and the linear relation was accepted without restriction.

We calculated K_c to a first approximation by the linear regression of eqn. 5. Table V contains equilibrium constants, ΔH determined by the linear least-squares fitting of log K_c *versus* $1/T$ and ΔS. Confidence limits for K_c are about 4%.

A rigorous study carried out by Martire and coworkers[27–29] showed that the found value of the equilibrium constant includes α, induced by non-complexing AD interactions or contact pairing. Hence K_c in eqn. 5 is overestimated.

To a second approximation we may infer, for reasons outlined above, that non-specific interactions involving iodododecane or iodododecyne and Lewis bases are similar. With a column of pure squalane and another column of pure iodododecane α', the interaction between iodododecane and a base, was evaluated approximately by eqn. 5. Removing α' from K_c we obtained the values given in Table VI.

TABLE V

RESULTS BY PURNELL'S METHOD FOR EQUILIBRIUM CONSTANTS, ΔH, ΔG AND ΔS

Base	K_c *(mol dm^{-3})**					$-\Delta H$ *(kcal mole^{-1})*	$s_{\Delta H}$** *(kcal mole^{-1})*	ΔG *(cal)*	$-\Delta S$ *(e.u.)*
	15°C	*25°C*	*35°C*	*45°C*	*55°C*				
Pyridine	3.42***	2.63***	2.05	1.63	1.30	4.6	0.3	−575	15.4
Propionitrile	1.17	1.06	0.87	0.77	0.66	2.8	0.15	−35	9.3
Dioxane	1.24	1.04	0.78	0.67	0.57	3.7	0.2	−23	12.3
Tetrahydrofuran	0.91	0.72	0.57	0.51	0.40	3.7	0.2	196	13.1
Diethyl sulphide	0.88	0.72	0.59	0.50	0.42	3.5	0.2	230	12.5
Diethyl selenide	1.03***	0.84	0.69	0.58	0.49	3.5	0.3	104	12.1
Acetone	1.10	0.94	0.79	0.67	0.60	2.95	0.1	37	10.0
2-Butanone	0.93	0.73	0.63	0.56	0.50	2.9	0.2	190	10.2
3-Methyl-2-butanone	0.90	0.67	0.55	0.51	0.45	3.15	0.2	238	11.4
Chloroacetone	0.78	0.65	0.58	0.51	0.49	2.2	0.2	257	8.4
Butadione	0.66	0.54	0.48	0.43	0.40	2.5	0.2	389	9.0
Trifluoroacetone	0.15	0.145	0.14	0.135***	0.13***	0.65	0.1	1150	5.9
Ethyl acetate	0.65	0.55	0.48	0.45	0.38	2.5	0.15	356	9.6
Methyl acetate	0.63	0.54	0.47	0.43	0.39	2.3	0.1	367	9.0
Ethyl thioacetate	0.64	0.55	0.44	0.42	0.37	2.5	0.15	356	9.6
Ethyl propionate	0.58	0.52	0.45	0.40	0.36	2.2	0.1	389	9.0
Acetaldehyde	0.47	0.44	0.36	0.30	0.25	3.1	0.2	489	12.0
Diethyl ether	0.33	0.29	0.24	0.21	0.18	2.9	0.1	737	12.2
Furan	0.18	0.17	0.14	0.13	0.12	1.7	0.2	1056	9.2
Benzene	0.22	0.205	0.19	0.18	0.165	1.4	0.2	944	7.9
Thiophene	0.23	0.21	0.19	0.17***	0.16***	1.8	0.3	826	9.1

* Confidence limits 4%.

** Standard deviation to linear regression.

*** Extrapolated values.

TABLE VI

$K_c - \alpha'$ VALUES AND CORRESPONDING ΔH VALUES

Base	$K_c - \alpha'$ *(mole dm^{-3})*⋆				$-\Delta H$ *(kcal mol^{-1})*	$s_{\Delta H}$⋆⋆ *(kcal mol^{-1})*
	15°C	*25°C*	*35°C*	*55°C*		
Propionitrile	0.63	0.59	0.48	0.39⋆⋆⋆	2.4	0.3
Dioxane	0.946	0.763	0.529	0.363	4.6	0.4
Tetrahydrofuran	0.726	0.530	0.40	0.28	4.5	0.3
Diethyl sulphide	0.667	0.494	0.389	0.28	4.05	0.3
Diethyl selenide	0.81⋆⋆⋆	0.590	0.47	0.31	4.2	0.4
Acetone	0.795	0.678	0.508	0.39	3.5	0.3
2-Butanone	0.58	0.427	0.345	0.262	3.6	0.4
3-Methyl-2-butanone	0.435	0.36	0.30	0.213	3.4	0.2
Chloroacetone	0.335	0.27	0.22	0.156	3.6	0.2
Butadione	0.317	0.267	0.19	0.16	3.4	0.4
Ethyl acetate	0.41	0.335	0.28	0.20	3.05	0.2
Methyl acetate	0.39	0.322	0.27	0.19	2.9	0.2
Ethyl thioacetate	0.402	0.335	0.235	0.20	3.15	0.3
Ethyl propionate	0.343	0.304	0.252	0.187	2.9	0.2
Acetaldehyde	0.23	0.19	0.16	0.11	3.5	0.2
Diethyl ether	0.22	0.185	0.15	0.10	3.8	0.2

⋆ Confidence limits 6–8 %.
⋆⋆ Standard deviation to linear regression.
⋆⋆⋆ Extrapolated values.

TABLE VII

RESULTS OF MARTIRE AND RIEDL'S METHOD FOR K AND ΔH

Base	K *(mole dm*$^{-3}$*)*				$-\Delta H$ *(kcal mol*$^{-1}$*)*	$s_{\Delta H}$** *(kcal mole*$^{-1}$*)*
	15°C	*25°C*	*35°C*	*55°C*		
Propionitrile	0.291	0.236	0.208	0.161	2.7	0.15
Dioxane	0.529	0.475	0.425	0.296	2.8	0.3
Tetrahydrofuran	0.454	0.385	0.325	0.235	3.1	0.3
Diethyl sulphide	0.393	0.354	0.347	0.283	3.2	0.2
Diethyl selenide		0.373	0.312	0.225	3.3	0.2
Acetone	0.402	0.314	0.275	0.234	2.5	0.2
2-Butanone	0.362	0.275	0.254	0.202	2.6	0.2
3-Methyl-2-butanone	0.351	0.274	0.249	0.191	2.8	0.2
Chloroacetone	0.191	0.175	0.146	0.134	1.8	0.3
Butadione	0.237	0.195	0.175	0.137	2.5	0.15
Trifluoroacetone		0.048	0.043	0.040	~0	
Ethyl acetate	0.29	0.262	0.22	0.17	2.3	0.15
Methyl acetate	0.287	0.252	0.222	0.177	2.2	0.10
Ethyl thioacetate	0.23	0.207	0.17	0.14	2.2	0.15
Ethyl propionate	0.301	0.244	0.221	0.183	2.3	0.15
Acetaldehyde	0.28	0.24	0.205		2.8	0.1
Diethyl ether	0.24	0.204	0.178		2.7	0.1
Furan	0.061	0.060	0.055	0.051	0.8	0.2
Benzene	0.054	0.050	0.047	0.042	1.1	0.2
Thiophene	0.072	0.070	0.062	0.045	1.2	0.2

* Confidence limits 4%.

** Standard deviation to linear regression.

TABLE VIII

RESULTS OF EON *et al.*'s METHOD FOR K_x^* AND ΔH

Base	K_x^* *(molar fraction)**					$-\Delta H$ *(kcal mole*$^{-1}$*)*	$s_{\Delta H}$** *(kcal mol*$^{-1}$*)*
	15°C	*25°C*	*35°C*	*45°C*	*55°C*		
Pyridine	6.52***	4.94***	3.80	2.97	2.33	4.95	0.2
Propionitrile	2.18	1.95	1.57	1.365	1.15	3.1	0.2
Dioxane	2.30	1.89	1.38	1.16	0.96	4.2	0.2
Tetrahydrofuran	1.67	1.28	0.99	0.865	0.65	4.3	0.2
Diethyl sulphide	1.57	1.25	0.99	0.81	0.65	4.15	0.1
Diethyl selenide	1.86***	1.47	1.175	0.955	0.78	4.1	0.1
Acetone	2.04	1.71	1.415	1.17	1.03	3.3	0.1
2-Butanone	1.69	1.29	1.09	0.95	0.82	3.3	0.2
3-Methyl-2-butanone	1.61	1.15	0.91	0.83	0.71	3.7	0.4
Chloroacetone	1.41	1.15	1.00	0.86	0.82	2.6	0.2
Butadione	1.17	0.93	0.81	0.70	0.64	2.85	0.2
Ethyl acetate	1.14	0.91	0.79	0.73	0.59	2.9	0.2
Methyl acetate	1.13	0.94	0.80	0.715	0.63	2.75	0.1
Ethyl thioacetate	1.11	0.92	0.71	0.66	0.56	3.2	0.3
Ethyl propionate	0.98	0.85	0.71	0.61	0.52	3.0	0.1
Acetaldehyde	0.845	0.78	0.62	0.50	0.40	3.65	0.3
Diethyl ether	0.51	0.43	0.33	0.27	0.21	4.3	0.3
Furan	0.26	0.24	0.18	0.16	0.135	3.2	0.3
Benzene	0.32	0.28	0.25	0.23	0.20	2.1	0.3
Thiophene	0.35	0.31	0.27	0.22***	0.20***	2.7	0.4

* Confidence limits 4%.

** Standard deviation to linear regression.

*** Extrapolated values.

The values of K, K_x^* and ΔH calculated by Martire and Riedl's and Eon *et al.*'s methods are listed in Tables VII and VIII. Comparison with Tables V and VII showed that the ΔH deviations were not significant. Martire and Riedl's method yielded values 0.3–0.5 kcal smaller and Eon *et al.*'s 0.5–0.7 kcal higher than Purnell's. These differences are linked with the nature of equilibrium constants: Martire and Riedl's constants are true thermodynamic ones, Eon *et al.*'s constants are thermodynamic ones in a given solvent and Purnell's constants are based on a concentration scale.

Using Harbison *et al.*'s process applied to pentane, hexane and heptane (eqn. 6), we found a mean value of 0.550 for χ_S^A. Including this value in eqn. 8, we obtained r, s and t by a weighted least-squares method. Corresponding χ_S^D, $\chi_A'^D$ and K_c values are listed in Table IX.

χ_S^D and $\chi_A'^D$ were about the same: in general, bases induced similar non-complexing interactions with iodododecyne and squalane. This result agrees with chemical theory and makes this method viable. On the other hand, χ_R^D (R = iodododecane) calculated from the equation

$$\ln \gamma_{D(R)}^{\infty} = \ln \frac{V_D}{V_R} + \left(1 - \frac{V_D}{V_R}\right) + \chi_R^D \tag{13}$$

was different to χ_S^D, making the previous approximation $\alpha' = \alpha$ wrong. Hence the corrected values of K in Table VI are smaller than the true values. Comparison of Tables V and IX showed reasonable agreement between K_c values.

It is worth noting that all methods gave the same basicity scale and similar values of C_A and E_A, Drago *et al.*'s acid parameters, and in particular, the same C_A/E_A ratio (see below). Hence our results are consistent.

Theoretically, Harbison *et al.*'s method yields better values of K_c because it takes into account interactions between all molecules present. Eon *et al.*'s method allows the disturbing effects of the size differences between the molecules to be suppressed: at 25°C, the correction term in eqn. 12 varies from -0.065 to -0.135 for K_x^* varying from 0.4 to 5.

In Martire and Riedl's method, great care must be taken with the choice of the reference solvent: the molecular polarizability, size and shape should be the same as those of the electron donor, as far as possible. No specific interaction must be observed between this reference compound and D.

The mathematical treatment, fitting through polynomial eqns. 6 and 8, as described for Harbison *et al.*'s method, requires more numerous and more accurate experimental data than other methods. In this work, in spite of the many columns used, the too large standard deviations of K_c and the too narrow temperature interval induced ΔH values with a bad confidence level.

Comparison with literature data

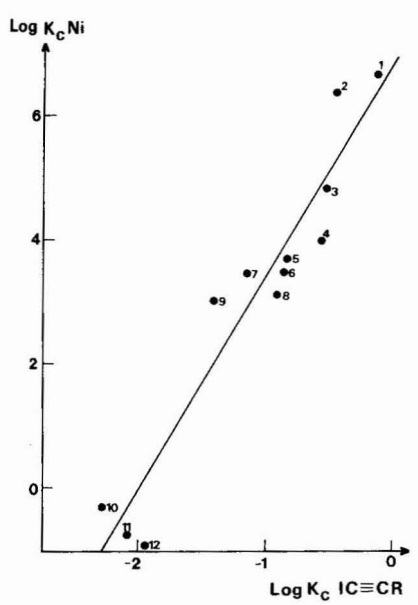
Our χ values are very similar to the literature data[12,30] (Table VII). By a simple chromatographic device, not very different from that of Purnell's, Schurig *et al.*[31] obtained equilibrium constants for 38 bases (12 of which were common to ours) with a Lewis acid, the dimeric 3-fluoroacetylcamphorate nickel(II), that was much stronger than iodododecyne, as shown by the K_c values (K = 782.9 at 75°C and 1.04 at 25°C

TABLE IX

RESULTS OF HARBISON *et al.*'s METHOD AT 25°C FOR χ PARAMETERS AND K_c

Solute	χ_S^D	χ_S^D	$\chi_A'^D$	χ_R^D	K_c *(mole dm*$^{-3}$*)*	sK_c *(mole dm*$^{-3}$*)*
Acetonitrile	3.76	3.53*	3.55	2.62	0.67	0.10
Propionitrile	3.10		3.07	2.08	0.79	0.10
Dioxane	1.326		1.12	0.75	0.58	0.05
Tetrahydrofuran	0.517		0.56	0.17	0.535	0.05
Diethyl sulphide	0.444		−0.116	0.145	0.145	0.05
Diethyl selenide	0.457		0.391	0.03	0.50	0.07
Acetone	2.20	2.14*	2.20	1.42	0.73	0.05
2-Butanone	1.536	1.55*	1.32	0.98	0.35	0.05
3-Methyl-2-butanone	1.142		1.16	0.66	0.40	0.04
Chloroacetone	2.476		2.48	1.72	0.14	0.10
Butadione	2.02		2.15	1.43	0.49	0.15
Ethyl acetate	1.37		1.32	0.90	0.345	0.02
Methyl acetate	1.68		1.44	1.15	0.25	0.05
Ethyl thioacetate	0.82		0.74	0.42	0.275	0.05
Ethyl propionate	0.736		0.98	0.40	0.46	0.03
Acetaldehyde	2.06		2.33	1.47	0.43	0.10
Diethyl ether	0.449		0.529	0.351	0.18	0.05
Benzene	0.558	0.536*	0.711	0.236	0.16	0.05
		0.583**				
Pentane	0.255	0.205*	0.650	0.452	0	
		0.246**				
Hexane	0.192	0.183*	0.647	0.391	0	
		0.187**				
Heptane	0.148	0.147*	0.640	0.359	0	
		0.149**				

* Ref. 25.
** Ref. 11.



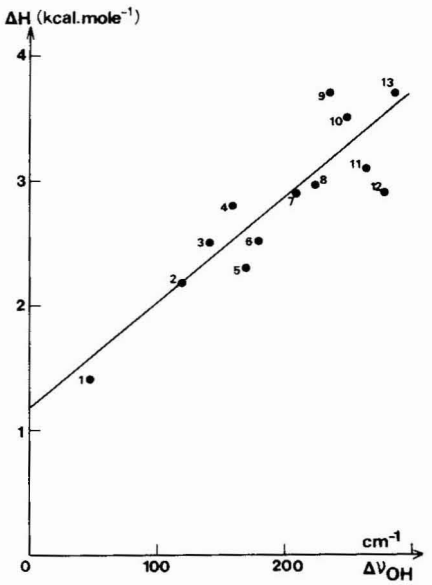


Fig. 2. ln K_c for base–dimeric 3-trifluoroacetylcamphorate of Ni(II) complexes at 75°C *versus* ln K_c for base–1-iodo-1-dodecyne complexes at 25°C. 1 = Dioxane; 2 = tetrahydrofuran; 3 = diethyl sulphide; 4 = 2-butanone; 5 = ethyl acetate; 6 = methyl acetate; 7 = acetaldehyde; 8 = ethyl propionate; 9 = diethyl ether; 10 = fûran; 11 = benzene; 12 = thiophene.

Fig. 3. ΔH of base–1-iodo-1-dodecyne complexes *versus* $\Delta\nu_{OH}$ of phenol in CCl_4. 1 = Benzene; 2 = chloroacetone; 3 = butadione; 4 = propionitrile; 5 = methyl acetate; 6 = ethyl acetate; 7 = 2-butanone; 8 = acetone; 9 = dioxane; 10 = diethyl sulphide; 11 = acetaldehyde; 12 = diethyl ether; 13 = tetrahydrofuran.

with dioxane). Fig. 2 represents the comparison between log K_c [Ni(II)] at 75°C *versus* log K_c (iodododecyne) at 25°C, the correlation is satisfactory ($r = 0.93$). This result is surprising, because in nickel(II) complexes "non-specific" interactions are negligible in comparison with the so-called charge transfer forces: there is no basic difference in nature between strong and weak association of the bases under study.

Comparison of the basicity scale obtained with iodododecyne by gas–liquid chromatography and with phenol by IR spectroscopy (Fig. 3) is an argument in favour of the accuracy of our ΔH values. Some discrepancies for dioxane and diethyl ether may be noted.

Chromatography gives, for a large set of bases, association enthalpies with iodododecyne under rigorously similar conditions. Further, bases are at infinite dilution, and the volume fraction of acid in the non-polar solvent varies from 0 to 1.

Application of Drago et al.'s relationship to iodododecyne

For each set of enthalpies, we checked the two-parameters equation $-\Delta H = E_A E_D + C_A C_D$ for ten bases whose E_D and C_D parameters are known. We obtained similar E_A and C_A values for iodododecyne (Table X).

As shown in the graphical representation of $-\Delta H/E_D$ *versus* C_D/E_D (Fig. 4), diethyl sulphide and diethyl selenide are very important for the fitness of the corre-

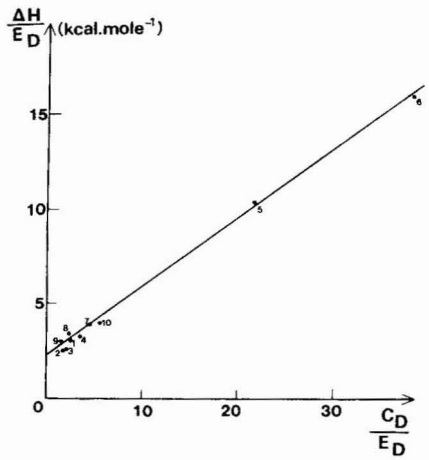


Fig. 4. $\Delta H/E_D$ *versus* C_D/E_D for base–1-iodo-1-dodecyne complexes. 1 = Acetone; 2 = ethyl acetate; 3 = methyl acetate; 4 = diethyl ether; 5 = diethyl sulphide; 6 = diethyl selenide; 7 = tetrahydrofuran; 8 = dioxane; 9 = benzene; 10 = pyridine.

lation ($r = 0.9987$). From E_A and C_A values and from their ratio we conclude that 1-iodododecyne is a less "soft" acid than iodine, according to the hard–soft acid–base (HSAB) principle of Pearson[32]. The contributions of electrostatic interactions to the enthalpy value are 13, 20, 50, 60, 70 and 80% for the associations of iodododecyne with diethyl selenide, diethyl sulphide, pyridine, ethers, ketones and esters, respectively.

Moreover, for carbonyl bases, the treatment of the data for variation of log K_c with $1/T$ shows that thermodynamic parameters are controlled by a linear free-energy relationship (LFER) with infinite β (isoenthalpic family)[33,34].

TABLE X

C_A AND E_A PARAMETERS OF DRAGO *et al.*'s EQUATION

Constant method	C_A	s_{C_A}*	E_A	s_{E_A}*	$\frac{C}{A}$	r**
Purnell	0.364	0.040	2.102	0.129	0.17	0.964
$K_c + \alpha - \alpha'$	0.416	0.076	2.571	0.198	0.16	0.937
Martire and Riedl	0.336	0.013	1.778	0.062	0.19	0.983
Eon *et al.*	0.418	0.086	2.521	0.214	0.17	0.932

* Standard deviation of the regression.
** Correlation coefficient.

Applicability of the diachoric solutions relationship

Purnell, Laub and co-workers[35–37] found that the equation

$$K_{R(M)} = K_{R(S)}\varphi_S + K_{R(A)}\varphi_A \qquad (12)$$

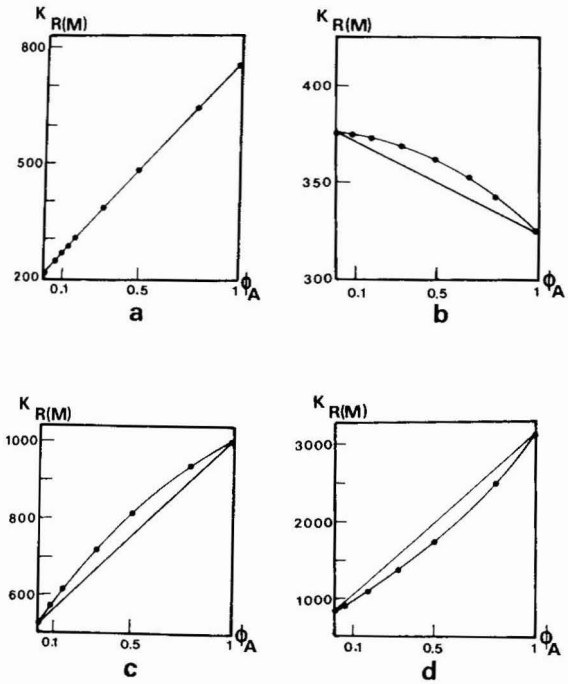


Fig. 5. Plots of $K_{R(M)}$ at 25°C *versus* φ_A. (a) Ethyl acetate; (b) hexane; (c) benzene; (d) diethyl sulphide.

is often obeyed for many chromatographic systems that they called diachoric. In fact, similarities can be found with eqn. 5 because $\varphi_A = V_A C_A$.

For iodododecyne–base–squalane systems we observed four types of curves for eqn. 12 (Fig. 5):

Type a: straight lines with 2% maximum deviations for ketones, esters and tetrahydrofuran.

Type b: curves with positive deviations and $K_{R(A)} < K_{R(S)}$ for alkanes.

Type c: curves with positive deviations and $K_{R(A)} > K_{R(S)}$ for acetaldehyde (weak deviation), benzene, thiophene and diethyl ether.

Type d: curves with negative deviations and $K_{R(A)} > K_{R(S)}$ for propionitrile (weak deviation), dioxane, diethyl sulphide and diethyl selenide.

As did Harbison *et al.*[12], we also noticed two possibilities:

(i) Eqns. 6 and 7 from conventional theories of solutions are well adapted to explain the results. In this instance, the diachoric relationship cannot be applied to all systems and deviations depend on relative values of the three parameters χ_S^D, $\chi_A'^D$ and χ_S^A.

(ii) The diachoric hypothesis is always applicable: in this instance an explanation must be found for the experimental deviations listed above, but this is difficult. One reason for the divergence is the dimerization of iodododecyne and another is the existence of 1:2 complexes.

We chose the first possibility and our K_c and ΔH values agree very well with it. However, we must bear in mind that in our study diachoric linearity is observed for about 50% of bases and, moreover, when deviations occur, they never exceed 4%.

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OPTIMAL CONDITIONS FOR THE COUPLING OF AROMATIC AMINES TO EPOXY-ACTIVATED SEPHAROSE 6B

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SUMMARY

The influence of pH, temperature, ligand concentration and reaction time on the coupling of aniline to epoxy-activated Sepharose 6B was studied. With the optimal temperature, ligand concentration and reaction time for the coupling of aniline, the influence of pH was investigated for the coupling of two aliphatic amines, *viz.*, L-leucine and 4-aminobutyric acid, to this gel.

INTRODUCTION

Epoxy-activated agarose is a convenient starting material for the preparation of affinity gels. If a bisoxirane such as 1,4-butanediol diglycidyl ether is used in its synthesis, two desirable properties are obtained: the agarose chains are cross-linked to give a stable gel and the reactive oxirane groups are attached to the matrix by long, non-charged, hydrophilic spacers.

Epoxy-activated agarose has been shown to couple with ligands containing amine[1–4], hydroxyl[5] or thiol[6] groups. However, the reaction conditions required for maximum coupling yields with these groups have not been studied in great detail. Only one systematic study[1], involving coupling with the α-amino group of glycyl-leucine, has been published. It was shown that a high pH (11) and a high temperature (50°C) are necessary in order to obtain high coupling yields in this instance. Since that study was published, most workers appear to have used these or even harsher conditions, irrespective of the nature of the functional group to be reacted with the oxirane groups on the gel and in spite of the fact that the stability of oxirane groups towards hydrolysis is diminished at high pH and high temperature[1,7].

The coupling reaction with amine groups is assumed to involve the unprotonated amine, which explains the relatively high pH required for aliphatic amine groups. However, aromatic amine groups, having a much lower basicity, might couple at lower pH. This might have the advantage of giving higher coupling yields as a result of the increased stability of the oxirane groups. Moreover, conditions might be found under which a ligand carrying both an aromatic and an aliphatic amine group is specifically coupled through the aromatic amine group. Finally, coupling at

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lower pH is, of course, indicated in those instances where the ligand is unstable at the high pH normally used.

In this paper, a study of the optimal conditions for the coupling of the simplest aromatic amine, *viz.*, aniline, to epoxy-activated Sepharose 6B (ES) in aqueous buffers is described. The influence of four variables, *viz.*, pH, temperature, ligand concentration and reaction time, was studied. The experimental strategy of a four-factor factorial design[8], followed by the method of steepest ascent[8], was chosen. In order to investigate the possibility of selective coupling through aromatic amine groups, coupling experiments with ligands containing aliphatic amine groups, *viz.*, L-leucine and 4-aminobutyric acid, were also performed. For convenience in the measurement of coupling yields, ^{14}C-labelled ligands were used.

EXPERIMENTAL

Aliquots of 0.5 g of epoxy-activated Sepharose 6B (Pharmacia, Uppsala, Sweden) were swollen on a glass filter and washed with distilled water and coupling buffer (0.2 *M* phosphate or 0.05 *M* borate), respectively. The filter-dried material was transferred into a reaction vial and 4 ml of a solution containing the ^{14}C-labelled ligand in coupling buffer was added.

Aniline, L-leucine and 4-aminobutyric acid (Merck, Darmstadt, G.F.R.) were used as ligands; they were labelled with the corresponding ^{14}C-compounds (Radiochemical Centre, Amersham, Great Britain) to a specific activity of about 0.8 μCi/mmole.

After incubation on a test-tube rotator for a given period of time at a given temperature, the gel was filtered off and washed successively with coupling buffer, distilled water, 0.5 *M* sodium hydrogen carbonate + 1 *M* sodium chloride (pH 8), distilled water, 0.1 *M* sodium acetate + 1 *M* sodium chloride (pH 4) and distilled water; three 10-ml portions of each solution were used.

The filter-dried gel was subsequently transferred into a counting vial, 10 ml of Aquasol (NEN, Boston, MA, U.S.A.) were added and the counting rate was measured in a scintillation counter (BF 5020, Berthold, Wildbad, G.F.R.); 10^5 counts were recorded for each gel and counting rates were corrected for the background.

These corrected counting rates (cps/0.5 g ES), pertaining to a heterogeneous counting system, were transformed into coupling yields (μequiv. ligand/g ES) by multiplication with a factor $2f$, which was separately determined by the following experiments. A 1-g amount of epoxy-activated Sepharose 6B, to which an unknown amount of labelled ligand was coupled, was divided into two equal parts. One part was directly counted as described above (corrected counting rate, C_1) and the other was dissolved by heating under reflux at 100°C in a total volume of 4 ml of 0.5 *M* hydrochloric acid. A 400-μl volume of the digest and 40 μl of water were added to 10 ml of Aquasol and counted (C_2). A 40-μl volume of the stock solution of known concentration c (μequiv./ml) of labelled ligand was added to 400 μl of a digest of 0.5 g of epoxy-activated Sepharose 6B in a total volume of 4 ml of 0.5 *M* hydrochloric acid, 10 ml of Aquasol were added and the sample was counted (C_3). From these experiments f can be found:

$$f = \frac{0.4\ C_2}{C_1\ C_3} \cdot c$$

The oxirane content of the gel was determined by the method described by Sundberg and Porath[1].

RESULTS AND DISCUSSION

In the initial coupling experiments with aniline, the conditions were chosen according to a four-factor factorial design[8]. The factor levels were as follows: pH, 5.8 and 8.1; temperature, 25 and 40°C; ligand concentration, 31.25 and 62.50 m*M* (corresponding to a 2.5- and 5-fold excess with respect to oxirane groups, respectively*); and reaction time, 17 and 25 h. All experiments were performed in duplicate. The results are given in Table I.

TABLE I

COUPLING YIELDS (μequiv./g) OF ANILINE TO EPOXY-ACTIVATED SEPHAROSE 6B IN 0.2 *M* PHOSPHATE BUFFERS

32 experiments in a 2^4 factorial design, with duplication.

pH	*Reaction time (h)*	*Temperature (°C)*			
		25		*40*	
		Ligand concentration (mM)			
		31.25	*62.50*	*31.25*	*62.50*
5.8	17	26.4, 18.4	41.2, 41.9	41.6, 45.0	54.0, 65.0
	25	28.5, 24.1	39.9, 51.4	41.3, 32.7	54.3, 57.1
8.1	17	25.5, 25.9	53.1, 51.2	49.2, 42.1	59.4, 60.8
	25	23.5, 22.9	41.7, 42.1	44.2, 37.5	47.8, 44.2

The data in Table I were submitted to a four-factor analysis of variance, the results of which are given in Table II.

From the results in Tables I and II one can draw the following conclusions, which hold for coupling conditions confined to the four-dimensional factor space, limited by the pre-set factor levels:

(i) the experimental error in the coupling yield is

$$\pm \sqrt{19.11} = \pm 4.4 \text{ μequiv./g ES;}$$

(ii) the pH has no significant influence on the coupling yield;

(iii) the reaction temperature and the concentration of the ligand have a strong influence on the coupling yield and their influence is interdependent; the coupling yield increases with increasing temperature and increasing concentration; and

(iv) the reaction time has a smaller but significant influence on the coupling

* We found the oxirane content of the gel to be higher than that stated by the manufacturer[7] (100 ± 2 instead of 50–70 μequiv./g ES).

TABLE II

RESULTS OF THE ANALYSIS OF VARIANCE OF THE DATA IN TABLE I

s^2 = Estimate of variance (= sum of squares/degrees of freedom); s_w^2 = estimate of variance within duplicate experiments, *i.e.*, estimate of experimental variance (= 272.48/16 = 17.03); $*s_w^2$ = better estimate of experimental variance: as the three- and four-factor interactions are not significant [$F^{5\%}$ (1.16) = 4.49], their variance estimates are pooled with that within duplicates, giving $*s_w^2$ = 401.24/21 = 19.11. As $F^{5\%}$ (1.21) = 4.32, the factors indicated with arrows in the last column are significant.

Factor	*Sum of squares*	*Degrees of freedom*	$F = \frac{s^2}{s_w^2}$	$*F = \frac{s^2}{*s_w^2}$
A = pH	2.15	1	0.13	0.11
B = temp.	1491.94	1	87.61	78.07←
C = conc.	2385.68	1	140.09	124.84←
D = time	142.38	1	8.36	7.45←
AB	12.38	1	0.73	0.65
AC	9.35	1	0.55	0.49
AD	109.15	1	6.41	5.71←
BC	106.22	1	6.24	5.56←
BD	73.51	1	4.32	3.85
CD	25.74	1	1.51	1.35
ABC	60.22	1	3.54	
ABD	17.85	1	1.05	
ACD	48.76	1	2.86	
BCD	0.07	1	0.00	
ABCD	1.86	1	0.11	
Between duplicates	4487.26	15		
Within duplicates	272.48	16		
Total	4759.74	31		

yield; increasing reaction times giving slightly decreasing coupling yields. Moreover, this effect of reaction time is significantly greater at higher pH.

Based on these conclusions, further coupling experiments with aniline were performed at pH 7.0 and at varying levels of the remaining three variables. The factor levels for the successive experiments were chosen according to the method of steepest ascent[8]. In Fig. 1 these levels are indicated on the abscissa; the ordinate gives the

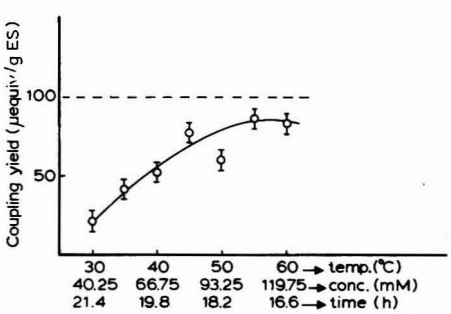

Fig. 1. Coupling yield of aniline to epoxy-activated Sepharose 6B in 0.2 *M* phosphate buffer (pH 7.0) under varying conditions, according to the method of steepest ascent. Broken line, oxirane content of the gel.

coupling yields found experimentally. Also indicated in Fig. 1 is the maximum coupling yield, as given by the oxirane content of the gel.

From the results in Fig. 1 it was decided to perform further experiments at the near-optimal temperature (55°C), ligand concentration (106.5 m*M*, corresponding to an 8.5-fold excess with respect to oxirane groups) and reaction time (17 h). These experiments were performed with aniline, L-leucine and 4-aminobutyric acid at varying pH with the aim of examining whether the coupling yield of aniline is indeed lower at higher pH (as a result of increasing hydrolysis of oxirane groups) and to investigate if at pH $\approx$ 7 the reaction conditions are such that aromatic amine groups are selectively coupled. The results of these experiments are shown in Fig. 2.

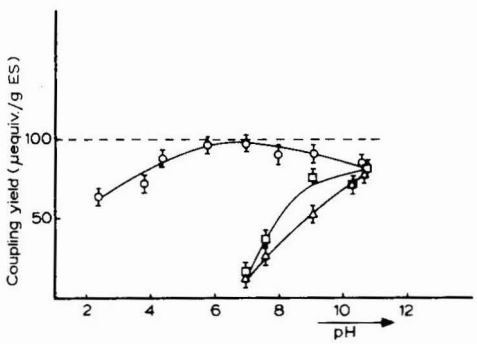

Fig. 2. Coupling yield of aniline (○), L-leucine (□) and 4-aminobutyric acid (△) to epoxy-activated Sepharose 6B in 0.2 *M* phosphate buffers (at pH $\leq$ 7.0) or 0.05 *M* borate buffers (at pH > 7). Reaction conditions: temperature, 55°C; ligand concentration, 106.5 m*M*; and reaction time, 17 h. Broken line, oxirane content of the gel.

It can be seen that at pH 6–7 the reaction of aniline with oxirane groups on the gel proceeds virtually to completion, whereas the aliphatic amines are not coupled at all at pH 6.5. Hence, for a ligand containing both amine functions, selective and 100% attachment through the aromatic amine group can be obtained by coupling at pH 6.5. At pH 7–10.5, the coupling yield at constant pH increases in the order 4-aminobutyric acid < L-leucine < aniline, in accordance with the decrease in the basicity of the corresponding amine groups (p*K* values 10.6, 9.7 and 4.6, respectively).

Coupling of aniline at pH 10.6 results in a reduction of about 20% in the coupling yield. It is probable that this reduction stems from a property of the gel, *i.e.*, increased hydrolysis of oxirane groups, as all three curves in Fig. 2 converge to about the same coupling yield with increasing pH.

Finally, we investigated if coupling of aniline at high pH (10.6) but at lower temperatures would result in higher coupling yields (the amount of hydrolysis of oxirane groups is known to increase with increasing pH and temperature). The results are shown in Fig. 3, and indicate that the reaction temperature has no significant influence on the coupling yield of aniline at pH 10.6. Thus, under these conditions (ligand concentration 8.5-fold excess, reaction time 17 h) the high pH (10.6) and not the high temperature (55°C) is primarily responsible for the 20% reduction in the coupling yield shown in Fig. 2.

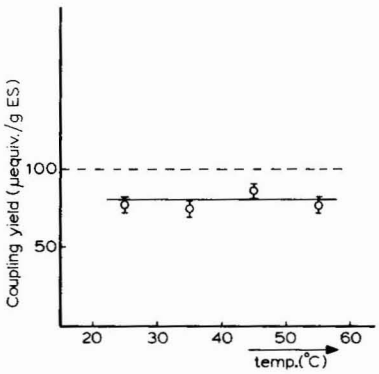

Fig. 3. Coupling yield of aniline to epoxy-activated Sepharose 6B in 0.05 *M* borate buffer (pH 10.6) at varying temperature. Reaction conditions: ligand concentration, 106.5 m*M*; and reaction time, 17 h. Broken line, oxirane content of the gel.

CONCLUSIONS

Aniline is coupled in 100% yield to epoxy-activated Sepharose 6B at pH 6–7 by performing the reaction at 55°C for 17 h using an 8.5-fold excess ligand concentration. Presumably the same holds for other ligands containing aromatic amine groups at a pH about 2 units higher than the corresponding p*K* value.

Under these optimal conditions for the coupling of aniline, aliphatic amine groups do not react. This allows the selective attachment through aromatic amine groups of a ligand containing both functions.

In coupling reactions performed at pH 10.6 with an 8.5-fold excess ligand concentration for 17 h and at temperatures between 25 and 55°C, only about 80% of the oxirane groups are substituted; there is a 20% loss due to hydrolysis of the oxirane groups.

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DYNAMIC CATION-EXCHANGE SYSTEMS FOR RAPID SEPARATIONS OF NUCLEOBASES AND NUCLEOSIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The retention behaviour of nucleobases and nucleosides in dynamic cation-exchange systems, consisting of a hydrophobic support as the stationary phase and water–ethanol mixtures containing small amounts of sodium dodecylsulphate as anionic detergent as the mobile phase was investigated.

The retention of nucleobases and nucleosides can be influenced over a wide range by variation of the pH and the concentration of the ethanol, anionic detergent and counter ion in the eluent. With respect to separation speed and selectivity, these dynamic cation-exchange systems are in many instances superior to conventional ion-exchange and reversed-phase systems. It is shown that, by optimizing the different retention parameters, the separation of fourteen nucleobases and nucleosides, simultaneously and under isocratic conditions, can be achieved in *ca.* 6 min. The performance of the phase system is demonstrated by the analysis of a calf thymus DNA hydrolysate.

INTRODUCTION

Among the various chromatographic techniques[1–3], column liquid chromatography has been a preferred method for analysing nucleobases and nucleosides. A particular advantage of liquid chromatography is the possibility of analysing directly the hydrolysed biological material, in contrast with gas chromatography, where volatile derivatives are required[4,5].

Until now, ion-exchange[3,6–8] and reversed-phase liquid chromatography[9,10] have been the most frequently applied separation methods for these nucleic acids.

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Although satisfactory for many applications, these phase systems sometimes have limitations with respect to separation speed and selectivity.

In recent years, different modes of ion-pair chromatography[11–16] have proved to be a useful alternative or supplement for the separation of ionizable substances such as sulphonic acids[12,14,15], catecholamines[17,18] and amino acids[19].

Ion-pair chromatography has also been tested for the separation of nucleic acid constituents and their analogues. Thus, for nucleotides[20,21], nucleobases[21], nucleosides[22], 5-fluorouracil nucleotides[22] and azapurines[21], ion-pair chromatography with quaternary ammonium salts has been applied. For the separation of nucleobases and nucleosides, camphorsulphonic acid[20] and heptanesulphonic acid[23] have been applied as pairing ions. In all of these studies only a few naturally occurring nucleobases or nucleosides were used. However, for the characterization of the different DNA and RNA types[24] or for the recognition of alterations in the purine and pyrimidine metabolic pathways, caused by some genetic diseases[25–27], there is a need for a rapid method of determining these nucleobases and their nucleosides. For the separation of these compounds in one run, dynamic anion exchange is not the method of choice in practice because of the high pH of the mobile phase needed to obtain ion pairing for all solutes. For this separation problem, dynamic cation-exchange chromatography is more promising.

In this paper we discuss a very efficient dynamic cation-exchange system with sodium dodecylsulphate as ion-pairing agent for extremely fast isocratic separations of eight naturally occurring nucleobases and many of their nucleosides in one run.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a reciprocating pump (Orlita type AE-10-4.4), a Bourdon-type manometer, a high-pressure injection valve (Rheodyne 7105) equipped with a sample loop of 20 μl and a Zeiss PMQ II UV spectrophotometer (260 nm). The stainless-steel columns had an I.D. of 4.5 mm and a length of 150 mm.

Materials

All solvents and chemicals were of analytical-reagent grade and used without any further pre-treatment. The alkyl-modified silica used as the column support was Hypersil ODS (Shandon, Great Britain), mean particle size 5 μm. The nucleobases and nucleosides were obtained from Sigma (St. Louis, MO, U.S.A.) and Merck (Darmstadt, G.F.R.). The following abbreviations for these substances are used: uracil (Ura); cytosine (Cyt); Thymine (Thy); adenine (Ade); guanine (Gua); hypoxanthine (Hyp); Xanthine (Xan); Uridine (Urd); cytidine (Cyd); adenosine (Ado); guanosine (Guo); Inosine (Ino); xanthosine (Xao); thymidine (dThy); and 5-methylcytosine (5-Cyt).

Procedures

The HPLC columns were packed according to the procedure recommended by the manufacturer of the C_{18} support (Shandon). The columns were washed with 100 ml of methanol and then equilibrated with the eluent until constant retention of the compounds under investigation was obtained.

RESULTS AND DISCUSSION

In dynamic ion-exchange systems one uses alkyl-modified silicas as the stationary phase and aqueous–organic solvent mixtures containing an ionic detergent as the mobile phase. Owing to the hydrophobic part of its molecule, such a detergent is strongly adsorbed on to the hydrophobic column packing and has the ability to exchange its associated counter ion[15,19] (*i.e.*, it can act as an ion exchanger). Such dynamic (solvent-generated) ion-exchange systems have definite advantages over conventional resin ion exchangers, such as excellent exchange kinetics and pressure-stable packings, which allows high separation speeds with highly efficient columns. Moreover, the type and capacity of the ion exchanger can be varied without changing the column packing (*i.e.*, via the mobile phase).

For the investigation of the applicability of dynamic ion-exchange chromatography for the separation of nucleobases and nucleosides, sodium dodecylsulphate (SDS) was chosen as a detergent to generate a cation exchanger on the hydrophobic support. In order to explore fully the possibilities of this dynamic cation-exchange system for nucleobases and nucleosides, the influence of the pH and the concentration of the organic modifier, counter ion and SDS of the mobile phase on the capacity ratio (k_i') was systematically investigated.

Fig. 1 shows the effect of the addition of SDS to the mobile phase on the retention of a number of nucleobases and nucleosides at constant pH and ethanol and Na^+ concentrations. It can clearly be seen that after addition of only 1.0 g/l of SDS, remarkably different retention behaviour is found for a number of compounds owing to additional retention via cation exchange. The order of elution of nucleobases and nucleosides on this dynamic cation-exchange system is similar to that found on cation-exchange resins[3,6], but is significantly different to the order obtained in reversed-phase elution chromatography[9,10]. The capacity ratio of most solutes increases with increasing amount of SDS and passes a maximum at about 0.1 % (w/w) of SDS. This dependence of k_i' on the SDS concentration in the mobile phase coin-

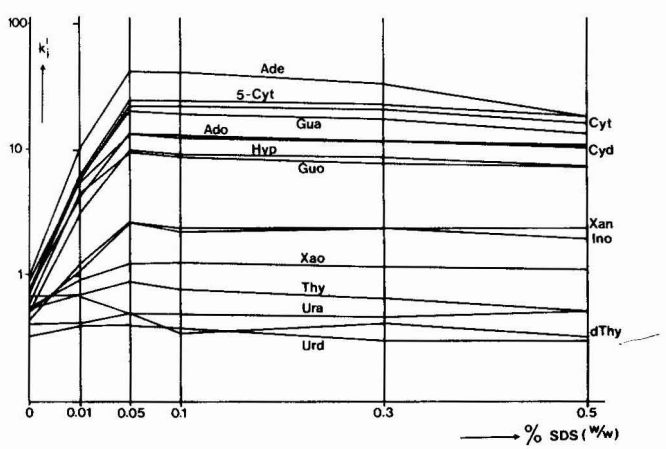

Fig. 1. Effect of the addition of SDS to the mobile phase on the capacity ratio (k_i') of nucleobases and nucleosides. Stationary phase: Hypersil ODS. Mobile phase: 0.05 *M* $HC.O_4$–ethanol (9:1, v/v) + SDS (0–0.5 %).

cides with the shape of the adsorption isotherm of SDS on the stationary phase[14,19]. Roughly, the average retention of the compounds discussed approaches constancy when the SDS concentration is $>0.1\,\%$. However, minor variations occur, which can be partly explained by solute–SDS micelles interaction in the mobile phase[15]. Fig. 1 shows that in some instances fine tuning of the selectivity can be accomplished by the choice of the SDS concentration.

The influence of the pH of the mobile phase on k_i' was investigated at constant SDS, ethanol and counter ion [Na^+] concentration, and is shown in Fig. 2. On going from low to high pH, the k_i' values of Ade, Ado, Gua, Guo, Cyd and Hyp show maxima in the pH region 2–3. For Xan and Xao no maximum appears in this pH region but the k_i' values still decrease with increasing pH. The k_i' values of the aforementioned solutes decrease sharply at higher pH and tend to become constant at pH > 6. For Thy, Ura and Urd pH seems to have hardly any influence on k_i' in the investigated pH range of 1–7. The pH of the mobile phase is a valuable parameter for influencing the absolute and relative retentions of nucleobases and nucleosides in dynamic cation-exchange systems.

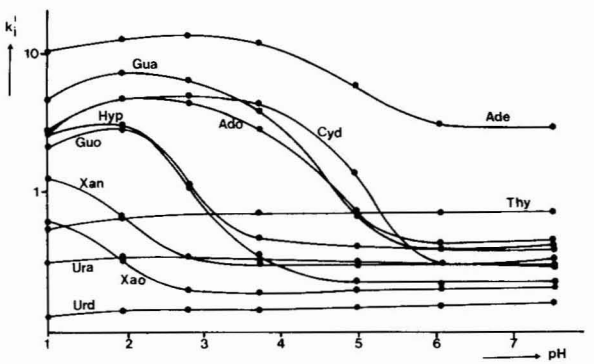

Fig. 2. Effect of the pH of the mobile phase on k_i' of nucleobases and nucleosides. Stationary phase: Hypersil ODS. Mobile phase: water–ethanol (9:1, v/v) + 0.05 *M* Na_3PO_4 + 0.72% (w/w) SDS, pH adjusted with 5 *M* $HClO_4$.

The dependence of the capacity ratio of nucleobases and nucleosides on the volume percentage of ethanol is shown in Fig. 3. Over the ethanol content region investigated (2–30%, v/v) log k_i' for all solutes increases linearly with decreasing volume percentage of ethanol, but with different slopes. This suggests that, apart from retention via cation exchange, physical distribution also contributes significantly to the overall retention of some solutes[15]. The strong influence of the concentration of the ethanol on the degree of retention can be used in gradient elution[19].

