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VOL. 116, NO. 1 JOURNAL OF CHROMATOGRAPHY JANUARY 7, 1976

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LIQUID COLUMN CHROMATOGRAPHY

A Survey of Modern Techniques and Applications

edited by **Z. DEYL, K. MACEK** and **J. JANÁK**

JOURNAL OF CHROMATOGRAPHY LIBRARY, Vol. 3

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Proceedings of the Third International Symposium on Isoelectric Focusing and Isotachophoresis held at the Department of Biochemistry, University of Milan, Milan, Italy, September 2-5, 1974

edited by P. G. RIGHETTI, *Department of Biochemistry, University of Milan, Italy.*

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The aim of this Third International Symposium and the resulting proceedings volume is to present a detailed treatment of the techniques of isoelectric focusing and isotachophoresis. Established procedures in the field as well as recent developments are elaborated upon, with particular emphasis given to the preparative aspects of both techniques.
Certain problems due to pH gradient stability and to isoelectric focusing in extreme pH are extensively discussed, and recent advances in the field of immuno-isoelectric focusing are presented. There are several reports on the applications of the two techniques in the biomedical sciences, both in research and in routine diagnosis: screening of human sera, sweat, human biopsies, isozymes in normal and pathological tissues. Recently discovered applications to the analysis of membrane and mitochondrial proteins, in bacterial taxonomy, and in separations of whole cells and subcellular particles are presented and evaluated. A new and exciting approach reported is the separation of nucleic acids, particularly the mRNA's for α- and β-globin chains.

The round table discussion at the end of the book summarizes the fundamental and practical aspects of isoelectric focusing and isotachophoresis. The discussion of eleven topics points out the advantages, as well as the limitations and pitfalls, of the two methods.

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SOLUBILITY PARAMETERS OF ALKYL DERIVATIVES OF PHENOL AND RESORCINOL*

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(Received July 17th, 1975)

SUMMARY

On the basis of saturated vapour pressure data, the values of solubility parameters and their temperature dependence in the range 25–200°C for about 50 alkyl derivatives of phenol and resorcinol were calculated and a rough estimation of their dispersion, polar and hydrogen bond components was made.

A comparison was made with the data obtained by using the distribution coefficient of phenols in apolar solvent–water systems and gas chromatographic retention data, the relationship with the phenol structure was investigated and calculation methods were carried out.

INTRODUCTION

Hildebrand's[1] solubility parameter in terms of the cohesive energy density and its breakdown into its various components[2,3] offers a basis for the general treatment of chromatographic retention[4]. However, in practice, the determination of solubility parameter values from the equilibrium data and *vice versa* is often difficult. This difficulty is caused by the restrictions of regular solution theory, whereas in real solutions forces other than dispersion forces play an important role (owing to the presence of polar groups and, exceptionally, $-CH_2-$ chains also. In the liquid phase, these difficulties have often been circumvented by the use of empirical solubility parameter values[5–8], but in the gaseous phase they have mostly been ignored (by taking the compressibility factor as equal to unity). In phenols, the intermolecular interaction is complicated and these simplifications may be effective to different extents. As solubility parameter data for phenols are scarce[2,3,8–10], it was our aim to calculate them by making use of various methods and, by comparing all of the results, to draw some conclusions about their general usefulness.

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* Presented at the 5th Soviet–Italian Chromatography Symposium, Tallinn, April 22–25, 1975.

THEORETICAL

Basic relationships[1,2,7]

$$\delta = \left(-\frac{E}{v}\right)^{\frac{1}{2}} = \left(\frac{(\Delta H_v - RT)z}{v}\right)^{\frac{1}{2}} \qquad (1)$$

$$\delta_T^2 = \delta_d^2 + \delta_p^2 + \delta_h^2 \qquad (2)$$

$$RT \ln k_x = v_x \left[(\delta_x - \delta_\mathrm{M})^2 - (\delta_\mathrm{S} - \delta_x)^2\right] \qquad (3)$$