The retention of solute ions in dynamic ion-exchange systems can be influenced reasonably predictably by the concentration of the counter ion in the mobile phase, as in conventional ion exchange[15,19]. This is demonstrated for a number of nucleobases and nucleosides in Fig. 4, using Na^+ as the counter ion. It can be seen that $1/k_i'$ is directly proportional to the amount of Na^+ added to the mobile phase over the range 10–150 m*M* of Na^+. As the retention in such dynamic ion-exchange systems is often the result of a mixed mechanism (physical distribution and ion exchange), significant

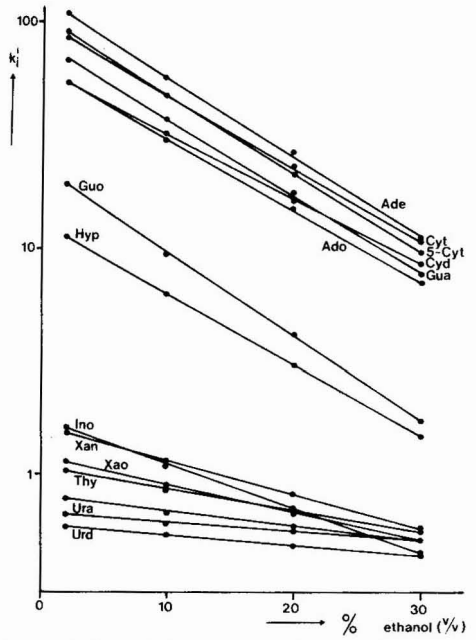

Fig. 3. Effect of the concentration of ethanol on k_i' of nucleobases and nucleosides. Stationary phase: Hypersil ODS. Mobile phase: 0.006 *M* $HClO_4$–ethanol (2–30%, v/v) + 0.72% (w/w) SDS, pH = 2.6.

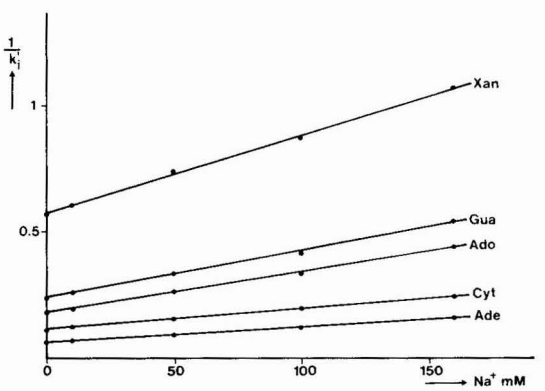

Fig. 4. Dependence of k_i' of nucleobases and nucleosides on the counter ion (Na^+) concentration in the mobile phase. Stationary phase: Hypersil ODS. Mobile phase: 0.1 *M* $HClO_4$–ethanol (9:1, v/v), pH = 1.0 + 0.1% (w/w) SDS + $NaClO_4$ (0–160 m*M*).

selectivity changes can be effected by variation of the counter ion concentration, depending on the relative magnitude of these separate distribution processes[12,15,19]. The counter ion concentration can be used as a parameter to produce gradients.

Apart from the retention characteristics of the nucleobases and nucleosides, the column efficiency was also measured as a function of the mobile phase composition. It was found that for all solutes the theoretical plate height (*H*) varies very little with the composition of the mobile phase. This is in contrast with results ob-

tained with conventional resin ion exchangers[6,8]. The plate height for solutes with $k_i' > 3$ ranges between 25 and 30 μm. For solutes with $k_i' < 3$ the plate height was larger, probably because of a significant contribution of the external peak broadening to the overall peak width.

For a few mobile phase compositions the dependence of H on the linear velocity, $\langle v \rangle$, was also measured. These measurements show that the H value ranges between 20 μm at $\langle v \rangle = 0.5$ mm/sec and 30 μm at $\langle v \rangle = 8$ mm/sec, indicating the excellent mass transfer in these dynamic cation-exchange systems. Further, the chromatographic characteristics of the columns did not change significantly over a 6-months period.

The results of the investigation of dynamic cation-exchange chromatography with SDS (Figs. 1–4) show that many parameters are available for adapting the mobile phase composition to a particular separation problem. The optimal mobile phase composition for a rapid separation of the nucleobases and nucleosides simultaneously and under isocratic conditions (still a difficult problem) can easily be determined from these figures. A low pH, about 10% of ethanol and 0.1% of SDS seems to be a good choice, as is demonstrated in Fig. 5, which shows the separation of a test mixture of eight nucleobases and seven nucleosides. Only Ura and dThy are not resolved with this phase system. However, the separation of this pair of solutes might be improved by small variations in pH or ethanol, SDS or Na^+ concentration. Further, the type of organic modifier and anionic detergent can have a significant effect on the retention characteristics of cationic compounds[19], but this was not investigated in this study.

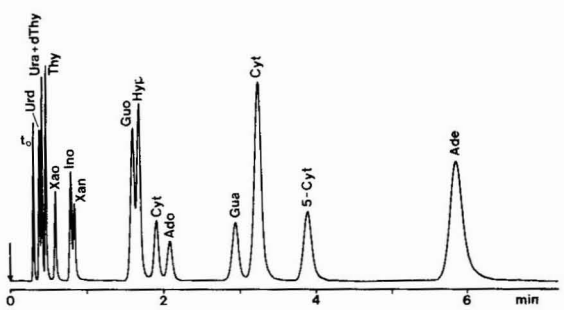

Fig. 5. Rapid separation of a test mixture of nucleobases and nucleosides under isocratic conditions. Stationary phase: Hypersil ODS. Mobile phase: 0.1 M $HClO_4$–ethanol (9:1, v/v) + 0.1% (w/w) SDS. Ambient temperature; $\Delta P = 170$ bar; $\langle v \rangle = 8$ mm/sec.

The applicability of the described dynamic cation-exchange system for biological samples is demonstrated in Fig. 6, which shows the analysis of a number of nucleobases in a calf thymus DNA hydrolysate.

CONCLUSIONS

Dynamic cation-exchange chromatography, with sodium dodecylsulphate as anionic detergent, is suitable for the separation of nucleobases and nucleosides. Owing to the excellent kinetics of the phase system and the large number of param-

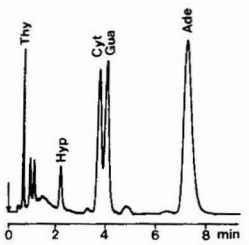

Fig. 6. Analysis of nucleobases in calf thymus DNA hydrolysate. Conditions as in Fig. 5 except ΔP = 130 bar and $\langle v \rangle$ = 6 mm/sec. Hydrolysis: 0.2 g of calf thymus DNA Type I (Sigma) was heated for 16 h at 80°C with 15 ml of 2 *M* HCl, and 1 ml of the supernatant was diluted with 9 ml of water–ethanol (8:1) and 20 μl of the resulting solution were injected on to the column.

eters available for influencing the retention, such as the pH and organic modifier, SDS or counter ion concentration in the mobile phase, it was possible to separate simultaneously almost all nucleobases and their nucleosides under isocratic conditions in 6 min. This result contrasts favourably, with respect to speed and selectivity, to the results obtained with conventional ion-exchange and reversed-phase liquid chromatography. Future work in our laboratory will be devoted to the applicability of these dynamic cation-exchange systems for the separation of modified nucleosides.

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STUDY OF PAPER AND THIN-LAYER CHROMATOGRAPHY OF PHENOLIC SUBSTANCES BY STATISTICAL METHODS

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SUMMARY

Classical paper chromatographic (PC) and thin-layer chromatographic (TLC) separations of phenolic compounds (including flavonoids) were classified into clusters according to their selectivities. Systems were compared two by two, by plotting the R_F values of a set of compounds in one system against the R_F values in the other system. One hundred and seventy correlation coefficients were computed and used in a hierarchical clustering method. The dendrograms obtained can be used by analysts to plan an efficient scheme for the identification or separation of phenolic substances. The potential of PC and TLC in this respect is discussed and compared with that of high-performance liquid chromatography.

INTRODUCTION

The literature contains descriptions of a wide variety of chromatographic systems for the analysis of phenolic substances of industrial, plant or animal origin. Most of these systems are based on paper chromatography (PC) and thin-layer chromatography (TLC). Gas chromatographic (GC) data also exist, and high-performance liquid chromatography (HPLC) is being used increasingly[1].

A qualitative comparison of HPLC with PC and TLC seemed appropriate for several reasons. There is no doubt that the quantitative analysis of phenolic substances can be carried out more accurately with HPLC than with PC and TLC systems. However, HPLC may not be competitive with PC and TLC in qualitative analysis (identification). PC and TLC offer the possibility of two-dimensional elution and selective detection, and simultaneous analysis on different systems can also easily be achieved. These factors can increase the information provided by the technique[2]. In the PC and TLC of phenolic substances, different chromatographic systems can be obtained by combining a variety of mobile phases with a single stationary phase. This will increase the information obtained only if changing the mobile phase also results in a significant change in selectivity, and this is only vaguely known for most systems at present (for an explanation of the selectivity of a chromatographic system, the reader is referred to ref. 3). The aim of this paper is to compare the selectivities that

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are obtained in the PC and TLC of phenolic compounds by varying either the mobile phase or the stationary phase. We therefore used a well defined set of published classical chromatographic systems. They are classified into "clusters" with analogous selectivities by means of statistical methods.

RESULTS AND DISCUSSION

Some classical papers on the PC and TLC of phenolic substances were chosen arbitrarily from the literature[4-7]. Two of them were concerned with PC[4,7], another with cellulose TLC[6] and the last[5] with TLC on mixed adsorbents. We compared the selectivities of the different systems used by one author, and when possible we also compared the selectivities of systems used by different authors.

Comparisons were made by plotting the R_F values of a set of phenolic compounds in one system against the R_F values in another system. Correlation (or absence of correlation) between the two was expressed by the correlation coefficient (ϱ). In our opinion, this coefficient is a good parameter of what we call qualitatively the "selectivity difference" between two chromatographic systems. These coefficients were used in a hierarchical clustering method as described by Massart and De Clercq[8], and the results were plotted in a "dendrogram".

Table I indicates which TLC and PC systems were compared in this way. Dendrograms showing the correlation of the systems of each publication separately are shown in Fig. 1. Fig. 2 shows correlations calculated from the overlap of data from two publications. These two figures were obtained from the algorithmic treatment of 119 correlation coefficients (119 different pairs of chromatographic systems were investigated using a computer program). Therefore, Figs. 1 and 2 represent the compression of a large amount of chromatographic data relating to phenolic compounds. They can be interpreted by chromatographers without requiring a thorough knowledge of statistics.

When there was a small spread of R_F values in a certain system, there was no point in looking for a correlation with another system. Therefore, we needed a measure of the spread of R_F values over the chromatograms. We divided the R_F scale into four equal parts and calculated for each system the number of R_F values in each part. We then compared this distribution with a hypothetical one in which the set of R_F values was distributed equally over the four parts (null hypothesis). As a measure of divergence from this null hypothesis we used the χ^2 value. These values are given in Table I. They were generally better (*i.e.*, smaller) in some publications (Jangaard, Van Sumere *et al.*) than in others (Reio, Jay *et al.*). As a measure of the eluting power of a chromatographic solvent system, the mean R_F value ($\bar{R}_F$) was calculated (see Table I). Based on the χ^2 values, we rejected two systems from Reio (RA and RF) for further comparison. Although they were not omitted from the dendrogram in Fig. 1, they were not used further in this work.

The resulting systems from this author could be divided into two selectivity groups, *i.e.*, [RB, RC, RD] and [RE] (see Fig. 1). Especially RB and RC showed a high correlation, and RE was the only system with completely different selectivity (see also the "scatter diagrams" in Fig. 3). Systems RE and RD had the lowest χ^2 value and when used in conjunction they should yield the largest amount of information for this set of phenolic compounds. One might argue that the subdivision of Reio's

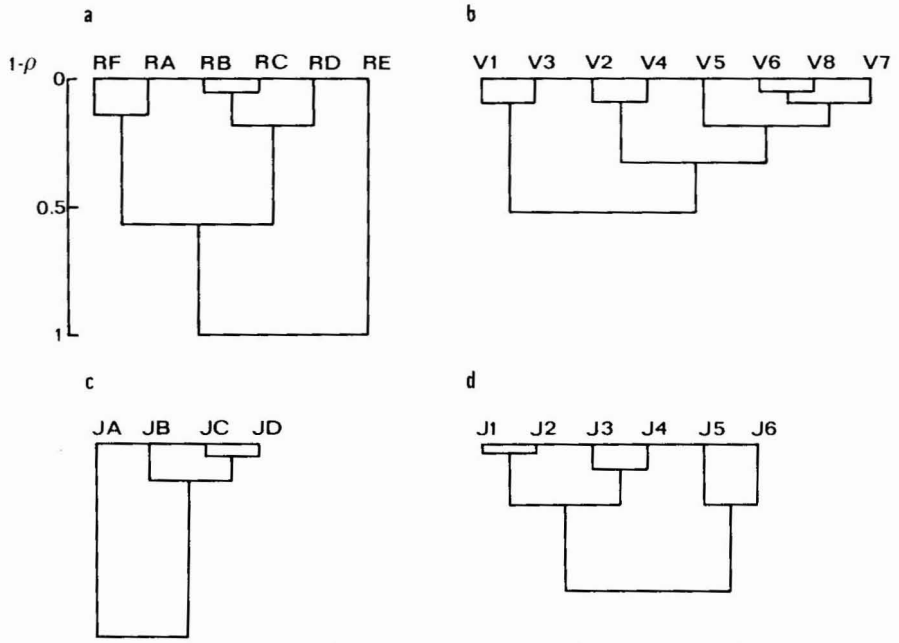

Fig. 1. Dendrograms from Reio's[4] (a), Van Sumere *et al.*'s[5] (b), Jangaard's[6] (c) and Jay *et al.*'s[7] (d) chromatographic systems (see also Table I for more information).

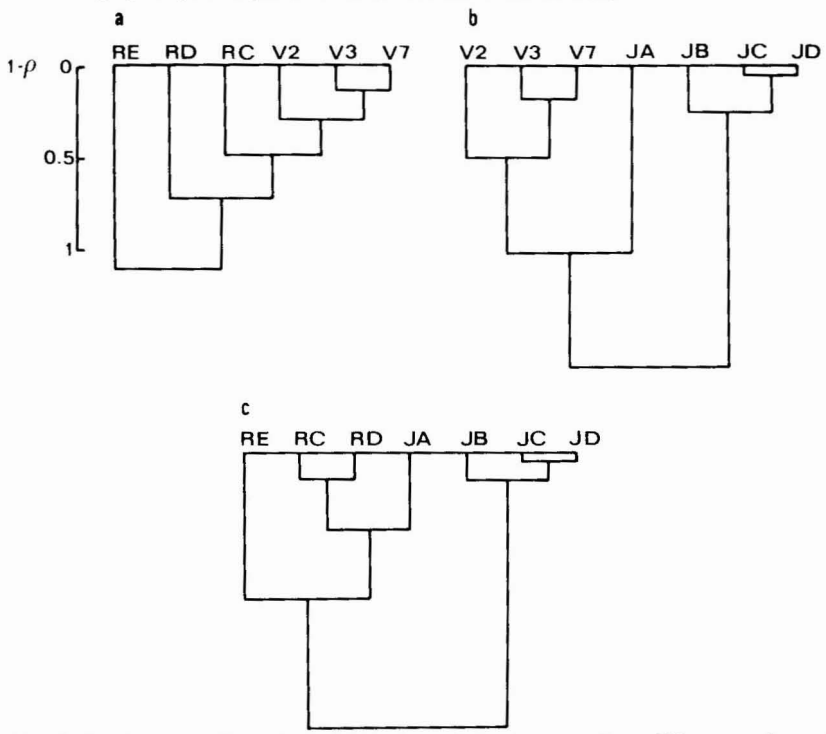

Fig. 2. Dendrograms from the cross-sections of R_F data sets from different authors; 35 R_F data were obtained from the cross-section Reio × Van Sumere *et al.* (a), 32 R_F data from Van Sumere *et al.* × Jangaard (b) and 51 R_F data from Reio × Jangaard (c).

TABLE I

PUBLISHED PC AND TLC SYSTEMS USED IN THIS STUDY

Reference	*Procedure*	*Systems*	*Mobile phase*	χ^2	$\bar{R}_F$
Reio[4]	228 phenolic compounds: PC	RF	Ethyl methyl ketone–acetone–formic acid–water	463	0.86
		RE	Water–ethyl methyl ketone–diethylamine	62	0.55
		RA	Methyl isobutyl ketone–formic acid–water	476	0.82
		RB	Chloroform–methanol–formic acid–water	151	0.68
		RC	Benzene–ethyl methyl ketone–formic acid–water	214	0.65
		RD	Benzene–formic acid–water	48	0.49
Van Sumere *et al.*[5]	81 phenolic compounds: TLC on silica gel (V1, V5); on steamed silica gel (V2, V6); on silica gel–cellulose (V3, V7); and on steamed silica gel–cellulose (V4, V8)	A = V1	Toluene–ethyl formate–formic acid	41.5	0.42
		B* = V2	Toluene–ethyl formate–formic acid	1.61	0.51
		C = V3	Toluene–ethyl formate–formic acid	29.8	0.47
		D* = V4	Toluene–ethyl formate–formic acid	5.90	0.49
		A = V5	Chloroform–acetic acid–water	10.9	0.43
		B* = V6	Chloroform–acetic acid–water	9.61	0.49
		C = V7	Chloroform–acetic acid–water	11.2	0.53
		D* = V8	Chloroform–acetic acid–water	22	0.55
Jangaard[6]	69 phenolic compounds, flavonoids and coumarins: cellulose TLC	JA [§]	Isopropanol–NH_3–water	18.8	0.38
		JB	20% potassium chloride	25.3	0.33
		JC [§]	2% formic acid	6.68	0.42
		JD	10% acetic acid	17.0	0.51
Jay *et al.*[7]	86 flavonoids: PC (J1, J2, J3, J4) TLC on polyamide (J5, J6)	J1	Acetic acid–water	27.9	0.63
		J2	Acetic acid–water–HCl	79.7	0.76
		J3	*n*-Butanol–acetic acid–water	149	0.83
		J4	*tert.*-Butanol–acetic acid–water	109	0.77
		J5	Benzene–methyl ethyl ketone–methanol	4.80	0.56
		J6	Benzene–petrol–methyl ethyl ketone–methanol	92.3	0.21

[§] In Jangaard's paper[6], the headings JA and JC were erroneously exchanged.

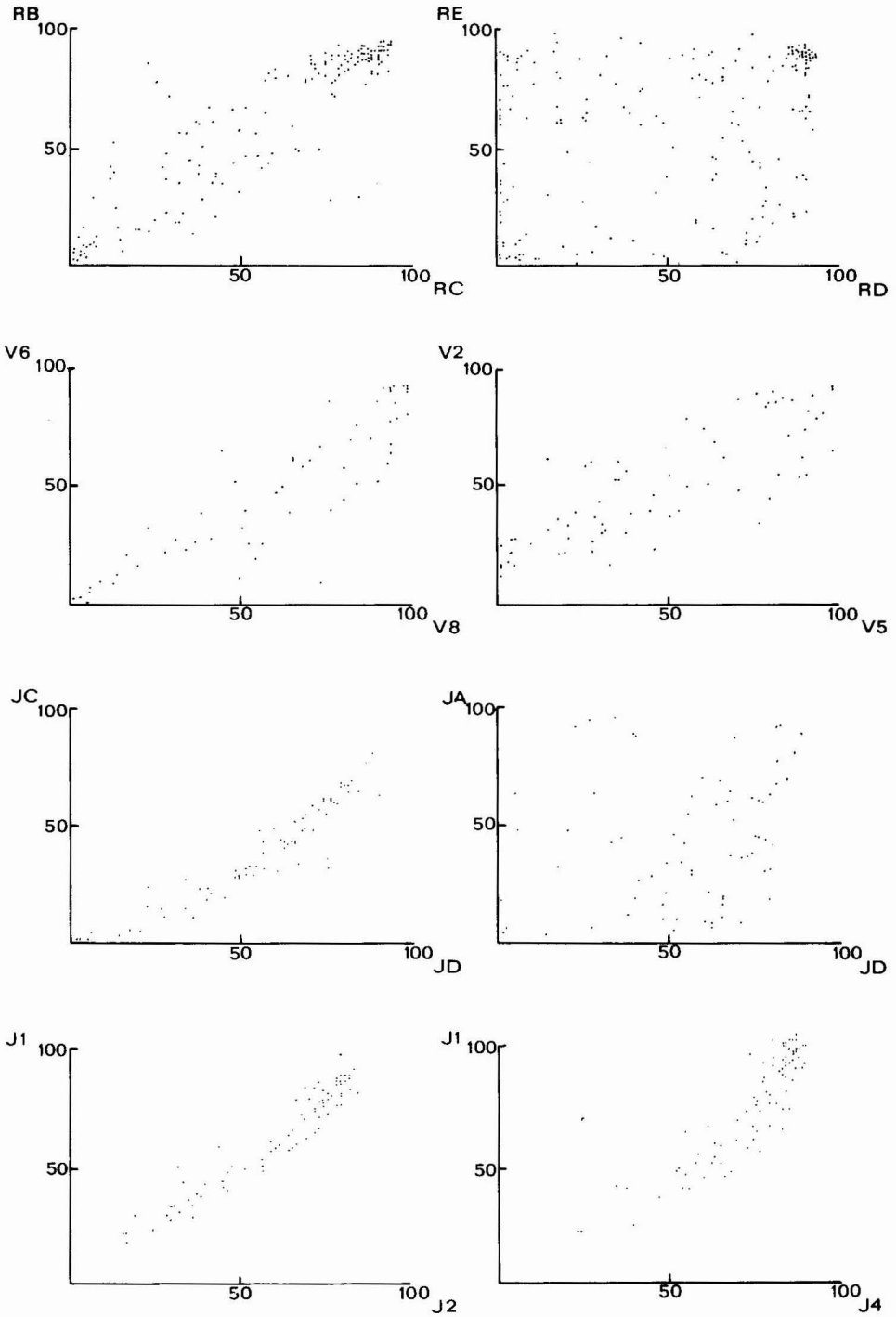

Fig. 3. Scatter diagrams obtained by plotting $R_F \times 100$ values from two different chromatographic systems. The left-hand scatter diagrams show highly correlated systems, and on the right-hand side less correlated systems from the same authors are shown.

systems into two groups, one group using an acidic solvent [RB, RC, RD] and the other using a basic solvent [RE], could have been induced by the fact that the subgroup of organic acids (77 R_F data) behaved completely differently from the subgroup of "neutral" phenolics (137 R_F data). We calculated the dendrograms for both subgroups, and only qualitative differences from the total group were noted: the difference between [RB, RC, RD] and [RE] was greater for the "acidic" subgroup than for the "neutral" subgroup. However, both subgroups yielded a dendrogram that was qualitatively comparable to that shown in Fig. 1 for the total group.

Jangaard's chromatographic systems could also be divided into two subgroups (see dendrogram B), *i.e.*, [JB, JC, JD] and [JA]. Surprisingly, three chemically completely different mobile phases fell into one subgroup. Two similar mobile phases (2% formic acid and 10% acetic acid) effected an uncorrelated elution behaviour. Jangaard suggested the use of the eluent pairs JA–JC, JB–JC, JD–JC and JD–JB in two-dimensional chromatography. This was completely in contradiction to the dendrogram that we calculated from his results.

On this basis we should rather suggest the combinations JA–JB, JA–JC and JA–JD for this purpose. Closer inspection of Jangaard's data revealed that when JA and JC were exchanged, Jangaard's results correlated with our findings. This meant that the mobile phase in the JA system was isopropanol–ammonia–water, whereas it was 2% formic acid in the JC system. Hence, in ref. 6, the column headings JA and JC were probably erroneously exchanged. Using a set of 66 phenolic compounds available in our laboratory, we tested this possibility. We chromatographed these compounds on MN300 + F_{254} cellulose TLC plates from Macherey, Nagel & Co. (Düren, G.F.R.) (the same type as used by Jangaard) with 2% formic acid and 10% acetic acid as mobile phases. The correlation between these two systems was clear ($\varrho = 0.83$). Even when an HPTLC stationary phase such as cellulose HPTLC + F_{254} (Merck, Darmstadt, G.F.R.), was used, these two eluents effected the same elution behaviour ($\varrho = 0.86$). Division of Jangaard's set of R_F values into "acidic" (40 R_F data) and "neutral" (39 R_F data) subgroups yielded qualitatively analogous dendrograms.

Van Sumere *et al.*'s systems could clearly be divided into several pairs, V1–V3, V2–V4, V6–V8 and V5–V7 (see Table I). These pairs were composed of two systems of steamed or non-steamed plates (see Fig. 3). This shows that the use of mixed layers had little or no effect on selectivity, whereas treatment of the plates with steam alters the selectivity to a certain extent. About the same variation in selectivity is obtained by changing the mobile phase from TEF to CAW: subgroups [V1, V2, V3, V4] and [V5, V6, V7, V8] were formed.

The dendrogram obtained with the acidic compounds subgroup (40 R_F data) was analoguous to that from the total group which is shown in Fig. 1. The neutral compounds subgroup (39 R_F data) behaved slightly different.

Cross-sections of chromatographic data sets from the different authors were also compared. We determined which phenolic compounds were found in two such collections, and these new, smaller data sets were used in the multiple correlation search. It was possible to do this for the systems Reio × Van Sumere *et al.*, Van Sumere *et al.* × Jangaard and Reio × Jangaard. Jay *et al.*'s data could not be used in this search, as they investigated compounds of a different nature (flavonoids), so that there was only a small overlap with the other authors' data.

Dendrograms of correlations between systems from the different publications are shown in Fig. 2. From each publication we selected three or four systems belonging to different selectivity groups. In one such group the choice was made on the basis of a favourable χ^2 value (best spreading of R_F values). The dendrogram from Van Sumere *et al.*'s data and Reio's data (Fig. 1) shows that generally there was little or no correlation between the two, except for Reio's system C, which correlated moderately well with the three systems of Van Sumere *et al.* This indicated an analoguous separation mechanism on RC and V2, V3, V7. From the RXJ dendrogram it was clear that JA and RC + RD had comparable selectivities, although the correlation was only moderate. It can be seen that all of these systems with a cellulose stationary phase could be divided into four groups with completely different selectivities, *i.e.*, [RE], [RC, RD], [JA] and [JB, JC, JD] (see Fig. 2, dendrogram c).

In accordance with the theories of Massart and De Clercq[8], it would be advantageous to use combinations of systems from these four groups when one is faced with the problem of product identification.

The VSX J comparison is also illustrated in a dendrogram in Fig. 2. The systems of Van Sumere *et al.* and of Jangaard were completely uncorrelated. Product identification in these systems is most likely to be successful when one uses a combination of systems chosen from the three groups [V2, V3, V7], [JA] and [JB, JC, JD].

As Jay *et al.*'s publication was concerned with a completely different set of phenolic compounds, namely the flavonoids, it is discussed separately. Using the dendrogram derived from Jay *et al.*'s system (Fig. 1), we could make subdivisions into three groups: [J1, J2], [J3, J4] and [J5, J6]. J1 and J2 had practically identical selectivities. The difference between the two eluents was their eluting power (J2 > J1; compare the $\bar{R}_F$ values in Table I). Systems J3 and J4 were completely comparable on the basis of selectivity and eluting power. There is also little difference between the two groups J1, J2 and J3, J4 (Fig. 3). Switching from PC to polyamide TLC changed the selectivity, but only to a limited extent.

The best chromatographic system for this set of flavonoids was undoubtedly J5 (lowest χ^2 and good $\bar{R}_F$ value). Maximum information on the nature of an unknown flavonoid should be obtained by chromatography on J1 and J5 when only two of the authors' systems were chosen. J3, J4 and J6 have χ^2 values too high for this set of flavonoids.

CONCLUSION

We detected only two sufficiently different selectivities in Reio's PC systems and in Jangaard's TLC separations. Jay *et al.*'s PC separations also split into two selectivity groups. They obtained two additional selectivities by using polyamide as a stationary phase. A study of Van Sumere *et al.*'s data set showed that replacing simple layers (silica) by mixed layers (cellulose/silica) did not change the selectivity. The water content of the layers had a great effect on the selectivity. In fact, one can select three systems from Van Sumere *et al.* with sufficiently different selectivities that have the same stationary phase. We found an almost complete lack of correlation between systems from the different authors used in our cross-correlation study.

The dendrograms in Figs. 1 and 2 can be used in the chromatography of phenolic compounds for the following purposes:

(1) to decide which combination of systems will yield the most information about the nature of an unknown phenolic compound (i.e., qualitative analysis); the use of systems with comparable selectivities can be avoided as they do not provide new information;

(2) to choose an ideal pair of mobile phases for two-dimensional chromatography;

(3) when newly developed stationary phases such as HPTLC cellulose plates are used; compilation of R_F data in these systems could be restricted to a set of selected mobile phases that yield sufficiently different selectivities.

The PC and TLC of phenolic compounds offer only two selectivities when one stationary phase is used. Changes of mobile phases are needed only in order to obtain a better spread of the compounds over the chromatogram (minimizing χ^2). Up to three different systems can be obtained with one stationary phase when the water content of the latter is changed (*e.g.*, Van Sumere *et al.*'s data). A change of stationary phase results in a complete change in selectivity (only one exception is noted, namely that substituting cellulose HPTLC for cellulose TLC only slightly affects the selectivity). This is what makes TLC advantageeous over HPLC in qualitative analysis. Indeed, with the former technique one can easily perform analyses on different systems at the same time. Only a few practical systems are known in the HPLC analysis of phenolic compounds. If the latter technique is to have the same capabilities as PC and TLC for the qualitative analysis of these compounds, other systems will have to be found.

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COMPARISON OF SUPPORTS CHEMICALLY MODIFIED BY ORGANOSILICON COMPOUNDS FOR GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

The adsorption activities towards polar solutes of eight supports (including five commercial samples) coated with squalane were compared, taking into account different types of treatment. One of the best commercial supports was found to be about the same as a support treated with a siloxane tetramer reagent mixed with various substances. Possible modified support surfaces obtained when different silanization reagents are used for the modification are discussed. The best results seemed to be achieved when polymer chains were bound to the surface of the support.

INTRODUCTION

The adsorption activity of diatomaceous supports was reported in the earliest papers on chromatography[1,2]. Poor peak symmetry and dependence of retention on amount of sample are the results of non-linear adsorption isotherms on the liquid–solid interface in the chromatographic column. These effects have an adverse influence on reproducibility of retention data and the separation of polar solutes on non-polar or low-polar stationary phases[3]. Non-reproducibility of retention data reduces the value of these parameters in qualitative analysis. Any inhomogeneity of the support surface leads to non-linear adsorption isotherms, and therefore most efforts towards the development of ideal supports for gas–liquid chromatography (GLC) should be directed to homogenization of support surfaces.

Some treatment techniques have been reported[1,4–6] in which polar organic modifiers, polymers or Ag were added to the support in order to cover the surface with a homogeneous film. All of these attempts failed because of a lack of strong chemical interaction of the modifier with the support surface. Chemical treatment (silanization) was found to be a powerful technique for eliminating active adsorption sites from the support surface[7,8]. Dimethyldichlorosilane (DMCS) and hexamethyldisilazane (HMDS) solutions were found to be the best reagents for this purpose[9]. Silanization in solutions at high temperatures and pressures is the preferred procedure for the manufacture of commercial silanized supports; many other reagents have also been reported to be useful for this process. No details have been published

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about the preparation of new high-quality supports, for example, Chromosorb HP, Chromosorb 750 and Chromaton N Super.

Silanization in the vapour phase at room[10] or elevated temperatures[11,12] has been proposed by some workers as the best technique. Unfortunately, no reports have appeared on comparisons of the different silanization techniques, which makes it impossible to discuss further improvements to supports for use in GLC. This paper describes an evaluation of some silanization techniques for the treatment of supports.

EXPERIMENTAL

Gas chromatography

The retention data for the packings were determined by using a Chrom-41 gas chromatograph (Laboratorní Přístroje, Prague, Czechoslovakia) with a flame-ionization detector (FID). Argon was used as the carrier gas. Glass columns (1.2 m × 4 mm I.D.) were filled with the packing coated with 5% of squalane (Schuchardt, Munich, G.F.R.), which was purified by passage through a silica gel column. The freshly prepared packings were conditioned for 10 h at 90°C. Samples (0.1 ml) were injected as saturated vapours of the solutes with a gas-tight Hamilton syringe. Relative retentions were determined at column temperatures from 40 to 60°C. Retention times were measured with an electronic timer with an accuracy of about 0.1 sec.

Relative retentions and relative molar heats of solutions were calculated in the usual manner. The mean relative standard deviation of the relative retention (r) was about 0.5% and the mean standard deviation of the relative molar heat of solution (ΔH_s^0) was 0.05 kcal/mole. The standard column temperature was 50°C. *n*-Heptane was chosen as the standard.

When the retention time of a solute changes with the amount of sample, the following equation was used in order to give a linear relationship[13]:

$$r = (A/\log h) + B \tag{1}$$

where A and B are constants and h is peak height. Relative retentions for three or four different values of h were determined and then the r values were plotted against $1/\log h$. Peak heights were measured in centimetres on the 25-cm recorder scale and h values were recalculated to the full scale of the recorder (10^{-11} A). The isobaric relative retention for $1/\log h = 0.3$ was taken for the comparison of the packings. These values are reproducible for the instrument with a constant gas flow.

The degree of non-linearity of the sorption isotherm is related to the A value. The more representative function A^* is used for this purpose[13]:

$$A^* = \frac{r_{0.4} - r_{0.3}}{r_{0.3}} \tag{2}$$

where the subscripts 0.3 and 0.4 relate to the corresponding values of $1/\log h$.

Preparation of supports

Chromaton N AW (Lachema, Brno, Czechoslovakia) was chosen as the initial support for silanization[14]. The following reagents were used for treatment: poly-

methylsiloxane PMS-500, hexamethylcyclotrisiloxane D_3, octamethylcyclotetrasiloxane D_4, DMCS, trimethylchlorosilane (TMCS) (all manufactured in the U.S.S.R.; reagent grade). A mixture of D_4, DMCS and TMCS (2:1:1) was also used.

A liquid-phase process was used for PMS-500 and DMCS treatments. The initial support was heated to 400°C, then a 5% solution of PMS-500 or DMCS in carbon tetrachloride was mixed with the support for 30 min. The slurry was heated at 150°C for 2 h and then the residue was held in a vacuum at 250°C in order to evaporate all reagents and volatile substances. The DMCS-treated support was contacted with water vapour at 300°C for 2 h in order to eliminate Si–Cl groups, and the resulting product was heated at 250°C in a vacuum for 2–3 h.

The treatment with D_3 was carried out in the gaseous phase at 150°C for 1 h. The initial support was heated under vacuum and the product after silanization was held in a vacuum at 250°C for 2 h. The modification with the mixture of reagents was carried out at 350°C for 2 h with subsequent heating in a vacuum for 2 h.

The following supports were used for preparing the packings: (1) Chromaton N AW (Lachema); (2) Chromaton N AW DMCS (Lachema); (3) Chromaton N AW treated with D_3; (4) Chromaton N AW HMDS (Lachema); (5) Chromaton N Super (Lachema); (6) Chromosorb G AW DMCS (Johns-Manville, Denver, CO, U.S.A.); (7) Chromaton N AW treated with D_4–DMCS–TMCS (2:1:1); and (8) Chromaton N AW treated with PMS-500.

One volume of Chromosorb G is about 2.5 times heavier than one volume of Chromaton; therefore, the packing with Chromosorb G has only 2.5% of the weight of squalane.

Because the non-polar and low-polarity solutes have low adsorption on diatomaceous supports, two very polar solutes were used in order to demonstrate the adsorption ability of the supports: *n*-propanol, which forms strong hydrogen bonds, and methyl ethyl ketone, which has a very high dipole moment; A^* for *n*-heptane is zero.

RESULTS AND DISCUSSION

The relationship between the relative retention of *n*-propanol and $1/\log h$ is shown in Fig. 1. The numbers on the lines correspond to the numbers of the supports specified in the previous section. Line 1 for the initial support, Chromaton N AW, is curved for the range of concentrations examined. The dependence of r on $1/\log h$ tends to be non-linear for peak heights with $1/\log h > 0.45$. This range refers to Henry's law for the partition coefficient. When the vapour pressure of the solute is extremely small, each solute molecule is bound to active sites on the support surface. These sites seem to be identical from the energetic point of view, and therefore the adsorption isotherm is approximately linear. The nature of these adsorption sites may be suggested as hydroxyl groups chemically bonded to the surface.

Hydroxyl groups were etherified after the silanization procedure. This reaction eliminates the more active adsorption sites from the support surface, as many workers have claimed. However, no reports have considered the alternative aspect, *i.e.*, new hydrocarbon groups appear on the support surface after silanization. This should lead to an increase in the degree of inhomogeneity of the support surface. The experimental data for the silanized supports in Fig. 1 show that the curved adsorption

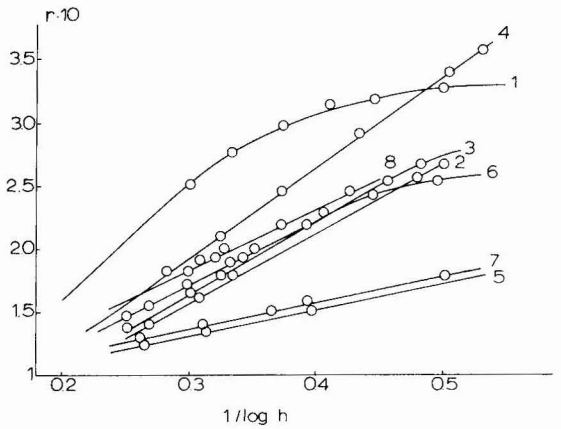

Fig. 1. Relationship between relative retention of *n*-propanol and 1/log *h* for the eight packings at 43.8°C.

isotherm for *n*-propanol extends over the whole range of vapour pressures for the solutes; no indication of the Henry's law region is observed for the silanized supports. Therefore, the relative retention of *n*-propanol is higher on silanized support No. 4 than on the initial support for extremely low vapour pressures.

The relationship in Fig. 1 gives two basic parameters for the sorption processes in the column: (i) the isobaric relative retention for any chosen filling of the interface surface and (ii) the slope of the relationship which indicates the degree of non-linearity of the sorption isotherm, A. The lower both values are, the better is the support.

The general picture for *n*-propanol also applies to the polar solute with a high dipole moment, methyl ethyl ketone (Fig. 2). The isobaric relative retentions at 1/log $h = 0.3$ and A^* values are presented in Table I, together with the relative isobaric heats of sorption for the solutes. A^* decreases when the column temperature increases (Fig. 3).

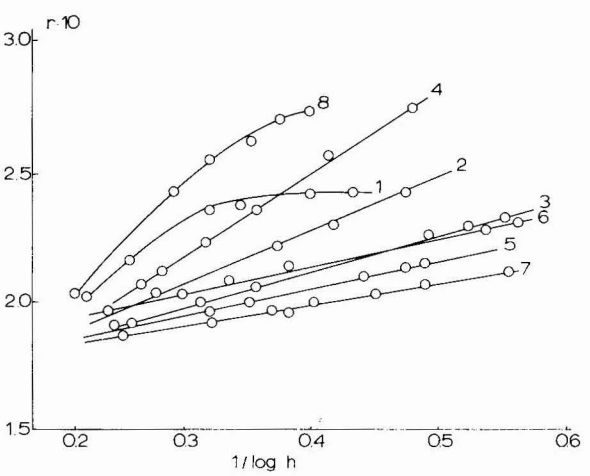

Fig. 2. Relationship between relative retention of methyl ethyl ketone and 1/log *h* for the eight packings at 43.8°C.

TABLE I

SORPTION PARAMETERS FOR THE EIGHT PACKINGS

Support No.	*n-Propanol*			*Methyl ethyl ketone*		
	*A**	ΔH_s^0 *(kcal/mole)*	*r*	*A**	ΔH_s^0 *(kcal/mole)*	*r*
1	3.55	0.25	0.252	1.70	1.25	0.240
2	3.50	0.85	0.170	1.16	1.50	0.216
3	3.57	0.90	0.172	0.90	1.35	0.207
4	3.68	0.30	0.193	1.50	1.35	0.230
5	1.55	1.20	0.137	0.68	1.60	0.200
6	3.08	0.75	0.176	0.74	1.55	0.214
7	1.55	1.20	0.143	0.42	1.60	0.189
8	2.69	0.25	0.184	2.10	0.60	0.251

Celite (Chromaton N AW is similar to Celite) has a surface covered by hydroxyl groups[15], which is the main reason for the high *r* values for *n*-propanol and low values of ΔH_s^0 on the packing with Chromaton N AW. The relative retention of *n*-propanol decreases markedly after silanization, which supports the theory of the elimination of hydroxyl groups from the surface. However, with different silanization techniques no linear isotherm resulted for *n*-propanol, which indicates the presence of different active sites on the support surface that can form hydrogen bonds. Elimination of hydroxyl groups from the support surface is insufficient for an ideal support to be obtained.

Treatment of the support with PMS-500 was tried in order to obtain the chemically bound layer of the stationary phase according to the principle of Aue and Younker[16]. When high temperatures are applied a chemically bound layer of the nonpolar liquid remains on the support surface, as was proved for polyethylene. Unfortunately, no such effects were observed on the support treated with PMS-500 at 250°C (line No. 8). This behaviour of PMS occurs with all stationary phases, and no improvements could be obtained for packings with this silicone.

Let us compare the packings by considering *r* and ΔH_s^0 values. According to these parameters, the hydrogen bonds are blocked to a minimum extent on packings 5 and 7. It is interesting to compare two silanizing agents (DMCS and HMDS) in order to understand the nature of the more effective deactivation of the support surface. ΔH_s^0 for *n*-propanol on packing 4 is 0.55 kcal/mole lower than that for packing 2. In general, both silanizing agents react with the surface hydroxyl groups, but DMCS has two active groups whereas HMDS has only one. This allows reaction with closely situated hydroxyl groups on the support surface when DMCS is used. It is surprising that ΔH_s^0 for *n*-propanol is about the same for the initial support Chromaton N AW and the HMDS-silanized support. This indicates that many hydroxyl groups are close together and some of them remain unsilanized with reagents such as HMDS.

It is interesting that two different types of supports treated with DMCS, Chromaton N AW and Chromosorb G, have similar sorption properties with respect to polar solutes. This shows the importance of the silanization procedure in spite of the nature of the support.

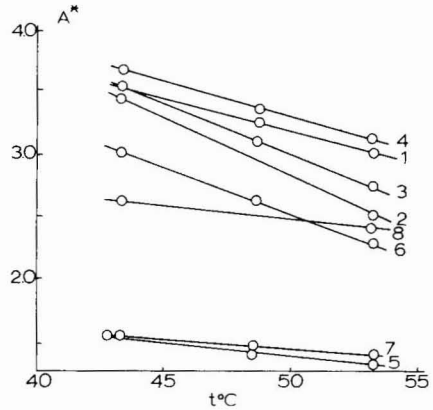

Fig. 3. Dependence of A^* values on column temperature with *n*-propanol as the solute.

When hydroxyl groups are eliminated by an effective silanization process, only other polar active sites remain on the support surface. The presence of these sites is the main reason for the selective adsorption of methyl ethyl ketone molecules. Supports 5 and 7 are the best for both polar solutes, but support 7 is better for methyl ethyl ketone. No great differences in ΔH_s^0 values for methyl ethyl ketone were observed for the different silanization processes, which indicates the relatively small importance of etherification of hydroxyl groups with respect to selective adsorption of methyl ethyl ketone.

The A^* values show the superiority of support 7 for methyl ethyl ketone (see also the temperature dependence of the A^* values). Support 7 therefore has the best homogeneity towards polar atomic groups.

Let us consider the ratio t_N/t_0 (where t_0 is the retention time of methane, which is assumed to be the non-sorbed solute, and t_N is the retention time of the solute) for pure supports in order to determine the hydrocarbon adsorption ability with respect to the surface. The t_N value is measured for an *n*-alkane. The data in Table II show that PMS-500 is the stationary phase rather than the modifier. Support 5 has a greater adsorption ability towards hydrocarbons than support 7, which indicates the different treatment processes used to prepare these supports. More hydrocarbon groups exist on the surface of support 2 (DMCS) than on support 4 (HMDS). It is

TABLE II

t_N/t_0 VALUES FOR DIFFERENT *n*-ALKANES ON THE SUPPORTS

Support No.	*Solute*			
	C_6	C_7	C_8	C_9
1	1.016	1.056	1.143	1.316
4	1.014	1.028	1.065	1.160
5	1.035	1.086	1.189	1.420
6	1.038	1.086	1.191	1.429
7	1.030	1.062	1.142	1.310
8	1.35	1.83	3.02	5.90

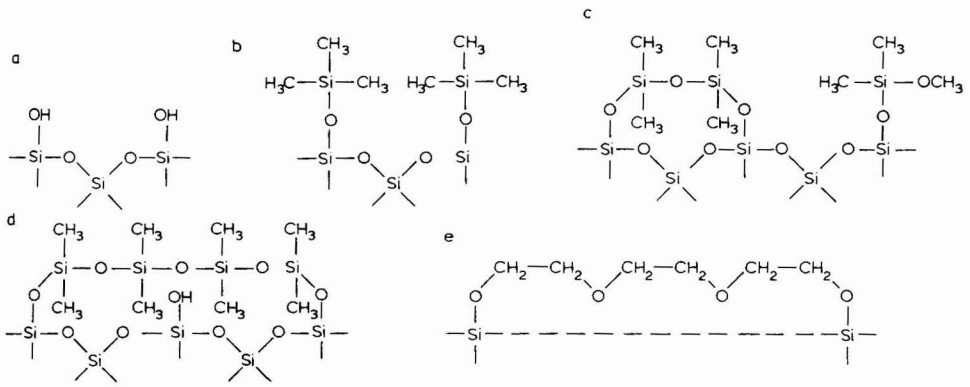

Fig. 4. Suggested structures of the support surface fragments (a) before and (b–e) after treatment with different reagents.

surprising that the sorption ability of hydrocarbons with respect to the HMDS-modified support is lower than that for the initial support, which indicates the complex nature of the modification process applied to the supports.

The possible explanations for the reactions with the different agents are illustrated in Fig. 4, where the silanization reagents bind with the support surface on hydroxyl group points. Fragment (a) shows a model for the support surface, and (b) shows the result of silanization with HMDS or TMCS. Fragment (c) is the support surface after treatment with DMCS and methanol vapour, and (d) and (e) show the supports after treatment with D_3 and D_4, respectively. This scheme shows that with increase in the part of the support surface shielded after the reaction, the more inert is the adsorption surface that results. This indicates the best means of developing an ideal support surface: treatment with a polymer which is chemically bound to the support surface and shielding of the whole surface by the polymer layer. An additional interesting point is the use of reagent mixtures, which are more effective than the individual reagents.

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ION-EXCHANGE PHENOMENA AND CONCOMITANT pH SHIFTS ON THE EQUILIBRATION OF REVERSED-PHASE PACKINGS WITH ION-PAIRING REAGENTS*

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SUMMARY

Breakthrough patterns of eluents containing tetrabutylammonium or cetyltrimethylammonium ions (two frequently used ion-pairing agents in high-performance liquid chromatography) have been studied, using reversed-phase columns with octadecyl chains permanently bonded to 10-μm silica particles. The occurrence of pH shifts either before or after the breakthrough of the ion-pairing agents has been demonstrated. An explanation for the breakthrough patterns is offered and evidence for two different binding mechanisms of these agents is presented.

INTRODUCTION

Ion-pair chromatography performed with reversed-phase columns and mobile phases containing ion-pairing agents has been the subject of a number of studies during recent years and has been reviewed by Tomlinson *et al.*[1]. Much attention has been focused on the mechanism underlying this type of chromatography and two models have been proposed: ion-pair formation in the mobile phase or at the interface of the stationary phase and the mobile phase has been proposed by some workers[2–4] and ion exchange of solute ions against the counter ion of the adsorbed reagent by others[5–11]. If the retention behaviour is to be explained by the latter mechanism, a relationship between the amount of reagent adsorbed and the retention volumes of the chromatographed solute ions should exist. Investigations on this relationship were carried out by Terwey-Groen *et al.*[12] and Van de Venne *et al.*[8].

During experiments performed in order to elucidate the retention mechanism of water-soluble corticosteroids, chromatographed with tetrabutylammonium (TBA) containing eluents, the breakthrough patterns of these eluents appeared to be strongly dependent on the history of the columns (*i.e.*, the previous eluents used). In a number of instances breakthrough of the TBA ions occurred in two distinct steps; pH measurements on the collected fractions indicated that these steps corresponded with shifts in the pH of the eluate. Similar observations were made in the breakthrough

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patterns of cetrimide-containing eluents used for studying the retention behaviour of weak acids in this type of chromatographic system.

We therefore decided to study this phenomenon of pH shifts and to investigate the breakthrough patterns with a flow-through detection device.

EXPERIMENTAL

Materials and reagents

Potassium dihydrogen phosphate, potassium bromide, methanol and sodium hydroxide were of analytical-reagent grade and were obtained from Merck (Darmstadt, G.F.R.). Dioctyl sodium sulphosuccinate (DOSS), 85% phosphoric acid ("reinst"), benzoic acid and lithium nitrate were also obtained from Merck. Tetrabutylammonium hydroxide (40%) ("prakt.") and tetrabutylammonium bromide ("puriss.") were purchased from Fluka (Basle, Switzerland). Potassium hydroxide came from EKA (Bohus, Sweden). Cetyltrimethylammonium bromide (cetrimide) was purchased from BDH (Poole, Great Britain) and boric acid ("analyzed" reagent) from Baker (Deventer, The Netherlands). Prednisolone 21-*m*-sulphobenzoate sodium was obtained from Lark (Milan, Italy).

Water was distilled twice from glass after deionization and used immediately.

Tetrabutylammonium dihydrogen phosphate solution was prepared from tetrabutylammonium hydroxide by neutralization with an equimolar amount of phosphoric acid. TBA-containing eluents were prepared by mixing equal weights of filtered methanol and a filtered aqueous solution containing $2 \cdot 10^{-2}$ *M* potassium dihydrogen phosphate and 10^{-2} *M* tetrabutylammonium dihydrogen phosphate (the pH of this aqueous solution was adjusted with potassium hydroxide).

Phosphate buffer eluents were prepared by mixing an equal weight of filtered methanol and a filtered aqueous solution containing $3 \cdot 10^{-2}$ *M* potassium dihydrogen phosphate (the pH of this aqueous solution was adjusted with potassium hydroxide).

The pH values of the eluents used, containing 50% (w/w) of methanol, were measured after calibrating the pH meter against methanol–water (1:1) buffers as described by Bates[13]. The pH meter readouts thus obtained are denoted by pH*. The pH of the aqueous solution used for the preparation of the methanol–water eluent was approximately 1.3 pH units lower than the pH* measured in the eluent itself.

Cetyltrimethylammonium (CTA) containing eluents were prepared in water–methanol mixtures (final ratio in the eluent = 1:1, w/w) with $5.49 \cdot 10^{-3}$ mol $\cdot$ kg^{-1} (= 0.2%, w/w) of cetrimide and $94.51 \cdot 10^{-3}$ mol $\cdot$ kg^{-1} of potassium bromide (the total bromide concentration is thus 0.1 mol $\cdot$ kg^{-1}); the eluent was buffered with boric acid buffer (0.025 mol $\cdot$ kg^{-1} in the eluent) and adjusted to the desired pH* with sodium hydroxide. Eluents without cetrimide were prepared in the same way; the cetrimide was replaced with an equimolar amount of potassium bromide (resulting in a potassium bromide concentration of 0.1 mol $\cdot$ kg^{-1} in the eluent).

Instrumental

The chromatographic experiments were performed with the following instrumental combination. A 6000A solvent-delivery system was connected to a U6K injection system, modified with a 200-μl sample loop; the two columns used in this study

were a μBondapak C_{18} column (30 cm × 3.9 mm I.D.), particle size 10 μm, and a Radial-Pak A cartridge (10 cm × 8 mm I.D.), in combination with an RCM-100 radial compression module (all from Waters Assoc., Milford, MA, U.S.A.). The packings of both columns were of the reversed-phase type, with octadecyl chains chemically bonded on 10-μm silica particles. Each column was installed between a U6K injection system and a Model 440 differential UV absorbance detector (Waters Assoc.) by means of a Valco rotary six-port 7000 p.s.i.g. valve (Chrompack, Middelburg, The Netherlands). The connection was made in such a way that a bypass could be used to flush the whole system except the column when the solvents were changed. The UV detector was connected to an R401 refractive index detector (Waters Assoc.).

The inlet of a Radiometer (Copenhagen, Denmark) G299A capillary glass pH electrode was connected to the outlet of the refractive index detector. The outlet of the capillary glass electrode was connected to a small conical glass vessel (provided with an outlet for the excess of eluted solvent) in which a Type 373-90 reference electrode (Ingold, Zürich, Switzerland) was placed. The capillary glass electrode and the reference electrode were connected to a Radiometer PHM 64 pH meter. The jacket around the capillary glass electrode was filled with potassium nitrate solution that was electrically connected to the reference electrode in order to avoid instability of the signal of the pH electrode[14].

During the chromatographic experiments the refractive index detector and the columns were kept at 25°C. Flame absorption spectrometry was carried out on a Perkin-Elmer 400 S spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.).

Procedures

The breakthrough pattern of TBA ions was studied in combination with the μBondapak C_{18} column; the breakthrough pattern of CTA ions was investigated with the Radial-Pak A cartridge. These breakthrough patterns were monitored at different pH* values of the eluents, with the UV absorbance detector, the refractive index detector and the pH detector on-line. The UV detection did not give any additional information to the results obtained with the other detectors.

For measuring potassium, TBA and CTA concentrations in the eluate, the pH detector was disconnected and fractions of the eluate were collected according to the response of the refractive index detector. For the determination of potassium the collected fractions were diluted with water to a concentration of 1–2 ppm of potassium and acidified with hydrochloric acid at a final concentration of 0.4 *M*; potassium concentrations were then measured by means of flame absorption spectrometry.

TBA and CTA concentrations were determined by titrating the collected fractions with DOSS in a two-phase system consisting of chloroform and an aqueous buffer of pH 2.8, using methyl yellow as the indicator[15]. The presence of bromide in the eluate was confirmed by the reaction with silver nitrate[16].

Injections of tetrabutylammonium bromide (TBABr), potassium bromide and phosphoric acid, each dissolved in the eluent, were carried out on the μBondapak C_{18} column, after equilibration of the column with a specific mobile phase. The hold-up time on the μBondapak C_{18} column was determined by measuring the retention time of lithium nitrate using the phosphate buffer eluent of pH* 5.8.

On the Radial-Pak A column the first change in refractive index on injection of a small amount of the eluent to which some methanol had been added was used as an indication of the hold-up time.

RESULTS AND DISCUSSION

The breakthrough patterns on equilibration of the columns with buffer solutions (without TBA or CTA) were found to depend on the history of the columns. If the column had previously been equilibrated with a phosphate buffer eluent of different pH*, the following pattern was observed. Before the hold-up time, the previous eluent is eluted. After the hold-up time, the eluate still exhibits the pH* of this previous eluent. A certain time after the hold-up time, the eluate shows a pH* shift to the pH* of the new eluent. Fig. 1 shows an example of the breakthrough pattern of the phosphate buffer eluent pH* 7.8 after equilibration of the column with the phosphate buffer eluent of pH* 5.8. From these phenomena it was concluded that the columns have some ion-exchange capacity. Apparently, during equilibration with a buffer, part of the residual silanol groups dissociates and the protons are replaced by buffer cations (in these experiments, potassium ions). The equilibration patterns of the columns with TBA- and CTA-containing eluents were also found to depend on the history of the columns.

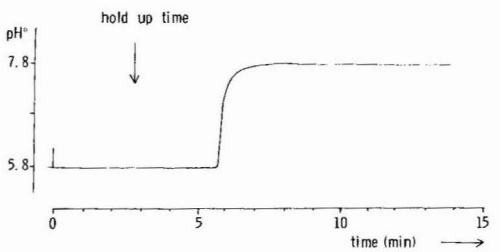

Fig. 1. Breakthrough pattern of the phosphate buffer eluent of pH* 7.8 after equilibration with the phosphate buffer eluent of pH* 5.8. Stationary phase, μBondapak C_{18}; flow-rate, 1.0 ml·min^{-1}.

According to chromatographic theory, the breakthrough volume of a component of the eluent should be the same as its retention volume after injection of a small amount of this component (provided that its distribution isotherm is linear). Injections of TBABr were therefore included in this study and the resulting chromatograms were correlated with the breakthrough patterns found with the TBA-containing eluents. For the interpretation of the chromatograms obtained after injection of TBABr, comparisons with the chromatograms resulting from injection of potassium bromide and phosphoric acid were made. Chromatograms obtained after injection of these compounds and elution with phosphate buffer eluent of pH* 7.3 are shown in Fig. 2. After injection of potassium bromide (Fig. 2a), a peak was observed on the refractive index detector just after the hold-up time. In the eluate corresponding with this peak, the presence of bromide and an increased potassium concentration were demonstrated. Injection of phosphoric acid (Fig. 2b) resulted in two peaks. The first, just after the hold-up time, demonstrated an increased potassium concentration; the second (negative) peak was accompanied by an increased proton concentration, as

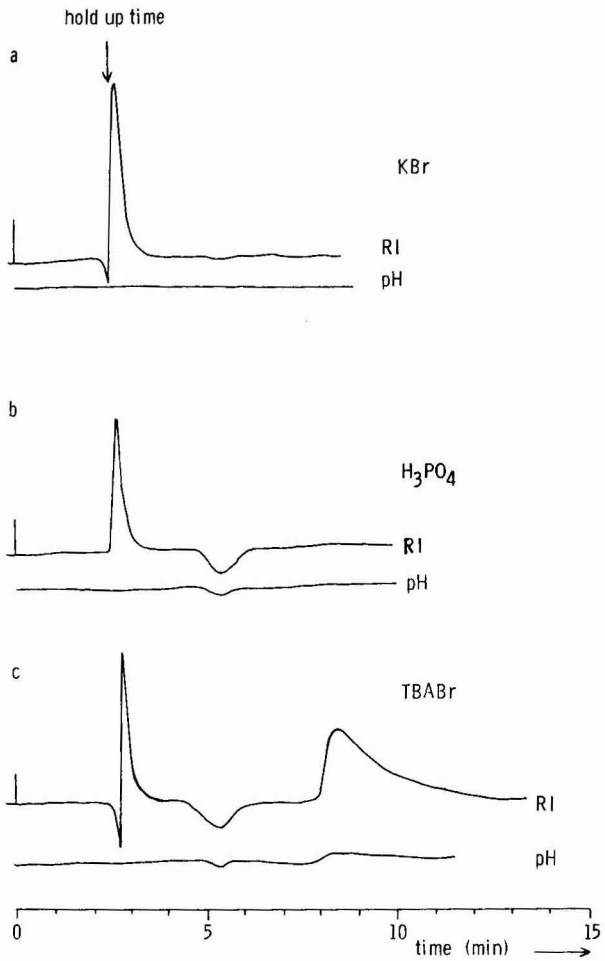

Fig. 2. Chromatograms of 50 μl of (a) a saturated solution of potassium bromide, (b) 20 μg of phosphoric acid and (c) 100 μg of TBABr, all dissolved in the eluent. Chromatographic conditions: μBondapak C_{18}; phosphate buffer eluent of pH* 7.3; flow-rate, 1.0 ml $\cdot$ min^{-1}.

can be seen from the pH detector response. It was therefore concluded that potassium ions are displaced from the stationary phase by protons, as in a cation-exchange system. The liberated potassium ions and the (injected) protons move through the column, each with their own velocity. After injection of TBABr, three peaks were observed with the refractive index detector (Fig. 2c). In the first peak, just after the hold-up, the presence of bromide ions and an increased potassium concentration were demonstrated. The second (negative) peak was similar to the second peak after the phosphoric acid injection (Fig. 2b). Titration of the eluate, corresponding to the third peak, with DOSS confirmed the presence of TBA ions. This last peak was accompanied by a decreased proton concentration. These phenomena can also be explained by cation-exchange processes. TBA is exchanged with potassium ions and protons; these protons in their turn are exchanged with potassium ions. The TBA peak is

eluted, accompanied by a decrease in the proton concentration, for protons will be taken up from the mobile phase by the stationary phase on the release of TBA ions.

At pH* 6.8 the picture is more complicated (Fig. 3), because of the reduction in the retention volume of TBA at the higher proton concentration and the asymmetry of the TBA peak. In the eluate corresponding to the first peak (at 2.8 min) the presence of extra potassium ions and of bromide ions was again demonstrated after injection of different amounts of TBABr. In the eluate of both the second and the third peaks (at amounts of TBA of 150–500 μg) the presence of TBA ions was demonstrated.

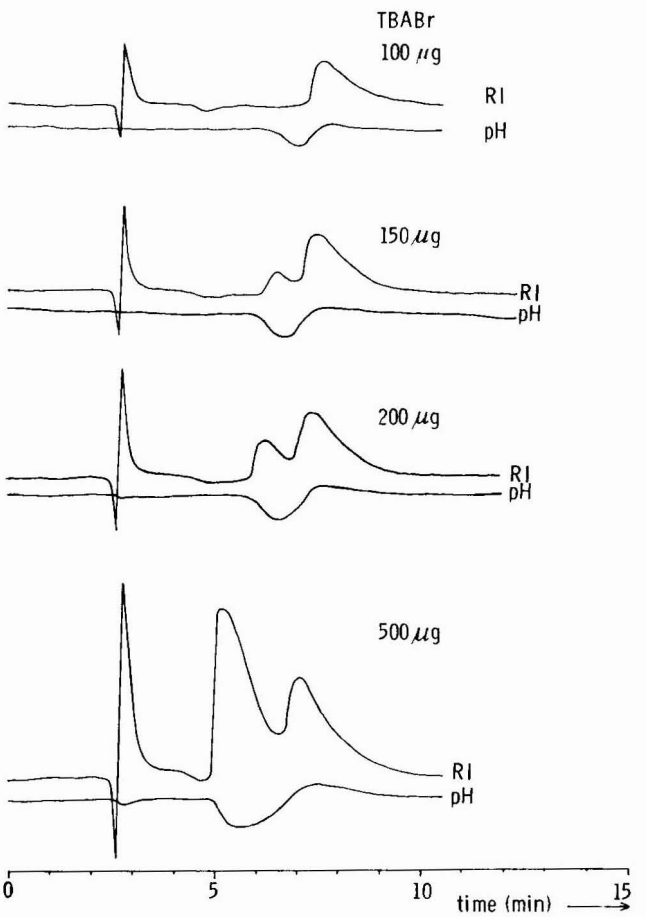

Fig. 3. Chromatograms of 100, 150, 200 and 500 μg of TBABr dissolved in the eluent. Chromatographic conditions: μBondapak C_{18}; phosphate buffer eluent of pH* 6.8; flow-rate, 1.0 ml · min^{-1}.

When 100 μg of TBABr is injected, the retention volume of the TBA zone is still larger than that of the acidic zone. On increasing the amount of TBABr injected, the retention volume of the TBA zone decreases because of the non-linearity of the distribution isotherm. The retention volume of the TBA zone can decrease to such an extent that its front coincides with the acidic zone. As a result, the TBA ions at the

back of the TBA zone move in a mobile phase solution with an increased pH*. The TBA ions at the front of the TBA zone move in a mobile phase solution with a decreased pH*. The alkaline reaction enhances the binding of the TBA ions and the acidic reaction decreases it. The velocity of the TBA ions at the back will therefore be lowered and that of the TBA ions at the front will be increased. Consequently, the TBA zone will split and be detected as a double peak. This splitting of the TBA zone was also observed at pH* values lower than 6.8. Because of the non-linearity of the distribution isotherm of TBA, coincidence of the acidic zone and the TBA zone is also possible at pH* values higher than 6.8, provided that a sufficiently large amount of TBABr is injected. It was established that injection of 1–2 mg TBABr with phosphate buffer of pH* 7.3 resulted in splitting of the TBA zone.

The chromatographic behaviour of injected TBABr explains the differences found in the breakthrough patterns of TBA-containing eluents. During equilibration with TBA-containing eluents protons and potassium ions are liberated continuously.

Fig. 4 shows the breakthrough patterns observed at different pH* values of the TBA-containing eluents after the column was equilibrated with eluents of the same pH* without TBA. Apparently, at this TBA concentration ($5 \cdot 10^{-3}$ M) in the eluent, at pH* 7.8 (Fig. 4c) protons and the TBA front move with almost the same velocity.

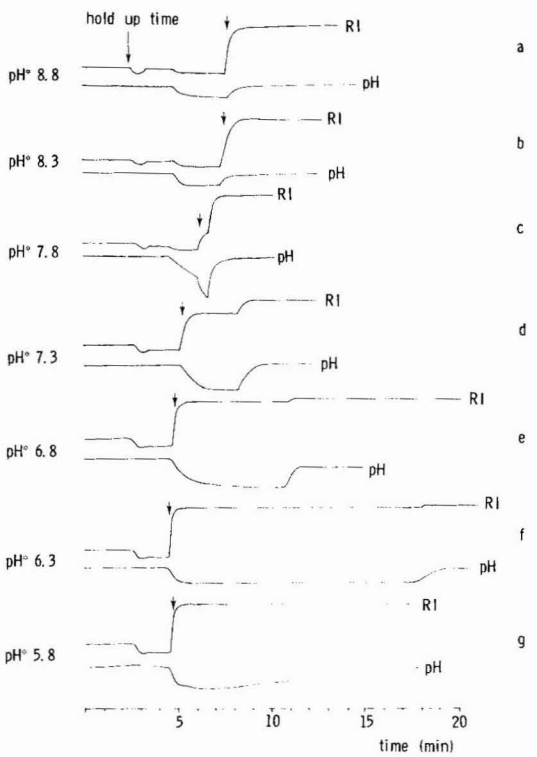

Fig. 4. Breakthrough patterns of the TBA-containing eluents after previous equilibration of the column with phosphate buffer eluents of the corresponding pH*: (a) pH* 8.8; (b) pH* 8.3; (c) pH* 7.8; (d) pH* 7.3; (e) pH* 6.8; (f) pH* 6.3; and (g) pH* 5.8. The arrows denote the breakthrough of TBA ions. Stationary phase, μBondapak C_{18}; flow-rate, 1.0 ml · min^{-1}.

At pH* 8.8 and 8.3 (Fig. 4a and b) the protons move faster than the TBA front. The TBA ions will be exchanged against protons and potassium ions and the protons in their turn against potassium ions. The patterns found at these two pH* values can be explained as follows. Until the hold-up time (2.7 min), the previous eluent will be eluted. After this hold-up time, a potassium-enriched zone (compared with the TBA-containing eluent) is eluted. The end of this potassium-enriched zone is the starting point of an acidic zone in the eluate; the pH* shifts to a lower value and the potassium concentration shifts back to that of the eluent. After a certain time, the TBA breaks through and the pH* shifts to that of the eluent. At pH* 7.3 and at lower pH* values (Fig. 4d–g) the protons move slower than the TBA zone. Consequently, the TBA ions will move through the column in a zone with lower pH* than the pH* of the eluent itself. At the front of this zone potassium ions will be exchanged against TBA ions and protons. The breakthrough patterns in Fig. 4d–g can now be explained as follows. Until the hold-up time the previous eluent is eluted. After the hold-up time, eluent in which the TBA ions are replaced by potassium ions is eluted. After a certain time the pH* will shift back to that of the eluent and the TBA concentration does the same. At pH* 5.8 this shift back to the pH* value of the eluent occurs gradually (Fig. 4g).

These observations on the breakthrough patterns are in good agreement with the observations concerning the injections. At pH* 8.8 and 8.3 (Fig. 4a and b), the time at which the pH* shifts to a lower value is almost the same as the retention time observed for the second peak after the injection of a small amount of phosphoric acid in these eluents without TBA. It was also found at pH* 7.3 and 6.8 (Fig. 4d and e), that the shift in pH* back to the pH* value of the eluent occurs at the retention time found for the acidic zone after injection of TBABr in TBA-containing eluents (results not shown).

If during the equilibration with TBA-containing eluents TBA ions and protons are exchanged against potassium ions, the potassium concentration in the first part of the eluate (*i.e.*, directly after the hold-up time) is expected to be increased. Fractions of that part of the eluate were collected and the potassium concentration was measured. The increases in the potassium concentration in that part of the eluate compared with that of the eluent itself are reported in Table I. The data indicate that

TABLE I

INCREASE IN POTASSIUM CONCENTRATION IN THE ELUATE, COLLECTED DIRECTLY AFTER THE HOLD-UP TIME, OF TBA-CONTAINING ELUENTS OF DIFFERENT pH*

pH of eluent*	*Increase in potassium concentration* ($mmol \cdot l^{-1}$)
5.8	2.2
6.3	2.2
6.8	2.8
7.3	3.0
7.8	3.0
8.3	3.4
8.8	3.8

the potassium concentration of the eluate collected directly after the hold-up time is significantly increased. If ion exchange were the only binding mechanism of TBA ions, an increase of approximately $5 \cdot 10^{-3}$ *M* should have been found, as this is the TBA concentration in all of the TBA-containing eluents. The values in Table I are significantly lower than this value of $5 \cdot 10^{-3}$ *M*, which indicates that at least one other binding mechanism also plays a role in the binding of TBA ions on the column packing material. These other binding mechanisms are based on the binding of TBA ions together with anions. These anions have to be phosphate ions as these are the only anions available. The binding of TBA ions and phosphate ions occurs either as ion pairs or in a double layer in which the TBA ions are bound by hydrophobic bonding to the octadecyl surface, their charge being compensated by phosphate ions. Recent investigations indicate that the double-layer model is probably correct[10,17,18].

The existence of at least two binding mechanisms was confirmed by stripping experiments. It was found that part of the TBA ions bound by the column could be stripped from the column with a methanol–water mixture. The remainder of the TBA ions could only be stripped with a buffer. Stripping with methanol–water will disturb the double layer by removing the phosphate ions. The TBA ions bound by ion exchange can be eluted only when cations are available in the stripping eluent. These TBA ions can therefore not be eluted efficiently with a methanol–water mixture. Table II gives the amounts of TBA ions bound to the column (as calculated from the breakthrough patterns) and the amounts of TBA ions stripped with methanol–water (1:1, w/w) and the phosphate buffer eluent of pH* 5.8.

TABLE II

AMOUNTS OF TBA BOUND TO THE COLUMN AFTER EQUILIBRATION WITH TBA-CONTAINING ELUENTS OF DIFFERENT pH* CALCULATED FROM THE BREAKTHROUGH VOLUMES AND THE AMOUNTS OF TBA WHICH CAN BE STRIPPED FROM THE COLUMN BY THE SUBSEQUENT ELUTION WITH METHANOL–WATER (1:1, w/w) AND WITH PHOSPHATE BUFFER ELUENT OF pH* 5.8 AND THE CAPACITY FACTOR (*k'*) FOR PREDNISOLONE 21-*m*-SULPHOBENZOATE SODIUM IN THE TBA-CONTAINING ELUENTS

*pH**	*TBA bound (μmol)*	*TBA stripped with methanol–water (μmol)*	*TBA stripped with eluent of pH* 5.8 (μmol)*	*k'*
5.8	8.5*	4.8	3.9	3.8
6.3	9.0*	5.8	5.5	4.2
6.8	10.5*	3.2	4.9	3.9
7.3	13.5*	2.7	15.1	3.4
7.8	21.0	1.0	14.8	3.3
8.3	24.0	0.6	22.5	3.0
8.8	28.5	0.9	24.9	2.6

* These figures apply to a condition in which the final equilibrium has not yet been fully established.

The data in Table I indicate that with increasing pH* the binding of TBA ions by ion exchange increases compared with the binding by other mechanism(s). The data in Table II indicate that the absolute amount of TBA ions bound by ion exchange increases with increasing pH*. This is in accordance with the expectation that

the silanol groups will show an increasing tendency to dissociate with increasing pH*. The data in Table II also indicate a decrease in the amount of TBA ions bound by a mechanism other than ion exchange with increasing pH*. This apparent decrease is stronger than might be expected from Table I. No fully satisfactory explanation can be offered for this discrepancy at this stage. However, the data in Tables I and II clearly indicate the existence of at least two binding mechanisms for the TBA ions. They also strongly suggest that ion exchange accounts for the major part of the reagent bound to the column packing material.

Comparable breakthrough patterns were found for CTA-containing eluents with the Radial-Pak A columns. The radial compression can compensate for the reduction in the volume of the packing on dissolution of the silica matrix during chromatography. These columns therefore remain free of voids and channels. This makes it possible to use eluents of higher pH than is permitted with the usual narrow-bore stainless-steel columns, without significant losses in column efficiency. In this study eluents with pH* values up to 11 were used.

The breakthrough patterns of CTA were studied at pH* 7, 9 and 11. At pH* 7 and 9, comparable results were obtained to those with TBA-containing eluents below pH* 7.8 (discussed above). After pumping through 150–200 ml of eluent, containing 0.2% (w/w) of cetrimide, the breakthrough volume was reached, as indicated by the response of the refractive index detector. This was confirmed by titration of the eluate fractions with DOSS.

At this moment the pH* shifted to a lower value. Because of the optimal buffer capacity of the boric acid buffer near pH* 9, the shift at this pH* could only be demonstrated with a non-buffered eluent. These large differences in volume before the pH* shifts back, observed in repeated experiments, are probably caused by the difficulty of removing all cetrimide from the stationary phase before a new experiment was started. It is assumed that some of the silanol groups can bind the CTA ions very tightly. If during the stripping process a smaller amount of CTA ions has been removed from the stationary phase, fewer protons will have the opportunity to be exchanged against CTA ions in the next experiment, and consequently the volume needed for the shift back in pH* will be smaller.

A difference with the TBA experiments is the fact that many more CTA ions are held up by the column. This difference cannot be explained by the use of different types of columns[19]. The amount of cetrimide bonded to the column material was calculated to be 824 μmol at pH* 7, 934 μmol at pH* 9 and 1044 μmol at pH* 11. This phenomenon of increased breakthrough volumes at increased pH* values was also observed in the TBA experiments (Fig. 4). This might be explained again by assuming an increased dissociation of the silanol groups at higher pH* values so more binding places are available for quaternary nitrogen compounds. At pH* 11 the breakthrough pattern of CTA was comparable to those of the TBA experiments above pH* 7.8. After the hold-up time the pH* gradually shifted to a lower pH* value. At the moment of the breakthrough of the CTA ions the pH* shifted back to the pH* of the eluent.

The results indicate that CTA ions, as TBA ions, are partly bound by an ion-exchange mechanism. Analogous to the TBA experiments, the potassium concentration was determined in the eluate of the CTA-containing eluents collected directly after the hold-up time. A small but not significant increase in the potassium concen-

tration was measured. This suggests that the contribution to the binding of CTA ions by ion exchange is comparatively small. A major part of the CTA ions will be bound by another, presumably hydrophobic, interaction with the stationary phase.

The retentions of a number of solutes were determined using both the TBA- and CTA-containing eluents.

In Tables II and III, two examples from the solutes studied are given (both solutes may be regarded as completely ionized over the pH range studied). It appears that the increase in the total amount of reagent that is bound to the column is accompanied by a decrease in the retention of anionic solutes. The same tendency was found for all of the anionic solutes studied. This clearly indicates that a correlation between the total amount of the reagent bound to the column and the retention of the anionic solutes does not make sense.

TABLE III

CAPACITY FACTOR (k') OF BENZOIC ACID IN CTA-CONTAINING ELUENTS OF DIFFERENT pH*

*pH**	*Total amount of CTA bound (μmol)*	k'
6.8	824	8.4
9.0	934	4.4
11.0	1044	3.1

When, however, the retention of prednisolone 21-*m*-sulphobenzoate sodium is compared with the amount of reagent bound to the column by a mechanism other than ion exchange, a positive correlation is found. When making this comparison, it should be kept in mind that there is some uncertainty about the amounts bound at higher pH values, and that this compound is partially retained by at least one other mechanism, as it also exhibits some retention when phosphate buffer eluents without TBA ions are used.

The data show that dissociation of silanol groups plays a role over a wide pH range. The explanation for this phenomenon probably lies in the fact that the pK_a values of silanol groups change progressively with the degree of neutralization of the packing material[20].

CONCLUSION

The existence of at least two binding mechanisms for TBA ions and CTA ions is of importance for the interpretation of the chromatographic behaviour of anionic solutes. The first binding mechanism is based on an ion-exchange process, in which TBA ions and CTA ions are exchanged against protons and potassium ions bound to residual silanol groups. The second binding mechanism is assumed to be based on the hydrophobic bonding of the TBA ions and the CTA ions to the stationary phase, their charges being compensated by a double layer of anions in the mobile phase. The exchange of anionic solutes against anions from the double layer could explain the

retention of anionic solutes. In this concept, the retention of anionic solutes must be correlated with the amount of TBA ions and CTA ions bound to the stationary phase by this second mechanism only, not with the total amount of adsorbed TBA and CTA. However, the amount bound by hydrophobic bonding could not be determined with sufficient accuracy with the techniques used in this study to make such correlations meaningful.

Finally, there is a practical implication of the results of our investigations. Our studies show that in a number of instances the moment of breakthrough of the ion-pairing reagent precedes the moment that equilibrium is reached. With TBA-containing eluents the column is comparatively quickly equilibrated; elution with 25 ml will usually be sufficient. When using CTA-containing eluents, the volumes needed for equilibration of the column are much larger; more than 1 l of eluent may then be needed.

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EXCLUSION CHROMATOGRAPHY OF ANIONIC DYES

ANOMALOUS ELUTION PEAKS DUE TO REVERSIBLE AGGREGATION

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SUMMARY

Several anionic dyes, especially the barbiturate and pyrazolone oxonol dyes, were shown by exclusion chromatography to form large, monodisperse, polymeric aggregates in dimethylformamide. Their size was approximately that of polystyrene of molecular weight 10^6 daltons. They were in labile equilibrium with monomer and dissociated in salt solution. The anomalous elution profile with a threshold characteristic of the dye and a peak increasingly retarded with increasing dye quantity was explained in terms of continuing concentration-dependent monomer–aggregate equilibrium during chromatography. This aggregation was contrasted with the association in water which accompanies a change in optical absorption spectrum and gives smaller particles. A vesicular structure of membrane made of associated dye molecules, with an electrical double layer was suggested on the basis of the monodisperse large size of the aggregates, their instability in salt solution and the ionic nature of the dyes. Exclusion chromatography allows the study of aggregation phenomena which cannot be followed by measurements of colligative properties or absorption spectra.

INTRODUCTION

Oxonol dyes[1] can serve in biological studies as indicators of membrane potential[2] and in photographic chemistry as antihalation or filter dyes and as spectral sensitisers[3]. A consideration of the state of aggregation of these dyes is relevant to these applications. While the aggregation of cationic dyes, particularly the cyanines, has been studied extensively[4], the aggregation of oxonol dyes is not so well known.

The difficulty of separating dyes by chromatography[5] is increased by their tendency to form pure aggregates as well as mixed complexes and aggregates. The source of anomalous smears and elution volumes in the exclusion (gel permeation) chromatography of oxonol dyes was the reversible formation of large aggregates. Studies of the aggregation of these anionic dyes or the use of exclusion chromatography to study dye aggregation have not previously been reported.

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EXPERIMENTAL

Chromatography of the dyes with dimethylformamide (DMF) solutions using Styragel (polystyrene) stationary phase was performed with a Waters high-pressure liquid chromatograph using three 4-ft. columns with given nominal exclusion limits of $1 \cdot 10^4$, $3 \cdot 10^4$ and $1 \cdot 10^5$ Å chain length polystyrene and a refractive index detector. The system was calibrated with polystyrene of known molecular weight (Fig. 1). The sample was injected as a 2-ml loop of varied concentration.

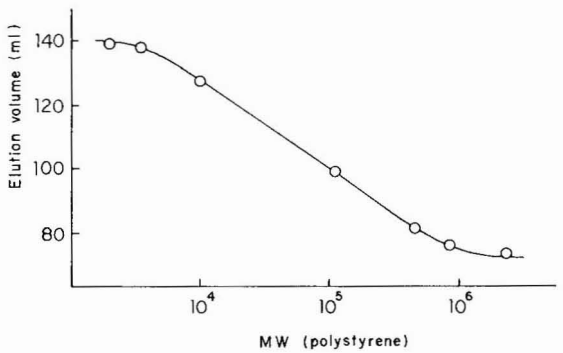


Fig. 1. Calibration of the Styragel columns with standard polystyrene of known molecular weight (MW).

Chromatography of the dyes with aqueous solutions using Sephadex and with dimethyl sulphoxide solutions using Sephadex was performed with vertical columns calibrated with Gaspar yellow 1350 (polymer) and copper sulphate (monomer). Where necessary the aqueous solutions were prepared by dissolving the solid in a little methanol or DMF and diluting with water. All measurements were made at room temperature.

Molecular weights according to the vapour pressure method were determined in the concentration range 0.1–1.0% using a Perkin-Elmer instrument.

RESULTS

The oxonol dyes in DMF, when added at a low concentration ($< 10^{-4}$%), chromatograph as large monodisperse aggregates with a size equivalent to that of polystyrene with molecular weight *ca.* 10^6 daltons. As the injected concentration is increased, the elution peak is retarded to a larger elution volume but the threshold of the leading edge is independent of the amount injected and is characteristic of the dye. Above 10^{-3}% initial concentration, the different amounts share a common leading edge while the trailing edge is further retarded with increasing amount injected. This behaviour is illustrated in Fig. 2 with a typical example, a barbiturate oxonol dye (No. 1, Table I). In Table I are listed the threshold values, characteristic of the dyes, for a number of anionic dyes. The molecular weight of the equivalent size polystyrene can be read from Fig. 1.

The aggregate formation was completed immediately on formation of the solution and there was no slow change on standing as evidenced by the chromatograms

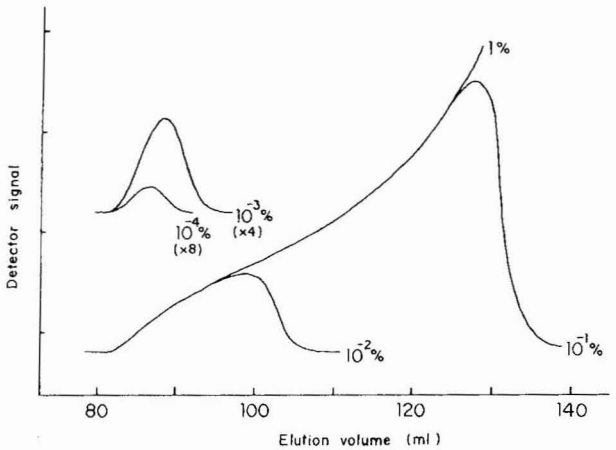
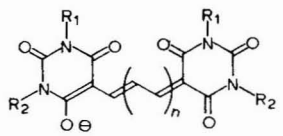


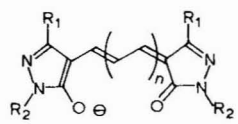
Fig. 2. The elution peak as a function of amount injected for a typical oxonol dye. The solvent is DMF with Styragel stationary phase. The percentage concentration of the dye injected is indicated. In parenthesis is the attenuation of the trace. The dye is No. 1 (Table I), structure I (n = 2, R_1 = R_2 = $-CH_3$), injected at concentrations of 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1} and 1 %, in 2-ml volume. For clarity the baseline is displaced for the two smallest amounts. The anomalous peak shape is attributed to a concentration-dependent reversible aggregation (see Fig. 5).

I



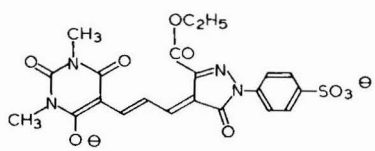
$(C_2H_5)_3\overset{\oplus}{N}H$

II



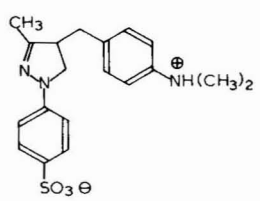
$M^{\oplus}$

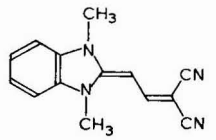
III

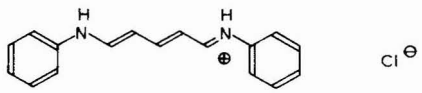


$2\,(C_2H_5)_3\overset{\oplus}{N}H$

IV



V 

VI 

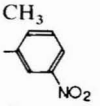
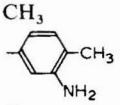
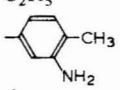
(dye No. 6). The addition of salts to the solution inhibited aggregate formation. In the presence of 1% lithium bromide, the dye No. 1 eluted completely as monomer at $1.3 \cdot 10^{-1}$% and $1.3 \cdot 10^{-3}$% injected concentrations, with an elution volume of 140 ml. The presence of 10% dimethylbarbituric acid (VII), which is not itself aggregated, in the injected sample of dye No. 1, a blue pentamethine barbiturate oxonol at 10^{-2}%, increased the elution volume from 82.5 to 90 ml. The elution volume of dye No. 6 was increased from 80 to 115 ml by 6% VII and to 135 ml by 18.5% VII when VII was added with the dye. Since VII was not added to the pumped solvent in these experiments, the disaggregation is not quantitatively determined, but is significant. The oxonol anions are very weak bases. Spectroscopy showed that dye No. 1 was not protonated by 1% oxalic acid in DMF.

While the barbiturate oxonols (I) showed essentially similar behaviour, the pyrazolone oxonols (II) appeared to form larger aggregates. For comparison, while IV showed aggregation, V and VI, which was deprotonated to the neutral molecule in the DMF, chromatographed as monomers. Gaspar yellow 1350, a covalent polymer, Gaspar cyan 802B and Pontamine blue gave elution volumes of 70 ml, 71–5 ml and 72–5 ml respectively. Covalent polymeric dyes with structure I ($n = 2$, $R_1 = H$, $R_2 = (CH_2)_n$) gave elution volumes of *ca.* 70 ml, and similar results were obtained with dye No. 3 copolymerised with succinyl chloride or hexamethylene diisocyanate.

In water the tendency to aggregate was less apparent, the molecular sizes being less than those of globular proteins of molecular weight 5000 daltons, with some exceptions such as dye No. 7 which gave large aggregates. In DMF, the absorption spectrum did not appear to change on aggregation. The specific optical absorption of dye No. 1 at 595 nm in DMF decreased in intensity only marginally between $4 \cdot 10^{-5}$% and $4 \cdot 10^{-2}$% concentration and no new peak appeared. In water on the other hand there was a dramatic and complex change in optical absorption with increasing concentration (Fig. 3). Above 10^{-4}% concentration a new peak appeared at lower wavelength, 520 nm, to reach a maximum absorption by 10^{-2}%. The 585 nm absorption decreased in intensity above 10^{-3}% concentration. These deviations from Beer's law are plotted for dye No. 1 in Fig. 3. The absorption spectra for dyes No. 1 and No. 4 at an intermediate concentration where both peaks are visible are shown in Fig. 4. The spectral change in water is not always as clearcut as with the barbiturate oxonol dyes. Dye No. 7 at $4 \cdot 10^{-2}$% and $4 \cdot 10^{-4}$% concentrations had the same absorption peak at 615 nm, although the higher concentration had a larger shoulder

TABLE I

ELUTION VOLUMES OF SOME ANIONIC DYES

Structure		*DMF (Styragel) (ml)**	*Water (Sephadex) (ml)***
No. 1	I, $n = 2$ $R_1 = R_2 = CH_3$	82.5	79
No. 2	I, $n = 2$ $R_1 = CH_3$ $R_2 =$ 	87.0	–
No. 3	I, $n = 2$ $R_1 = CH_3$ $R_2 =$ 	80.0	70
No. 4	I, $n = 2$ $R_1 = R_2 = C_2H_5$	86.0	73
No. 5	I, $n = 2$ $R_1 = C_2H_5$ $R_2 =$ 	85.0	90
No. 6	I, $n = 1$ $R_1 = R_2 = CH_3$	80.0	–
No. 7	II, $n = 2$ $R_1 = CH_3$ $R_2 = p\text{-}C_6H_4SO_3^-K^+$ $M^+ = K^+$	71.0	50
No. 8	II, $n = 2$ $R_1 = NH_2$ $R_2 = C_6H_5$ $M^+ = (C_2H_5)_3N^+H$	75.0	135 (G-75)
No. 9	II, $n = 1$ $R_1 = CH_3$ $R_2 = C_6H_5$ $M =$ 	80.0	90
No. 10	III	82.5	–
No. 11	IV	81.5	–

* Threshold value, see text. See Fig. 1 for calibration.

** Sephadex G-25 except where otherwise stated. With G-25 monomer ($CuSO_4$) eluted at 90 ml and high polymer (Gaspar yellow 1350) at 49 ml. With G-75 monomer eluted at 135 ml and high polymer at 33 ml.

on the low wavelength side. Aggregation of these dyes is also seen when the solvent is dimethyl sulphoxide.

The apparent molecular weight of ionic dyes calculated from colligative properties increases only up to a point on their aggregation. Assuming separation of the counter-ion from the aggregated dye, the maximum value for dye No. 1, an ion pair,

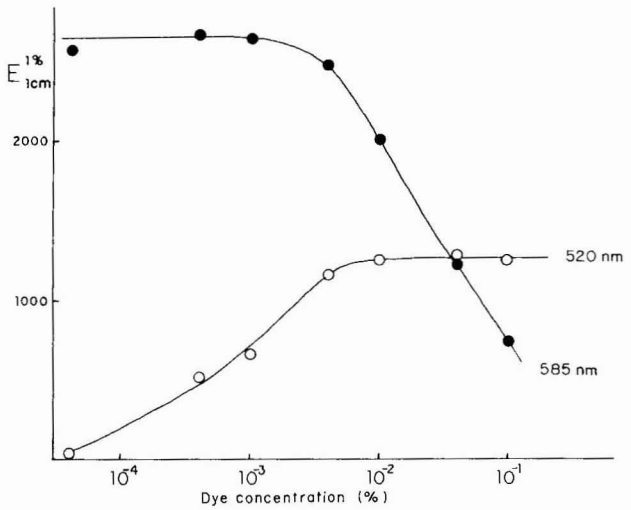


Fig. 3. The optical absorption of dye No. 1 (Table I) at the two absorption maxima, $\lambda_{max.}$ = 585 nm and $\lambda_{max.}$ = 520 nm in water as a function of concentration. These absorption changes are attributed to reversible aggregation of the dye.

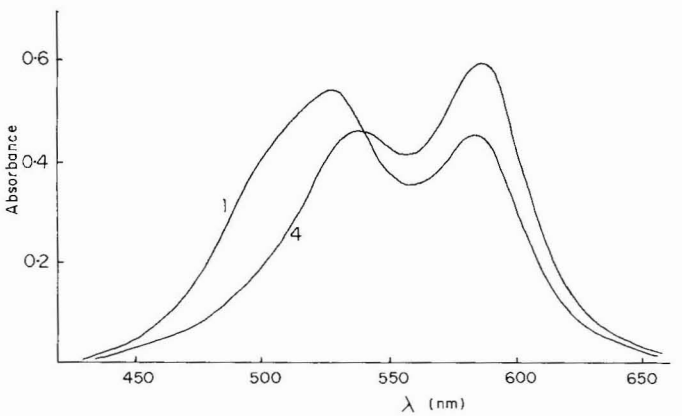


Fig. 4. The optical absorption of dyes No. 1 and No. 4 (Table I) in water at intermediate concentrations where both peaks are visible. The concentration is 0.04%, pH 6, 0.01-cm path length cuvette.

is 475 and for dye No. 7, an ion quadruplet, 228, although incorporation of counterions in the aggregate would give higher results. Molecular weights determined by the vapour pressure method for dye No. 7 were 180 in water and 273 in DMF. Dye No. 1 gave 327 in DMF.

DISCUSSION

That a number of dyes, particularly the pyrazolone and barbiturate oxonol dyes, form monodisperse polymeric aggregates in solution in DMF as well as dimethyl sulphoxide and to a lesser extent water has been shown by exclusion (gel perme-

ation) chromatography. The rapid formation of the aggregates, their instability in salt solutions even at fairly low concentrations of salt and the exclusion chromatography elution volumes and peak shapes support the formation of large, labile, monodisperse aggregates, unstable in salt solution and in equilibrium with monomer. This model explains the anomalous skewed elution peaks. Since the dyes are themselves salts, there is a dye concentration above which the aggregates, stable in DMF, are dissociated to monomer. A low injected concentration (*ca.* 10^{-4}%) is completely aggregated and chromatographs as a monodisperse polymer. Above 10^{-3}% concentration there is appreciable dissociation to monomer, particularly at the center of the peak where the concentration is highest. Aggregate concentration is optimal at the front and rear of the peak and since the aggregate travels faster than the monomer its transport counteracts backward diffusion at the rear of the peak and extends the front of the peak towards a lower elution volume. This behaviour is illustrated schematically in Fig. 5. This model skews the peak in the opposite sense to a reversible aggregation independent of ionic strength.

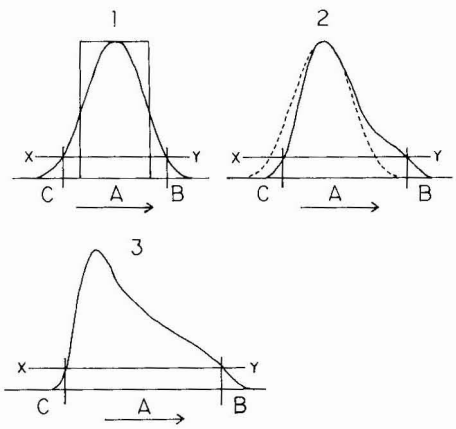


Fig. 5. The proposed origin of the anomalous exclusion chromatography elution peaks for ionic dyes which aggregate at low salt concentration only. The ordinate represents the dye concentration and the abscissa the distance travelled through the column. Direction of motion is indicated by the arrows. The lines XY represent the concentration above which dissociation of the aggregates occurs. In 1 the plug has begun to spread by diffusion in the solvent. Regions B and C of low concentration are aggregate while A is largely monomer. In 2, after some transport, the aggregate travels faster than monomer, B draws ahead of A but C catches up A and disaggregates. C is replenished by back diffusion and B is increased by diffusion. Continuation of this process gives rise to the shape in 3, and the experimental curves in Fig. 2. The final shape depends on the amount injected and the distance travelled.

Unlike the association of these dyes in water, which gives smaller particles and is accompanied by changes in the optical absorption peaks, this high aggregation in DMF occurs without significant change in absorption spectrum. It cannot be followed by colligative properties.

The dyes which aggregated were ionic. The largest aggregates were formed by the triionic species VII, and the neutral species V and VI were not aggregated in these conditions. The results suggest that the associated ions form aggregates and that dissociation of ion pairs with increasing salt (dye) concentration is accompanied by

disaggregation. The monodisperse and large size of the aggregates, equivalent to that of polystyrene of molecular weight 10^6 daltons, suggests the formation of vesicles (microsacs) by the packing of parallel dye chains perpendicular to the surface in a membrane the surface of which is an electrical double layer. This structure would be analogous to that well documented for lipids in water[6], although in this case there is a dye monolayer rather than a lipid bilayer. This aggregate structure may be different from those formed in other solvents at different concentrations.

Effects of the association of oxonol dyes have been prevented by the addition of zwitterionic detergents[7], and this improved their performance when used as photographic filter dyes. In chromatography the smear due to aggregation of these dyes is completely removed by the presence of 1 % lithium bromide in which the dyes elute as monomers rather than polymeric aggregates. Thus other species, such as the dye as part of or attached to a covalent polymer, may be clearly resolved.

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MICROSCALE PREPARATION OF PENTAFLUOROBENZYL ESTERS

ELECTRON-CAPTURE GAS CHROMATOGRAPHIC DETECTION OF INDOLE-3-ACETIC ACID FROM PLANTS

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SUMMARY

A microscale method is described for the preparation of the pentafluorobenzyl ester of an organic acid, indole-3-acetic acid, using α-bromopentafluorotoluene as the esterifying agent and the volatile base, N-ethyl-piperidine. The resultant reaction mixture may be used directly for gas chromatography employing an electron-capture detector, or greater sensitivity and selectivity can be attained by negative ion chemical-ionization gas chromatography–mass spectrometry. The method is applicable to assay of indole-3-acetic acid in resinous plant material such as olive leaves.

INTRODUCTION

Pentafluorobenzyl (PFB) esters of organic acids are convenient for gas chromatographic (GC) analysis using an electron-capture detector (ECD)[1–3]. Previously described methods for preparation of PFB esters utilized large reaction volumes, an inorganic water-soluble base such as K_2CO_3, and partitioning the derivative into a water-immiscible solvent or further purification. Such procedures precluded microscale derivatization and also resulted in poor recoveries of a labile aromatic compound such as indole-3-acetic acid (IAA). We describe a microscale procedure for the preparation of PFB esters and illustrate its use in the analysis of IAA from plant material. Purification subsequent to derivatization is not usually required, but for those cases requiring purification a C_{18} reversed-phase high-performance liquid chromatographic (HPLC) procedure is described.

The N-heptafluorobutyryl and N-trifluoroacetyl derivatives of IAA have been utilized[4,5] but these required a second step for derivatization of the carboxyl group and reaction yields were low owing to sensitivity to trace amounts of water[6]. Trichloroethyl esters of IAA have also been used, but adaptation to assay of IAA has

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not been described[7]. The derivatization procedure we report results in a compound sufficiently volatile for GC analysis and measurable by an ECD. In addition, the method provides quantitative yields of the PFB ester without anhydrous conditions and using reagents stable to normal storage conditions.

MATERIALS AND METHODS

Reaction conditions

Optimal derivatization conditions were established by monitoring the reaction using silica gel 60 (E. Merck, Darmstadt, G.F.R.) thin-layer chromatography (TLC) (chloroform–methanol–water, 85:14:1) and visualization of indoles with Ehmann's reagent[8]. Derivatization was carried out in 300-μl actinic glass "Microflex" (Kontes, Vineland, NJ, U.S.A.) tubes in a block heater at 60°C.

Plant material

Young olive leaves (*Olea europaea* L. cv. Manzanillo) (15 g) were ground within 15 min of collection in acetone–water (70:30) containing the isotope-labeled IAA. The resultant homogenate was filtered, the residue reextracted by grinding in an additional volume of acetone–water (70:30), and the combined filtrates freed of acetone *in vacuo*. The sample was made to pH 2.5 with sulfuric acid and extracted three times with chloroform. The combined organic phases were extracted with 1 *N* $NaHCO_3$, brought again to pH 2.5 and reextracted three times with chloroform. The organic phases were pooled, dried over anhydrous granular Na_2SO_4, filtered, reduced to near dryness, and dissolved in methanol for chromatography on a Varian LC-5020 high-performance liquid chromatograph equipped with a guard column of 20–40-μm RP-8 and an analytical Varian MicroPak MCH-10 column. Eluent was monitored at 254 nm and 1-ml samples collected. IAA was eluted isocratically using methanol–water–acetic acid (25:75:5) as solvent with a flow-rate of 2 ml/min. Recently we have found with acidic plant extracts that good separation can be obtained using methanol–water–tetrahydrofuran (25:75:5), thus eliminating problems associated with the removal of acetic acid. Samples containing IAA were pooled, reduced in volume *in vacuo*, transfered to a "Microflex" tube and reduced to dryness under a stream of nitrogen. The sample was dissolved in 50 μl of redistilled acetone and 1 μl of N-ethylpiperidine (Pfaltz & Bauer, Flushing, NY, U.S.A.) added followed by 5 μl of α-bromopentafluorotoluene (Aldrich, Milwaukee, WI, U.S.A.). After reaction for 45 min at 60°C, the sample was either diluted with acetone for analysis by GC–ECD or chromatographed by C_{18} reversed-phase HPLC with isocratic elution with methanol–water (70:30).

The above procedure served for assay of "free" IAA. When it was desired to assay free plus ester and amide-linked IAA[9] it was necessary to hydrolyze the extract with 7 *N* NaOH for 3 h at 100°C prior to extraction and purification as previously described[9]. In experiments utilizing GC–ECD, 4.6 nmol (0.26 μCi) of [2-^{14}C]IAA (Radiochemical Centre, Amersham, Great Britain) was added to 15 g fresh weight of plant material as internal standard. For samples to be assayed by GC–selected ion monitoring–mass spectrometry (GC–SIM–MS) 7.2 μg of [4,5,6,7-$^2H_4$]IAA[10] was added as internal standard in addition to the [2-^{14}C]IAA.

Preparation of standards

PFB ester of IAA, for use as a standard, was synthesized using 2.9 mmol (0.5 g) of IAA and 4.8 mmol each of N-ethyl-piperidine and α-bromopentafluorotoluene. The product was dissolved in diethyl ether and partitioned against 0.1 *M* $NaHCO_3$, column chromatographed on silica gel 60 (solvent as for TLC), recrystallized from ethanol–water (70:30), and dried *in vacuo* over P_2O_5 for three days. The resultant light yellow needles (m.p. 128–130°C), absorbed at 219 nm (log ε = 4.51) and at 280 nm (log ε = 3.75). Color production with Ehmann's reagent was identical to that of an equimolar amount of IAA[8,11]. Further characterization of the standard was by GC–MS.

Gas–liquid chromatography

For electron-capture detection a Packard Model 419 equipped with a ^{63}Ni ECD was used. Operating conditions were injector port and detector at 270°C, column oven 250°C and nitrogen as carrier gas at 30 ml/min through a 1.3 m × 4 mm glass column packed with 1% OV-17 on Gas-Chrom Q (Supelco, Bellefonte, PA, U.S.A.). The detector was operated in pulse mode with 10-μsec period and 10-μsec width. For MS work a 10 ft. × 2 mm glass column packed with 3% SP2250 on 80–100 Supelcoport (Supelco) was used coupled to a Hewlett-Packard 5985a GC–MS instrument. Temperature was programmed from 200–260°C at 10°C/min after a 3-min isothermal hold and He carrier gas was at 30 ml/min. Some studies also utilized a flame-ionization detector with a Hewlett-Packard 402 and a 4 ft. × 2 mm 3% OV-17 on Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.) glass column held isothermally at 225°C with nitrogen as carrier gas at 30 ml/min.

RESULTS AND DISCUSSION

Mineral salts are incompatible with HPLC and GC, thus requiring the substitution of a volatile organic base for the K_2CO_3 employed by earlier workers[1–3]. Table I compares the efficacy of K_2CO_3 and a variety of organic bases. Highest yields were obtained using N-ethyl-piperidine, and as little as 0.5 μg/ml was sufficient to permit quantitative derivatization as judged by TLC (Table II). The use of 2.5 μl (a large excess) of α-bromopentafluorotoluene was also necessary for quantitative derivatization (data not shown). Routinely, 5 μl of α-bromopentafluorotoluene and 1 μl of N-

TABLE I

COMPARISON OF VARIOUS ORGANIC BASES WITH K_2CO_3

Reaction contained 50 μg IAA, 50 μl α-bromopentafluorotoluene and the indicated base.

Base	*Yield (%)*
0.5 *M* K_2CO_3 (50 μl in water)	0
0.25 K_2CO_3 (100 μl in acetone–water, 50:50)	90
Pyridine (50 μl)	2
Piperidine (50 μl)	1
N-Ethyl-piperidine (50 μl)	100
2-Ethyl-piperidine (50 μl)	5

TABLE II

AMOUNT OF N-ETHYL-PIPERIDINE REQUIRED IN DERIVATIZATION REACTION

All reactions contained 5 μl α-bromopentafluorotoluene, 50 μg IAA and 50 μl acetone.

Volume of N-ethyl-piperidine	*Yield (%)*
1.0 nl	10
5.0 nl	10
10.0 nl	15
50.0 nl	60
0.1 μl	70
0.5 μl	100
1.0 μl	100
5.0 μl	100
10.0 μl	100
50.0 μl	100

ethyl-piperidine were added to 50 μl of acetone containing up to 0.3 μmole of organic acid giving a molar ratio of reagent:base:reactant of 110:24:1. For larger amounts of organic acids the amount of α-bromopentafluorotoluene and N-ethyl-piperidine can be doubled while retaining the same volume of solvent.

Derivatization of 50 μg of IAA in 50 μl of acetone with 5 μl of α-bromopentafluorotoluene and 1 μl of N-ethyl-piperidine as a function of time is shown in Fig. 1. In the first 30 sec, without heating, the reaction goes to 60% of completion and, with heating at 60°C, a near quantitative yield is obtained in 45 min. TLC of the reaction mixture after 45 min showed no unreacted IAA and this procedure would detect as little as 25 ng of unreacted IAA[8] in the 10 μl spotted. Thus, the reaction proceeds to, at least, 99.75% completion. Quantitative yields were further established by reversed isotope dilution analysis[12]. A 10-μl aliquot of a [^{14}C]IAA-containing reaction mixture

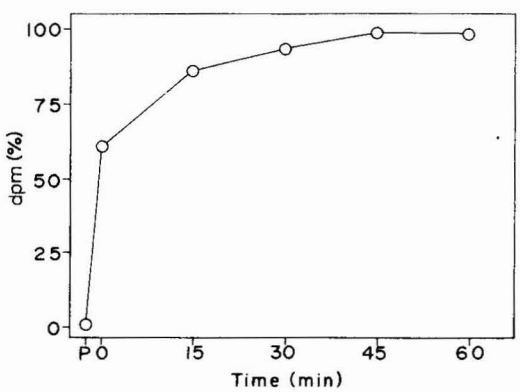


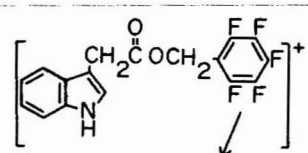
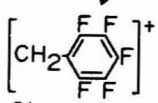
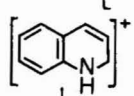
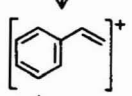
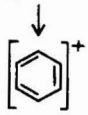
Fig. 1. Time course of derivatization. A 50-μg amount of IAA plus 100 nCi [^{14}C]IAA was derivatized at 60°C. At the indicated time 5 μl was removed and added to a vial containing 1 ml diethyl ether and 1 ml 100 m*M* $KHCO_3$. Data are percentage of dpm found in the upper phase. Data point indicated by "P" was sampled prior to the addition of reagent and the zero time point was taken within a few seconds after derivatization reagent was added.

was added to 1 mg of IAA-PFB and purified by TLC and C_{18} reversed-phase HPLC. The specific activity of the purified product as determined by UV extinction and liquid scintillation counting indicated that 97% of the label co-chromatographed with the authentic standards. Thus, only 3% of the IAA is not recovered as the derivative and this includes unrecovered counts due to: (1) radiolabeled impurities in the [^{14}C]IAA, (2) less than quantitative derivatization and (3) degradation during derivatization.

The identity of the reaction product was established by combined GC–MS on a Hewlett-Packard 5985a instrument using electron-impact ionization at 70 eV and positive-ion detection. The mass fragmentation pattern is given in Table III and shows fragments expected of both a pentafluorobenzyl ester and of a 3-substituted indolealkanoic acid.

TABLE III

POSITIVE ION 70 eV ELECTRON-IMPACT FRAGMENTATION PATTERN FOR INDOLE-3-ACETIC ACID PENTAFLUOROBENZYL ESTER OBTAINED WITH A HEWLETT-PACKARD 5985a GAS CHROMATOGRAPH–MASS SPECTROMETER

m/z	*Abundance (%)*	*Fragment*
355 (M^+)	3.0	
181	8.7	
130	Base peak	
103	7.1	
77	7.9	

Analysis of IAA isolated from olive leaves by GC–ECD of the C_{18} HPLC purified derivatization mixture is shown in Fig. 2. The IAA peak is well separated from contaminant peaks. Direct injection of the diluted reaction mixture, without HPLC, yields a similar chromatogram except that the solvent peak is longer and additional small peaks appear before the IAA (Fig. 3). If specific activity is to be determined prior to the GC step, then the post-derivatization HPLC step provides assurance that underivatized IAA and most degradation products are not counted. However, in our work with olive leaves the values obtained with or without this step

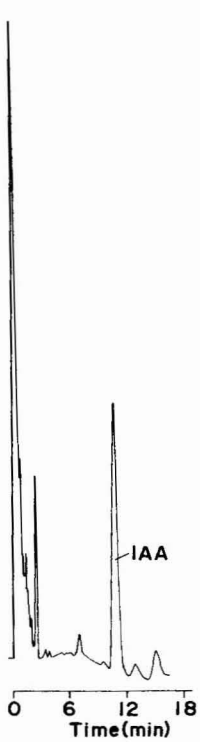


Fig. 2. GC–ECD of a sample from olive leaves purified as described in text and using the post derivatization C_{18}-HPLC step. Based on external standardization, peak represents 133 pg of IAA injected as its PFB ester.

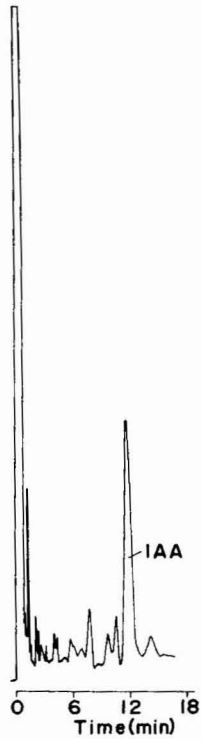


Fig. 3. GC–ECD of a sample from olive leaves purified as described in text. Sample was injected after dilution to 10 ml with acetone and without further purification. Peak represents 119 pg of IAA injected as its PFB ester.

were similar. Most accurate quantitation is provided by using the double standard method previously described[13].

GC–MS was used to validate the assay of IAA as the PFB ester in two ways: first, a GC–SIM–MS assay was compared with the above assay on two identical aliquots of the same lot of olive leaves. The first aliquot was analyzed by an ECD and the second aliquot was prepared for GC–SIM–MS by adding 7.2 μg of [$^2H_4$]IAA to the homogenate and using post-derivatization HPLC to reduce the mass of material entering the mass spectrometer. The MS assay was that described by Magnus *et al.*[10] except that the ions at m/z 130 and 355 were monitored for the naturally occurring IAA and ions at 134 and 359 were monitored for the ^2H-labeled internal standard. The ratios of ion intensities at 130:134 and 355:359 were used for quantitation. As is seen in Table IV the two methods agree well and the values obtained by GC–ECD for corn seed agree with previous reports. The differences observed are within those expected for biological variation.

A repetitive scan analysis of the samples used for GC–SIM–MS showed no extraneous fragment ions greater in quantity than 2% of base peak in both the alkali-hydrolyzed and non-hydrolyzed samples thus providing assurance as to the identity and composition of the GC peaks.

A further advantage of the halogenated derivative is that it permits assay by

TABLE IV

AMOUNT OF NATURALLY OCCURRING INDOLE-3-ACETIC ACID AS DETERMINED BY GC–ECD AND BY OTHER ISOTOPE DILUTION METHODS

Plant material	*Amount of IAA µg/g fresh weight*	*Analysis procedure*
Olive leaves (non-hydrolyzed)	1.2	GC–ECD
	1.9	GC–SIM–MS
Olive leaves (7 *N* NaOH treated)	2.6	GC–ECD
	3.2	GC–SIM–MS
Olive callus tissue (non-hydrolyzed)	0.2	GC–ECD
(7 *N* KOH treated)	0.9	GC–ECD
Sweet corn kernels (non-hydrolyzed)	0.8	GC–ECD
	0.5–1.0	Ref. 9
	1.3	Ref. 14

negative ion chemical-ionization MS (Fig. 4), thus increasing the sensitivity attainable by GC–SIM–MS. Negative-ion spectra using either ammonia or methane as reagent gases were obtained using a Finnigan 4000 instrument equipped with pulsed positive/negative-ion detection. These results are presented in Table V. Using GC–SIM–MS negative-ion chemical-ionization MS and ammonia as reagent gas permitted the detection of 5 pg of IAA as its PFB ester.

The procedure described for the formation of PFB esters is rapid, simple to use, results in high yields, and should be adaptable to study a variety of organic acids

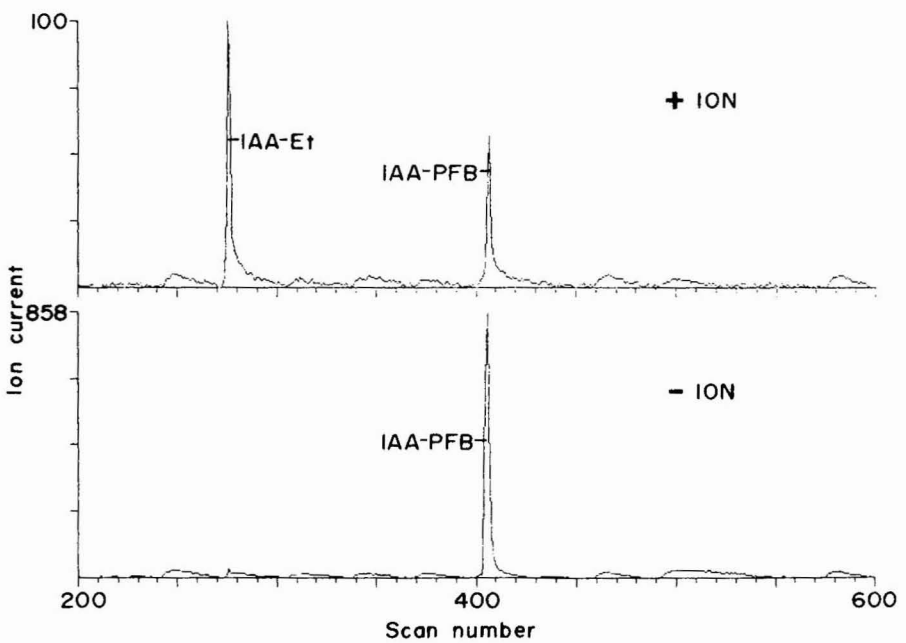


Fig. 4. Pulsed positive/negative-ion ammonia chemical-ionization total ion chromatogram obtained on a Finnigan 4000 instrument. An injection of 100 ng IAA ethyl ester (IAA-Et) and 100 ng IAA pentafluorobenzyl ester (IAA-PFB) in 1 µl chloroform was made onto a 20 m SE-54 J & W quartz capillary. Injector was at 250°C, oven at 130°C for 2 min then 15°C/min to 280°C. Injection was splitless for 0.8 min then split 40:1. Scans shown were taken from 4.7 min (scan 200) to 14 min (scan 600).

TABLE V

NEGATIVE-ION CHEMICAL-IONIZATION FRAGMENTATION FOR INDOLE-3-ACETIC ACID PENTAFLUOROBENZYL ESTER OBTAINED ON A FINNIGAN 4000 CAPILLARY GAS CHROMATOGRAPH–MASS SPECTROMETER AND USING THE INDICATED REAGENT GAS

Reagent gas	*Fragment ion (m/z)*	*Relative abundance (%)*
Methane	335	1.5
	174	100
Ammonia	335	1.5
	196	15
	174	100

(*cf.* refs. 1 and 15). The use of N-ethyl-piperidine allows great variation in the amount of base used and owing to its volatility under GC conditions, it does not interfere with analysis. The applicability of the method to a highly resinous and fatty tissue such as olive leaves, suggests its general suitability for study of organic acids and plant hormones which are present only in minute amounts.

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HUMIC ACID CHARACTERIZATION OF COLOMBIAN SOIL BY DISC ELECTROPHORESIS AND INFRARED SPECTROSCOPY FOLLOWING GEL FILTRATION

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SUMMARY

Humic acid extracted from Columbian soil was separated into fractions by means of gel filtration on Sephadex G-25 and G-75. A high proportion of acid has a molecular weight greater than 50,000 daltons.

Analysis of these fractions by disc electrophoresis on polyacrylamide gel and by infrared spectroscopy shows a highly aromatic character for humic and inorganic materials bound to the small fractions.

INTRODUCTION

The aim of this study was to characterize the humic acid of a Colombian soil by non-degradative methods. This particular soil was chosen as it is found in a large area in Colombia, which, even though having a high organic and nitrogen content, shows a low fertility.

The Colombian soil shows a slow mineralization of organic matter[1] and a developed humic system[2]. The high humidification grade is due principally to moisture and clay alofanic materials; however, little is known about the composition and structure of the humic system. The soil under examination contains a high percentage of organic matter and also a high percentage of humic acid[3]. It has been classified as Inceptisoil Cumulic Humitropept[4] and has been studied with respect to its productivity with the addition of nitrogenated compounds[4], its phosphate fixation[5] and its content and distribution of minor components[6]. Its humic acid has already been studied by the usual chemical methods[3].

For the characterization of humic acid we applied the non-degradative techniques described previously[7], owing to its particular properties. The techniques used

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were gel filtration followed by disc electrophoresis under the usual conditions or with urea.

Humic acid must not be treated as a statistically uniformly distributed system, but as a system divisible into homogeneous fractions with different chemical properties as shown by electrophoretic methods. Gel filtration was used to separate humic acid and obtain more homogeneous fractions than total humic acid. These fractions were then submitted to infrared spectroscopy and disc electrophoresis. The resolving power of disc electrophoresis on polyacrylamide gel allows the direct characterization of some properties[8]. Urea (6.0 *M*) was used in the electrophoresis to reduce the weak interactions among the humic acid subfractions. The characterization of the gel was performed with three different staining techniques, one general and two more specific.

EXPERIMENTAL

The soil was Serie Bermeo, collected in the Municipio de Facatativa, Departamento de Cundinamarca, Colombia, at 2640 m above sea-level and an average temperature of 13°C. It had the following general properties: structure, granular; consistency, firm; macro-organisms, rare; roots, copious; internal drainage, quick; external drainage, moderately quick; declivity, 3–7%; erosion, absent; class, Inceptisoil; subclass, Tropept; high group, Humitropept; and subgroup, Humitropept Cumulic[4].

The physico-chemical properties were as follows[3]: pH, 5.4; organic C, 11.2%; organic matter, 19.31%; N, 0.81%; C/N, 13.8; exchange power, 61.0 mequiv. per 100 g; total basicity, 3.47; base saturation, 5.68%; exchange bases (mequiv. per 100 g), Na^+ 0.69, K^+ 0.65, Ca^{2+} 1.22, Mg^{2+} 0.91; and phosphorus content, 30.19 kg P_2O_5/ha.

The humic substance had the following chemical properties: humic acid (HA), 4.5%; fulvic acid (FA), 1.75%; C of HA, 53.85%; C of FA, 31.95%; and C (HA)/C (FA), 1.69. The humic acid had the following chemical properties: moisture, 7.5%; ash, 8.7%; C, 56.64%; N, 2.55% and C/N, 22.21.

Extraction and separation

Dry soil was sieved with a 2-mm mesh. The extraction was achieved with 0.1 *M* sodium hydroxide and 0.1 *M* $Na_4P_2O_7$ solution (150 g of soil per 3 l) with stirring under nitrogen for 1 day[9,10]. After decantation the supernatant was acidified with concentrated sulphuric acid to pH 2–3. The precipitated humic acid was recovered by centrifugation at 4200 *g* for 15 min.

Purification

The humic acid was dissolved in 0.1 *M* sodium hydroxide solution and reprecipitated with concentrated sulphuric acid three times. Finally, humic acid was dialysed against distilled water until all sulphate had been removed (barium sulphate test) and then lyophilized.

Gel filtration

Humic acid (50 mg in 2 ml of 0.05 *M* Tris chloride buffer, pH 9.0) was loaded on to a Sephadex G-25 column (60 × 2 cm), with the same buffer as eluent, to avoid interactions between the gel and humic acid[11,12]. Fractions of 5 ml were collected and monitored at 578 nm (Beckman ACTA CIII). The fractions were collected in six pools, and each was dialysed against distilled water and lyophilized.

An aliquot of the first batch (25 mg) was re-suspended in the starting buffer (1 ml) and loaded on to Sephadex G-75. Three pools were collected. It should be noted that the two Sephadex gels, especially Sephadex G-25, strongly retain a humic acid fraction (*ca.* 5% of total) that cannot be washed from the gel.

Electrophoresis

The method used has been reported previously[7]; 16% acrylamide was used. The apparatus was a Minivolt vertical model. The concentration of urea, when used was 6.0 *M*.

For characterization of the bands we used a reported staining method[8], with Blue Alcian (BA) as a general stain for acid mucopolysaccharides, Fuchsin (F), decoloured (Zacharius method[13,14]), as a stain for polysaccharides and all other molecules with neighbouring hydroxyl groups and Prussian Blue (PB) as a stain for fractions with reducing properties. All the gels were uniformly distributed despite the slightly different gel length obtained after electrophoresis; this was allowed for by calculating electrophoretic mobilities (EM), defined as[15]

$$\mathrm{EM} = \frac{\text{band migration (after staining) (mm)}}{\text{electrophoretic front migration (at the end of the run) (mm)}} \cdot \frac{\text{gel length (at the end of the run) (mm)}}{\text{gel length (at the end of destaining) (mm)}} \cdot 100$$

Infrared spectroscopy

The IR spectra of total humic acid and of each pool collected were obtained using lyophilized samples; a sample of 0.50 mg per 70 mg of potassium bromide was obtained for dilution. The IR spectra were recorded in the range 4000–600 cm^{-1} with a Perkin-Elmer 247 spectrophotometer.

RESULTS AND DISCUSSION

Gel filtration on Sephadex G-25 of starting humic acid and electrophoretic examination of pools collected

The gel filtration separation is shown at the top of Fig. 1, and below is shown the electrophoretic pattern obtained under normal conditions or with 6.0 *M* urea with the three different stains: Blue Alcian, Basic Fuchsin and Prussian Blue. The initial humic acid and only three pools ($P1_{25}$, $P4_{25}$ and $P6_{25}$) are shown because the differences between one pool and its immediate neighbours are not marked, whereas comparisons are more significant when distant pools are considered. The differences between the gels are only qualitative because quantification of an electrophoretic band (with a scanning photometer) is very difficult: first, the humic acid retains its

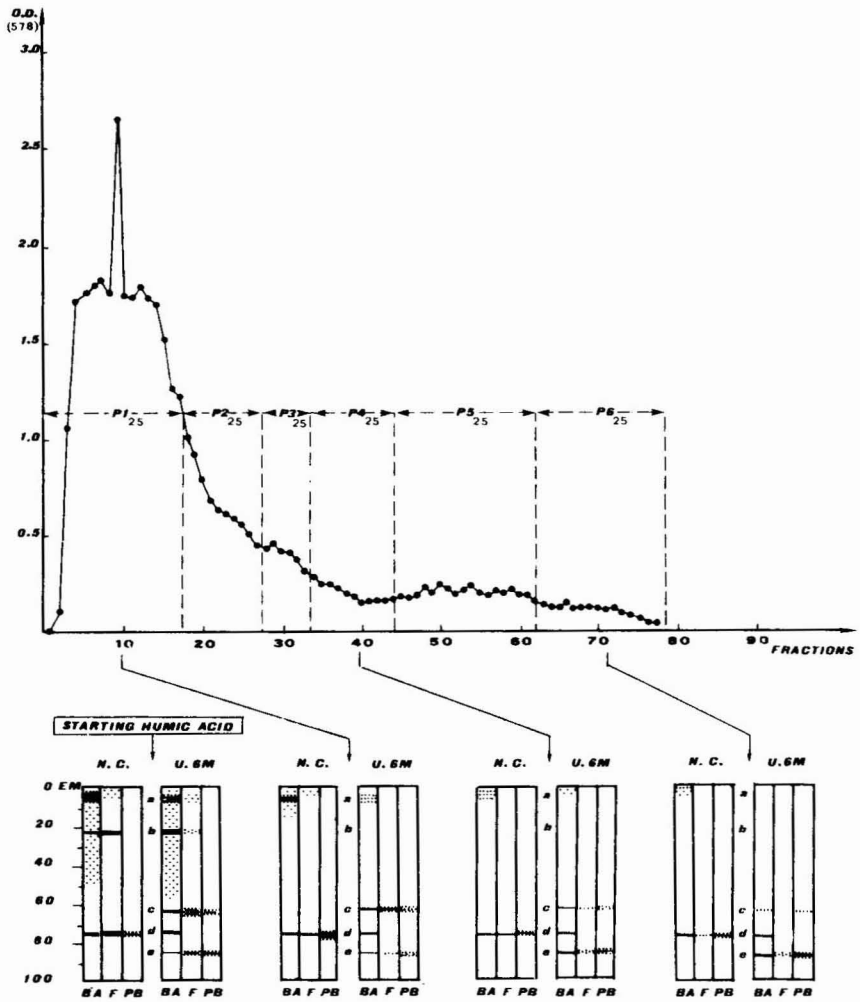


Fig. 1. Gel filtration (Sephadex G-25) of initial humic acid (top) and electrophoretic patterns of the initial acid and of some pools collected from gel filtration (bottom) (see Results and discussion). N.C. = Normal conditions; U. 6M = 6.0 *M* urea; BA = Blue Alcian stained gel; F = Fuchsin stained gel; PB = Prussian Blue stained gel.

own colour, which interferes with the specific stain, even with the use of an appropriate filter (in the Fuchsin staining method there is a slight humic acid decoloration, probably due to the strong oxidation with periodate); secondly, different electrophoretic fractions have different stain binding powers. Gel filtration on Sephadex G-25 shows a very low resolving power and the largest amount of humic acid is eluted without retention on the gel (immediately after one void volume); probably this fraction has a molecular weight of more than 5000 daltons.

Also, if the molecular weight discrimination of Sephadex G-25 is in the range 5000–500 daltons, it is impossible to obtain information on the molecular weight of the pool collected, because appropriate standards do not exist and the last fractions

are eluted after the passage of three void volumes of eluent. This is caused by an abnormal retention by the gel of humic acid, also under the choosen conditions[11,12] (pH of buffer and ionic strength). A proportion of the sample is completely retained by the gel and cannot be recovered. This fraction is retained not because it has a molecular weight smaller than 500 daltons, but probably because Sephadex G-25 strongly binds some part of humic acid. This is supported by a general comparison between the electrophoretic pattern of the initial humic acid and the pools collected: in fact, a loss of fraction b (22 EM units) and generally of a dispersed fraction of low mobility ranging from 0 to 55 EM units can be observed, while the small fractions of high mobility are all present in each pool.

Thus it could be suggested that the apparent low resolving power of gel filtration may be caused by a double effect, *viz.*, normal molecular sieving and abnormal adsorption.

From examination of the electrophoretic patterns with the three stains, the following observations can be made.

Blue Alcian. This stain is good for the general characterization of all of the fractions; only fraction a is slightly stained, but retains its own colour. Observing the gel under normal conditions, the above-described loss of fractions after gel filtration and the constant presence of the same fractions in all of the pools eluted must be noted. Fraction a (5 EM units) shows a slight decrease in intensity from $P1_{25}$ to $P6_{25}$.

Comparison with urea-treated gels with the same stain indicates that urea reveals humic acid more clearly in the electrophoretic gel and separates the fraction into a more complete pattern, with five definite fractions.

Band d in the gel under normal conditions is probably the sum of bands d and e in the urea-treated gel; band a with urea treatment should generate band c, as shown by the decrease in intensity of band a compared with the use of normal conditions. These results are in good agreement with the electrophoretic pattern obtained on humic acids from soils of different origin[8]; moreover, a decrease in the intensity of band c (62 EM units), constancy of that of fraction d (75 EM units) and an increase in that of band e (85 EM units) occurs on elution on Sephadex G-25.

Thus, even if gel filtration is unable to separate humic acids into homogeneous fractions, it probably allows the progressive enrichment of small fractions.

Basic Fuchsin. This stain allows the characterization of polysaccharides and of other compounds with neighbouring hydroxyl groups. In contrast with results obtained for humic acids of different origin[8], all of the fractions are stained, both under normal conditions and on urea treatment, except for band d present in urea-treated gel; probably the humic acid is associated with a cellulose matrix in almost all of the fractions; band d obtained on urea treatment shows that only a small amount of this cellulose matrix is released from humic acid; other conclusions are in agreement with those for Blue Alcian.

Prussian Blue. This stain, for fractions with reducing properties, indicates that only bands c and e in the urea-treated gels show reducing properties. These bands, as previously observed, correspond to small molecules of high charge density; moreover, it is impossible to establish if the reducing power of bands c and e is due to oxidizable polysaccharides or other groups, whereas this does not apply to humic acids from other soils[8].

Finally, a progressive decrease of bound stain along the pools must be noted;

this observation, which is not explained by the gel filtration and electrophoretic data, is classified on the basis of the IR results below.

Gel filtration on Sephadex G-75 of Pl_{25} and electrophoretic examination of pools collected

Gel filtration on Sephadex G-75 is shown at the top of Fig. 2, and below is shown the electrophoretic pattern of the pools collected. As only three pools were collected, these are all shown. The fraction retained on Sephadex G-75 is negligible, but the large elution volume (three void volumes) indicates that an abnormal interaction also exists between Sephadex G-75 and humic acid.

The gel filtration results show that the largest fraction of Pl_{25} has a molecular weight greater than 50,000 daltons and a well defined peak appears after two void

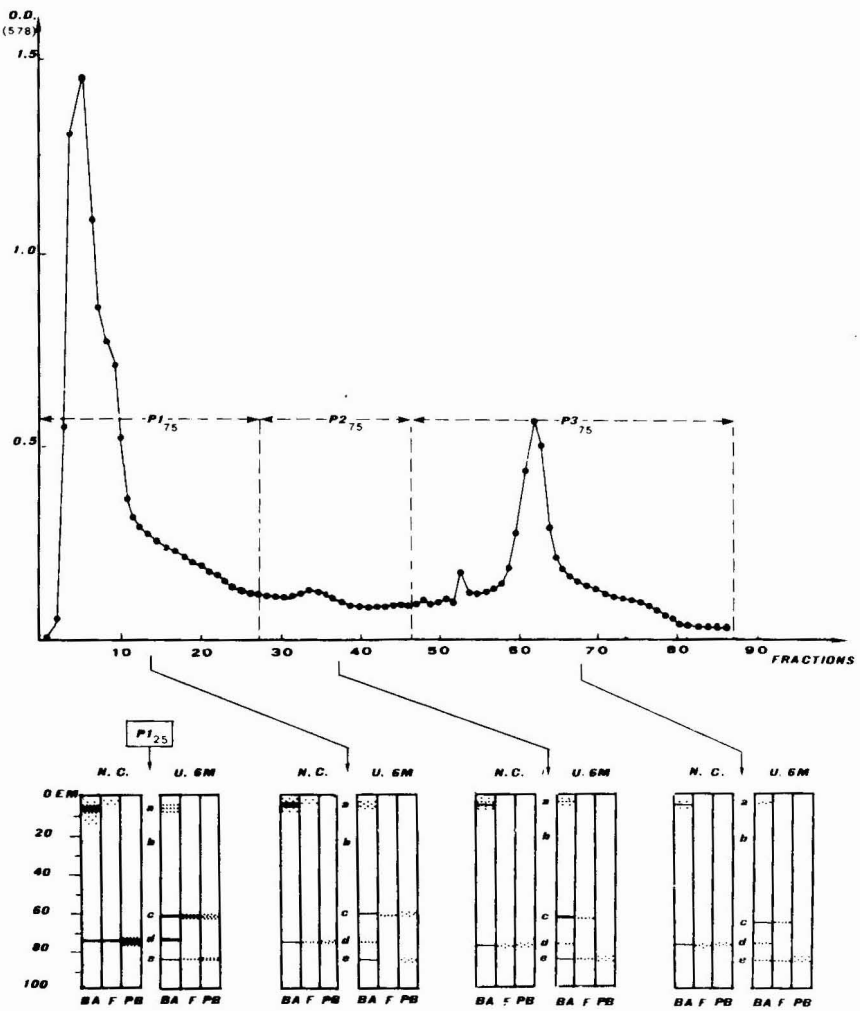


Fig. 2. Gel filtration (Sephadex G-75) of the pool 1 from Sephadex G-25 (Pl_{25}) (top) and electrophoretic patterns of Pl_{25} and of the pools collected from gel filtration (bottom). Abbreviations as in Fig. 1.

volumes of eluent. This should indicate that in the region of 10,000 daltons there is a uniform molecular type; however, the electrophoresis of $P3_{75}$ shows that the uniformity is based only on molecular weight, because this pool has a high heterogeneity.

Examination of the electrophoretic pattern of each pool indicates that, in general, gel filtration is a poor separation method for humic acid. It should be noted that fraction e of $P1_{75}$in urea-treated gel is stained by Prussian Blue but not by Basic Fuchsin, indicating that the reducing power of this band is probably due to chemical groups other than oxidizable polysaccharides (*i.e.*, *p*- and *o*-diphenols easily oxidizable to quinones). The fact that band c in $P2_{75}$ and $P3_{75}$ is stained by Fuchsin but not by Prussian Blue confirms this hypothesis. Moreover, there is an enrichment of band c in $P2_{75}$ and band a is particularly intense in $P1_{75}$, obviously corresponding to the humic acid fraction of highest molecular weight and lowest negative charge. Other observations are in agreement with results obtained from Sephadex G-25 gel filtration.

Infrared spectroscopy

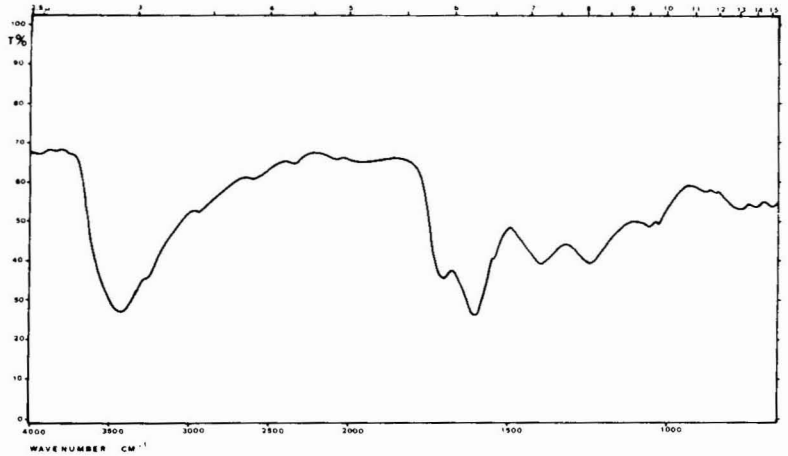
Fig. 3A shows the IR spectrum of the initial humic acid, and Fig. 3B shows the IR spectra of some samples derived from gel filtration on Sephadex G-25, *i.e.*, $P1_{25}$, $P2_{25}$, $P4_{25}$ and $P6_{25}$. The choice was made for the same reason as for electrophoresis.

Fig. 4 shows similar results for Sephadex G-75. The following conclusions can be drawn: four principal absorption bands occur in all of the spectra, at 3400, 1600, 1400 and 1050 cm^{-1}.

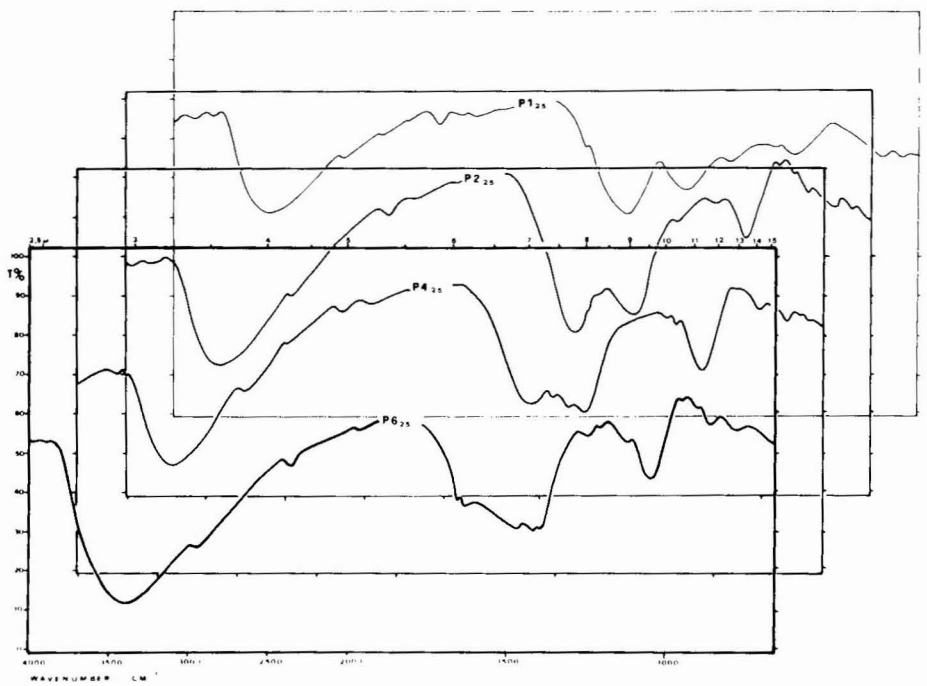
The 3500 cm^{-1} band represents OH from different classes such as phenols, alcohols and organic acids; it is impossible to establish the exact origin of the signal[16,17]. The 1600 cm^{-1} band is characteristic of humic-type molecules and corresponds to double bonds such as C=N, C=O and C=C. The free carboxylic group absorbs at 1725 cm^{-1} and the carboxylate anion at 1600 cm^{-1}. About 1540 cm^{-1} some spectra show a shoulder of various size; from Schnitzer and Khan's interpretation[18] it can be attributed to bound peptides. The 1400 cm^{-1} band corresponds to CH, CH_2 and CH_3 groups; this attribution is confirmed by an absorption band at 2950 cm^{-1}. Carboxylate anion also absorbs at the same frequency (1400 cm^{-1}). The 1050 cm^{-1} absorption band[18] must be attributed to inorganic material, and the Si–OSi band absorbs at 1070 cm^{-1}.

Infrared spectra obtained from Sephadex G-25 pools. In the spectrum of initial humic acid it can be seen that the intensity of the 1600 cm^{-1} band (aromatic C=C) is greater than that of the 1390 cm^{-1} band. In the $P1_{25}$ spectrum these intensities are very similar to those of total humic acid but the 2950 cm^{-1} band, corresponding to aliphatic C–H, is absent. In the $P2_{25}$ spectrum the intensities of the 1600 and 1390 cm^{-1} bands are identical and the 2950 cm^{-1} band slowly increases. In the $P4_{25}$ spectrum the relative intensities of the bands change, the 1390 cm^{-1} band becoming greater relative to the 1600 cm^{-1} band and the 2950 cm^{-1} band increasing even more. The $P6_{25}$ spectrum shows the same characteristics as $P4_{25}$. Hence it can be concluded that $P1_{25}$ is principally an aromatic fraction, while an increase of aliphatic character along the pools was detected.

None of the spectra from Sephadex G-25 shows the 1725 and 1200 cm^{-1} bands, confirming that part of humic acid is lost in the separation (fraction b on

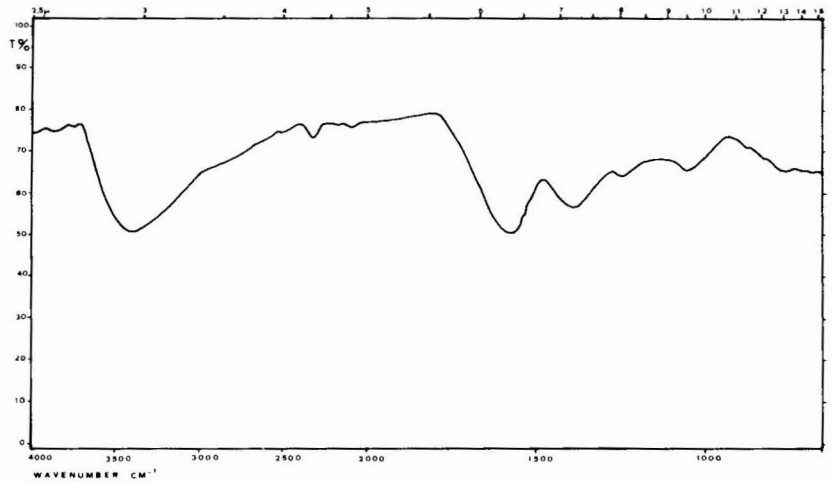


A.

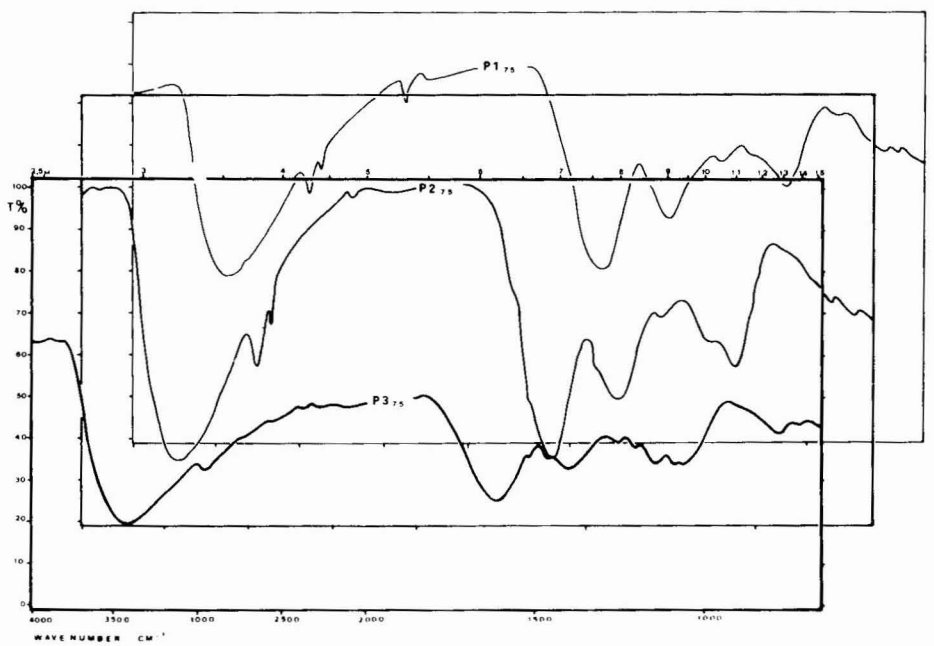


B.

Fig. 3. Infrared spectra of (A) initial humic acid and (B) pools collected from gel filtration on Sephadex G-25.



A.



B.

Fig. 4. Infrared spectra of (A) $P1_{25}$ and (B) pools collected from gel filtration on Sephadex G-75.

electrophoresis) and should be rich in carbonyl groups and C–O bonds (the 1200 cm^{-1} band is assigned to C–O stretching).

The 1050 cm^{-1} band (Si–O bond) present in the initial humic acid increasing with increasing elution volume, showing that the small fractions are more strongly bound than higher fractions to the inorganic matrix. This is confirmed by the decreasing staining power of these fractions, as can be seen in the electrophoretic patterns[7].

$P1_{25}$ shows a great difference to the other pools, justifying the choice of Sephadex G-75 for subsequent separation.

Infrared spectra obtained from Sephadex G-75 pools. In the $P1_{75}$ and $P2_{75}$ spectra the 1050 cm^{-1} band is well defined, whereas it is absent from the $P3_{75}$ spectrum. According to gravimetric experiments (data omitted), the small fractions have a greater ash content and so the IR spectra of Sephadex G-75 pools should have a small 1050 cm^{-1} band, as suggested from the Sephadex G-25 results. This discrepancy can be explained by assuming that the band is also due to other groups, such as C–O–C. The interpretation of IR spectra from gel filtration fractions by Russel and Anderson[19] confirms this hypothesis. They suggested that the bonds of silicates and humates could be superimposed in some IR regions. Moreover, in the spectra of these pools, as could be predicted from Sephadex G-25 data, the 1725 and 1200 cm^{-1} bands are absent. It should be noted that the $P3_{75}$ spectrum is very similar to that of $P1_{25}$, the starting pool for the second gel filtration.

CONCLUSIONS

The general application of gel filtration to the preparative separation of humic acids has serious limitations: the fractions obtained show high heterogeneity and information on its molecular weight is uncertain owing to the absence of appropriate standards and to the interaction between humic acid and Sephadex, which causes a loss of some fractions. However, combination with disc electrophoretic and IR spectroscopic data allows the elucidation of properties of the fractions obtained.

These methods are very powerful, providing a large amount of information with little consumption of sample (*ca.* 1 mg). For these reasons it is advisable to find a preparative method with better resolution than gel filtration, whereas electrophoresis could be proposed as general technique. However, some workers[20] have reported that isotachophoresis is more efficient, separating the humic acid into up to eleven distinct fractions, but this method requires complex apparatus.

From gel filtration it could be established that a high proportion of total humic acid has a molecular weight greater than 50,000 daltons. Bendeck[3] reported that 93% of the humic acid of the soil studied had a molecular weight smaller than 100,000 daltons; hence this fraction of molecular weight ranging from 100,000 to 50,000 daltons, of high condensation and aromatic character (as proved by the IR spectra), can account for the low soil fertility, in spite of the large amount of organic matter.

The explanation of the low availability of organic material could be extended even to the small fractions, tightly bound to inorganic material; however, this conclusion requires confirmation by performing the same analysis on humic acids of different origin.

These comparisons with other soils are also desirable for establishing the common properties of humic acids of different origin in order to clarify the general

humification process; conversely, the differences in properties should explain particular origins of the humic acids and account for soil fertility.

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Note

Column loadability and particle size in preparative liquid chromatography

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In a previous article[1] the specific loadability, defined as the mass of solute per gram of packing material above which the column plate number tends to decrease sharply, was found to depend on the particle size of two different fractions obtained from one batch of silica material. A similar effect was found by Bombaugh and Almquist[2]. An explanation for this dependence was not given, but changes in the silica arising from the grinding and sieving procedures were mentioned as a possible cause. In this paper we present some more experimental results pertaining to the relation between column plate number, particle size and loadability for a given solute and a discussion of this relation.

EXPERIMENTAL

Apparatus

A home-made liquid chromatographic system was used comprising a high capacity pump (V 410; Burdoza, Giessen, G.F.R.), a flow-through Bourdon-type manometer serving also as a pulse dampener and a variable-wavelength UV detector (PM 2 ALC; Zeiss, Oberkochen, G.F.R.). The following columns of stainless-steel 316 tubing were used: 10 cm × 17 mm O.D. × 10.8 mm I.D., honed to a smooth inner surface and filled with 5–8 μm Si 60; 25 cm × 17 mm O.D. × 10.8 mm I.D., honed and filled with 20–25 μm Si 60; 50 cm × 17 mm O.D. × 10.8 mm I.D. and 50 cm × 16 mm O.D. × 10 mm I.D. used as delivered and both filled with 20–25 μm Si 60. The two 50-cm columns were coupled[3] to give a 100-cm column. A central injection system was used on all columns. The bottom terminators for the two 50-cm columns had a 150° top angle conical shape with an embedded 6-μm frit (5 mm diameter), while the bottom terminator for the 10-cm column had a 150° top angle complete conical shape connected to the outlet capillary tubing (the packing material in this case was retained by a plug of PTFE wool). Samples were introduced by means of a sampling valve (70-10; Rheodyne, Berkeley, CA, U.S.A.) fitted with a 50-, 100- or 1000-μl loop. All connections between the valve, the column(s) and the detector consisted of Swagelok 1/16 in. zero dead-volume unions.

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Materials

Acetone, tetrabromoethane and chloroform (E. Merck, Darmstadt, G.F.R.) were used in the column packing procedure. Dichloromethane (Baker, Deventer, The Netherlands) was used as solvent. Silica gel Si 60 (E. Merck) was used as the packing material; it was ground and classified by means of an air classifier (Alpine M.Z.R., Augsburg, G.F.R.) to particle size ranges of 5–8 μm and 20–25 μm. The tested solute was 2,4-dimethylphenol.

Procedures

All columns were filled using a balanced density slurry technique as described previously[1]. Two methods were used for the calculation of the standard deviation of an elution profile: (a) the standard deviation was taken as half of the peak width at 0.607 of the maximum plate height (denoted by the subscript 0.6); (b) the standard deviation was taken as 1/4.29 of the peak width at 0.1 of the maximum peak height (denoted by the subscript 0.1). Column plate numbers were calculated from the retention time of the peak maximum, t_{Ri}, and the standard deviations $\sigma_{0.6}$ and $\sigma_{0.1}$ using:

$$N_{0.6} = (t_{Ri}/\sigma_{0.6})^2 \quad \text{and} \quad N_{0.1} = (t_{Ri}/\sigma_{0.1})^2$$

The $N_{0.1}$ values are given since it is thought[1] that they give a better insight into the separation capabilities of a column for preparative purposes.

RESULTS AND DISCUSSION

The processes involved in the dilution of a large mass of solute from the top to the end of a column are the isotherm non-linearity broadening and the column dispersion. How these two broadening effects interact and add up to give the final profile cannot be described analytically, only by approximate calculation[4] or by simulation. For dispersionless chromatography (infinite plate number) and where the sample is injected as a spike, Huber and Gerritse[6] showed that it is only the solute outlet concentration which determines the final peak shapes

$$t_R(c_{i,m}) = t_{R,0}\left(1 + q \cdot \frac{dc_{i,s}}{dc_{i,m}}\right)$$

where $t_R(c_{i,m})$ is the retention time for the concentration $c_{i,m}$, $c_{i,m}$ and $c_{i,s}$ are the concentrations of solute i in the mobile and the stationary phase respectively, $t_{R,0}$ is the retention time of an unretained component, q is the phase ratio, and $dc_{i,s}/dc_{i,m}$ is the derivative of the isotherm at the mobile phase concentration $c_{i,m}$. For other input profiles in dispersionless chromatography this relation still holds[5]. Also for real chromatography (finite plate number) it can be shown[7] that different input profiles (*e.g.*, variation of injection volume, but avoiding volume overload) result in identical elution profiles. So it is understood that it is only the outlet concentration which determines the magnitude of the broadening due to isotherm non-linearity (see also ref. 8).

When loadability studies are carried out by increasing the mass of a solute in a

constant injection volume, one is searching for the mass of solute which yields the maximum allowable increase of peak width over the normally observed peak width for a very low mass of solute (and consequently the maximum allowable loss of resolution). Above this mass, the isotherm non-linearity broadening starts to dominate the column dispersive broadening, giving rise to a sharp decrease in column efficiency for higher loading. This effect, however, is in itself related to concentration, rather than to mass. When the isotherm non-linearity broadening is not operative (*e.g.*, for low loading), it is the column dispersion which determines the maximum concentration of the elution profile. Consequently, the column dispersion determines the loading above which the isotherm non-linearity broadening becomes effective, as a specific maximum outlet concentration may not be surpassed: for columns of the same size, a greater mass of solute must be introduced into a column with a low plate number than into a column with a high plate number.

A further "advantage" of the higher dispersion in a column of low plate number is the effect of a high sample load on the peak shape. A deviation in the $dc_{i,s}/dc_{i,m}$ value from the infinite dilution value will occur when a specific (high) outlet concentration is obtained. This deviation can severely affect the peak shape on a column of high plate number while only a small change in peak shape may occur on one of low plate number.

Following this reasoning we can now understand:

(a) Why columns of different plate numbers, filled with the same amount of packing material, must have quite different specific loadabilities. This is shown in Fig. 4 in ref. 1: for columns of the same size, but different particle size, different specific loadabilities are found.

(b) Why for columns of different tube size and different particle size but equivalent plate number, the same specific loadability should be found. This aspect is shown in Fig. 1A and B, where two different columns with approximately the same plate number are compared. Indeed, the specific loadabilities for the packing materials (5–8 μm and 20–25 μm fractions from one batch of silica Si 60 material) are

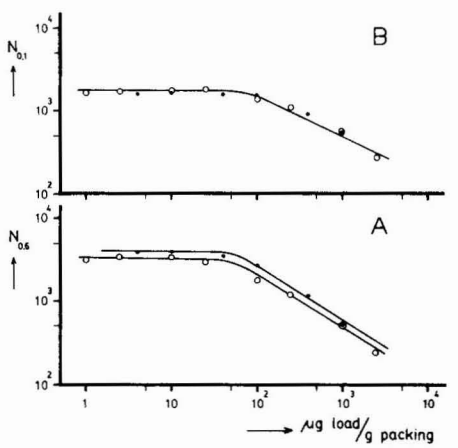

Fig. 1. Plots of column plate number $N_{0.6}$ (A) and $N_{0.1}$ (B) *versus* specific loading for 2,4-dimethylphenol (capacity factor, $\kappa = 3$) on columns of approximately equal plate numbers. Solvent: dichloromethane. Columns: ○, 100 cm (50 cm × 10.8 mm I.D. + 50 cm × 10 mm I.D.), 39.5 g of 20–25 μm Si 60; ●, 10 cm × 10.8 mm I.D., 4.9 g of 5–8 μm Si 60.

equivalent. This aspect was also reported by Wehrli[9], but the columns compared by him have quite different plate numbers for low solute loadings, and also no comparison was made on the basis of the amount of packing material in the columns.

Fig. 2A and B demonstrate the different specific loadabilities found for 2,4-dimethylphenol on the 20–25 μm silica, when different column sizes, and consequently different plate numbers, are used. Different solvents were also used in these two columns, but the capacity factor was approximately the same for 2,4-dimethylphenol. It is clearly useless to ascribe a specific loadability value to a column packing material if the plate number is not specified. Therefore, in Fig. 3A and B the observed column plate numbers are plotted against the total column loading. It is seen that the loadability increases with decreasing plate number on a given column. Although no other columns and packing materials have been tested in this respect, it appears possible, that a loadability borderline can be drawn for each column, into which all the different lines merge. This borderline represents the situation where column dispersion no longer plays the major rôle: the elution profile is determined by isotherm non-linearity broadening. The loadability borderline is entirely determined by the distribution isotherm of the solute in the phase system. Thus, the effect of the loading on the separation efficiency can be visualized from a graph of the observed plate number, N, *versus* the specific loading.

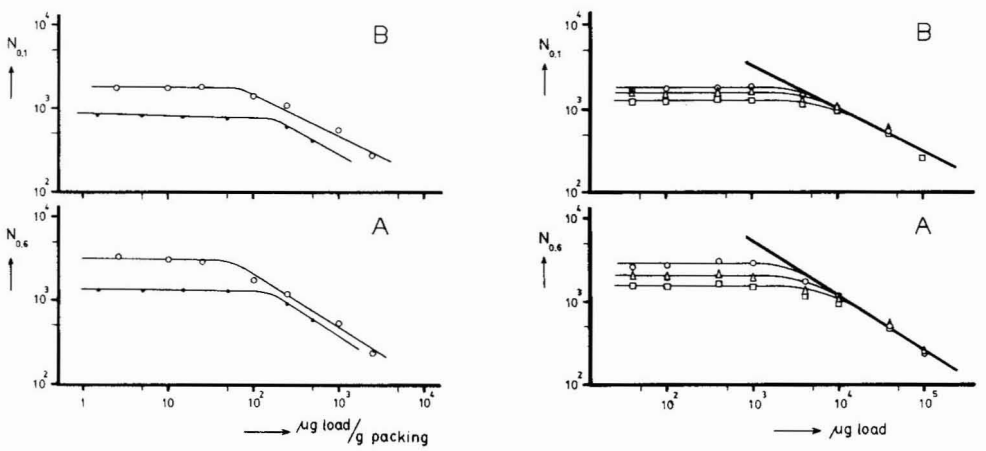

Fig. 2. Plots as in Fig. 1 but for columns of different plate numbers. Columns and phase systems: ○, 100 cm (50 cm × 10.8 mm I.D. + 50 cm × 10 mm I.D.), 39.5 g of 20–25 μm Si 60, solvent, dichloromethane; ●, 25 cm × 10.8 mm I.D., 9.6 g of 20–25 μm Si 60, 1% butanol in 2,2,4-trimethylpentane as solvent (see ref. 1).

Fig. 3. Plots of column plate number $N_{0.6}$ (A) and $N_{0.1}$ (B) *versus* loading for 2,4-dimethylphenol ($\kappa = 3$) using different solvent flow-rates. Column and phase system: 100 cm (50 cm × 10.8 mm I.D. + 50 cm × 10 mm I.D.); 20–25 μm Si 60; solvent, dichloromethane. Linear velocities: ○, 1.8 mm/sec; △, 5.5 mm/sec and □, 8.4 mm/sec.

In Fig. 4A and B the specific loadability results are compiled for the above 10- and 100-cm columns. Such a graph, made for various columns of different plate numbers, again shows an envelope or borderline, which characterizes the particular combination of solute and phase system. For an actual separation, where a certain plate number is required, one can never use a specific loading higher than the value

indicated by the borderline at the point corresponding to this required plate number. Therefore, such a loadability borderline for a given solute and a given phase system gives an indication of the preparative utility of the phase system and may be a helpful guide to the choice of column and flow-rate. The borderline is in principle predictable from the distribution isotherm of the solute.

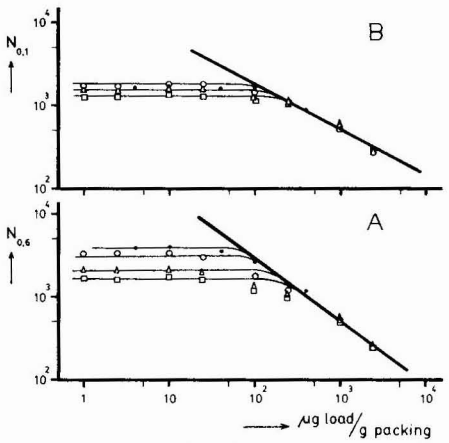

Fig. 4. Plots as in Fig. 1 for the phase system silica Si 60 (E. Merck)–dichloromethane. Columns and linear velocities: ●, 10 cm × 10.8 mm I.D., 5–8 μm, 2.1 mm/sec; ○, △, □, 100 cm × ± 10 mm I.D., 20–25 μm, 1.8 mm/sec (○), 5.5 mm/sec (△) and 8.4 mm/sec (□).

CONCLUSIONS

The reporting of specific loadabilities for a solute on a given packing material must be accompanied by specification with respect to the column and particle size and/or column plate number (*i.e.*, the operating conditions).

A borderline, observed in graphs of the observed plate number, N, *versus* the specific loading, is a useful aid to selecting optimum conditions in preparative liquid chromatography.

The higher loadabilities found for coarser particles, reflected in a relative increase in plate height (as compared to the loadability for smaller particles in the same column), are a necessary result of this testing procedure and have nothing to do with the different distribution parameters found for the coarser particles.

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Note

Improved microlitre syringe for gas–liquid chromatography

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In chromatography, various problems and difficulties are encountered in the injection of samples into the apparatus, such as the variation in the injected volume for sampling at a desired volume, the necessity for rapid discharging, pumping out of air bubbles from the syringe, bending of the plunger of the syringe, and so on. This paper describes a device with an automatic plunging system by which the required volume of a liquid can be sampled rapidly and injected without any technical skill, with high accuracy and reproducibility.

EXPERIMENTAL

Device

The device (Fig. 1) is composed of a microlitre syringe and a tubular holder with spring systems. The syringe was a modified Model No. 701-N with capacity of 10 μl (Hamilton, Reno, NV, U.S.A.). The spring system which drives the plunger of the syringe forward is located in the hind part of the holder. The plunger head and the push piece can be freely controlled from outside the holder through the slots by moving knobs 1 and 2, respectively. The spring is compressed by sliding knob 1 to the hind part of the slot, and locked when the pin protruding from the top of the push

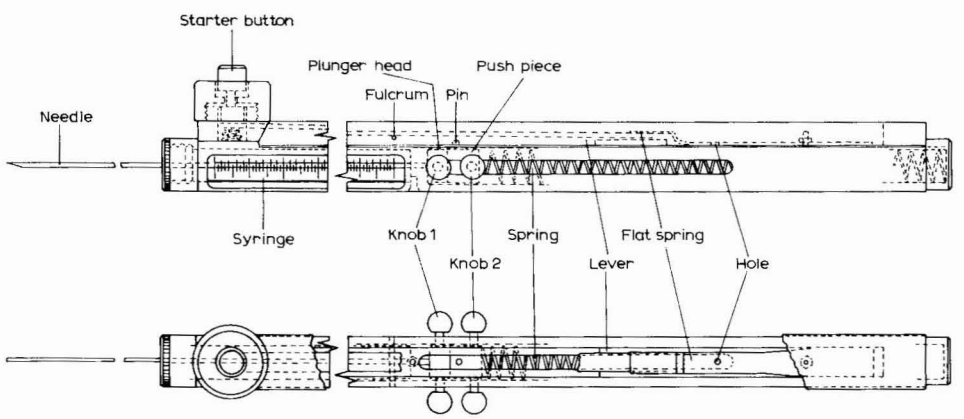

Fig. 1. Structure of improved microlitre syringe.

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piece is engaged with the hole in the flat spring. When the starter button is pushed down, the rear end of the lever is pushed up, and simultaneously the flat spring also is pushed up, releasing the pin of the push piece from the hole.

When, in the sampling, the knobs 1 and 2 are moved slowly along the slots as far back as possible without the spring system being locked, and then released, the air bubble introduced from the needle is discharged by the compressed spring. After the spring is locked by sliding knob 1, the required volume of the sample liquid is transferred to the syringe by sliding knob 2 to the scribe line. The needle is inserted through the rubber closure of the injection port, and the starter button is pushed down immediately. Then the needle is withdrawn as quickly as possible.

Accuracy

Volumes of 5.0 μl and 10.0 μl of water were continuously discharged into capillary tubes (5.0 cm $\times$ 0.8 mm I.D.), one side of which had been sealed. Each volume was calibrated from the difference of the weights obtained on a directing balance (Mettler Model H 20).

Reproducibility

A glass column (1.5 m $\times$ 3 mm I.D.) packed with 2% silicon OV-225 on Gas-Chrom Q (80–100 mesh) was installed in a gas chromatograph equipped with an electron-capture detector (^{63}Ni) (Shimazu 5A EF). The temperatures of the column, the injection port and the detector were maintained at 175, 200 and 250°C, respectively. The flow-rate of the nitrogen carrier gas was 60 ml/min. Under these conditions, a linear calibration curve of γ-BHC was obtained in the range 10–100% in the recorder scale.

Volumes of 5.0 μl of the 10-ppb γ-BHC solution were continuously injected to the gas chromatograph.

Efficiency on washing

A 5.0-μl volume of the γ-BHC solution which showed a recorder response near full scale was first injected into the gas chromatograph. Subsequently, only the solvent, *n*-hexane was repeatedly injected.

RESULTS AND DISCUSSION

Mean volumes of the water discharged when the water had been sampled in the syringe ten times at a volume of 5.0 μl and twelve times at 10.0 μl were 5.008 μl and 9.998 μl, respectively. Each standard deviation was 0.013 μl and 0.029 μl, which correspond to 0.26% and 0.29% for the mean volumes.

Gas chromatograms obtained by repeated injections of the γ-BHC solution (10 ng/ml) are shown in Fig. 2. In two series of six and seven injections of volumes of 5.0 μl each of the solution using two different syringes, each deviation was 0.41% and 0.33% in the height of the peaks showing mean recorder response of 94.56% and 94.76%, respectively, which correspond to 0.43% and 0.35% for the discharged volumes. The slightly higher deviation in the volume discharged in gas chromatography than in measuring weights of the discharged water may be due to the variable amount of the material adhering around the needle, the difference of loading of the material onto the column, etc. The result, however, shows that the required volume of the sample liquid was injected into the gas chromatograph.

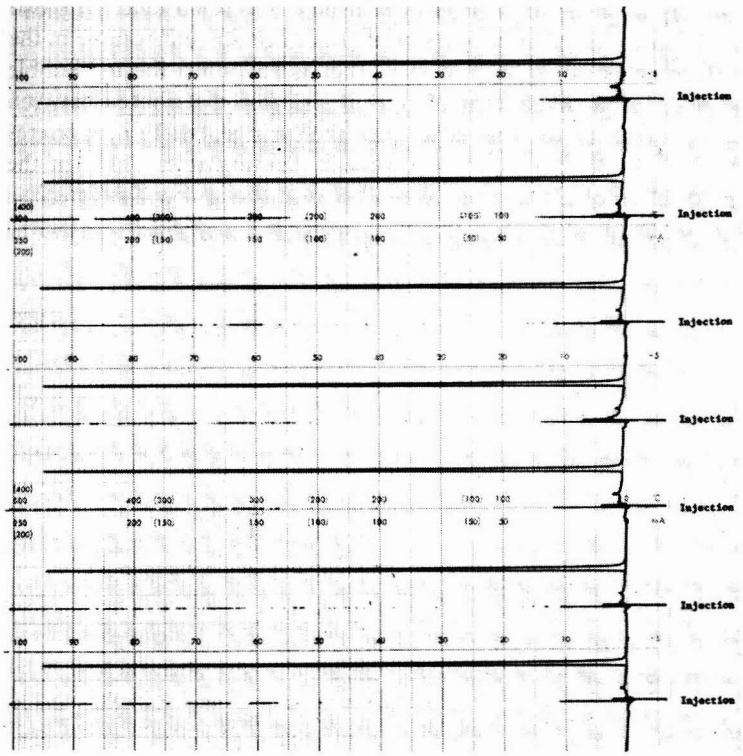

Fig. 2. Gas chromatograms of γ-BHC obtained by repeated injection of the desired volume of the sample injection. Sample liquid: 10 ppb γ-BHC solution in *n*-hexane. Volume injected: 5.0 μl.

After an injection of the amount of γ-BHC showing a recorder response near full scale, the syringe was completely washed by two pumpings of the plunger using the solvent as shown in Table I. The washing procedure is easily operated by repeated movement of both knobs 1 and 2 without locking the spring.

TABLE I

EFFICIENCY OF SPRING SYSTEM ON WASHING OF MICROLITRE SYRINGE

Sample liquid: 10 ppb γ-BHC solution in *n*-hexane. The syringe was washed three times with 10 μl of *n*-hexane.

Injection	*Recorder response (%)*	*Peak ratio*	*Amount of substance injected/ amount of substance sampled (%)*
Sample liquid (5 μl)	91.1	100	85.3
First washing solution	14.2*	15.6	13.3
Second washing solution	1.5**	1.7	1.4
Third washing solution	0***	0	0

*,**,*** These values correspond to the residual amounts in the syringe after sample injection, first washing and second washing, respectively.

When the syringe is washed, the efficiency will be determined chiefly by two procedures. One is to minimize the amount of sample liquid or solvent residue in the syringe and needle after each discharge, and the other is to pump the air bubble rapidly out of the syringe. In particular, the air bubble is often distributed between residual liquid and solvent transferred thereafter into the syringe, where it interferes with the diffusion of the sample liquid to the solvent, leading to a delay in the washing. This device could resolve these problems because the spring system is capable of moving the plunger rapidly.

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Note

Use of reversed-phase thin-layer chromatography for the identification of black inks from board felt markers and ball-point pens

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The general availability of a wide range of chemically bonded silica particles has given rise to a fast developing branch of chromatography, reversed-phase chromatography. The difficult problem of binding together these particles on a glass plate has recently been overcome by manufacturers and a technique for preparing home-made plates is available[1]. It is therefore now possible to perform thin-layer chromatographic (TLC) separations of very polar compounds easily and rapidly. The plates can be re-used after suitable washing[1].

The main advantages of this hydrophobic material are the virtual absence of interferences from vapours adsorbed from ambient air, the good reproducibility of the retention data, the absence of spot tailing in almost all instances, the ease of adjusting the R_F value in the practical range of 0.2–0.8 by adjusting the composition of simple mixtures of water and an organic solvent and the good stability in the pH range 2–9.

The main drawback is the low energy of the surface, which makes it poorly wet by water-rich solvents. Development is very slow with mixtures that contain more than 40% water[1,2].

In spite of its attractive features, reversed-phase TLC is still little used. We report here an analysis developed for a forensic laboratory and used by us as a demonstration of the principle and separation power of chromatography for chemistry students, namely the separation of the constituents of black inks. Whereas conventional china ink is made with carbon particles[3], many black inks, especially for board markers or ball-point pens, consist of mixtures of dyestuffs. TLC separation makes the identification of the ink used easy as the natures of the individual dyes can be established using a spectrophotodensitometer.

TLC separation of synthetic dyes has been performed on various systems such as Kieselguhr[4], cellulose[5] and silica coated with *p*-methylbenzenesulfonate[6]. Analyses of ball-point pen inks by paper chromatography have been reported[7,8] and a high-performance liquid chromatographic (HPLC) separation has been achieved by Colwell and Karger[9] on silica using 2% formamide in methanol as the mobile phase.

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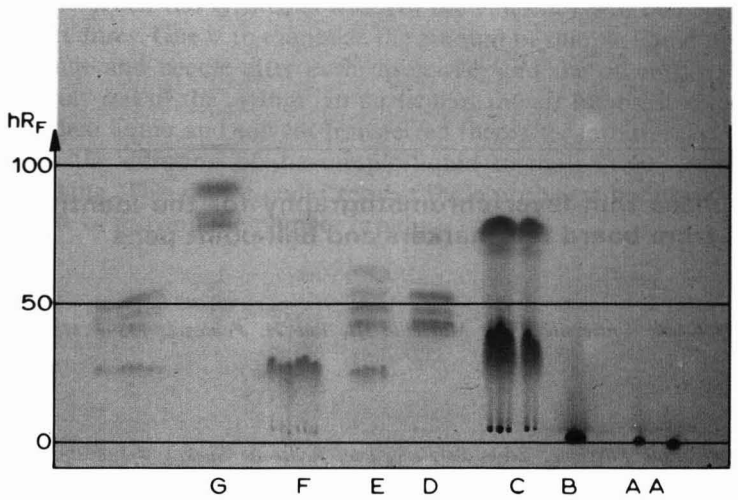


Fig. 1

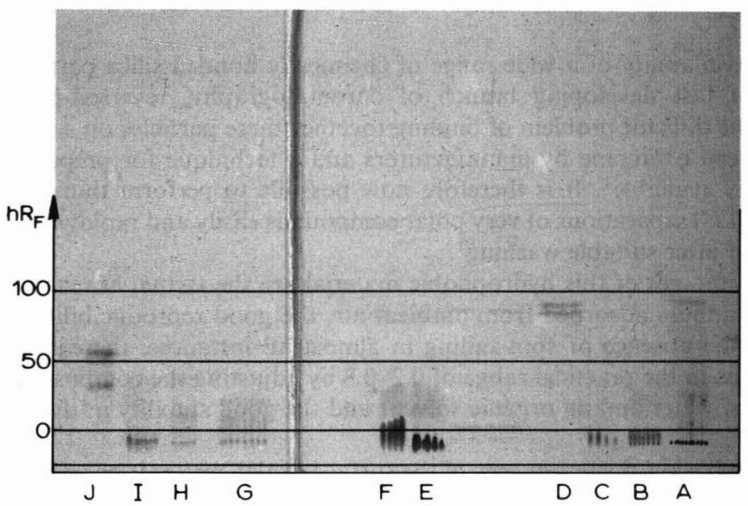


Fig. 2

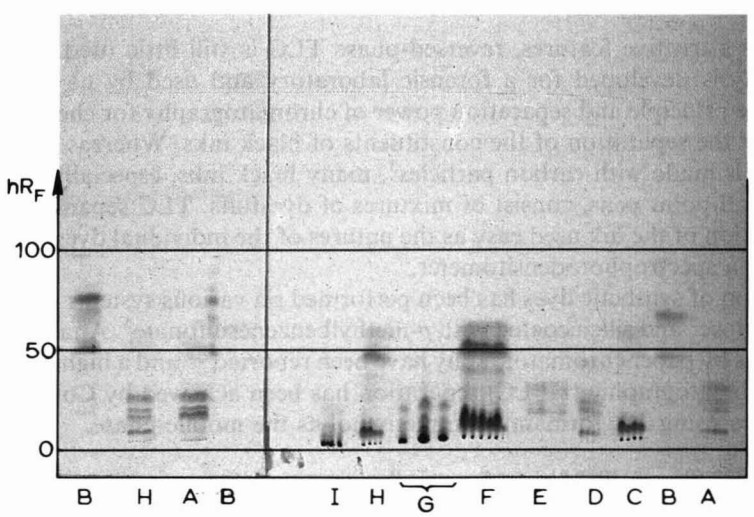


Fig. 3

To our knowledge there has been no report of the separation of these dye mixtures on TLC plates coated with alkyl-grafted silica, although dye mixtures have been separated successfully by reversed-phase TLC on paraffin-impregnated cellulose, silica gel and cellulose[10].

EXPERIMENTAL

TLC plates prepared with silica particles chemically bonded to ethyl, *n*-octyl or *n*-octadecyl groups were obtained from Merck (Darmstadt, G.F.R.) under the trade names RP-2, RP-8 and RP-18, respectively. The average particle size was 7 μm (ref. 2). Developments were carried out in a Camag (Muttenz, Switzerland) N-chamber. Because of the relatively low volatility of the solvents used, there was no need for a sandwich system.

The solvents were of spectrograde quality and were used without further purification. The developing solvent was prepared by careful mixing of 3.5 cm^3 of a phosphate buffer solution and 6.5 cm^3 of ethanol. The buffer solution (pH 2.8) was prepared from 0.1 *M* sodium dihydrogen orthophosphate solution, the pH being adjusted by adding orthophosphoric acid.

Samples were deposited 1 cm from the edge of the plate and the plate was immersed 0.5 cm in the developing solvent. Development was carried out to a distance of 4 cm, which usually took about 1 h.

Densitograms were obtained using a TRD 2 spectrophotodensitometer (Vernon, Paris, France) equipped with a tungsten lamp. No filter was used.

RESULTS AND DISCUSSION

An aqueous solution of the sample cannot be used, as it would not wet the sorbent layer. Two application techniques were used. With small-tipped markers and pens, simple pressure of the felt tip or pen on the layer yielded a convenient spot of diameter 0.2–0.5 mm. With large-size board markers or pens with cartridges, one drop of ink was mixed with a few drops of methanol and the sample was applied on the layer using a glass capillary.

We found that the use of plates with a concentration zone is inadequate. As the solvent migrates through the inactive zone, pushing forward the sample to the chromatographic bed, some separation takes place and strong tailing of some compounds occurs. Not all sample components reach the small gap at the same time and the separation is poor, with broad and tailing spots. To our knowledge, this is the first reported failure of the concentration zone system.

Fig. 1. Photograph of a developed TLC plate (Merck RP-18). Conditions as described in text. Samples: A, china ink; B, Waterman black; C, Veleda (felt); D, Somarco (felt); E. Somarco (ball point); F, Bic (ball point); G, Reynolds (felt).

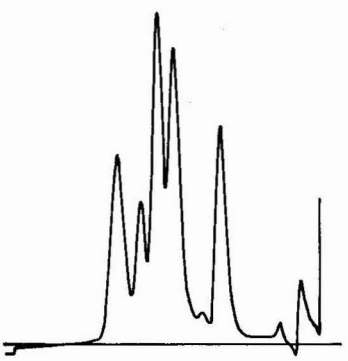
Fig. 2. As Fig. 1 for A, Pentel (felt); B, unknown ink from an Omniscribe recorder; C, Immont blue; D, ink from a Vernon recorder; E, Bic (blue ball point); F, Waterman cartridges (for fountain pen); G, Somarco (ball point); H, Stylist (felt); I, unknown; J, Reynolds (felt).

Fig. 3. As Fig. 1 for black felt pens: A, Somarco; B, Reynolds; C, Staedtler; D, Somarco; E, Bic; F, Plan Master; G, Onyx; H, Stabilo; I, black ink from Waterman.

As the black inks studied are unknown mixtures of dyestuffs, the best chromatographic system is the one which gives the largest number of spots. The best results were obtained with RP-18 plates. The pH of the solvent has a critical effect on the number of spots and their resolution. A strongly acidic buffer was found to be necessary. Alcohols gave better results than acetonitrile; ethanol was preferred to methanol because of its lower volatility.

Figs. 1–3 show some of the results. Obviously black china ink exhibits one spot only, with infinite retention. The black inks from different manufacturers exhibit a number of different coloured components, with a wide range of different dyes and concentrations. Some of these dyes are strongly retained, others weakly.

Densitometry of the developed plates also provided valuable information, and was much more accurate than visual examination of the plate. Figs. 4 and 5 show such densitograms for two different ink samples. The relative percentages of each dye permit the derivation of a "fingerprint" for the various inks studied. The identification and authentication of small ink samples was possible. Positive authentication could be achieved, if needed, by running a UV and visible spectrum of each dye component of the mixture. The amount of information supplied by the TLC densitogram is close to 100 bits with the system used, which is already large compared with the number of brands of black ink available[11].



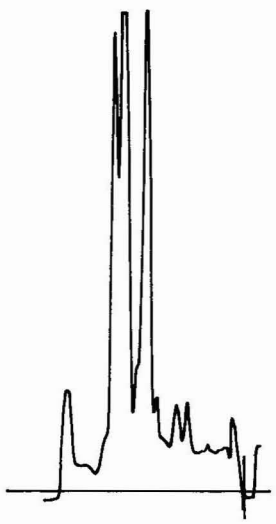


Fig. 4. Photodensitogram of sample E in Fig. 1 (Somarco ball point).

Fig. 5. Photodensitogram of sample B in Fig. 3 (Reynolds black felt).

It is easy to run such separations for a class of up to about 20 students. Each student can put a spot with a pen on a TLC plate and a clear chromatogram is obtained in 15 min, exhibiting arrays of brightly coloured spots which illustrate dramatically the resolving power of chromatography, its fields of application, its principle and its etymology. The sharpness of resolution also permits some further work in the laboratory on comparison between the performances of various plates, the influence of development length, etc. TLC is much quicker and simpler than HPLC as a preliminary introduction or for limited laboratory work and it is much cheaper;

many runs can be made simultaneously, clean-up is not necessary and plates can be re-used a number of times.

A comparison of Figs. 1 and 4 and Figs. 3 and 5 offers some insight into what actually takes place in LC columns. Figs. 4 and 5 show excellent chromatograms, with almost symmetrical peaks and no tailing, and would be considered acceptable by all chromatographers. Nevertheless, as shown by the photographs in Figs. 1 and 3, there is definitively some tailing, although very slight. The dye density behind the spots is very small and the effect is markedly enhanced by the non-linear response of the eye, a phenomenon too often overlooked[12]. This shows that a very small amount of dye, probably of any other polar compound chromatographed, remains sorbed for a long period on alkyl-bonded silica phases. We have not investigated this phenomenon in detail but have shown that the amount of dye sorbed is too small to be measured; it does not affect the chromatographic properties of the phase. It is possible that this sorption takes place mainly on the external surface of the particles, where the organic layer bonded to the silica surface is prone to be worn by friction between particles. This would explain our observations, but this assumption has not been tested.

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Note

Thin-layer chromatography of chlorinated catechols on a silica gel 60 layer

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(Received January 12th, 1981)

Thin-layer chromatography (TLC) has been widely applied to phenolic compounds, and the TLC of chlorinated cresols[1], the soil metabolites of MCPA[2], chlorinated guaiacols[3,4] and chlorinated catechols[5] has been carried out previously in our laboratory. In these studies several solvents and solvent mixtures were screened in an attempt to select the most suitable solvent systems for particular separations. Sattar *et al.*[5] studied the TLC of catechol, 3,4-, 3,5- and 3,6-dichlorocatechols, 3,4,5-trichlorocatechol, tetrachlorocatechol and 5-chloro-3-methylcatechol on five different layer materials with 60 different solvent systems. It was found that pure silica gel and the solvent system benzene–ethanol–acetic acid (85:10:5) or benzene–acetone–acetic acid (80:15:5) are suitable for the separation (clean-up) of chlorinated catechols from other compounds. However, for the separation of the individual components an alumina layer has been recommended.

Chlorinated catechols are present in pulp bleachery effluents in the paper industry and thus occur as potent environmental residues[6–10]. As some polychlorinated catechols are known to be toxic to fish[11] and also bioaccumulate in fish[12], the present work was carried out in an attempt to synthesize four new chlorinated catechols, study the characteristic colour reactions of all chlorocatechols and determine their R_F values with several solvent systems. However, the main purpose was to calculate the standard deviations (s)[3,4] and the relative differences[2–4] in the R_F values in order to select the most suitable solvent systems for particular separations of catechol and all possible chlorinated catechols.

EXPERIMENTAL

Samples

The compounds studied were as follows (see Fig. 1): catechol (I), 3-chlorocatechol (II), 4-chlorocatechol (III), 3,4-dichlorocatechol (IV), 3,5-dichlorocatechol (V), 3,6-dichlorocatechol (VI), 4,5-dichlorocatechol (VII), 3,4,5-trichlorocatechol (VIII), 3,4,6-trichlorocatechol (IX) and tetrachlorocatechol (X). II–VI, VIII and IX were synthesized from the corresponding chlorinated salicylaldehydes by the method described by Dakin[13]. VII and X were prepared by direct chlorination of catechol (I) (commercial product, Fluka, Buchs, Switzerland) with SO_2Cl_2 in diethyl ether[14] and with chlorine in glacial acetic acid[15], respectively. The structures of II–X were confirm-

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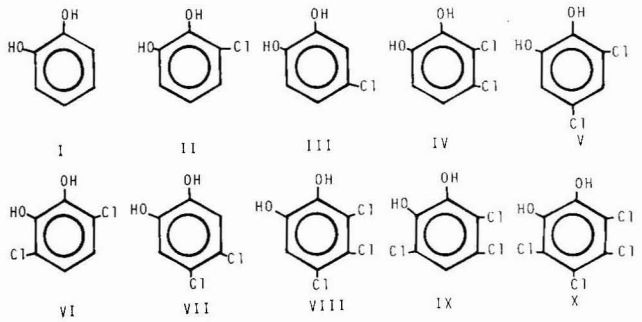

Fig. 1. Structures of catechol (I), 3-chlorocatechol (II), 4-chlorocatechol (III), 3,4-dichlorocatechol (IV), 3,5-dichlorocatechol (V), 3,6-dichlorocatechol (VI), 4,5-dichlorocatechol (VII), 3,4,5-trichlorocatechol (VIII), 3,4,6-trichlorocatechol (IX) and tetrachlorocatechol (X).

ed by ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectroscopy[16]. The purities of the samples were checked by gas chromatography on an SE-30 glass capillary column (25 m × 0.3 mm I.D.) and flame-ionization detection after derivatization of the samples with ethereal diazoethane solution[6].

Thin-layer chromatography

Pre-coated plates with a silica gel 60 layer and a concentrating zone (20 × 20 cm, layer thickness 0.25 mm; Merck, Darmstadt, G.F.R.) were used. The samples (10 μg of each catechol) were applied on a line 1.5 cm from the bottom of the concentrating zones the first spot being 2 cm from the side of the plate and the following nine spots at 1.7-cm intervals. The other experimental conditions were as used earlier[3].

Solvent systems

Screening of 20 different solvents or solvent mixtures was carried out in order to find those which give the sharpest spots and the most reasonable R_F values. The compositions (by volume) of the ten most suitable solvent systems were as follows:

(1) Benzene–acetone–acetic acid (80:15:5)
(2) Light petroleum (b.p. 40–60°C) acetone–acetic acid (70:25:5)
(3) Light petroleum (b.p. 40–60°C)–ethyl acetate–acetic acid (80:15:5)
(4) *n*-Hexane–ethyl acetate–acetic acid (70:25:5)
(5) Benzene–chloroform–acetic acid (60:30:10)
(6) Benzene–ethanol–acetic acid (85:10:5)
(7) Chloroform–ethyl acetate–acetic acid (80:15:5)
(8) Chloroform–diethyl ether–acetic acid (85:10:5)
(9) Benzene–acetic acid (85:15)
(10) Chloroform–acetic acid (80:20)

RESULTS AND DISCUSSION

Colour reactions

The colours of the spots were compared 1 h, 24 h and 10 days after spraying the plates with a chromogenic reagent. The colours of the spots are given in Table I.

When the plates with a concentrating zone and solvent mixtures containing

TABLE I

CHARACTERISTIC COLOUR REACTIONS OF CATECHOL (I) AND CHLORINATED CATECHOLS (II–X) DIFFERENT TIMES AFTER SPRAYING TLC PLATES WITH A 2% SOLUTION OF 3,5-DICHLORO-*p*-BENZOQUINONECHLORIMINE IN TOLUENE

Amount of each compound applied: 10 μg.

Compound	*Acidic developing solvent*		
	1 h	*24 h*	*10 days*
I	Red-violet	Brown	Grey-brown
II	Violet	Brown	Grey-brown
III	Red-violet	Brown	Grey-brown
IV	Grey-violet	Brown	Grey-brown
V	Grey-brown	Violet-brown	Grey-brown
VI	Violet-blue	Violet-black	Grey-brown
VII	Yellow-brown	Red-brown	Grey-brown
VIII	Pale green	Pale grey	Pale grey
IX	Pale green	Pale grey	Pale grey
X	Pale green	Pale grey	Pale grey

acetic acid were used, almost all of the compounds studied formed sharp spots. Only with a few solvent mixtures did the spots of trichlorocatechols (VIII and IX) and tetrachlorocatechol (X) show tailing.

One hour after spraying, the spots of II–VII had different colours but, for example after 10 h, their spots were brownish. On the other hand, the spots of VIII–X were much lighter than those of the other compounds studied and their colours changed from greenish to pale grey.

R_F values

The R_F values of the spots are given in Table II. The average R_F values ($\bar{R_F}$), the standard deviations (s) and the averages of the relative differences ($\bar{x}$), Σx values

TABLE II

R_F VALUES OF CATECHOL (I) AND CHLORINATED CATECHOLS (II–X) ON A SILICA GEL 60 LAYER WITH DIFFERENT SOLVENT SYSTEMS

Solvent system	*Compound*										*Development time (min)*
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	
1	0.35	0.36	0.35	0.35	0.37	0.42	0.34	0.32	0.36	0.34	40
2	0.40	0.42	0.42	0.41	0.47	0.48	0.44	0.41	0.44	0.39	35
3	0.20	0.22	0.18	0.18	0.23	0.29	0.18	0.18	0.25	0.20	35
4	0.28	0.29	0.27	0.25	0.29	0.35	0.25	0.24	0.29	0.26	40
5	0.14	0.22	0.14	0.19	0.20	0.28	0.13	0.15	0.22	0.20	50
6	0.42	0.43	0.42	0.42	0.42	0.45	0.40	0.38	0.42	0.39	50
7	0.37	0.40	0.36	0.36	0.40	0.44	0.35	0.33	0.37	0.35	60
8	0.37	0.39	0.37	0.37	0.39	0.46	0.35	0.32	0.35	0.34	60
9	0.21	0.32	0.22	0.28	0.28	0.39	0.18	0.23	0.33	0.30	40
10	0.45	0.53	0.43	0.49	0.50	0.61	0.42	0.42	0.50	0.48	60

TABLE III

AVERAGE R_F VALUES ($\bar{R}_F$), STANDARD DEVIATIONS (s) OF THE R_F VALUES, THE SUMS (Σx) AND THE AVERAGES ($\bar{x}$) BETWEEN THE R_F VALUES AND THE NUMBER OF ZERO VALUES IN EACH x_{ij} MATRIX OF I–X ON A SILICA GEL 60 LAYER WITH DIFFERENT SOLVENT SYSTEMS

The value of each element, x, in x_{ij} matrixes is calculated by dividing the difference of two R_F values by their average.

Solvent system	$\bar{R}_F$	s	Σx	$\bar{x}$	*Number of* x_{ij} *(= 0.000)*
6	0.42	0.020	2.447	0.054	10
1	0.36	0.026	3.403	0.076	5
2	0.43	0.029	3.558	0.079	3
7	0.37	0.032	4.292	0.095	4
8	0.37	0.038	4.918	0.109	5
4	0.28	0.032	5.519	0.123	4
10	0.48	0.059	6.053	0.135	2
3	0.21	0.037	8.350	0.186	7
9	0.27	0.064	12.595	0.280	1
5	0.19	0.047	13.016	0.289	3

and the number of zero values in each x_{ij} matrix are presented in Table III.

With solvents 1, 2, 6 and 7 the variations in the R_F values in each run were small (small s, Σx and $\bar{x}$ values) (see Table III). With these solvent systems a relatively high elution power was also observed ($\bar{x}$ = 0.36–0.43) and thus they are suitable for the group separation of I–X. The smallest s (0.020), Σx (2.447) and $\bar{x}$ (0.054) and the highest number of the zero values in the x_{ij} matrixes (see Table III) were observed for benzene–ethanol–acetic acid (85:10:5) (solvent 6). Hence, this solvent mixture is the most suitable for the separation of this group from its mixtures with other compounds[5]. Benzene–acetone–acetic acid (80:15:5) (solvent 1) and light petroleum (b.p. 40–60°C)–acetone–acetic acid (70:25:5) (solvent 2) would also be useful for this purpose, although the number of zero values in the x_{ij} matrixes for these solvents are relatively small.

The highest variations in the R_F values were found for solvents 5 and 9 (see Table III). The Σx and $\bar{x}$ values of these systems are nearly as high but the absolute separation power is clearly different. Hence, benzene–acetic acid (85:15) (solvent 9), which gives the highest s value (0.064), can be recommended for the separation of the individual components.

None of the solvent systems studied is suitable for the separation of all of the individual components (I–X) by one-dimensional TLC.

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Note

Improved method of separating and quantitating hemoglobin compositions by isoelectric focusing

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Isoelectric focusing on polyacrylamide gels is a popular analytical tool for separating and identifying many different component mixtures[1–4]. The many advantages and some disadvantages of this technique have been described in detail elsewhere[1]. One of these disadvantages is that after completing the electrophoresis, the polyacrylamide gels must be removed from the gel tubes. The gels are either placed in special 10-cm cuvettes for scanning in a spectrophotometer or they may be fixed and stained prior to scanning. We have found that removing isoelectric focusing gels from the tubes is sometimes difficult, especially when using "soft" gels. Cutting gels to fit into the cuvette is also not very precise. Quantitation of the amount of materials in the polyacrylamide gels is determined by measuring the area under each peak of the resultant chromatogram.

In this communication, we describe a simple, rapid method of running long polyacrylamide isoelectric focusing gels and quantitating the amounts of materials present without removing the gels from the tubes. Another advantage of this method is that we have increased the width of the pH gradient over the gels and thus improved the separation of the hemoglobins.

EXPERIMENTAL

Equipment. The isoelectric focusing equipment used was the standard cylindrical unit for gel tubes. To accomodate the longer gel tubes used, new, lower buffer chambers were built. These chambers were made of plastic cylinders cemented to a square base with the appropriate polymer solvent. The cylinder was 7 3/4 in. × 5 in. O.D. × 4 1/2 in. I.D. The base was 6 in. × 6 in. × 1/2 in.

The power supplies used were either a Büchler Model 3-1014A or a Hewlett-Packard Model 6521A. Measurements of pH were made on a Radiometer Model 26 pH meter.

Chemicals. Carrier ampholytes were obtained from LKB (Stockholm, Sweden). We used Ampholine pH 6–8, batch numbers 25, 26, and 27. Electrophoresis-grade acrylamide and N,N′-methylene bisacrylamide were purchased from Eastman (Rochester, NY, U.S.A.). All other chemicals were analytical-reagent grade from various sources.

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Methods. Two electrophoresis gel tubes, 10 cm × 8 mm O.D. × 5 mm I.D., were connected by a 1.25-cm piece of 1/4 in. × 1/16 in. Tygon tubing. The bottom of the joined tubes were covered with parafilm and then inserted into the electrophoresis apparatus. A stock solution of 6.67% acrylamide containing 0.33% bisacrylamide was made and stored refrigerated for up to a month. A 45-ml volume of this stock solution was placed in a 50-ml graduated cylinder to which 2.5 ml of the ampholine, 25 μl of N,N,N′,N′-tetramethylethylenediamine (TEMED) and water were added to a total volume of 50 ml. A 25-mg amount of ammonium persulfate was placed in a 125-ml erlenmeyer flask to which the 50 ml of solution was added and swirled. This solution was rapidly transferred with a Pasteur pipette into the 20-cm gel tubes. Water was layered over the top and the gels were allowed to polymerize for 1 h. The upper reservoir buffer was 0.5% monoethanolamine and the lower reservoir buffer was 0.2% sulphuric acid. The gels were pre-electrophoresed at 200 V for 1 h and the buffers were discarded. Samples (50–100 μl in 15% sucrose) were layered onto the top of the gel. A 5% sucrose solution was layered over the top of the samples to fill the tubes. New buffers were put into the cathode and anode compartments. The electrophoresis was started at 200 V for 1/2 h, then 300 V for the next 1/2 h and finally set at 400 V for 17 h. The power supply was set on constant voltage. As the electrophoresis progressed, the current gradually decreased. All electrophoresis experiments were performed in the cold room at 4°C.

At the end of each run the pH gradient was determined on one of the gels. The gel was cut into 1-cm slices and soaked in 1 ml of deionized, distilled water. The 20-cm tubes which contained samples were cut with a razor blade at the junction between the two tubes. The tubing that connected the two tubes was completely removed and each of the two 10-cm electrophoresis tubes and gels was placed directly into the Gillford Model 240 gel scanner cuvette holder. The tubes were scanned at 2 cm/min at 540 nm and the absorbance was recorded on a strip chart recorder.

RESULTS

A representative sample of the pH distribution along the gels is shown in Fig. 1. By extending the length of the gel, we have expanded the pH gradient, especially in the pH 7–8 region which works well for hemoglobin separations. A typical hemo-

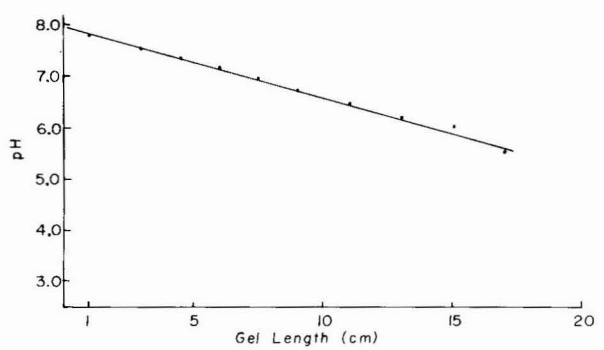

Fig. 1. pH Gradient using LKB ampholyte pH 6–8. Gel length is measured from the bottom of the meniscus.

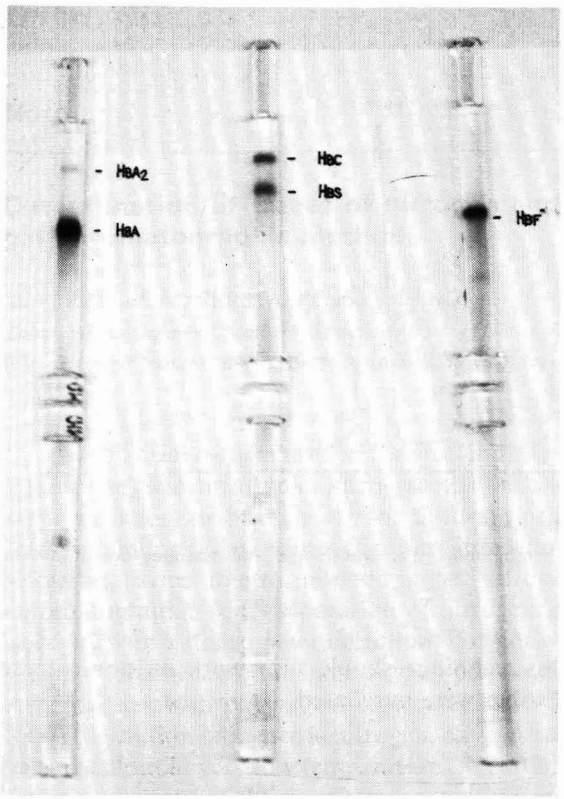

Fig. 2. The separation of human hemoglobins is shown. Note that two 10-cm gel tubes are connected by a short piece of tubing. The two tubes are removed from the Tygon tubing for quantitation of the amounts of material present.

globin separation is shown in Fig. 2. Here we have shown the separation of human hemoglobin into its major and minor components. In Fig. 3 we show the scan of the separation through the gel tube. The hemoglobin zones are separated readily and quantitated by calculating the area under each peak of the chromatogram.

DISCUSSION

We have modified the widely used technique of analytical isoelectric focusing on polyacrylamide gels to make quantitation of the separate zones more rapid and convenient. This has been accomplished by joining two 10-cm gel tubes together. Each tube exactly fits into the cuvette holder of the Gilford spectrophotometer scanning attachment. We have found that by lengthening the tube, we get a better pH gradient and hence a better separation of the hemoglobin components. Connecting more 10-cm tubes to give a 30- or 40-cm tube would be practical and feasable. By building a taller anode compartment, even longer gel tubes could be used. The advantage to connecting two or more 10-cm tubes together by tubing is that each of the pieces fit directly into the gel scanner, thus eliminating the need for removal of the gel

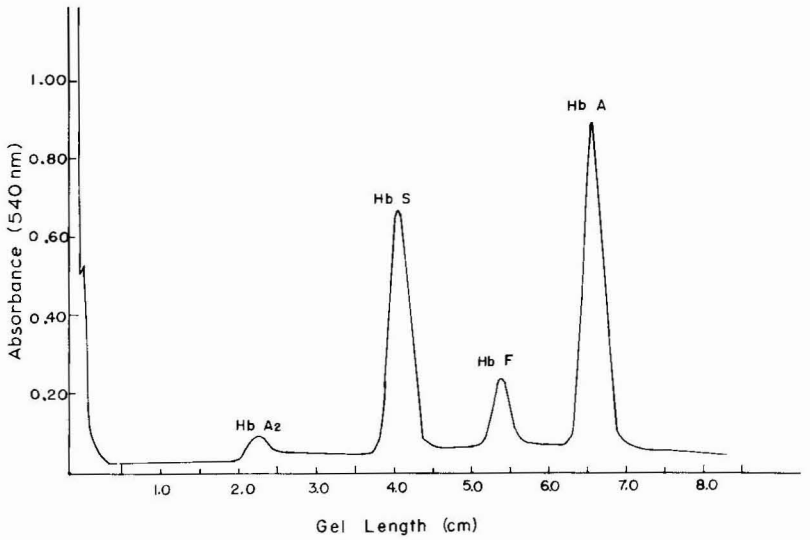

Fig. 3. Isoelectric fractionation of human hemoglobins. The wavelength and scanning conditions are described in the *Methods* section.

from the tube for quantitation. This technique should also work on non-colored proteins in the UV region if special tubes were purchased.

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Note

Determination of traces of nitrogen and argon in oxygen by a simple gas chromatographic method

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Industrial oxygen used in the steel industry may contain traces of nitrogen and argon. The concentration of nitrogen must be below 1000 ppm otherwise the steel can show weakness or brittleness. The accurate determination of the nitrogen concentration in industrial oxygen is therefore important. For other purposes it may also be interesting to be able to measure higher nitrogen concentrations in O_2. Direct gas chromatographic (GC) separation of nitrogen and oxygen is possible on molecular sieve 5A with katharometer detection, but not when the nitrogen concentration is at the ppm level because the huge oxygen peak overlaps the later eluting nitrogen peak. Argon elutes with oxygen on molecular sieve 5A. On Chromosorb 102 (pellicular SiO_2) the elution sequence is nitrogen, oxygen and then argon. This separation has to be carried out at very low temperature (-78°C). This is not very practical, especially for routine analyses. At room temperature, nitrogen and oxygen are not separated on Chromosorb 102. Gas analysis in general was thoroughly discussed in a Varian special edition by Thompson[1]. The special case of the determination of nitrogen and argon was described in 1959[2], and more recently by Karlsson[3] using hydrogen as carrier with BTS catalyst and molecular sieve 13X as packing material. We find that the conditions for introducing the sample are critical and must be described more clearly, and that other column materials are preferable. We also prefer helium as carrier gas because with hydrogen the water uptake of the separating column changes retention times (as mentioned by Karlsson) and thus adversely affects reproducibility of peak area measurements with the katharometer detection used.

EXPERIMENTAL

The GC instrument was a Varian 1400 fitted with a micro katharometer. The detailed set-up for the analytical method to determine nitrogen and argon is shown in Fig. 1.

Several oxygen-absorbing chemicals were evaluated. Some commercial materials had batches with good performance but some of the same brand failed to absorb all the oxygen in the samples. RSL-Fixox is a material specially selected for the purpose of the described analysis (RSL, Eke, Belgium or Alltech, Deerfield, IL, U.S.A.). To activate the RSL-Fixox, the column is mounted in the GC oven and connected to an empty stainless steel column led out of the oven. The oven tempera-

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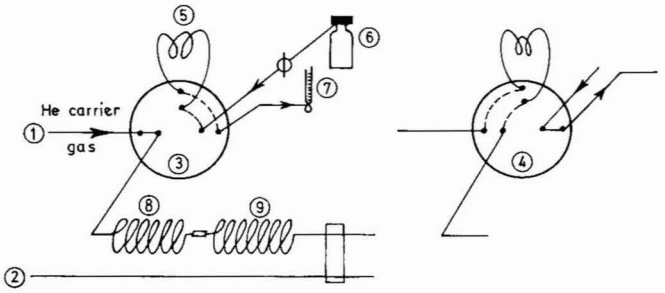

Fig. 1. Instrumental set-up for trace analysis of N_2 and Ar in O_2. Varian 1400 gas chromatograph with micro katharometer. 1 = Helium carrier gas at a flow-rate of 18 ml/min; 2 = helium reference gas at a flow-rate of 18 ml/min; 3 = six-port Valco 7000 HPLC sample loop injector in load position; 4 = six-port Valco 7000 sample loop injector in injection position; 5 = sample loop (*ca.* 2 ml) in stainless steel of *ca.* 50–60 cm × 3.2 mm O.D.; 6 = lecture bottle of calibration gas or unknown; 7 = soap bubble flow-meter; 8 = stainless-steel column (1 m × 0.62 cm I.D.) filled with RSL-Fixox; 9 = stainless-steel column (3 m × 0.62 cm I.D.) filled with molecular sieve 5A.

ture is raised to 250°C and hydrogen is passed through for *ca.* 2 h at 30 ml/min. Water vapour is a reaction product. The end of activation can be ascertained accurately when no more water is eluted (mirror test!). RSL-Fixox shrinks somewhat during this activation step and it is generally necessary at this stage to add some packing to fill the column completely. The oxygen-absorbing column has to be regenerated in the same way when the Ar/N_2 peak ratio of the reference gas sample starts to increase. The advocated RSL-Fixox column (100 × 0.62 cm I.D.) can absorb *ca.* 0.5–0.6 g of oxygen. Regeneration is however needed after every 20 analyses in the conditions as described.

For reproducible results it is important to rinse the sample loop sufficiently (*e.g.* 30 sec at 50 ml/min) and to keep the sample gas rate constant.

To fill the sampling gas pressure cylinders they were coupled via a three-way valve to a gas pressure line. Evacuating and filling the cylinder three times by appropriately manipulating the valve then ensures correct sampling. A minimum pressure of 0.2 kg/cm^2 was required in our case.

The separation of nitrogen and argon is better at room temperature than at 30°C. We prefer the latter temperature, however, because this is easier to control in a closed oven with a heated detector. In the Varian 1400 with micro katharometer, used for the present work, 30°C is obtained normally with the oven fan on and the detector at 230°C.

RESULTS AND DISCUSSION

To determine nitrogen and argon concentrations a reference gas sample —in our case 99.7% O_2, 0.2% Ar (2000 ppm) and 0.1% N_2 (1000 ppm)— is injected three times, and then the unknown sample is also injected three times. The peak heights or peak areas are measured (manually or with an electronic integrator) and the amounts of nitrogen and argon in the unknown are calculated. The Ar/N_2 peak ratio can vary as a function of the current intensity through the bridge. Best results on the Varian 1400 with micro katharometer were obtained with a bridge current of 250 mA.

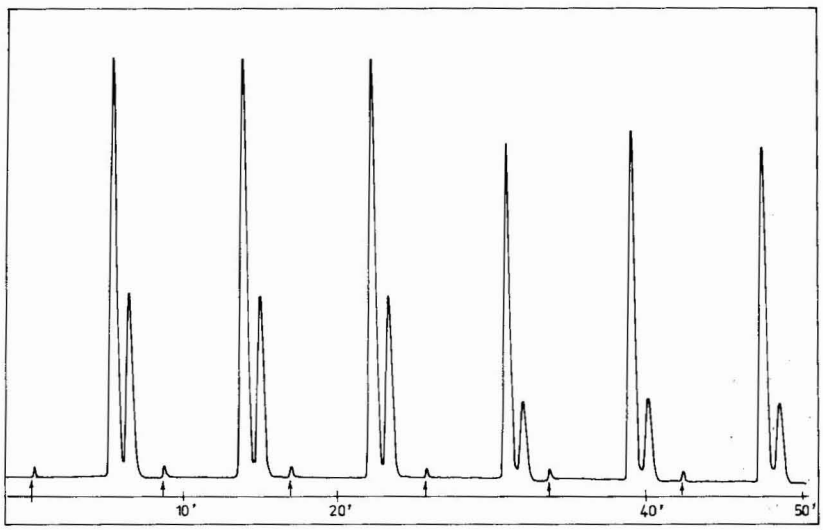

Fig. 2. Chromatograms obtained with calibration gas (first three injections) and with an unknown (second group of three) at 30°C. First peak Ar, second peak N_2. Total time 50 min.

An example of such an analysis is shown in Fig. 2. Typical results based on peak height measurements are as follows:

Calibration gas. Ar: 137.5; 137.2; 137.2; $\bar{x}$, 137.3; σ, 0.12%; N_2: 59.5; 59.7; 60.2; $\bar{x}$, 59.8; σ, 0.60%.

Unknown gas sample. Ar: 107.0; 109.0; 107.0; $\bar{x}$, 107.7; σ, 1.07%. Result: 1583 ppm; N_2: 26.5; 27.0; 26.2; $\bar{x}$, 26.6; σ, 1.52%. Result: 461 ppm.

The standard deviation can easily be kept below 1.5%. It is obvious that the same method could be used to determine nitrogen and argon in other oxygen-containing samples, such as air.

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Note

Determination of butyltin species in water by gas chromatography with flame photometric detection

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Organotin compounds are used in three major ways, *viz.*, as thermal stabilizers for polyvinyl chloride, as catalysts in the production of polyurethane foams and as biocides[1]. The increasing annual usage of organotins raises the possibility of environmental pollution. Organotins are a class of compounds about which more information is sought under Canada's Environmental Contaminants Act[2] regarding toxicology and environmental fate. We chose to examine the aquatic environmental fate of bis(tri-*n*-butyltin) oxide (TBTO), and this article describes the method we have established for the determination of TBTO and some of its possible[1] de-alkylation products in natural waters.

Our method is a refinement of an earlier method[3] which involved (1) extraction of TBTO, Bu_2Sn^{2+}*, $BuSn^{3+}$ and Sn^{4+} from water, (2) derivatization with a methyl Grignard reagent to form the various $Bu_nMe_{4-n}Sn$ species, and (3) analysis by gas chromatography–mass spectrometry. This method is suitable for the parent TBTO; however, it is not suitable for Bu_2Sn^{2+}, $BuSn^{3+}$ and Sn^{4+} since the derivatives Bu_2Me_2Sn, $BuMe_3Sn$ and Me_4Sn are fairly volatile compared with solvents such as hexane and benzene, and appreciable quantities of the derivatives are lost during routine concentration procedures such as "rotary" and "vortex" evaporating of solvents. The same problem is encountered with (1) the series of ethyl derivatives, $Bu_nEt_{4-n}Sn$ when $n \leq 2$, and (2) the series of *n*-propyl derivatives, $Bu_nPr_{4-n}Sn$ when $n \leq 2$ (ref. 4).

Our approach is to make *n*-pentyl derivatives of TBTO and its de-alkylation products. The species $Bu_nPe_{4-n}Sn$ are all sufficiently non-volatile compared with hexane or benzene that none are lost in solvent "stripping", yet they are volatile enough to be analyzed by gas chromatography (GC). We used a modified flame photometric detector which has been shown to be sensitive to organotins[5–9].

EXPERIMENTAL

Materials

TBTO (97%), tetrabutyltin (97%), dibutyltin dichloride (96.5%), butyltin trichloride (95%), tin (99.99%), 48% HBr and *n*-pentylmagnesium bromide in diethyl

* Bu = *n*-butyl, Pe = *n*-pentyl, Me = methyl, Et = ethyl, Pr = *n*-propyl.

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ether were obtained from Ventron (Danvers, MA, U.S.A.); 2-hydroxy-2,4,6-cycloheptatrien-1-one (tropolone) was from Aldrich (Milwaukee, WI, U.S.A.), and was recrystallized from diethyl ether before use (m.p. 52°C, uncorr.). All organic solvents were pesticide grade from Caledon Labs., Georgetown, Ontario, Canada.

Preparation of $Bu_nPe_{4-n}Sn$ standards

$Bu_nPe_{4-n}Sn$ species where $n \geqslant 1$ were prepared according to the method of Meinema *et al.*[3] except that the derivatization was carried out under refluxing conditions. The derivatives were purified by passage through a 1 m × 2 cm I.D. column of 5% water-deactivated Florisil, with hexane as eluent. Fractions of 5 ml were collected and the presence of organotins confirmed by thin-layer chromatography with dithizone developer[3]. Appropriate fractions were pooled, dried with magnesium sulfate and stripped of solvent; standard solutions of the derivatives in hexane were prepared by weighing and subsequent decadic dilution. All standards were ≥98% pure by GC with electron capture (ECD), flame ionization (FID) and flame photometric detectors (FPD); the standards were stable in the dark at room temperature for at least six months.

For the synthesis of Pe_4Sn, solutions of Sn^{4+} were prepared by dissolution of Sn in hot concentrated HCl and subsequent dilution to an appropriate volume such that the final concentration of HCl was 10% (v/v). These aqueous solutions were extracted with freshly prepared solutions of 1% tropolone in benzene. This procedure ensured quantitative (100 ± 3%) extraction of Sn^{4+} from the aqueous phase as shown by the pyrocatechol violet assay for Sn[10]. The organic phase was then treated with Grignard reagent and Pe_4Sn was purified as described above.

Analysis for butyltins in water

Twenty-five ml of 10-ppm solutions of TBTO (Bu_3Sn^+) and its debutylated metabolites Bu_2Sn^{2+} (as dibutyltin dichloride), $BuSn^{3+}$ (as butyltin trichloride) and (acidified) Sn^{4+} in distilled water, tap water or Hamilton Harbour (Lake Ontario) water were acidified with 25 ml of 48% HBr. The acidified solutions were extracted (2 × 25 ml) with freshly prepared solutions of 1% tropolone in benzene, and the extracts were derivatized as described above. The analyses of each of the organotins, and of solutions of all the organotins together, were done five times to check the efficiency of extraction from water and the yields of the derivatization reactions.

The $Bu_nPe_{4-n}Sn$ derivatives were detected with a Tracor 550 gas chromatograph equipped with a Melpar FPD and a Spectra-Physics 4000 integrator. The FPD was modified in three ways: (i) for maximum photomultiplier response to tin, the interference filter was replaced with a round sheet of metal foil[5] which had a 0.5 cm diameter hole half way between the center and the top of the foil, (ii) the detector inlet ports for hydrogen and air were reversed to avoid solvent flameout[11] with no change in sensitivity or linearity of response and (iii) copper tubing was wound around the photomultiplier tube to allow thermostating at 25°C. A 2 m × 2 mm I.D. glass column containing 3% OV-225 on Chromosorb WHP (80–100 mesh) was used under the following conditions: inlet and outlet temp., 220°C; detector temp., 240°C; nitrogen carrier, hydrogen and air flow-rates of 25, 100 and 80 ml/min, respectively (these flow-rates were optimal for the FPD in the reversed configuration[11], and a separate supply of oxygen was not required). Optimal column temperatures were

TABLE I

ANALYSES OF BUTYLTINS IN WATER BY FPD

Species	*% Extraction from water*			*% Yield of derivatization*	*Minimum detectable amount (pg)*	*Linear response range (pg)*
	Distilled	*Tap*	*Hamilton Harbour*			
Bu_3Sn^+	98 ± 5	101 ± 6	96 ± 4	100 ± 3	1.5×10^2	1.5×10^2–3.0×10^4
Bu_2Sn^{2+}	100 ± 3	95 ± 7	97 ± 6	96 ± 6	1.2×10^2	1.2×10^2–3.0×10^4
$BuSn^{3+}$	96 ± 8	99 ± 5	103 ± 8	95 ± 7	1.0×10^2	1.0×10^2–2.0×10^4
Sn^{4+}	94 ± 9	104 ± 6	98 ± 7	99 ± 4	6.0×10^1	6.0×10^1–2.5×10^4

Initial organotin concentration in water, 10 ppm.

135°C for Bu_3PeSn, 145°C for Bu_2Pe_2Sn, 155°C for $BuPe_3Sn$ and 165°C for Pe_4Sn. Ten aliquots of each butyltin solution, at each concentration tested, were injected to check for (i) reproducibility of injections, (ii) active sites on the column which would necessitate "priming" and (iii) detector poisoning.

Determinations of the purity of the $Bu_nPe_{4-n}Sn$ derivatives were also made with an ECD and an FID mounted on the same gas chromatograph, and under the same general conditions. Mass spectra of the derivatives were obtained with a Finnigan 3200 gas chromatograph–mass spectrometer equipped with a Ribermag 1000 data system.

RESULTS AND DISCUSSION

Table I shows the results of the butyltin analyses. The extractions are quantitative no matter the source of the water. Extractions of each butyltin and Sn^{4+} from aqueous solutions of all four species together were also quantitative. It is important to prepare the tropolone–benzene solution immediately before extraction of the aqueous phase (we recommend less than 5 min). We have noted, for example, that a tropolone–benzene solution prepared 18 h earlier extracted only 20% of Sn^{4+} from a 10-ppm solution. We have also noted that poor extraction efficiencies for Sn^{4+} were obtained if the tropolone were added to the aqueous phase before extraction with benzene, rather than the reverse. Not all the butyltins required tropolone for extraction. For example, TBTO can be quantitatively extracted from water into benzene or hexane alone; however, in the interests of documenting a general method we recommend the use of tropolone for all butyltin species.

The yields of the derivatization reactions were also quantitative, even in the case in which all four tin species are together. TBTO can be quantitatively pentylated at room temperature, but $BuSn^{3+}$ and Sn^{4+} require refluxing for quantitative reaction.

Fig. 1 shows a chromatogram of all four derivatives. The minimum detectable amounts injected were about 100 pg with linear responses over concentration ranges of at least 100. These detection limits could probably be improved significantly with different instrumentation; for example, using a modified version of a newer commercially available FPD, Aue and Flinn[9] were recently able to detect as little as 0.04 pg of

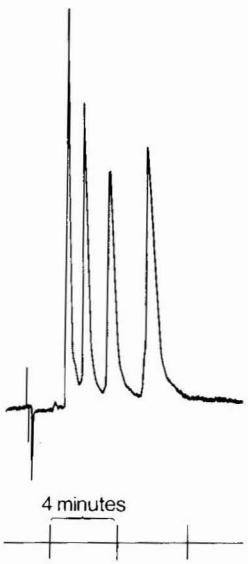

Fig. 1. Isothermal chromatogram (135°C) of about 4 ng each of, in order of elution, Bu_3PeSn, Bu_2Pe_2Sn, $BuPe_3Sn$ and Pe_4Sn. FPD conditions as described in the text.

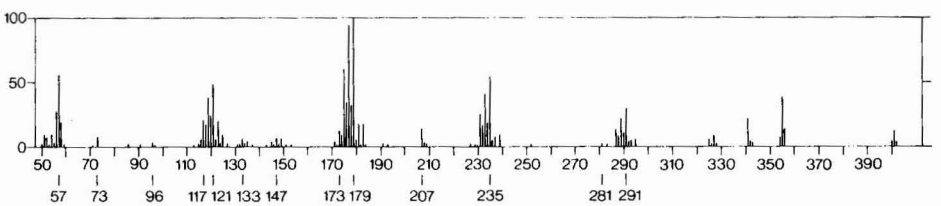

Fig. 2. Mass spectrum of tetrabutyltin, Bu_4Sn.

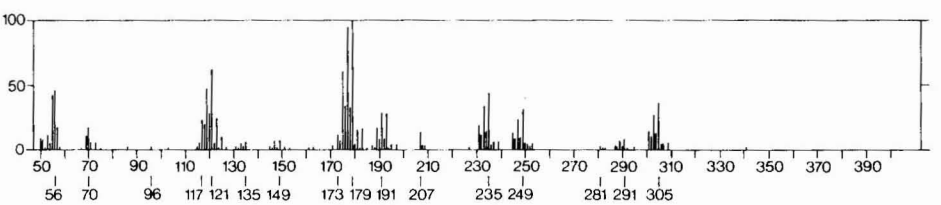

Fig. 3. Mass spectrum of tributylpentyltin, Bu_3PeSn.

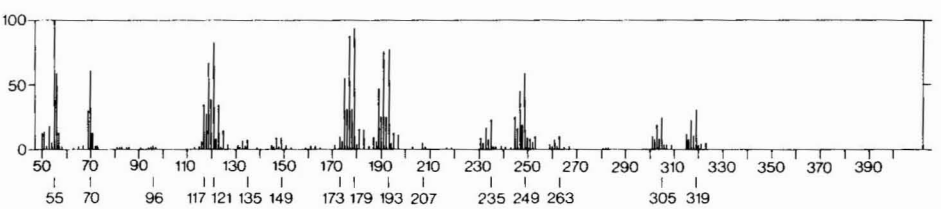

Fig. 4. Mass spectrum of dibutyldipentyltin, Bu_2Pe_2Sn.

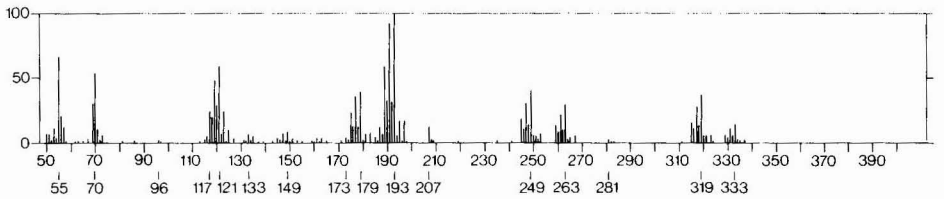

Fig. 5. Mass spectrum of butyltripentyltin, $BuPe_3Sn$.

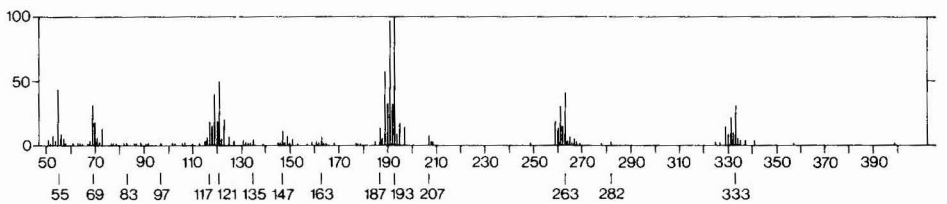

Fig. 6. Mass spectrum of tetrapentyltin, Pe_4Sn.

tetrapropyltin. Our FPD can be poisoned by injections of more than 10^2 ng, but does recover slowly with time. Tailing of organotin peaks such as seen in Fig. 1 has been ascribed to the detector rather than the column[6]; our results support this contention in that priming the OV-225 column with $Bu_nPe_{4-n}Sn$ standards did not reduce the tailing. The reproducibility of peak area with multiple injections was excellent.

Figs. 2–6 are mass spectra of the four derivatives and tetrabutyltin which is shown for comparison. Mass spectra could be obtained with about 25 ng of derivative. The typical tin cluster is evident as are losses of butyl and pentyl groups, but the striking feature is the disappearance of the cluster centered at *m/e* 179 ($BuSnH_2^+$) and the appearance of the cluster centered at *m/e* 193 ($PeSnH_2^+$) as *n* decreases in the series $Bu_nPe_{4-n}Sn$.

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Note

Identification of carbamate derivatives formed during chloroform extraction of tricyclic antidepressants from urine

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During the isolation and identification procedure of octriptyline metabolites from monkey urine, two metabolites were subsequently identified as the methoxy and ethoxy carbamate derivatives. As unknown metabolites they were chromatographed in a series of thin-layer chromatographic (TLC) systems and then analyzed by gas chromatography–mass spectrometry (GC–MS) using the GC conditions given in Table I. The mass spectrum of the methoxy carbamate had a base peak at m/z 102, characteristic peaks at m/z 244, 231, 216, 117 and 44, and a small molecular ion at m/z 333. The ethoxy carbamate spectrum had a base peak at m/z 116, characteristic peaks at m/z 244, 231, 216, 102 and 44, and a molecular ion at m/z 347.

TABLE I

RETENTION TIME OF METHOXY AND ETHOXY CARBAMATE DERIVATIVES OF OCTRIPTYLINE

Sample	*GC conditions*	*Retention time (min)*
Methoxy carbamate	1.83 m × 2 mm I.D. 1.5% OV-17 column programmed from 180 to 285°C at 7°C/min	11.6
Ethoxy carbamate	1.83 m × 2 mm I.D. 1.5% OV-17 column programmed from 190 to 250°C at 5°C/min	13.7

The methoxy and ethoxy carbamate derivatives of octriptyline were synthesized and their mass spectra, GC retention times, characteristic proton nuclear magnetic resonance (^{1}H NMR) peaks, and carbonyl infrared (IR) absorption bands were all identical to those of the isolated derivative.

Sheehan and Haythorn[1] reported that nortriptyline and desipramine were chemically altered under various urinary extraction conditions. An ion at m/z 116 was found to be the base peak and second largest in the mass spectra of nortriptyline and

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desipramide derivatives, respectively. This appeared very similar to the ethoxy carbamate which we had isolated. Our compound originated from a secondary N-alkylmethylamine. The only similarity in our procedures and that of Sheehan and Haythorn was the chloroform used to extract urine. We postulated that phosgene in chloroform was responsible for forming the carbamate derivatives. To test this, both octriptyline and nortriptyline were extracted from aqueous solutions using the procedure of Sheehan and Haythorn. Each was extracted with chloroform containing ethanol preservative and with chloroform not containing ethanol preservative. Subsequent TLC (ethyl acetate–methanol–ammonium hydroxide, 17:2:1) showed ultraviolet light sensitive spots having the same R_F values as authentic nortriptyline and octriptyline. However, the extracts using chloroform without ethanol preservative had additional ultraviolet light sensitive spots near the solvent front. The silica gel from one of these zones was extracted with methanol and a GC–MS profile obtained. The methoxy carbamate derivatives of both octriptyline and nortriptyline were identified.

Our studies and those of Sheehan and Haythorn show that derivatives of tricyclic antidepressants can be formed under various extraction procedures. Our studies further suggest that the derivatives are carbamates formed during chloroform extraction from phosgene which can be present in chloroform if the ethanol preservative is missing. When possible, chloroform extraction of tricyclic antidepressants should be avoided.

MATERIALS AND METHODS

The derivatives were isolated as part of an identification procedure for metabolites in monkey urine[2]. The monkeys had been orally dosed with an aqueous solution of [^{14}C]octriptyline (specific activity 19.4 μCi/mg). The ethoxy carbamate was isolated from a chloroform extraction of urine at pH 5. The urine had been treated previously with glucuronidase/sulfatase (Calbiochem, Los Angeles, CA, U.S.A.) at pH 5 and 37°C for 24 h. The chloroform extract was evaporated to dryness and the extract residue was placed on a 5-g silica gel column in chloroform. The ethoxy carbamate was eluted from the column with chloroform.

The methoxy carbamate was isolated from other monkey urine which also was treated previously with glucuronidase/sulfatase at pH 5. This mixture was placed on an Amberlite XAD-2 column, and then eluted with methanol. The methanol extract was evaporated to dryness and chromatographed on a silica gel column. The methoxy carbamate was eluted with methanol–chloroform (5:95).

Thin-layer chromatography was on silica gel (250 μm, HF; Woelm, Eschwege, G.F.R.) plates. The plates were then scanned for the presence of radioactivity zones (Actigraph III, Searle Analytic), which were scraped and the silica gel eluted with methanol.

GC (Searle Analytic) analysis was performed on a 1.83 m × 2 mm I.D. 1.5% OV-17 column which was temperature programmed.

GC–MS analyses (Kratos-AEI MS-30 mass spectrometer) were performed at 20 eV with a source temperature of 180°C and molecular separator temperature of 206°C.

The nuclear magnetic resonance spectra were determined in [$^2H_4$]methanol

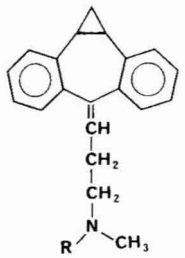

Fig. 1. Structures of octriptyline and ethoxy and methoxy carbamate derivatives. R = H: octriptyline (Ia); R = $\overset{O}{\overset{\|}{C}}$–O–$CH_2$–$CH_3$ ethoxy carbamate (IIa, *syn*; IIb, *anti*); R = $\overset{O}{\overset{\|}{C}}$–O–$CH_3$ methoxy carbamate (IIIa, *syn*; IIIb, *anti*). *Syn* conformer has cyclopropane and *exo*-propylidine amino group on same side of 7-membered ring. *Anti* conformer has them on opposite sides.

using a Varian XL-100-15 instrument, and the infrared absorption spectra were determined in chloroform solution using a Beckman IR-12.

SYNTHESIS

The synthesis of ethyl[3-(1a,10b-dihydrodibenzo[*a*,*e*]cyclopropa[*c*]cycloheptan-6(1H)-ylidene)propyl]ethylcarbamate (II, Fig. 1) is as follows. To a solution of octriptyline (free base, 3.0 g, 9.3 mmole; I) in 50 ml of chloroform and 1.4 ml of triethylamine was added ethylchloroformate (1.14 g, 10.5 mmole). After 15 min, the solution was washed successively with water, dilute hydrochloric acid and saturated sodium bicarbonate. The chloroform solution was dried over magnesium sulfate and solvents were removed. The residual oil was distilled to yield 700 mg of a 70:30 mixture of conformers IIa and IIb at 203°C (0.06 mmHg). IR($CHCl_3$) 1695, 1602 cm^{-1}. NMR (C^2HCl_3, ppm), 7.17 (8H, m), 5.60 (1H, t, J = 7.5 Hz, IIa), 5.77 (1H, t, J = 7 Hz, IIb), 4.10 (2H, q, J = 7 Hz, IIa), 4.03 (2H, q, J = 7 Hz, IIb), 3.38 (2H, t, N-CH_2), 2.83 (3H, s), 2.6–2.2 (5H, cmplx band, allylic-CH_2 and 3 cyclopropyl protons, IIa and IIb), 1.4 (1H, two m, IIa and IIb), 1.18 [^3H, t, J = 7, $CH_3(CH_2O)$], 0.63 (1H, td, J = 5.5, 3.5 Hz). NMR ($C^2H_3O^2H$) 5.55, 4.06, and 2.80 correspond to the 5.60, 4.10, and 2.83 signals in C^2HCl_3 spectrum, otherwise identical. Mass spectrum, M^+ 347, 0.5% of base. Analysis calculated for $C_{23}H_{25}NO_2$:C, 79.50; H, 7.25; N, 4.03. Found: C, 79.79; H, 7.41; N, 4.01.

The synthesis of methyl[3-(1a,10b-dihydrodibenzo[*a*,*e*]cyclopropa[*c*]cycloheptan-6(1H)-ylidene)propyl]methylcarbamate (III) from octriptyline (3.23 g, 10 mmole) and methylchloroformate (1.16 g, 12.3 mmole) gave 950 mg of IIIa and IIIb at 205°C (0.1 mmHg). IR($CHCl_3$) 1700, 1600 cm^{-1}. NMR (C^2HCl_3) 7.17 (8H, m), 5.72 (1H, t, J = 7.5 Hz, IIIb), 5.58 (1H, t, J = 7.5 Hz, IIIa), 3.65 (3H, s), 3.38 (2H, t), 2.81 (3H, s), 2.6–2.2 (5H, cmplx band), 1.4 (1H, 2m), 0.6 (m, trace). Mass spectrum, M^+ 333, 0.9% of base. Anal. calcd, for $C_{22}H_{23}NO_2$:C, 79.25; H, 6.95; N, 4.20. Found: C, 79.16; H, 7.03; N, 4.15.

The cyclopropylcycloheptane ring has stable *syn* and *anti* conformers which are differentiated by the ^1H NMR spectra. The *syn* conformer (a) exhibits a vinyl

proton resonance upfield from the *anti* conformer (b). Additionally, the *anti* conformer exhibits an upfield cyclopropyl proton which is absent in the *syn* conformer. Octriptyline is the phosphate salt of the *syn* conformer.

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Note

Separation of modified bases and ribonucleosides with cytokinin activity using fused silica capillary gas chromatography

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Derivatives of adenine and adenosine containing various moieties at the 6-amino nitrogen exhibit cytokinin or cell division activity in plant tissue[1,2]. Many of these compounds, both bases and ribonucleosides, have been found free or unbound as natural constituents of plant tissues and microorganisms. Cytokinin ribonucleosides have also been identified as constituents of tRNA from animals, plants, and microorganisms. The closely related structures and sub-micromolar concentrations of these compounds in tissue extracts and tRNA hydrolysates have made them difficult to separate and identify. Among the primary methods used to separate cytokinins have been gas chromatography (GC) using packed columns[3–5] and high-performance liquid chromatography[6–8]. Glass capillary column GC permits high resolution of most classes of volatile compounds; however, there has been very little work on the separation of volatile derivatives of cytokinins with this technique. In a recent study, Claeys *et al.*[9] used a glass capillary column to resolve permethyl derivatives of *cis*- and *trans*-zeatin and isopentenyladenine isolated from bacterial culture media. In the present investigation, we have employed high-resolution GC using a recently developed inert fused silica type capillary column to separate trimethylsilyl (TMS) derivatives of reference compounds representing a range of cytokinin bases and ribonucleosides known to occur as natural products.

EXPERIMENTAL

Reference compounds

trans-Zeatin, isopentenyladenine, isopentenyladenosine and phloretin were purchased from Sigma (St. Louis, MO, U.S.A.)*. Dihydrozeatin was purchased from Calbiochem (La Jolla, CA, U.S.A.). The remaining cytokinins were provided by Dr.

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Preparation of samples

Prior to silylation, compounds (25 μg each) were dried for 24 to 72 h over phosphorus pentoxide at 10^{-2} mmHg, or methylene chloride was added to the sample and then evaporated under a stream of nitrogen to remove water. Both procedures gave satisfactory results. Silylation was carried out at 60°C for 30 to 45 min in sealed reaction vials after adding 10 μl of dry pyridine (Supelco, Bellefonte, PA, U.S.A.) to dissolve the sample and 40 μl of N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (Regis, Chicago, IL, U.S.A.). Typically, 2-μl samples were analyzed.

Gas chromatography

Silylated compounds were analyzed using a microprocessor-controlled Hewlett-Packard Series 5880A instrument with a flame ionization detector. A 30 m × 0.25 mm I.D. fused silica capillary column coated with SE-54 (J & W Scientific, Rancho Cordova, CA, U.S.A.) was used. The injection port and detector temperatures were 220 and 280°C, respectively, and the column was temperature-programmed at the outset from 200 to 265°C at 4°C/min. Helium was used as carrier gas at a split ratio of 60:1 and a flow-rate through the column of 0.70 ml/min.

Gas chromatography–mass spectrometry

GC–mass spectrometry (MS) was carried out with a Hewlett-Packard Model 5985 instrument and a 20 m × 0.5 mm I.D. glass capillary column coated with SE-54 (Supelco). The mass spectrometer was operated at an ionization voltage of 70 eV and the source temperature was maintained at 150°C.

RESULTS AND DISCUSSION

Fused silica capillary columns are virtually inert due to lack of metal oxides and contaminants frequently present in glass. These columns have been found to be especially useful with nitrogen and sulfur compounds, such as amines and sulfides, which may degrade on other types of glass[10]. Since cytokinins contain amino nitrogen and in some cases sulfide groups a fused silica capillary column was selected for use with this class of compounds.

Cytokinins were chromatographed on an SE-54 fused silica capillary column with a single set of programmed temperature conditions following the evaluation of several programs and conditions. The structures and relative retention data for the cytokinins are given in Table I. All of the compounds, with the exception of two ribonucleosides, were resolved under the conditions used. A representative chromatogram obtained with compounds closely related structurally is shown in Fig. 1. Among the cytokinins resolved by this technique were dihydrozeatin, *trans*-zeatin and corresponding ribonucleosides, dihydroribosylzeatin and *trans*-ribosylzeatin. Separation of these pairs of compounds is frequently difficult to achieve using conventional packed column GC[11], especially on columns with stationary phases having low enough bleed to be used successfully in conjunction with MS. Bleed from capil-

TABLE I

STRUCTURES AND RETENTION DATA FOR CYTOKININS CHROMATOGRAPHED AS TMS DERIVATIVES ON SE-54 FUSED SILICA CAPILLARY COLUMN

R_1	R_2	R_3	*Common name*	*Relative retention**
$-CH_2-CH=C(CH_3)_2$	H	H	Isopentenyladenine	0.44
$-CH_2-CH_2-CH(CH_3)(CH_2OH)$	H	H	Dihydrozeatin	0.71
$-CH_2-C(H)=C(CH_3)(CH_2OH)$	H	H	*cis*-Zeatin	0.75
$-CH_2-C(H)=C(CH_2OH)(CH_3)$	H	H	*trans*-Zeatin	0.80
$-CH_2-CH=C(CH_3)_2$	H	Ribose**	Isopentenyladenosine	1.28
$-CH_2-CH_2-CH(CH_3)(CH_2OH)$	H	Ribose	Dihydroribosylzeatin	1.72
$-CH_2-C(H)=C(CH_3)(CH_2OH)$	H	Ribose	*cis*-Ribosylzeatin	1.81
$-CH_2-C(H)=C(CH_2OH)(CH_3)$	H	Ribose	*trans*-Ribosylzeatin	1.96
$-CH_2-C_6H_4-OH$ (*o*)	H	Ribose	*o*-Hydroxybenzyladenosine	2.71
$-CH_2-CH=C(CH_3)_2$	CH_3S-	Ribose	2-Methylthioisopentenyladenosine	1.96
$-CH_2-C(H)=C(CH_3)(CH_2OH)$	CH_3S-	Ribose	*cis*-2-Methylthioribosylzeatin	2.87
$-CH_2-C(H)=C(CH_2OH)(CH_3)$	CH_3S-	Ribose	*trans*-2-Methylthioribosylzeatin	3.18

* Internal standard TMS-phloretin [2′,4′,6′-trihydroxy-3-(*p*-hydroxyphenyl) propiophenone] = 1.00.

** Ribose attached as β-D-ribofuranosyl group.

lary columns is usually minimal and does not interfere with GC–MS analyses. In addition, the *cis* and *trans* isomers of zeatin, ribosylzeatin, and 2-methylthioribosylzeatin were all resolved on the capillary column. Recent work on the development of high-performance liquid chromatographic techniques for the separation of geometrical isomers of cytokinins[12,13] showed that reversed-phase columns resolved *cis* and *trans* isomers of zeatin and ribosylzeatin, but *cis*- and *trans*-2-methylthioribosylzeatin were not separated[13]. Conventional packed column GC was also used to separate the TMS derivatives of the latter compounds[5], but there was undesirable peak broadening during packed column analysis of higher-molecular-weight cytokinin ribonucleosides, especially methylthio derivatives. Generally, narrow band widths and symmetry characterize capillary column peaks and extend the sensitivity of compound detection beyond that obtained with packed columns[14], although no direct comparisons were made in the present work. Two compounds, *trans*-ribosylzeatin and 2-methylthioisopentenyladenosine, could not be separated on the SE-54 capillary column. Coinjection of these compounds using either programmed temperature or isothermal conditions resulted in a single peak in each case.

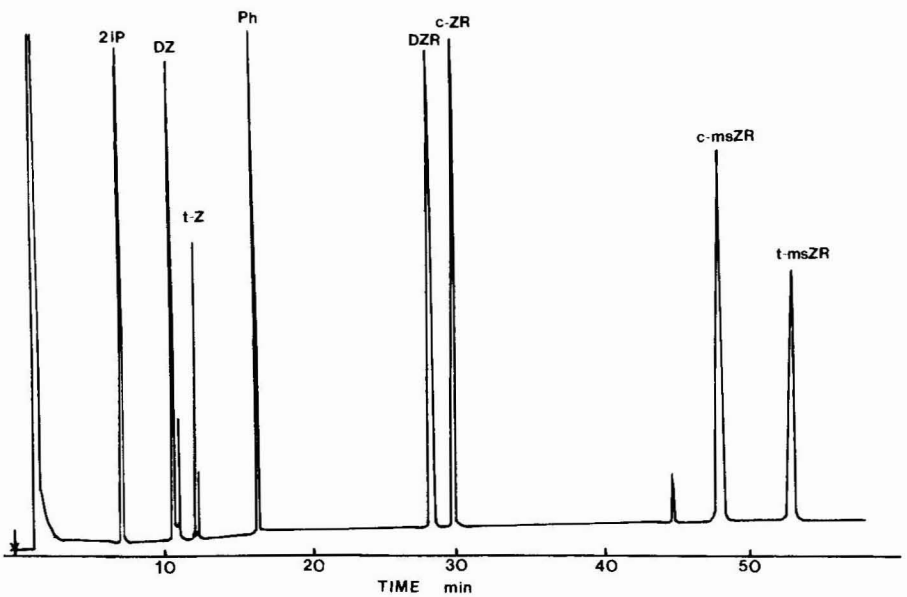

Fig. 1. Chromatogram of a mixture of silylated cytokinins including pairs of closely related compounds on a 30 m × 0.25 mm I.D. SE-54 fused silica capillary column programmed from 200 to 265°C at 4°C/min. Abbreviations: 2iP = isopentenyladenine; DZ = dihydrozeatin; t-Z = *trans*-zeatin (contaminant in commercial dihydrozeatin); Ph = phloretin (internal standard); DZR = dihydroribosylzeatin; c-ZR = *cis*-ribosylzeatin; c-msZR = *cis*-2-methylthioribosylzeatin; t-msZR = *trans*-2-methylthioribosylzeatin.

Silylation of cytokinin ribonucleosides usually gave sharp singlet peaks during GC analysis. However, silylation of the zeatins, namely *trans*-zeatin, *cis*-zeatin, and dihydrozeatin, frequently resulted in the formation of doublet or multiplet peaks even after rigorous drying of the samples prior to the derivatization reaction. In order to obtain information on the principal derivative formed from these bases, freshly silyl-

ated *trans*-zeatin was analyzed by GC–MS. The major peak yielded a molecular ion at *m/e* 363 and prominent ions at *m/e* 349, 348, 274, 273, 272, 261, 260, 258, 257, and 232 which corresponded with data reported by Purse *et al.*[4] for $(TMS)_2$-*trans*-zeatin. The secondary peaks were not identified. Composition of the silylated zeatin-type bases changed upon prolonged storage at $-20°$ as indicated by GC retention data.

The derivative formed upon silylation of *trans*-ribosylzeatin was also examined by GC–MS. The spectrum revealed a molecular ion at *m/e* 639 and characteristic ions at *m/e* 624, 550, 549, 537, 536, 406, 321, 320, 292 and 290. The fragmentation pattern of this compound was consistent with $(TMS)_4$-*trans*-ribosylzeatin and was very similar to that recently published[15] for $(TMS)_4$-*cis*-ribosylzeatin.

ACKNOWLEDGEMENTS

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Note

Analysis of pyridine bases isolated from a high-temperature coal tar by capillary gas chromatography *

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The composition of the pyridine base fraction of high-temperature bituminous coal tar can be determined reliably and precisely by gas chromatography[1–7]. For the separation of multi-component mixtures of isomeric alkylpyridines, polar stationary phases[1,7] and the method of capillary gas chromatography have been used[8,9].

It has already been shown that the analysis of a complex mixture of basic plant biosubstrates can be carried out on an Amine 220 (1-ethanol-2-heptadecenyl-2-isoimidazole) phase[9,10]. The relative retention (pyridine = 1) of alkylpyridines de-

TABLE I

RELATIVE RETENTION INDICES (R) OF PURE ALKYLPYRIDINES ON A COLUMN PACKED WITH CELITE 545 WITH 20% OF AMINE 220 (PYRIDINE = 1.00)

Compound	*R*	*Compound*	*R*
2-Methylpyridine	1.42	2,4,6-Trimethylpyridine	4.25
3-Methylpyridine	2.22	2,3,6-Trimethylpyridine	4.59
4-Methylpyridine	2.31	2,3,5-Trimethylpyridine	7.45
2,6-Dimethylpyridine	1.93	2,4,5-Trimethylpyridine	8.57
2,5-Dimethylpyridine	3.12	4-Methyl-2-ethylpyridine	4.83
2,4-Dimethylpyridine	3.26	6-Methyl-3-ethylpyridine	5.54
2,3-Dimethylpyridine	3.48	2,4-Dimethyl-6-ethylpyridine	5.73
3,5-Dimethylpyridine	4.89	2,6-Dimethyl-3-ethylpyridine	7.31
3,4-Dimethylpyridine	6.27	2,6-Dimethyl-4-ethylpyridine	7.39
2-Ethylpyridine	2.23	2-Butylpyridine	7.00
3-Ethylpyridine	3.96		
4-Ethylpyridine	4.24		

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

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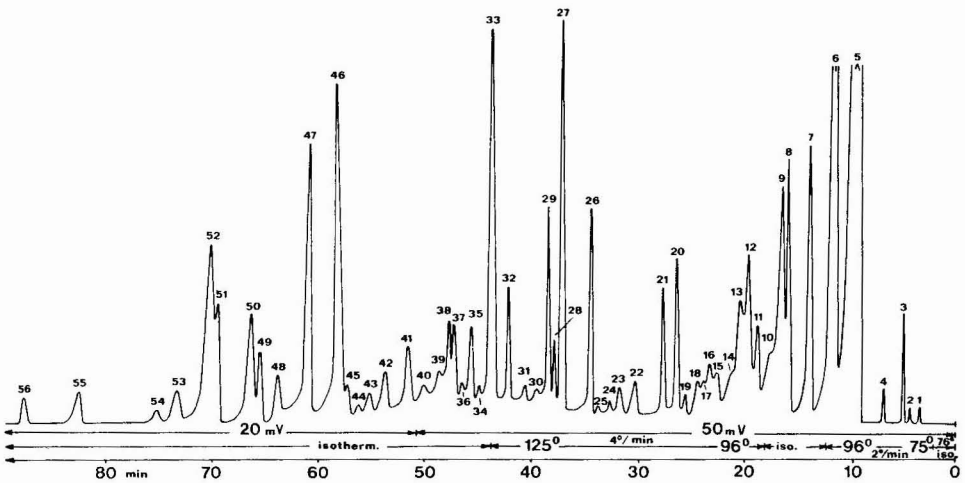

Fig. 1. Chromatogram of basic fraction of high-temperature bituminous coal tar (stainless-steel capillary column coated with Amine 220).

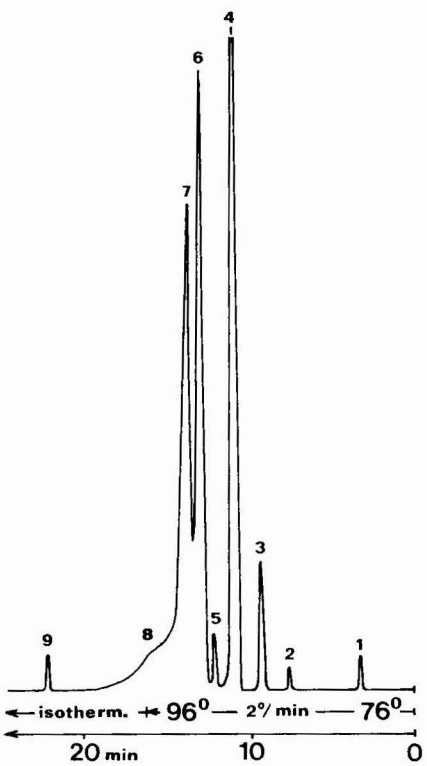

Fig. 2. Chromatogram of β-picoline fraction of tar bases (stainless-steel capillary column coated with Amine 220).

termined on a column packed with Celite 545 prepared by treatment with 1% potassium hydroxide solution using Amine 220 stationary phase indicate the good selectivity of Amine 220 for the separation of their isomers, as demonstrated in Table I.

These results correlate with those obtained using a capillary column on a Chrom 41 (Laboratory Instruments, Prague, Czechoslovakia) gas chromatograph equipped with a flame-ionization detector. The pressure of the carrier gas (nitrogen) at the inlet of the column was 29.3 kPa. A stainless-steel capillary column (25 m × 0.26 mm I.D.) was coated with Amine 220 stationary phase, the splitting ratio being 1:100 and the evaporator temperature 250°C (523°K). As the sample substances had a wide range of boiling points, temperature programming was applied.

A low-boiling mixture of pyridine derivatives isolated from a high-temperature bituminous coal tar (Fig. 1) and of a β-picoline fraction prepared by rectification were analysed (Fig. 2).

The main fraction of the crude pyridine bases consists of pyridine, methylpyridines, 2,6-dimethylpyridine, 2,4-dimethylpyridine, aniline, methylaniline, quinoline,

TABLE II

ELUTION SEQUENCE OF PYRIDINE BASES OF COAL TAR ON A STAINLESS-STEEL CAPILLARY COLUMN COATED WITH AMINE 220

Sample size: 0.15 μl.

Sequence in chromatogram (Fig. 1)	*Substance identified*	*Sequence in chromatogram (Fig. 1)*	*Substance identified*
1–4	Unidentified	25	Unidentified
5	Pyridine	26	Aniline
6	2-Methylpyridine	27–29	Unidentified
7	2,6-Dimethylpyridine	30	N-Methylaniline
8	3-Methylpyridine	31	N,N-Diethylaniline
9	4-Methylpyridine	32	Unidentified
10	Unidentified	33	2-Methylaniline
11	2,5-Dimethylpyridine	34	Unidentified
12	2,4-Dimethylpyridine	35	4-Methylaniline
13	2,3-Dimethylpyridine	36	Unidentified
14	3-Ethylpyridine	37	3-Methylaniline
15	4-Ethylpyridine + 2,4,6-trimethyl pyridine	38	2,6-Dimethylaniline
		39	Unidentified
16	2,3,6-Trimethylpyridine	40	Unidentified
17	2-Ethyl-5-methylpyridine	41	2,5-Dimethylaniline +
18	2-Ethyl-4-methylpyridine + 3,5-dimethylpyridine		2,4-dimethylaniline
		42	3,5-Dimethylaniline
19	3-Ethyl-6-methylpyridine	43	3,4-Dimethylaniline
20	3,4-Dimethylpyridine + aromatic hydrocarbon	44	Unidentified
		45	2,3-Dimethylaniline
21	Aromatic hydrocarbon	46	Quinoline
22	2,3,5-Trimethylpyridine + 2,6-dimethyl-3-ethylpyridine + 2,6-dimethyl-4-ethylpyridine	47	2-Methylquinoline + 8-methylquinoline
		48	Isoquinoline
23	2,4,5-Trimethylpyridine + 2,3,4-trimethylpyridine	49–51	Unidentified
		52	2,8-Dimethylquinoline
24	N,N-Dimethylaniline	53–56	Unidentified

methylquinoline and some aromatic hydrocarbons. The use of a capillary column with temperature programming column made it possible to identify 2,3,6- and 2,4,6-trimethylpyridines in the β-picoline fraction. These substances have not previously been identified in the crude pyridine base fraction of high-temperature coal tar[11,12].

TABLE III

ELUTION SEQUENCE OF ALKYLPYRIDINES IN PICOLINE FRACTION OF TAR ON A STAINLESS-STEEL CAPILLARY COLUMN COATED WITH AMINE 220

Sample size: 0.6 μl.

Sequence in chromatogram (Fig. 2)	*Substance identified*	*Sequence in chromatogram (Fig. 2)*	*Substance identified*
1	Unidentified	6	3-Methylpyridine
2	Unidentified	7	4-Methylpyridine
3	Pyridine	8	2,4-Dimethylpyridine + 2,5-dimethylpyridine
4	2-Methylpyridine		
5	2,6-Dimethylpyridine	9	2,4,6-Trimethylpyridine + 2,3,6-trimethylpyridine

The results obtained are also summarized in Tables II and III, and demonstrate the applicability of this method for the process control of the manufacture of pyridine bases based on coal tar.

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Note

Gas–liquid chromatographic determination of the iridoid content in *Harpagophytum procumbens* D.C.

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Several methods for the determination of the iridoid content (harpagoside, harpagide, procumbide) in *Harpagophytum procumbens* D.C. (*Pedaliaceae*) have been described. Among these, high-performance liquid chromatography (HPLC) with UV detection[1–4] appears to be very useful for the rapid and accurate determination of harpagoside in crude extracts of the drug. However, it does not allow the determination of harpagide which could be, together with harpagoside, implicated in the biological activity of the plant; moreover, the presence of great amounts of harpagide in the drug or in the extracts could indicate that an enzymatic or a chemical degradation has occurred during sample collection, extract preparation or storage.

A colorimetric method[5] can also be applied to the quantitative analysis of the crude extracts. The final results of this procedure, which concern the total iridoid content (principally, harpagoside and harpagide), are expressed as harpagoside but the linearity of the colorimetric response of harpagide with the vanillin–sulphuric reagent was not investigated.

On the other hand, gas–liquid chromatographic (GLC) analysis of a great number of iridoids (except harpagoside and harpagide) as silyl derivatives has been performed by Inouye *et al.*[6].

This paper describes a rapid GLC method for the determination of harpagide, and consequently of harpagoside, in crude extracts of *H. procumbens*.

EXPERIMENTAL

High-performance liquid chromatography

The liquid chromatograph supplied by Waters Assoc. (Milford, MA, U.S.A.) was equipped with a pump (Model 6000A), a sample loop (Model U6K), a UV detector (Model 440) operating at 254 or 280 nm and a 300 × 3.9 mm I.D. stainless-steel column pre-packed with μBondapack C_{18} (mean particle size 10 μm). The mobile phase was ethanol–water (4:6) at a flow-rate of 1 ml/min (retention time of harpagoside, 7 min).

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Gas-liquid chromatography

The Packard-Becker (Delft, The Netherlands) Model 421 gas chromatograph was equipped with a packed column (1 m × 0.125 in. I.D.) of 3% SE-30 on Chromosorb W HP (100–120 mesh).

The internal standard solution was prepared by dissolving 50 mg of cholesterol in 25 ml of anhydrous pyridine.

The reference solutions were prepared by dissolving 4, 5 and 6 mg of harpagide in 5 ml of the internal standard solution; 0.5 ml of N-trimethylsilylimidazole (TSIM, Macherey, Nagel & Co., Düren, G.F.R.) was added to 0.5 ml of each of the three reference solutions. Before injection, the mixtures were heated for 30 min in a sealed vial equipped with a Mininert® valve (Pierce, Rockford, IL, U.S.A.).

To prepare sample solutions, 20 mg of the crude dried extract (aqueous or methanolic) of the drug (corresponding to *ca.* 0.5 mg of harpagide) were dispersed in a mixture of 0.5 ml of the internal standard solution and of 0.5 ml of TSIM, then treated as described for the reference solutions.

Another amount (20 mg) of the crude extract was treated by 1 ml of an ammonia solution at 25% ($d = 0.91$) and allowed to stand at room temperature for 4 h; the solution was then evaporated at 45°C *in vacuo* and the residue silylated as described for the non-hydrolysed extract.

RESULTS AND DISCUSSION

Even as its silyl derivative, harpagoside is extensively decomposed during GLC analysis, probably because of the presence of the ester bond between cinnamic acid and harpagide. This problem can be resolved as follows. Harpagide is determined in the crude extract using cholesterol as internal standard; harpagide yields a single peak under these silylation and chromatographic conditions. The crude extract is hydrolysed under alkaline conditions in order to transform harpagoside quantitatively into harpagide. The total harpagide content is finally determined by the proposed GLC procedure (Table I, Fig. 1). In this way, the content of harpagoside and harpagide can be calculated separately without interferences: the crude extract (before or after alkaline hydrolysis) gives no peak before silylation at the retention time of the silyl

TABLE I

THE PERCENTAGES OF HARPAGOSIDE AND HARPAGIDE IN A COMMERCIALLY AVAILABLE AQUEOUS SPRAY-DRIED EXTRACT OF *H. PROCUMBENS*: A COMPARISON BETWEEN GLC, HPLC AND COLORIMETRIC METHODS

	Crude extract without treatment	*Crude extract after alkaline hydrolysis*
% total iridoids (harpagoside + harpagide) by colorimetry	2.70 ± 0.10	Irreproducible results
% harpagoside by HPLC	2.45 ± 0.03	< 0.03
% harpagide by GLC	0.50 ± 0.02	2.30 ± 0.08
Calculated % harpagoside following GLC procedure	$\frac{(2.30 - 0.50) \text{ mol. wt. harpagoside}}{\text{mol. wt. harpagide}} = 2.44$	

derivative of harpagide. The comparison between HPLC and GLC methods does not show any significant difference with respect to the content in harpagoside (Table I). The results obtained with the colorimetric method could not be interpreted as absolute values. The reaction of harpagide with the vanillin–sulphuric reagent, in contrast to that of harpagoside under the same experimental conditions, gives a poor consistency; so, in comparison with the chromatographic methods, the results are systematically too low (Table I).

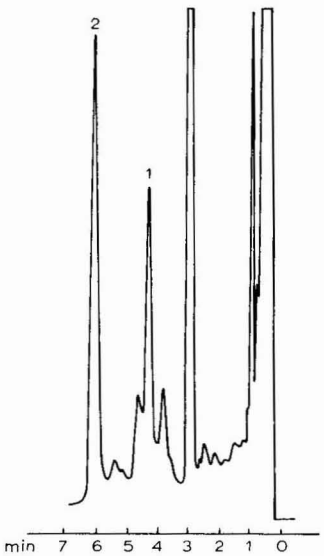

Fig. 1. Gas–liquid chromatogram of a silylated aqueous spray-dried extract of *H. procumbens* after alkaline hydrolysis. Conditions: detector and injector temperature, 280°C; oven temperature (isothermal), 250°C; nitrogen flow-rate, 30 ml/min. Peaks: 1 = harpagide (silyl derivative); 2 = cholesterol (silyl derivative) as internal standard.

For these reasons, GLC seems to be the only reliable method for the separate determination of harpagide and harpagoside in *H. procumbens* and in other drugs which contain the same iridoids.

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Note

Taurine levels in cat plasma

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Taurine is important in the elimination of bile[1]. The bile acids are conjugated with glycine and taurine in the liver, yielding water-soluble bile acids that can form salts with sodium and potassium. These glycocholate and taurocholate salts give rise to a detergent effect in the intestine that aids in the digestion of fats[2].

Taurine is not considered to be an essential amino acid for the well being of man, as it can be synthesized *in vivo* from cysteine or methionine[3]. But taurine is an essential amino acid for cats as they are unable to synthesize it from the other sulphur-containing amino acids. Exclusion of taurine from a cat's diet will result in total retina degeneration —blindness[4].

The taurine molecule is incapable of being included in the peptide chain of a protein because of its structure. It is "loosely attached" to proteins and, thus, can be easily lost, for example, as in the process of pellet preparation of cat dried-food, by being leached out.

The level of taurine in a cat diet can be ascertained by analysis of deproteinised cat plasma with an amino acid analyzer. However, it has been previously determined that estimates of taurine in vertebrate brain tissue can vary widely depending upon the methods used for isolating and quantitating the amino acid[5]. Such a wide variation in taurine levels has been found to occur when analysing plasma that had been deproteinized with 5-sulphosalicylic acid (SA). This reagent has proved to be unsatisfactory because a fine precipitate slowly forms when the "deproteinised" plasma is stored at 5°C or ambient temperature. Thus, this communication deals with protein precipitation from plasma using SA, and describes a simple procedure for conversion of an initial finely divided precipitate (that is held in suspension) into flakes that can be more effectively removed from the free amino acid solution when loading onto an amino acid analyzer ion-exchange column.

EXPERIMENTAL

A Beckman 120B amino acid analyzer was used for the taurine analyses and titanous chloride-reduced ninhydrin reagent was used for colour development[6]. The analyses were carried out with Durrum DC-1A cation-exchange resin (57 cm) and the amino acids were eluted with pH 3.25 sodium citrate buffer. Sulphosalicylic acid was obtained from Ajax Chemicals, Sydney, Australia.

The red cells in the drawn blood (5 ml) were spun down in a Epindorf bench

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centrifuge and a 0.4-ml aliquot of plasma was transferred to a clean plastic centrifuge tube. A 0.2-ml volume of the 12.5% SA solution was added. The samples were spun for 15 min and the supernatant transferred to a clean tube and spun again for 10 min. The cloudy supernatant was transferred to a thick-walled Pyrex glass tube (4 × 1 in.) and 0.2 ml of the ethanol–water (80:20) mixture added. The tube contents were evaporated to dryness on a rotary evaporator and to the residue was added 0.2 ml of

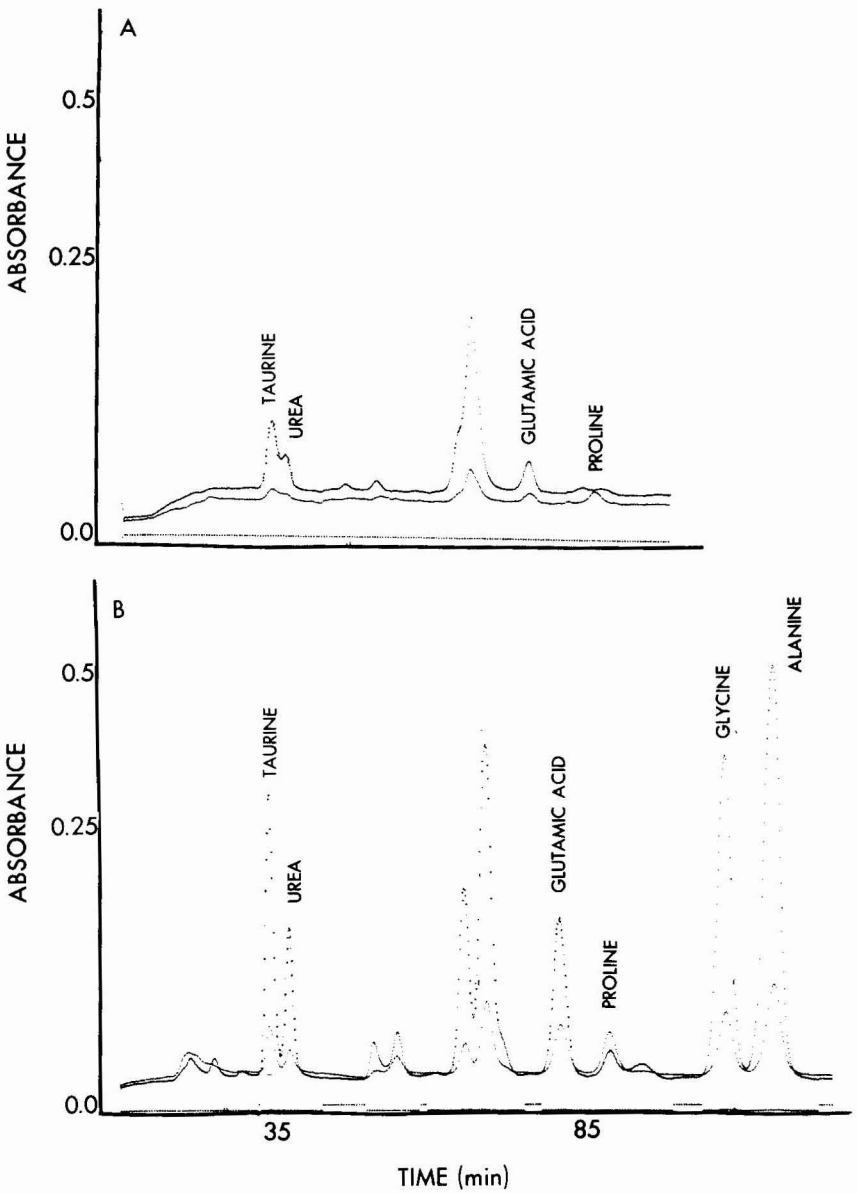

Fig. 1. Analyses of (A) a 0.2-ml aliquot of cat plasma, and (B) 0.4 ml of the same plasma. Sample A was treated with SA. Sample B had been treated with SA and 80% ethanol.

pH 2.2 sodium citrate buffer. All the contents of the tube were transferred to the amino acid column with a disposable pipette. A 0.2-ml wash with pH 3.25 sodium citrate buffer was also added to the column. A water pump vacuum line was placed inside the open end of the column close to the PTFE disc, capping the resin, and with a disposable pipette a jet of pH 3.25 buffer was forced against the PTFE disc and the suspension removed with the vacuum line. The analysis is then begun after filling the column void volume with pH 3.25 buffer.

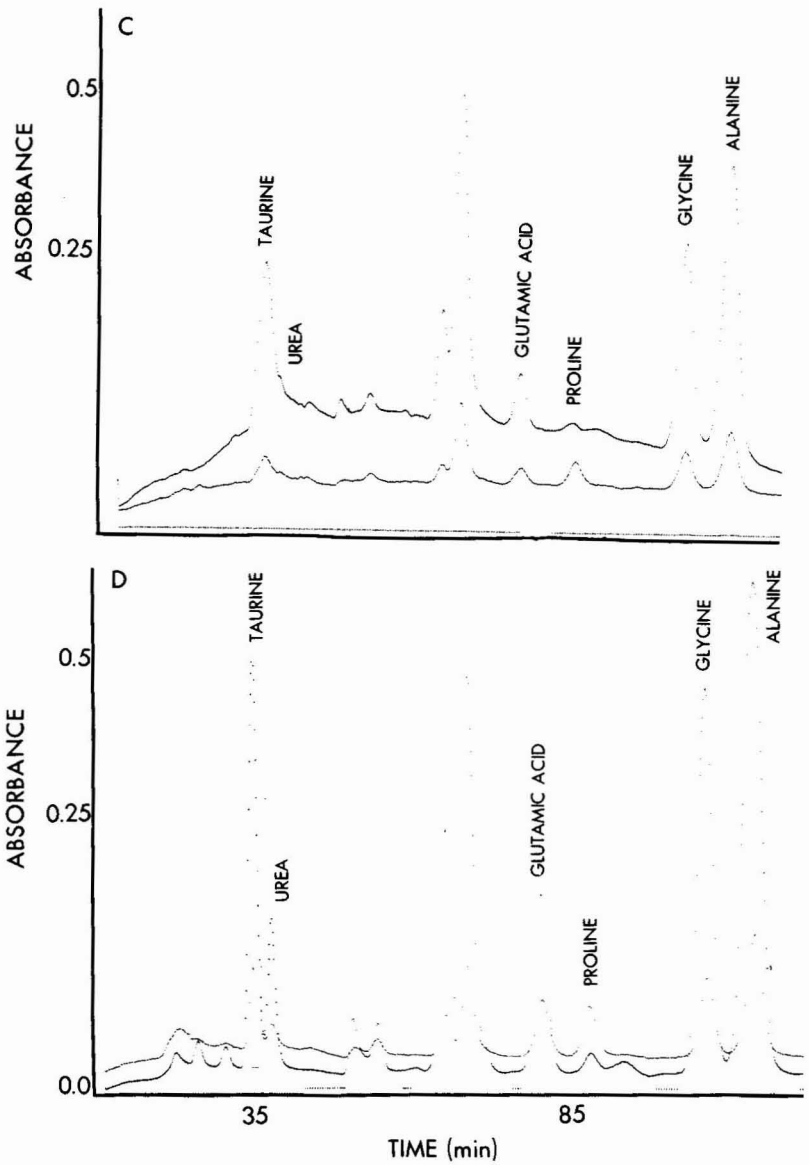

Fig. 2. Analyses of 0.4-ml aliquots of the same cat plasma. Sample C was treated with SA. Sample D had been treated with SA and 80% ethanol.

RESULTS AND DISCUSSION

The results of analysis of cat plasma to determine free taurine levels are shown in Figs. 1 and 2. The chromatograms labelled C and D were obtained with duplicate aliquots of plasma, but the sample in chromatogram C had not been treated with 80% ethanol. In chromatogram D the advantage of the 80% ethanol treatment is clearly demonstrated; the taurine peak is completely resolved from urea and is much larger, the baseline printout of the recorder is also much improved. Plasma from the same cat was used to obtain chromatograms A and B. A 0.2-ml volume of plasma had been used to obtain chromatogram A and a 0.4-ml volume to obtain chromatogram B. This experiment had been carried out to determine whether resolution of taurine and baseline printout could be improved by using less sample for the analysis. As can be seen, no advantage was gained by reducing the volume of plasma analysed.

The differences between SA alone and SA plus 80% ethanol-treated samples is not only demonstrated by the complete resolution of taurine and urea, but also throughout the chromatogram development. Further, in every analysis undertaken with identical aliquots of cat plasma the samples treated with SA and 80% ethanol provided higher levels of recovery of taurine and other amino acids than samples treated with SA only.

Chromatograms E, F and G in Fig. 3 show the reproducibility obtainable with triplicate aliquots of cat plasma that were deproteinised with SA and 80% ethanol. The peaks upon integration gave the equivalent of 63.0 nmoles, 63.0 nmoles and 62.8 nmoles for taurine in samples E, F and G respectively.

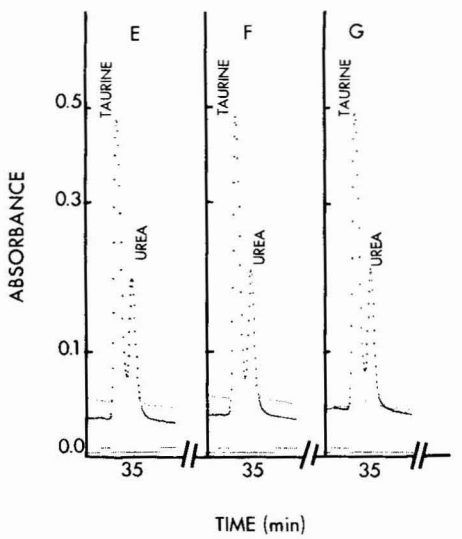

Fig. 3. Chromatograms E, F and G are triplicate analyses of cat plasma that had been treated with SA and 80% ethanol.

Deproteinisation of plasma using picric acid was not attempted. However, the reliability and quantitation accuracy obtained with the picric acid procedure is well documented[7,8]. The advantage of using SA followed by 80% ethanol deproteinisation over the picric acid method is one of convenience; it is quicker to carry out and does

away with the necessity of passing the sample down a Dowex 50 resin column to remove the yellow-coloured picric acid, before amino acid analysis.

As mentioned above, the domestic cat is very vulnerable to retina degeneration through the absence of taurine in its food. This condition can readily arise in cat breeding establishments, especially when the cat food is predominantly in the dry pellet form. Indeed, some plasma from domestic cats, when analysed, showed no taurine, and in each of these cases retina degeneration was detected[9].

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Note

pH-Gradient-Dünnschicht-Chromatographie von Benzodiazepinen

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Die Literatur über Benzodiazepine ist deren Bedeutung entsprechend recht umfangreich. Sie wird in mehreren Übersichtsartikeln zusammenfassend dargestellt[1–3]. Dabei findet die Chromatographie bei verschiedenen pH-Werten nur wenig Berücksichtigung, obwohl die Verbindungen ein pH-abhängiges Verhalten erwarten lassen.

Im Rahmen unserer Arbeiten mit der Gradient-Dünnschicht-Chromatographie (Gradient-DC)[4–10] erschien es uns deshalb sinnvoll, die Trennmöglichkeiten von Benzodiazepinen auf pH-Gradient-Schichten zu untersuchen.

EXPERIMENTELLER TEIL

Geräte

Gradient-DC-Streicher 85 (neues Modell; Desaga, Heidelberg, B.R.D.) mit nivellierbarem Streichtisch für fünf Platten 20 × 20 cm.

Prüfung des Streichgerätes auf Dichtigkeit

In den Gradient-DC-Streicher 85 wird die Diagonaltrennwand fest eingesetzt und das Gerät auf die Startplatte gestellt. Dann füllt man eine der beiden Trogkammern mit *ca.* 50 cm^3 Wasser in einem Zug und beobachtet den Wasserdurchtritt in die andere Kammer. Bei funktionstüchtigen Geräten sollte eine Dichtigkeit für einige Sekunden gegeben sein.

Herstellung der pH-Gradient-Schichten

In zwei 100-cm^3 Erlenmeyerkolben werden je 18.5 g TLC-Kieselgel 60 GF_{254} (Merck, Darmstadt, B.R.D.) eingewogen. Aus Büretten misst man dann jeweils 50 cm^3 0.5 *N* Schwefelsäure und 0.5 *N* Natronlauge ab und gibt sie dem entsprechenden Kieselgel zu. Die Kolben werden verschlossen und unter Festhalten der Stopfen *ca.* 10 sec intensiv geschüttelt. Anschliessend werden die beiden Suspensionen gleichzeitig in je einen Teilerraum des Gradientstreichgerätes eingebracht und die Flüssigkeitsoberflächen mit je einem Spatel nivelliert. Danach zieht man die Trennwand des Teilers heraus und setzt die Mischwelle mit Hilfe der Kunststoffklammer ein. Mittels des Motorantriebes wird dann *ca.* 15 sec nur in einer Richtung gemischt und danach die Verbindung zum Motor gelöst. Dann wird der Hebel des Streichgerätes um 90° (Belüftung!) nach oben gedreht und die Masse gleichmässig auf die fünf DC-Platten

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ausgestrichen. Die Schichten werden bis zum Verschwinden des wässrigen Glanzes auf dem nivellierten Streichtisch gelassen, danach *ca.* 15 min im Warmluftstrom vorgetrocknet und anschliessend 30 min auf 110°C erhitzt.

Herstellung von uniformen Kieselgelschichten

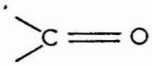
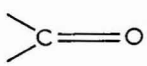
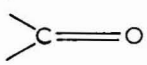
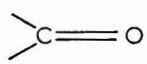
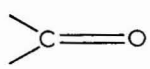
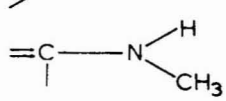
Verwendung des gleichen Streichgerätes, jedoch ohne Diagonalteiler und Mischwelle. In einem 250-cm^3 Erlenmeyerkolben werden 30 g TLC-Kieselgel 60 GF_{254} mit 80 cm^3 demineralisiertem Wasser gemischt und in üblicher Weise ausgestrichen und getrocknet.

Substanzen

Die verwendeten Benzodiazepine sind in Tabelle I aufgeführt. Sie wurden alle von der Firma Hoffmann-La Roche (Basel, Schweiz) freundlicherweise zur Verfügung gestellt.

TABELLE I

STRUKTUREN DER UNTERSUCHTEN BENZODIAZEPINE

Benzodiazepine	R_1	R_2	R_4	R_5	R_7
Medazepam	$-CH_3$	$-CH_2-$	–		-Cl
Flunitrazepam	$-CH_3$		–		$-NO_2$
Diazepam	$-CH_3$		–		-Cl
Clonazepam	-H		–		$-NO_2$
Nitrazepam	-H		–		$-NO_2$
Flurazepam	$-CH_2-CH_2-N(C_2H_5)_2$		–		-Cl
Chlordiazepoxid	–		$\rightarrow O$		-Cl

Untersuchungslösung

Es wurde eine frisch bereitete 0.1 %ige Lösung der Benzodiazepine in Toluene verwendet, wobei zur vollständigen Auflösung bis zu einem Gehalt von etwa 1 % (v/v) Methanol zugesetzt wurde. Zu Vergleichszwecken dienten ebenfalls 0.1 %ige Lösun-

gen der Einzelsubstanzen im gleichen Lösungsmittel. Zur Chromatographie im Verlauf des Gradients wurde eine ältere Lösung verwendet, die schon Zersetzungsprodukte enthielt.

Auftragen

0.1 cm^3 der Probelösung wurden mit dem Desaga Autoliner 75 als *ca.* 17 cm langes, schmales Startband aufgetragen, entweder quer zum Gradient oder in der Richtung des Gradients. Von den Lösungen der Einzelsubstanzen wurden je 2 mm^3 am alkalischen Ende des Startbandes punktförmig aufgetragen. (Nicht alle auf eine Schicht)

Chromatographiebedingungen

Luftfeuchtigkeit. Die Luftfeuchte wurde nicht speziell eingestellt, sie betrug 30–35 % rel.

Fliessmittel und Entwicklung. Bei der Chromatographie quer zum Gradient wurde als Fliessmittel Chloroform–Aceton (90:10) bei Kammersättigung verwendet, wobei zweimal über eine Laufstrecke von 10 cm entwickelt wurde. Die Chromatographie im Verlauf des Gradients sowie die zum Vergleich durchgeführte Entwicklung auf einer uniformen, neutralen Kieselgelschicht wurden in der BN-Kammer unter Durchlaufbedingungen mit Toluene–Propanol-2 (90:10) als Fliessmittel durchgeführt. Die Zeit betrug 90 min, nachdem die Front eine Höhe von 18 cm erreicht hatte.

Detektion. Zur Detektion wurde die Schicht mit 8 *N* Schwefelsäure besprüht und die auftretenden Fluoreszenzen bei Bestrahlung mit langwelligem UV-Licht (365 nm) (Desaga HP-UVIS) beobachtet. Photographiert wurde mit UV- und Gelbfilter mit dem Film Agfaortho 25 professional.

ERGEBNISSE UND DISKUSSION

Fig. 1 zeigt das chromatographische Verhalten der Benzodiazepine bei verschiedenen pH-Werten. Die Auftragung des Gemisches und die Entwicklung erfolgten quer zum Gradient (T-Gradient-Technik)[5]. Zum Vergleich wurde am alkalischen Ende der Schicht das Gemisch noch einmal punktförmig aufgetragen. Man erkennt für alle Substanzen höhere R_F-Werte im alkalischen und niedrigere im sauren pH-Bereich. Dieses Verhalten ist typisch für basische Verbindungen. Die sich bei einer Protonierung im Sauren ergebende Polaritätszunahme macht sich im Chromatogramm in einer verringerten Wanderungsgeschwindigkeit bemerkbar. Die Reihenfolge dieser R_F-Wert-Abnahme beim Übergang vom basischen zum sauren Milieu entspricht in etwa der Reihenfolge der pK_a-Werte[11]. Zuerst lagert Flurazepam aufgrund seiner basischen Seitenkette (R_1 in Tabelle I) ein Proton an. Als nächste Substanzen folgen Medazepam und Chlordiazepoxid. Die übrigen Benzodiazepine haben kleinere pK_a-Werte, dementsprechend liegt der Wendepunkt der Substanzzonen weiter im Sauren. Die Carbonylgruppe in 2-Stellung verhindert die Protonenaufnahme am N-1, das Protonierungszentrum liegt am zweiten Ringstickstoffatom in 4-Stellung[12].

Der pK_a-Wert selbst ist aus dem Chromatogramm nicht abzulesen, da für die Lage der Kurvenwendepunkte nicht allein die Säuredissoziationskonstanten, sondern auch die Adsorptions- bzw. Verteilungsgleichgewichte eine Rolle spielen. Flunitrazepam und Diazepam, welche unter den angewandten Bedingungen im alkalischen

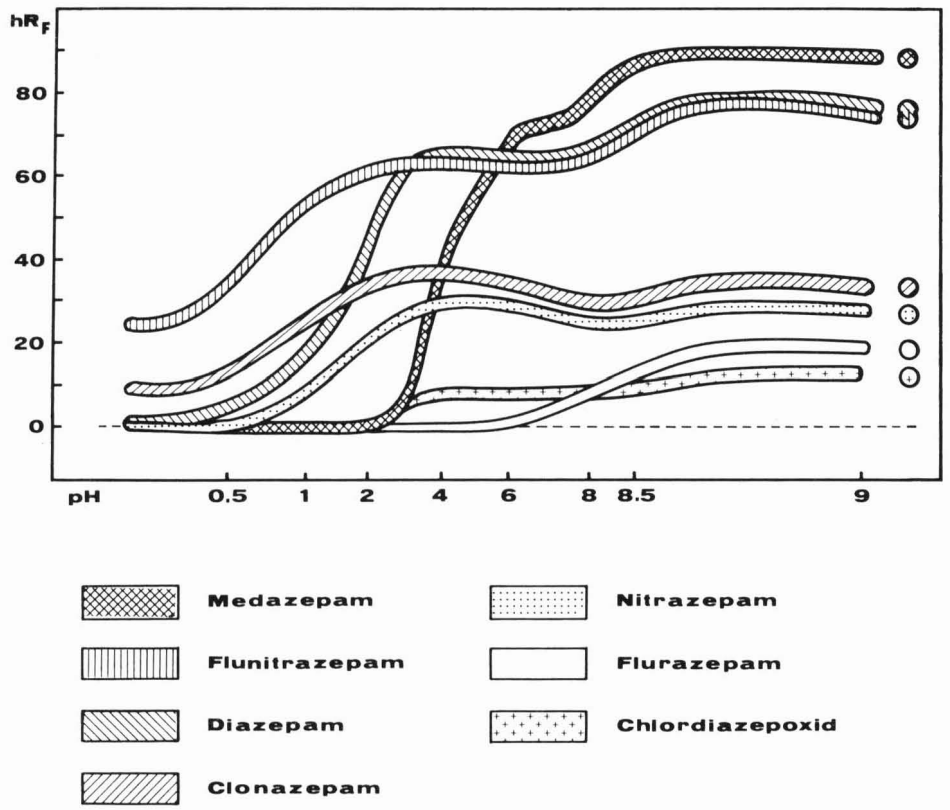


Fig. 1. Dünnschicht-Chromatogramm von Benzodiazepinen im pH-T-Gradient.

Milieu nicht getrennt werden, haben im sauren Bereich stark unterschiedliche R_F-Werte. Umgekehrt bleiben alle Benzodiazepine bis auf Flunitrazepam und Clonazepam im Sauren auf der Startlinie, während im Basischen ihre Trennung gelingt. So lässt sich mit Hilfe der pH-T-Gradient-Technik schnell erkennen, wo optimale Trennbedingungen vorliegen. Die Gradient-Chromatographie hat aber auch einen weitaus höheren Informationsgehalt als die übliche "punktförmige" Chromatographie auf uniformen Schichten. Denn die erhaltenen Kurven zeigen das chromatographische Verhalten der Substanzen über einen grösseren pH-Bereich übersichtlich und unmittelbar auf. Sie können deshalb bei der Identitätsprüfung von Benzodiazepinen eine wesentliche Hilfe darstellen. Auch die Erkennung von Verunreinigungen wird erleichtert, da es unwahrscheinlich ist, dass ihre R_F-Werte über den gesamten pH-Bereich identisch mit denen der Hauptsubstanzen sind.

Schliesslich wurde ein Benzodiazepingemisch, welches schon Zersetzungsprodukte enthielt, auf die basische Seite einer pH-Gradient-Schicht aufgetragen und in Richtung des sauren Bereichs chromatographiert. Man erhält eine Auftrennung in 14 Zonen (Fig. 2A), während im Vergleichschromatogramm (Fig. 2B), das die Auftrennung des Gemisches unter sonst gleichen Bedingungen auf einer normalen, uniformen Kieselgelschicht zeigt, nur 6 Zonen zu erkennen sind.

Im Gradient-Chromatogramm (Fig. 2A) sind die Substanzzonen teilweise zu

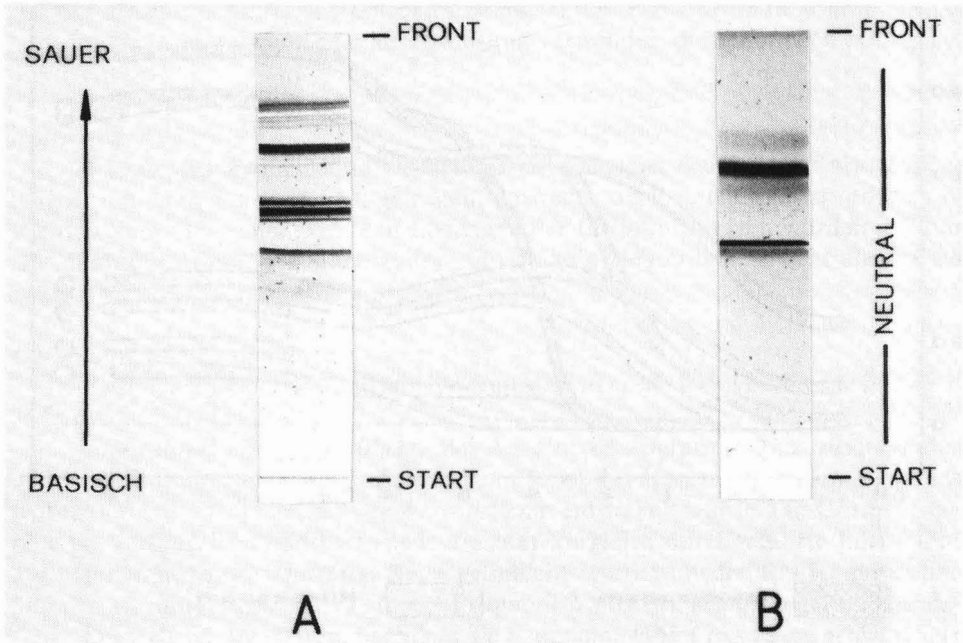


Fig. 2. Dünnschicht-Chromatogramme von Benzodiazepinen mit Zersetzungsprodukten. A: Chromatographiert wurde im pH-Gradient von basisch nach sauer. B: Chromatographiert wurde auf einer uniformen, neutralen Kieselgelschicht. Im Vergleich zu Fig. 2A tritt kein Fokussierungseffekt auf.

schmalen Bändern zusammengeschoben. Dieser Fokussierungseffekt ergibt sich bei Substanzen, deren Wanderungsgeschwindigkeit bei einem bestimmten pH-Wert drastisch abnimmt, so dass eine Konzentrierung der Substanz an dieser Stelle erfolgt. Dieser Fokussierungseffekt führt zu einer erheblichen Erniedrigung der Nachweisgrenze, was in der Spurenanalyse nützlich sein kann. Dies kann auch für die Lösung präparativer Probleme von Vorteil sein.

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Note

Reversed-phase ion-pair high-performance liquid chromatography of the plant hormones indolyl-3-acetic and abscisic acid

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The analysis of endogenous plant hormones usually requires their purification as trace components of plant tissue extracts. Modern techniques of high-performance liquid chromatography (HPLC) are being increasingly utilized in both the preparation and analysis of plant extracts. For the naturally occurring auxin indolyl-3-acetic acid (IAA) and the growth inhibitor 2-*cis*-4-*trans*-abscisic acid (*c,t*-ABA) HPLC methods using ion-exchange[1,2] and reversed-phase[2–4] columns have been described. Since both IAA and *c,t*-ABA are weak carboxylic acids reversed-phase chromatography can be performed either with a mobile phase of low pH using the ion-suppression principle[5] (see refs. 2–4) or with a mobile phase with a pH greater than 5 (when the acids are in their ionic dissociated form) if the ion-pairing[5,6] technique is used. This paper describes the application of reversed-phase ion-pair chromatography to the analysis of IAA and *c,t*-ABA in partially purified plant tissue extracts.

EXPERIMENTAL

Chromatographic techniques

Chromatography was performed using equipment manufactured by Pye Unicam (Cambridge, Great Britain): LC-XPD dual reciprocating pump, Model 7125 sample injection valve fitted with a 20-μl injection loop, and LC–UV variable-wavelength absorption detector. The column was 250 × 4.6 mm I.D. stainless steel packed with Partisil-10 ODS bonded reversed-phase material (particle size 10 μm, Whatman, Maidstone, Great Britain). The mobile phases were: (for ion-supression reversed-phase chromatography) 5% acetic acid (pH 3.0)–methanol (70:30 and 60:40); for reversed-phase ion-pair chromatography, 0.01 *M* tetramethylammonium phosphate or tetrabutylammonium hydrogen sulphate (in 0.001 *M* K_2HPO_4/KH_2PO_4, pH 6.6)–methanol (90:10–60:40). IAA, *c,t*-ABA and a mixture of *c,t*-ABA with the non-naturally occurring isomer 2-*trans*-4-*trans*-ABA (*t,t*-ABA) were purchased from Sigma (London) (Poole, Great Britain). Injections were made in 10 μl methanol and the column effluent monitored at 254 nm (ABA isomers) or 278 nm (IAA). A flow-rate of 1 ml/min was used throughout. The time equivalent of the void volume was determined by injecting 10 μl methanol and measuring the time from injection to the first deviation from the baseline.

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Plant tissue extraction and purification

Extracts were prepared from stem bark of dormant apple (*Malus domestica* Borkh., rootstock MM104) trees and leaf petiole of tobacco (*Nicotiana tabacum* cv Xanthi-nc). After the periderm had been removed the inner bark of apple stems was excised, frozen by immersion in liquid nitrogen and ground in a pestle and mortar. 1 g of the frozen powder was extracted overnight at −15°C with 5 ml redistilled methanol, the extract centrifuged and the supernatant added to 15 ml 0.5 *M* K_2HPO_4 (pH 8.5). After partitioning against 2 × 15 ml and 1 × 5 ml redistilled diethyl ether the aqueous phase was adjusted to pH 3 with 3 *M* H_3PO_4 and partitioned against 2 × 15 ml ether. The pH 3 ether-soluble fraction was reduced to dryness under a stream of nitrogen at room temperature, redissolved in 1 ml redistilled methanol and applied to a column of insoluble polyvinyl-pyrrolidone (50 × 10 mm I.D.) equilibrated with methanol[7]. The column was eluted with 12 ml redistilled methanol and the eluate reduced to *ca.* 1 ml volume by rotary film evaporation at room temperature before transfer to a 1.5-ml microvial and reduction to dryness under a stream of nitrogen. The residue was dissolved in 50 μl redistilled methanol and 10 μl injections were used for analysis. The same procedure was used for 100-mg amounts of lyophilized tobacco petiole.

RESULTS AND DISCUSSION

Chromatography on the Partisil-10 ODS column in aqueous methanol–acetic acid resolved IAA and the isomers of ABA (Table I). In 0.001 *M* phosphate (pH 6.6)–methanol (80:20) IAA was unretained whereas the isomers of ABA eluted later and

TABLE I

REVERSED-PHASE HPLC SEPARATION OF INDOLYL-3-ACETIC ACID AND ABSCISIC ACID ISOMERS ON C_{18} PARTISIL-10 COLUMN

TMA = Tetramethylammonium phosphate; TBA = tetrabutylammonium hydrogen sulphate. Flow-rate: 1 ml/min. Time equivalent of void volume: 3.2 min.

Mobile phase	*Retention time (min)*		
	IAA	*t,t-ABA*	*c,t-ABA*
Ion suppression			
5% Acetic acid (pH 3.0)–methanol (70:30)	12.8	19.0	27.0
5% Acetic acid (pH 3.0)–methanol (60:40)	8.0	9.5	12.0
Ion pairing			
0.001 *M* Phosphate (pH 6.6)–methanol (80:30)	2.9	4.0	5.7
0.01 *M* TMA in 0.001 *M* phosphate (pH 6.6)–methanol (80:20)	6.9	11.8	18.2
0.01 *M* TMA in 0.001 *M* phosphate (pH 6.6)–methanol (90:10)	9.6	24.7	45.2
0.01 *M* TBA in 0.001 *M* phosphate (pH 6.6)–methanol (60:40)	7.0	7.3	9.1
0.01 *M* TBA in 0.001 *M* phosphate (pH 6.6)–methanol (80:20)	26.7	35.1	54.3

were resolved, suggesting that adsorption or other effects also influenced the elution pattern. Including 0.01 *M* tetramethyl- or tetrabutylammonium counterion greatly increased the retention times of IAA and the isomers of ABA while maintaining the order of elution observed in ion-suppression reversed-phase chromatography (Table I). The more lipophilic counterion, tetrabutylammonium, increased the retention time of IAA relatively more than those of the ABA isomers (Table I).

Reversed-phase ion-pair chromatographic procedures gave excellent separations of IAA and *c,t*-ABA from other UV-absorbing peaks when applied to partially purified apple (Figs. 1 and 2) and tobacco (Fig. 3) extracts. Apple bark extracts contained very little *c,t*-ABA which moreover was not fully separated from adjacent UV-absorbing peaks in reversed-phase chromatography in aqueous methanol–acetic acid mixtures; an estimated tissue level of $\leq$ 8 ng/g fresh weight could with reversed-phase ion-pair chromatography be reduced to $\leq$ 5 ng/g. The purity of the peaks co-chromatographing with IAA or *c,t*-ABA was not established. The wavelength ratios of suspected IAA or *c,t*-ABA peaks were, however, similar to those observed for

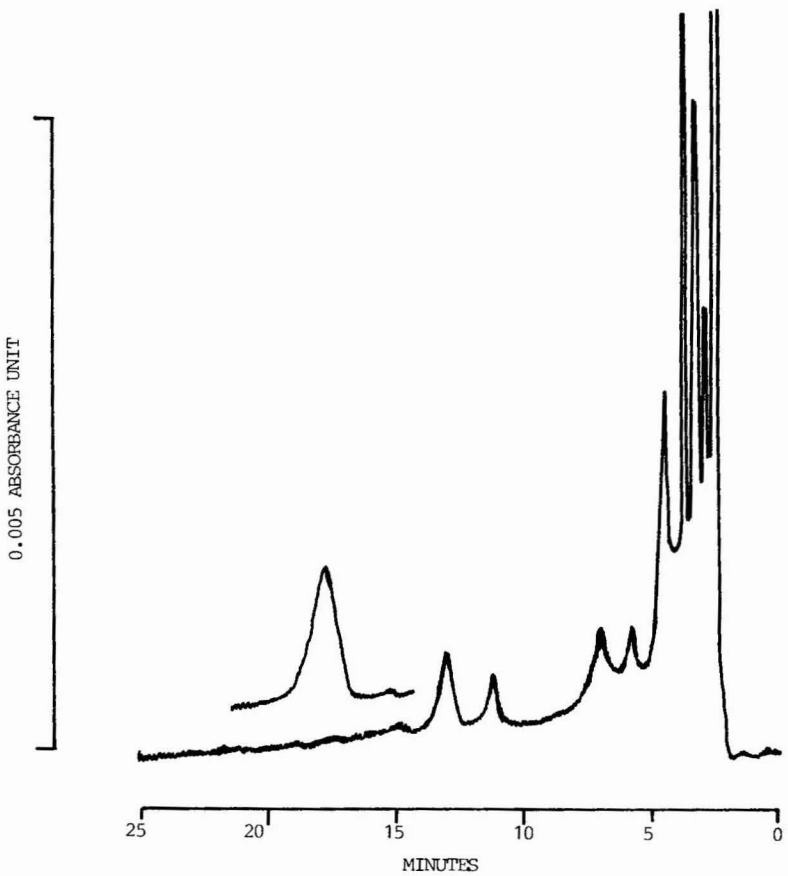

Fig. 1. Chromatogram of extract of apple (*Malus domestica* Borkh.) stem bark. Column: Partisil-10; mobile phase: 0.01 *M* tetramethylammonium phosphate in 0.001 *M* phosphate (pH 6.6)–methanol (80:20). 10-μl extract injected. UV absorption monitored at 254 nm. Upper trace: 8 μl extract and 2 μl (25 ng) *c,t*-ABA.

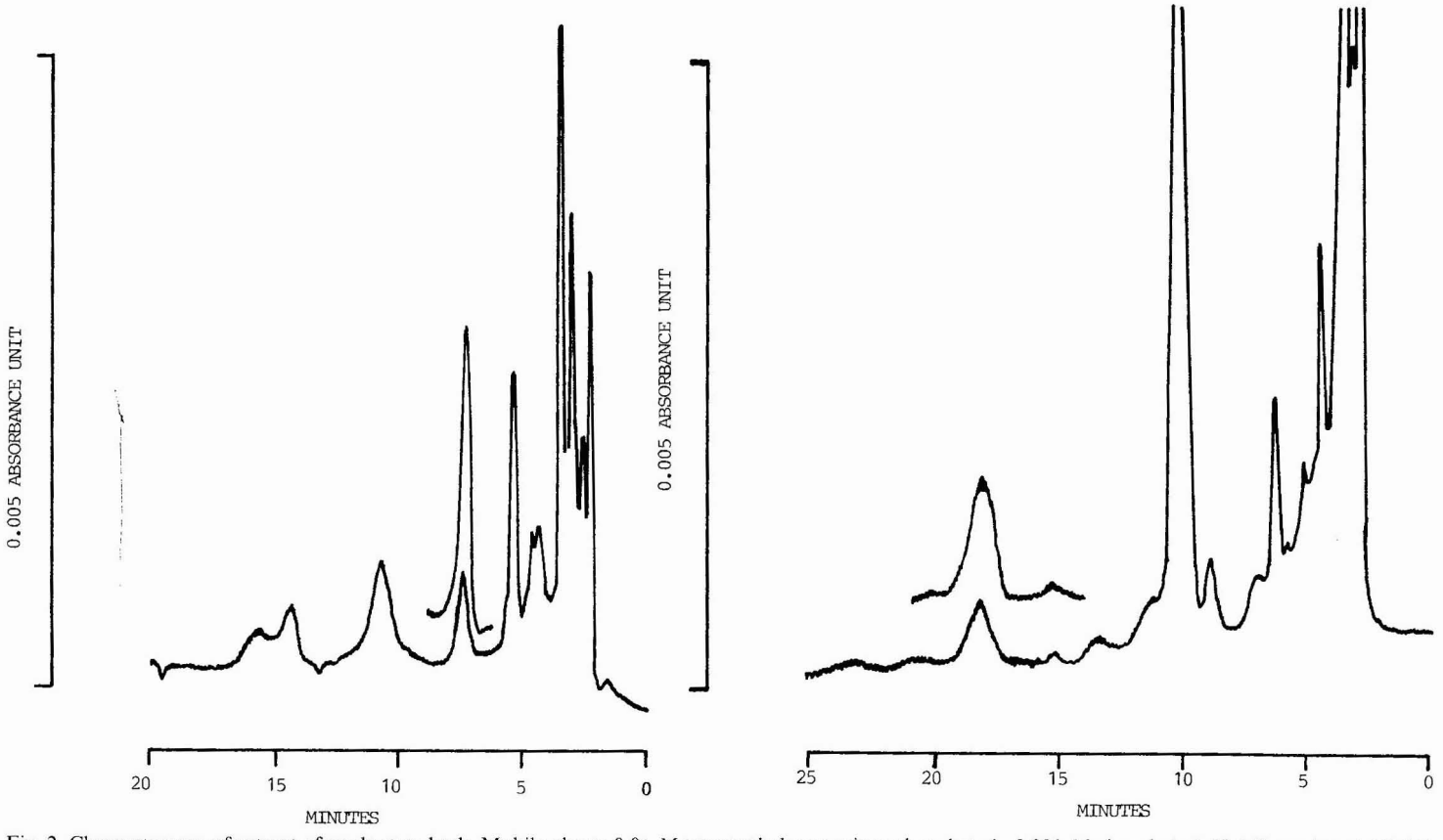

Fig. 2. Chromatogram of extract of apple stem bark. Mobile phase: 0.01 *M* tetramethylammonium phosphate in 0.001 *M* phosphate (pH 6.6)–methanol (85:15). 10-μl extract injected. UV absorption monitored at 278 nm. Upper trace: 8 μl extract and 2 μl (25 ng) IAA.

Fig. 3. Chromatogram of extract of tobacco (*Nicotiana tabacum*) petiole. Mobile phase: 0.01 *M* tetramethylammonium phosphate in 0.001 *M* phosphate (pH 6.6)–methanol (80:20). 10-μl injected. UV absorption monitored at 254 nm. Upper trace: 8 μl extract and 2 μl (12.5 ng) *c,t*-ABA.

authentic samples: for *c,t*-ABA peak height at 254 nm/peak height at 278 nm was 2.1 (observed 1.9); for IAA peak height 254 nm/peak height 278 was 0.4 (observed 0.33). Further manipulation of mobile phase composition or the nature or concentration of the counter ion may result in superior chromatographic separations and may be required to meet the demands posed by extracts of other plant tissues. The results presented here demonstrate, however, the potential value of reversed-phase ion-pair chromatography as a supplement to reversed-phase chromatography in the ion-suppression mode of analysis of weak acid solutes.

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CHROM. 13,642

Book Review

Applied headspace gas chromatography, edited by B. Kolb, Heyden & Son, London, Philadelphia, Rheine, 1980, X + 185 pp., price US$ 29.30, £13.00, DM 61.00, ISBN 0-85501-488-1.

This book contains 21 papers selected from those presented at the *GC Headspace Symposium* (held on October 5th, 1978, in Beaconsfield, Great Britain) and the *2nd International Colloquium on GC Headspace Analysis* (held from October 18th to 20th, 1978, in Überlingen, G.F.R.). Both events were organized by Perkin-Elmer. The basis for the Editor's selection of papers was, in his own words, "the desire to provide a representative coverage of the most typical applications of headspace-gas analysis". Although all of the contributions are more or less on the same topic, the different standards of the individual papers and the diversity of the subject matter dealt with make it expedient to mention each paper separately.

Ch. 1: a paper entitled "Physicochemical applications of headspace gas chromatography", demonstrating the use of headspace-gas analysis (HSGA) to the determination of the Raoult's-law activity coefficients of volatile components of mixtures of known composition and to the measurement of the adsorption isotherms of volatile compounds in defined inert gas–solute–adsorbent systems. Unfortunately, the discussion on quantitative HSGA, which actually is simply a demonstration of how three proportionality constants are combined into a single one, is very misleading. Ch. 2: a comparison of the performances of wall-coated open tubular and ordinary packed columns regarding the sensitivity of gas chromatographic (GC) analysis. The experimental findings might have been easily predicted theoretically. Ch. 3: a method for the determination of air pollutants by trapping them in a cooled adsorbent, transferring the latter with the analyte into an equilibration vial, heating the contents in the presence of a displacer and applying HSGA. The theoretical treatment is misleading. Ch. 4: a competent discussion of analytical problems occurring in the field of industrial hygiene. Examples are presented of using the method described in Ch. 3; the reference model system/absolute calibration is applied. Ch. 5: an elegant, high-sensitivity method for the determination of carbon monoxide in air and blood. Ch. 6: an attractive variant of HSGA, in which the sensitivity of analysis is increased by sampling a relatively large volume of headspace gas at reduced pressure and subsequently compressing the sample prior to its injection into the gas chromatograph. Ch. 7: simple capillary GC analyses of headspace-gas samples taken from above samples of heavy fuel oils in thermostated vials. Ch. 8: an unusual, but probably questionable method of measuring the profiles of volatile compounds in wine, consisting in stripping the volatiles with a stream of gas, absorbing them from the gas in aqueous ethanol, extracting them from the absorbent with Freon 11 and analysing the final condensed extract by GC. One might ask why the authors did not extract the wine directly with Freon 11. Ch. 9: an interesting assay of acetohydroxy acids and

diketones in beer by employing conventional HSGA and an electron-capture detector. Ch. 10: a useful, though not unknown method of measuring "aromagrams" of spices. Ch. 11: determination of acetaldehyde, ethanol, acetone, and diacetyl in yoghurt by ordinary HSGA. Ch. 12: a knowledgeable treatise on the application of quantitative HSGA in the plastics packaging industry, demonstrating the determination of vinyl chloride monomer and acetonitrile in various fluidic and non-fluidic materials by the standard additions method. Ch. 13: determination of monomeric acrylonitrile in polymers and the co-existing food-simulating solvents by the standard additions method. The use of a charcoal-trapping/HSGA method for the determination of acrylonitrile in air is also demonstrated. Ch. 14: determination of monomeric acrylonitrile in polyacrylonitrile by dissolving the latter in a solvent and applying HSGA. Ch. 15: determination of residual solvents in packaging films by dissolving the material in a large excess of a suitable solvent and applying HSGA; calibration was carried out by the reference model system/internal standard method, employing the same solvent. Ch. 16: coupling of a modified F-40 Perkin-Elmer gas chromatograph with a data-processing system. Ch. 17: determination of trichloroethylene and its metabolites in blood and urine by HSGA, employing the reference model system/internal standard method. Ch. 18: an interesting and promising application of HSGA to measuring the profiles of volatile fatty acids present in different cultures of bacteria. The possibilities of using this method for the diagnosis of infection are shown. Ch. 19: GC measurement of the contents of C_2–C_4 hydrocarbons in the breath of animals and humans in order to study *in vivo* the ability of different chemicals to influence the peroxidation of lipids. Ch. 20: examples of the application of HSGA in forensic investigations. Ch. 21: examples of the application of HSGA to testing various materials for their health hazard.

As with every book, this one has weak points in addition to its merits. Apart from reports that evidently were not aimed at providing absolute quantitative data (Ch. 7, 8, 10, 16 and 18–21), there are papers in which the authors speak of quantitative HSGA but give no quantitative data (Ch. 2 and 9) and/or in which the procedure of quantitation proper is unclear (Ch. 3, 5 and 6). In other papers (Ch. 11, 14 and 17), the procedures involving the use of the internal standard method, although having given satisfactory results in particular instances, could hardly be employed generally in quantitative HSGA. It seems that only the authors of Ch. 4, 12, 13 and 15 fully recognized the intricacies and problems of quantitative HSGA. On the other hand, the book provides an interesting profile of applications of HSGA and characterizes the present standard of commercial instrumentation and the manner in which it is employed by most of its users. In this respect the Editor certainly achieved his goal. I believe that many practising chromatographers will find the book helpful.

Brno (Czechoslovakia) JOSEF NOVÁK

Author Index

Instructions to Authors

JOURNAL OF CHROMATOGRAPHY

JOURNAL OF CHROMATOGRAPHY, BIOMEDICAL APPLICATIONS

CHROMATOGRAPHIC REVIEWS

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General

The JOURNAL OF CHROMATOGRAPHY publishes papers on all aspects of chromatography, electrophoresis and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental development and their applications.

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

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

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The title of the paper should be concise and informative. Since titles are widely used in information retrieval systems, care should be taken to include the key words. The title should be followed by the author's full names, academic or professional affiliations, and the address of the laboratory where the work was carried out. If the present address of an author is different from that mentioned, it should be given in a footnote. Acknowledgements of financial support are not to be made in a footnote to the title or name of the author, but should be included in the Acknowledgements at the end of the paper.

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

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

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The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. One original and two photocopies are required. Attention should be given to any lettering (which should be kept to a minimum) and to spacing on axes of graphs in order to ensure that numbers, etc., remain legible after reduction of the printing. Axes of a graph should be clearly labelled. The figures should preferably be of such a size that the same degree of reduction can be applied to all of them. Standard symbols should be used in line drawings, the following are available to the printers and can also be used in the legends:

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The tables should be typed (in double spacing) on separate pages, and numbered in Roman numerals according to their sequence in the text. A brief descriptive heading should be given above each table. Below the heading the experimental conditions should be described. The layout of the tables should be given serious thought, so that the reader can grasp quickly the significance of the results.

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Widely accepted symbols and forms of abbreviation should be used. If there is any doubt about a particular symbol or abbreviation, the full expression followed by the abbreviation should be given the first time it appears in the text. Abbreviations used in tables and figures should be explained in the legends. In general, the recommendations of the International Union of Pure and Applied Chemistry (I.U.P.A.C.) should be followed.

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2 L.R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 R.D. Marshall and A. Neuberger, in A. Gottschalk (Editor), *Glycoproteins*, Part A, Elsevier, Amsterdam, 2nd ed., 1972, Ch. 3, p. 251.
4 R.H. Doremus, B.W. Roberts and D. Turnbull (Editors), *Growth and Preparation of Crystals, Proc. Int. Conf. Crystal Growth, Coopertown, N.Y., August 27–29, 1958*, Wiley, New York, 1958.

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Vol. 209, No. 3

journal of chromatography news section

SYMPOSIUM PROGRAM

SIXTEENTH INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY
"CHROMATOGRAPHY '81"

The Sixteenth International Symposium on Advances in Chromatography will be held September 28–October 1, 1981, at the Aula of the Autonomous University of Barcelona in Bellaterra, Spain.

A total of 72 papers will be presented at the Symposium representing contributions from 20 countries. A special feature of this meeting will be an exposition of the latest instrumentation and books.

Registration should be made in advance. The programs, registration forms, and hotel reservation forms can be obtained from:

Professor Ramon Segura,
Catedra de Fisiologia,
Universidad Autonoma de Barcelona,
Bellaterra (Barcelona),
Spain
Tel. (3) 692-0200 ext. 1919

or

Professor Albert Zlatkis,
Chemistry Department,
University of Houston,
Central Campus,
4800 Calhoun,
Houston, TX 77004,
Tel. (713) 749-2623

The detailed program of the Symposium is given below.

MONDAY, SEPTEMBER 28, 1981

Monday Morning
Lecture Hall: Rectorate

9:30 Professor Ramon Segura
Welcome from the City of Barcelona
Presentation of the M.S. Tswett Chromatography Medal
Address by Secretary of Health of Catalan Government

CONTEMPORARY CHROMATOGRAPHY

L.S. Ettre, presiding

10:00 Gas chromatography, mass spectrometry, and high-resolution two-dimensional electrophoresis in the study of human disease. E. Jellum, I. Bjørnson, R. Nesbakken, A. Torvik, E. Johansson and S. Wold (University of Oslo, Oslo, Norway)

10:30 Capillary columns – An overall perspective. D.H. Desty (B.P. Research Center, Sunbury-on-Thames, Great Britain)

11:00 Semi-preparative separation of complex mixtures by HPLC. I. Halász (Universität des Saarlandes, Saarbrücken, G.F.R.)

11:30 Particle size distribution of colloidal latices by sedimentation field-flow fractionation. F.-S. Yang, M.N. Myers, K.D. Caldwell and J.C. Giddings (University of Utah, Salt Lake City, UT, U.S.A.)

Monday Afternoon
Lecture Hall: Rectorate

CAPILLARY GAS CHROMATOGRAPHY

R.E. Sievers, presiding

2:00 High efficiency zone electrophoresis in open tubular glass capillaries. K.D. Lukacs and J.W. Jorgenson (University of North Carolina, Chapel Hill, NC, U.S.A.)

2:20 Separation of aromatic hydrocarbon mixtures by glass capillary column gas chromatography. R.G. Mathews, J. Torres and R.D. Schwartz (Pennzoil Products Company, Shreveport, LA, U.S.A.)

2:40 A new procedure to pretreat and deactivate glass surfaces for high temperature capillary column chromatography. A. Soustre, P. Chebrous and M. Rigaud, C.H.U. Dupuytren, Limoges, France; and J. Rosello and E. Gelpi (Instituto de Quĭmica Bio-Orgánica (C.S.I.C.), Barcelona, Spain)

3:00 The role of surface silanol and siloxane groups in effecting the chromatographic performance of certain types of fused-silica glass capillary columns S.R. Lipsky and W.J. McMurray (Yale University, New Haven, CT, U.S.A.)

3:20 Intermission

J.A. Rijks, presiding

3:35 Data noise in the laboratory and solution by specialized laboratory computer programs. R.E. Kaiser (Institut für Chromatographie, Bad Dürkheim, G.F.R.)

3:55 Characterization of solvent-refined coal liquids by chromatographic methods. F.K. Schweighardt and I.S. Kingsley (Air Products and Chemicals, Inc., Allentown, PA, U.S.A.); and W. Bertsch (University of Alabama, University, AL, U.S.A.)

4:15 Characterization of Hungarian coals by extraction and chromatographic methods. G. Alexander and I. Hazai (Hungarian Academy of Sciences, Budapest, Hungary)

4:35 Analysis of polar compounds on PEG glass capillary columns. The influence of KF and Na_3PO_4 on the change of partial molar free energy. R.V. Golovnya and A.L. Samusenko (Academy of the Sciences of the U.S.S.R., Moscow, U.S.S.R.)

TUESDAY, SEPTEMBER 29, 1981

Tuesday Morning
Lecture Hall A

GAS CHROMATOGRAPHY – THEORETICAL ASPECTS

J.C. Giddings, presiding

9:30 Selectivity in sorption and detection in chromatography of encironmental pollutants. R.E. Sievers (University of Colorado, Boulder, CO, U.S.A.)

9:55 Correlation and prediction of ECD response from molecular parameters. E.C.M. Chen and W.E. Wentworth (University of Houston, Houston, TX, U.S.A.)

10:20 Temperature gradient tubes in capillary gas chromatography. J.A. Rijks (University of Technology, Eindhoven, The Netherlands)

10:45 A new criterion for polarity of stationary phases in gas chromatography. J. Ševčík and M.S.H. Löwentap (Analytical Laboratory Systems, Deidesheim, G.F.R.)

11:10 Gas–liquid chromatographic retention behavior of hindered aliphatic esters. J.R. Chrétien (Université de Paris VII, Paris, France) and J.K. Haken (The University of New South Wales, Kensington, Australia)

11:35 Studies on improving the reproducibility of simulated distillation by gas chromatography. D.J. Abbott and A.G. King (Esso Petroleum Co. Ltd., Abingdon, Great Britain)

Tuesday Afternoon
Lecture Hall A

GAS CHROMATOGRAPHY – BIOMEDICAL APPLICATIONS

C.J.W. Brooks, presiding

2:00 An evaluation of substituted polyphenyl ethers as polar stationary phases in gas chromatography. C.F. Poole, H. Buttler and S. Agnello (Wayne State University, Detroit, MI, U.S.A.), W.-F. Sye and A. Zlatkis (University of Houston, Houston, TX, U.S.A.) and G. Holzer (Colorado School of Mines, Golden, CO, U.S.A.)

2:25 Sample purification by a C_{18}-bonded reversed-phase cartridge for the quantitative analysis of corticosteroids in adrenal cell cultures by GC–MS. L.C. Ramirez, C. Millot and B.F. Maume (Université de Dijon, Dijon, France)

2:50 Analysis of oxocarboxylic acids in urine and serum as methoximes by the use of the thermionic specific detector. H.M. Liebich, A. Pickert and J. Wöll (Medizinische Universitätsklinik, Tübingen, G.F.R.)

3:15 The simultaneous determination of diclofenac sodium and its hydroxylated metabolites by capillary column gas chromatography with electron capture detection. P.H. Degen and W. Schneider (Ciba-Geigy Ltd., Basle, Switzerland)

3:40 Intermission

E.C. Horning, presiding

3:55 Determination of chlorinated dibenzo-*p*-dioxin contaminants in 2,4-D products by gas chromatography–mass spectrometric techniques. W.P. Cochrane, J. Singh, W. Miles and B. Wakeford (Agriculture Canada, Ottawa, Canada)

4:20 Determination of C_2–C_5 hydrocarbons in the atmosphere in the lower ppb and upper ppt level. J. Rudolph, D.H. Ehhalt, A. Khedim and C. Jebsen (Institute for Atmospheric Chemistry, Jülich, G.F.R.)

4:45 Gas chromatographic behaviours of the chemical bonded microparticulate silica. C. Wang, A. Xia, G. Wang, L. Chen and P. Lu (Chinese Academy of Sciences, Dalian, China)

Tuesday Afternoon
Lecture Hall B

HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

R.E. Kaiser, presiding

2:00 Fluorescence-inducing procedures for HPTLC. L. Zhou, H. Shanfield and A. Zlatkis (University of Houston, Houston, TX, U.S.A.)

2:25 Chemical modification of HPTLC plates by *in situ* reaction with monochlorosilanes. M. Ericsson and L.G. Blomberg (University of Stockholm, Stockholm, Sweden)

2:50 Electronically differentiated signals from HPTLC plates. Applicability and performance in quantitative assays. V. Such, J. Traveset and R. Gonzalo, Lacer, S.A., and E. Gelpi (Instituto de Biofisica y Neurobiologia, (C.S.I.C.), Barcelona, Spain)

3:15 Application of TLC spectrodensitometry to the quantitative analysis of herbicide formulations. P. Hitos (Laboratorio Central de Ensayos y Análisis Agrícolas, Madrid, Spain)

3:40 Intermission

D.C. Fenimore, presiding

3:55 HPTLC – Determination of fluorescence-labeled cortisole. W. Funk, Boll, Kerler, and Dammann (Fachhochschule Giessen–Friedberg, Giessen, G.F.R.)

4:20 The influence of food-simulating liquids on charge transfer complex formation of plastic additive. G. Haesen, B. Le Goff and Ph. Glaude (Gemeenschappelijk Centrum voor Onderzoek, Petten, The Netherlands)

4:45 Continuous thin-layer chromatography of sugars of clinical interest in samples of urine impregnated on paper. J.R. Alonso-Fernandez, M.D. Boveda, C. Parrado, J. Peña and J.M. Fraga (Universidad de Santiago de Compostela, Santiago, Spain)

5:10 Thin-layer chromatography in melts of organic compounds – a new effective chromatographic technique for determination of organic compounds. V.G. Berezkin and S.L. Bolotov (Academy of Sciences of the U.S.S.R., Moscow, U.S.S.R.)

WEDNESDAY, SEPTEMBER 30, 1981

Wednesday Morning
Lecture Hall A

GENERAL CHROMATOGRAPHY

F. Bruner, presiding

9:30 Capillary supercritical fluid chromatography. M. Novotny and S.R. Springston (Indiana University, Bloomington, IN, U.S.A.) and P.A. Peaden, J.C. Fjeldsted and M.L. Lee (Brigham Young University, Provo, UT, U.S.A.)

9:55 Determination of traces of hydrogen halides in the atmosphere by collection on sampling tubes and subsequent derivatization. B. Vierkorn-Rudolph and K. Bachmann (Technische Hochschule Darmstadt, Darmstadt, G.F.R.)

10:20 Quantitative analysis of α-dicarbonyl compounds in cigarette smoke. P. Moree-Testa and Y. Saint-Jalm (Société Nationale d'Exploitation Industrielle des Tabacs & Alumettes, Paris, France)

10:45 The alkylation of organic acids for gas chromatography trace analysis – A reassessment of Claisen's carbonate method. W. Dünges (Institut für Biochemie der Deutschen Sporthochschule, Köln, G.F.R.)

11:10 The migration behaviour of hydrogen ion and its role in isotachophoresis of cations. P. Boček, P. Gebauer and M. Deml (Czechoslovak Academy of Sciences, Brno, Czechoslovakia)

11:35 New results in the gas chromatographic separation of the enantiomers of hydroxy acids and carbohydrates. W.A. König, I. Benecke and S. Sievers (Universität Hamburg, Hamburg, G.F.R.)

Wednesday Morning
Lecture Hall B

GENERAL CHROMATOGRAPHY

J. Sjövall, presiding

9:30 Dimethylformamide and carbon disulphide desorption efficiencies for organic vapors on gas sampling charcoal tubes analysed with a GC backflush technique. I. Johansen and J.F. Wendelboe (The Danish National Institute of Occupational Health, Hellerup, Denmark)

9:55 Combined TLC spectrofluorometric method for the quantitation of orthophthalaldehyde-hydroxy and methoxy indole derivatives of biological interest. J.M. Arque, A. Leiva, M. Lopez, X. Navarro and R. Segura (Universidad Autonoma de Barcelona, Bellaterra, Spain)

10:20 Comparison of the distribution constants of benzene in various sorbent–gas systems determined from gas chromatographic retention data and the corresponding sorption isotherms. J. Vejrosta, M. Roth and J. Novák (Czechoslovak Academy of Sciences, Brno, Czechoslovakia)

10:45 HPLC of nucleotides on bonding-phase adsorbents: general methods and their development. A.N. Wulfson and S.A. Yakimov (Academy of Sciences of the U.S.S.R., Moscow, U.S.S.R.)

11:10 Manganese(II)–bis(3-heptafluorobutyryl-1R-camphorate): a versatile resolving agent for cyclic ethers in complexation gas chromatography. V. Schurig and R. Weber (Universität Tübingen, Tübingen, G.F.R.)

Wednesday Afternoon
Lecture Hall A

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

P.R. Brown, presiding

2:00 Determination and control of resistance to mass transfer in liquid chromatography for high speed analyses. K. Ogan and R.P.W. Scott (Perkin-Elmer, Norwalk, CT, U.S.A.)

2:25 Optimization of HPLC separations. S.D. Mott and E. Grushka (Hebrew University, Jerusalem, Israel)

2:50 Universal liquid chromatography methods: high sensitivity, low wavelength, full gradient methods, and further sequential isocratic step applications. V. Berry (Polaroid Corp., Cambridge, MA, U.S.A.)

3:15 Effects of instrumental parameters on very-high speed liquid chromatography. J.L. DiCesare, M.W. Dong and J.G. Atwood (Perkin-Elmer, Norwalk, CT, U.S.A.)

3:40 Intermission

V. Berry, presiding

3:55 Stationary phase characterization in HPLC: a test for trace metal activity in alkyl-bonded silica gel. M. Verzele and C. Dewaela (Rijksuniversiteit Gent, Gent, Belgium)

4:20 An approach to the prediction of optimum solvent composition in liquid chromatography. M. Richard, P. Cunningham and D. Nurok (Purdue University, Indianapolis, IN, U.S.A.)

4:45 Temperature dependence of HETP for the columns with small amount of liquid stationary phase. J. Rayss and A. Waksmundzki (Maria Curie-Sklodowska University, Lublin, Poland)

5:10 Study of the dependence of liquid chromatographic performance upon pore structure of the porous polydivinylbenzene microspheres. L. Deng, X.-Z. Hu, Q.-H. Wu, Q.-C. Heng, Y.-L. Li and Y.-L. Zhang (Academia Sinica, Beijing, China)

Wednesday Afternoon
Lecture Hall B

GAS CHROMATOGRAPHY – GENERAL

C.F. Poole, presiding

2:00 Application of the split/splitless injector to environmental analysis. W. Vogt, K. Jacob and A.-B. Ohnesorge (Ludwig-Maximilians-Universität München, München, G.F.R.)

2:25 The properties of an automatic sampler with controlled injection speed of the sampler. J. Eyem (Bruker-Franzen Analytik GmbH, Bremen, G.F.R.)

2:50 Columns for amine analysis by gas–liquid chromatography. A.-M. Olsson, J.Å. Jönsson and L. Mathiasson (University of Lund, Lund, Sweden)

3:15 Comparison of column packings for trace analysis of free amines by gas–liquid chromatography. L. Mathiasson and P. Lövkvist (University of Lund, Lund, Sweden)

3:40 Intermission

W. Bertsch, presiding

3:55 Large volume injection of samples dissolved in a non-eluting solvent: application to the determination of antipyrine using normal phase HPLC. P.R. Guinebault and M. Broquaire (LERS-Synthélabo, Paris, France)

4:20 The relationship between polynuclear aromatic hydrocarbon structures and their HPLC retention data. A. Bylina, L. Gluziński, K. Leśniak, B. Radwański and P.A. Penczek (Polish Academy of Sciences, Warsaw, Poland)

4:45 Preparation and appreciation of small-bore reversed-phase high-performance liquid chromatographic column. J. Wang, R. Yao, J. Li, M. Bao and P. Lu (Chinese Academy of Sciences, Dalian, China)

THURSDAY, OCTOBER 1, 1981

Thursday Morning
Lecture Hall A

LIQUID CHROMATOGRAPHY – BIOMEDICAL APPLICATIONS

E. Grushka, presiding

9:30 Mobile phase effects on the reversed-phase retention behavior of selected groups of purine and pyrimidine compounds. M. Zakaria and P.R. Brown, (University of Rhode Island, Kingston, RI, U.S.A.) and E. Grushka (The Hebrew University, Jerusalem, Israel)

9:55 An LC method for demoxepam using a post-column photo-chemical reactor and fluorescence detection. A.H.M.T. Scholten, P.L.M. Welling, U.A.Th. Brinkman and R.W. Frei (Vrije Universiteit, Amsterdam, The Netherlands)

10:20 High-performance liquid chromatography and field desorption mass spectrometry quantification of picomole amounts of endogenous neuropeptides in biologic tissue. D.M. Desiderio, S. Yamada and F.S. Tanzer (University of Tennessee, Memphis, TN, U.S.A.)

10:45 Analyses of free stool porphyrins by HPLC. H.D. Meyer, K. Jacob and W. Vogt (Ludwig-Maximilians-Universität München, München, G.F.R.)

11:10 High-performance liquid chromatography of the oligonucleotides. J.B. Crowther and R.A. Hartwick (Rutgers University, New Brunswick, NJ, U.S.A.)

11:35 Fluorescence labelling in grease analysis of biological samples. W. Voelter (Universität Tübingen, Tübingen, G.F.R.) and R. Huber and K. Zech (Byk Gulden Lomberg Chemische Fabrik GmbH, Konstanz, G.F.R.)

Thursday Afternoon
Lecture Hall A

LIQUID CHROMATOGRAPHY – APPLICATIONS

E. Gelpi, presiding

2:00 Analysis of isomeric ethynylestradiol glucuronides in urine. M. Axelson, D.J. Collins, B.-L. Sahlberg and J. Sjövall (Karolinska Institutet, Stockholm, Sweden)

2:25 HPLC of *cis*-dichlorodiammine platinum(II) using chemically bonded and solvent generated anion exchange. L.A. Sternson, C.M. Riley and A.J. Repta (University of Kansas, Lawrence, KS, U.S.A.)

2:50 Plasma catecholamines in essential hypertension and pheochromocytoma determined using ion-pair reversed-phase chromatography with amperometric detection; methodology and clinical interpretation of data. A.M. Krstulovic and D. DiRico (Manhattanville College, Purchase, NY, U.S.A.) and S.W. Dziedzic and L. Bertani-Dziedzic (The City University of New York, New York, NY, U.S.A.)

3:15 Correlation of Hammett substituent constants with reversed-phase "ion-pair" liquid chromatographic adsorption parameters of charged organic compounds. S.N. Deming and R.C. Kong (University of Houston, Houston, TX, U.S.A.)

3:40 Intermission

L.S. Ettre, presiding

3:55 Interaction of solute-surfaced silanols for the separation of basic amine compounds. B.A. Bidlingmeyer, J. Del Rios, J. Little and J. Korpi (Waters Assoc., Milford, MA, U.S.A.)

4:20 Programmed flow preparation system. S. Hara, K. Oka, T. Ohkuma and Y. Dobashi (Tokyo College of Pharmacy, Tokyo, Japan)

4:45 Effects of radial compression on column performance in terms of reduced chromatographic parameters in HPLC. M.J. Brusich, D.P. Herman and L.R. Field (University of Washington, Seattle, WA, U.S.A.)

5:10 Closing of symposium

NEW BOOKS

Theory and mathematics of chromatography, by A.S. Said, Hüthig, Heidelberg, 1981, 210 pp., Price DM 75.00, US$ 38.00, ISBN 3-7785-0616-1.

Recent advances in capillary gas chromatography, edited by W. Bertsch, W.G. Jennings and R.E. Kaiser, Hüthig, Heidelberg, 1981, 592 pp., price DM 75.00, US$ 38.00, ISBN 3-7785-0711-7.

Optimization in HPLC, by R.E. Kaiser and E. Oelrich, Hüthig, Heidelberg, 1981, 278 pp., price DM 66.00, US$ 33.00, ISBN 3-7785-0657-9 (German edition has been reviewed in *J. Chromatogr.*, 195 (1980) 164).

Chromatography methods in inorganic analysis, by G. Schwedt, Hüthig, Heidelberg, 1981, 226 pp., price DM 75.00, US$ 38.00, ISBN 3-7785-0690-0 (German edition has been reviewed in *J. Chromatogr.*, 206(1981) 630).

Trace-organic sample handling, edited by E. Reid, Ellis Horwood (Wiley), Chichester, 1981, 383 pp., price £ 30.00, ISBN 0-85312-187-7.

Gmelin, Handbook of inorganic chemistry, Sc, Y, La–Lu – Rare earth elements, Part D 3, Springer, Berlin, Heidelberg, New York, 8th ed., 1981, XIV + 324 pp., price DM 793.00, ca US$ 467.90, ISBN 3-540-93432-4.

Fluorimetrie, by M. Zander, Springer, Berlin, Heidelberg, New York, 1981, 144 pp., price DM 68.00, ca.US$ 40.20, ISBN 3-540-10512-3.

Proceedings of the 5th symposium on chemical problems connected with the stability of explosives (Båstad, May 28–30, 1979), Parts 1 + 2, edited by J. Hansson, Sectionen för Detonik och Förbränning, Sundbyberg, Sweden, 1981, XII + 580 pp., price SwKr. 250.00.

CALENDAR OF FORTHCOMING MEETINGS

July 13–17, 1981
Plymouth, NH, U.S.A.
Second Gordon Research Conference on Analytical Pyrolysis
Contact: Dr. Henk L.C. Meuzelaar, Chairman, 1981 Gordon Research Conference on Analytical Pyrolysis, Biomaterials Profiling Center, 391 S. Chipeta Way, Suite F, Research Park, Salt Lake City, UT 84108, U.S.A.

July 26–31, 1981
Liverpool, Great Britain
6th International Symposium on Carotenoids
Contact: Dr. G. Britton, Department of Biochemistry, University of Liverpool, POB 147, Liverpool L69 3BX, Great Britain.

July 28–31, 1981
New Hampton, NH, U.S.A.
30th Anniversary Meeting of the Gordon Research Conference on Statistics in Chemistry and Chemical Engineering
Contact: Dr. Alexander M. Cruickshank, Director, Gordon Research Conferences, Pastore Chemical Laboratory, University of Rhode Island, Kingston, RI 02881, U.S.A. Tel. (401) 783–4011.

Aug. 17–21, 1981
Meriden, NH, U.S.A.
Gordon Research Conference on Ion Exchange
Contact: Dr. A.M. Cruickshank, Director, Gordon Research Conferences, Pastore Chemical Laboratory, University of Rhode Island, Kingston, RI 02881, U.S.A. Tel. (401) 783–4011, or (401) 783–3372. (Further details published in Vol. 209, No. 2)

Aug. 20–21, 1981
Helsinki, Finland
Symposium on Harmonisation of Collaborative Analytical Studies
Contact: Dr. H. Egan, Laboratory of the Government Chemist, Cornwall House, Stamford Street, London SE1 9NQ, Great Britain.

Aug. 23–28, 1981
Espoo, Finland
Euroanalysis IV – Triennial Conference of the Federation of European Chemical Societies
Contact: Professor L. Niinistoe, Department of Chemistry, Helsinki University of Technology, SF-02150 Espoo 15, Finland.

Aug. 23–28, 1981 New York, NY, U.S.A.	**National American Chemical Society Meeting** Contact: American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
Aug. 30–Sept. 5, 1981 Vienna, Austria	**XI International Congress of Clinical Chemistry – IV European Congress of Clinical Chemistry** Contact: Congress Secretariat: Interconvention, P.O. Box 35, A-1095 Vienna, Austria. Tel. (0222) 421352.
Sept. 1–3, 1981 Houston, TX, U.S.A.	**"EXPOCHEM '81"** Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel. (713) 749–2623.
Sept. 1–4, 1981 Siofok, Hungary	**3rd Danube Symposium on Chromatography** Contact: Hungarian Chemical Society, H-1368 Budapest, P.O.B. 240, Hungary. Tel. Budapest 427–343. (Further details published in Vol. 189, No. 2).
Sept. 7–11, 1981 High Tatras, Czechoslovakia	**International Symposium on Clathrate Compounds and Molecular Inclusion Phenomena** Contact: Doc. Ing. Anna Sopková, CSc., Special Group of Inorganic Chemistry in Eastern Slovakia, Slovak Chemical Society at Slovak Academy of Sciences, Moyzesova 11, 041 67 Košice, Czechoslovakia.
Sept. 20–25, 1981 Philadelphia, PA, U.S.A.	**8th Annual Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies (FACSS)** Contact: Richard J. Knauer, Publicity Chairman, ARMCO INC., P.O. Box 1697, Baltimore, MD 21203, U.S.A.
Sept. 28–Oct. 1, 1981 Barcelona, Spain	**16th International Symposium Advances in Chromatography** Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel. (713) 749-2623. (Further details published in Vol. 205, No. 2).
Sept. 28–Oct. 4, 1981 Moscow, U.S.S.R.	**International Symposium on Chemical Physics** Contact: Prof. N.M. Emmanuel, Institute of Chemical Physics, Academy of Sciences of U.S.S.R., Vorobyevskoye Chaussee 2-6, SU–117334 Moscow, U.S.S.R.
Sept. 29–Oct. 2, 1981 Basle, Switzerland	**ILMAC 81; 8th International Exhibition of Laboratory, Chemical Engineering, Measurement and Automation Techniques in Chemistry** Contact: D. Gammeter, Secretariat ILMAC 81, Postfach, CH-4021 Basle, Switzerland. Tel. 061 20 20 20.
Oct. 22–23, 1981 Montreux, Switzerland	**Workshop on Liquid Chromatography – Mass Spectrometry** Contact: Prof. Dr. R.W. Frei, Free University, Department of Analytical Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands (Further details published in Vol. 207, No. 3).
Oct. 27–29, 1981 London, Great Britain	**Petroanalysis 81** Contact: Miss I.A. McCann, Conference Officer, Institute of Petroleum, 61 New Cavendish Street, London W1M 8AR, Great Britain. (Tel: 01–636 1004, Telex: 264380)
Nov. 9–10, 1981 Berlin, G.F.R.	**Symposium on Practical Aspects of HPLC** Contact: Dr. I. Molnár, Wissenschaftliche Geratebau Dr. H. Knauer GmbH, Hegauer Weg 38, D-1000 Berlin 37, G.F.R. (Further details published in Vol. 207, No. 2).

PUBLICATION SCHEDULE FOR 1981

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

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

INFORMATION FOR AUTHORS

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

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

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

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

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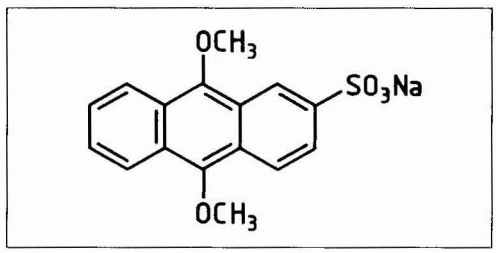
9,10-DIMETHOXYANTHRACENE-2-SULFONIC ACID SODIUM SALT, Na-DAS

1981



Fluorimetric determination of amine-containing drugs by ion-pair extraction[1)2)]

The determination of drugs in biological materials requires high sensitivity which can be obtained by fluorimetric measurements. Since most substances are non-fluorescent or weakly fluorescent, it is necessary to transform these molecules into a fluorescent form. Na-DAS offers a fluorescent counter ion for the ion-pair extraction of ionizable organic compounds. The following features of Na-DAS lead to the determination of an anticholinergic drug in blood plasma in a very low concentration range:

- ideal dissociation constants of the corresponding ion-pairs
- excellent extraction properties of the formed complexes
- high fluorescent intensities

An automated analysis system was developed, which makes it possible to determine amines in pharmaceutical products in the parts per billion (10^{-9}) range with high reproducibility.

Post-column derivatization system using the fluorimetric ion-pair technique

The automated system has now been adapted as a post-column reactor and first reported for the assay of hyoscyamine in low doses[4)]. The amines separated by HPLC are mixed in an air-segmented flow with Na-DAS, the ion-pairs formed are extracted and detected by their fluorescence (383/446 nm). The limit of detection e.g. for hyoscyamine was about 200 pg (signal-to-noise ratio = 3:1), the improvement therefore being at least 200-fold in comparison with UV detection[1)]. In the meantime a number of drug substances have been investigated and determined by this method[4)5)6)7)]. The usefulness of this derivatization technique in view of routine quality control and for dissolution rate testings has recently been demonstrated[8)].

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2) D. Westerlund, K.H. Karset, Anal. Chim. Acta *67*, 99 (1973)
3) J.C. Gfeller and G. Frey, Z. Anal. Chem. *291*, 332 (1978)
4) J.C. Gfeller, G. Frey, J.M. Huen and J.P. Thevenin, J. High Resolut. Chromatogr. Chromatogr. Commun. *4*, 213 (1978)
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7) J.F. Lawrence, U.A.Th. Brinkman and R.W. Frei, J. Chromatogr. *171*, 73 (1979)
8) J.C. Gfeller, G. Frey, J.M. Huen and J.P. Thevenin, in "Proceedings of 10th Chromatogr. Symp., Venice, 1979", Verlag Elsevier, Amsterdam (1980)

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