$$RT \ln (t_x/t_{st}) = (v_{st} - v_x)\,\delta_\mathrm{S}^2 - 2\,(v_{st}\cdot\delta_{yt} - v_x\cdot\delta_x)\,\delta_\mathrm{S} \qquad (4)$$

where

δ = solubility parameter, $(\mathrm{cal/cm^3})^{\frac{1}{2}}$;
E = energy of vaporization, cal/mole;
ΔH_v = heat of vaporization, cal/mole;
v = molar volume, cm^3;
z = compressibility factor (taken as unity);
R = universal gas constant, cal/deg·mole;
T = temperature, °K;
k = molar distribution coefficient;
t = gas chromatographic retention time.

Subscripts T, d, p, h are total value and its dispersion, and polar and hydrogen bonding components, respectively; x and st = the partitioned substances; M and S = mobile and stationary phases, respectively; w and c = water and critical, respectively.

The dispersion component was calculated from a homomorph plot for cycloparaffin hydrocarbons[11] and that of the hydrogen bond from the value of bond energy of 4300 cal per bond[12]; the values of δ_p were found from the difference. As error analysis for eqns. 1, 3 and 4 has shown, the relative errors in the values of E, t_x and k_x may not exceed 2, 30 and 50%, respectively, when calculating the values of δ or δ_x with an absolute error of less than 0.1 unit. It seems that calculations from eqns 3 and 4 are more precise. In fact, however, the latter relationships are good enough only for regular solutions.

Initial data

The heats of vaporization given in Table I were calculated from the saturated vapour pressures[13,14] by using the Douglas–Avakyan differentiation method[15].

All non-available ΔH_v values of phenols that contain up to 14 carbon atoms per molecule were calculated in the temperature range 20–200°C by the method of linear interpolation and extrapolation (with an error of about ± 700 cal/mole). The remainder of the data were obtained from the following relationships.

For alkylresorcinols (the data of Lille[16] were treated): $v^{20} = 1.202M - 44.32$, $M = 110$–376, $2s = 0.70$, $n = 9$; $\mathrm{d}v^t/\mathrm{d}t = 8.28\cdot10^{-4}\cdot M - 0.050$, $t = 25$–200°C, $2s = 4.2\cdot10^{-4}$, $n = 9$. At 20–22°C: for 2-isomers, $\log k = 0.4900\cdot n_C - 0.8000$, $2s = 0.49$; for 4-isomers, $\log k = 0.6128\cdot n_C - 1.1866$, $2s = 0.15$; for 5-isomers, $\log k = 0.5007\cdot n_C - 1.2473$, $2s = 0.12$. Here, M = molecular weight, k = weight distribu-

TABLE I

INITIAL VALUES OF HEATS OF VAPORIZATION (kcal/mole) AND BOILING POINTS (°C)

Alkylphenol, PhOHR				*Alkylresorcinols, Ph-1,3-(OH)$_2$R*			
R	ΔH_v^{298}	ΔH_v	t_B	R	ΔH_v^{298}	ΔH_v^{413}	t_B
—	13.09	11.96	182		21.69	17.48	273
2-C_1	13.62	11.94	191	2-C_1	16.00	20.48	264
2-C_2	13.65	12.54	207	2-C_4	19.09	27.74	—
2-C_4	16.57	13.69	237	2-C_6	20.77	28.97	324
3-C_1		12.37	203	4-C_1	16.94	20.96	269
3-C_2	16.06	13.45	214	4-C_5	21.56	27.71	
3-C_3	16.33	14.66	228	4-C_6	23.41	30.98	340
3-C_4		14.44	249	5-C_1	22.87	18.44	289
4-C_1		12.62	202	5-C_2	24.48	19.79	308
4-C_2	16.52	13.48	219	5-C_7	35.50	25.72	393
4-C_3		13.88	232	2,5(C_1)$_2$	17.63	21.94	280
4-C_4	19.09	15.07	250	5-C_1-2-C_2	17.96	22.62	—
4-C_5	20.27	15.73		5-C_1-2-C_6	22.52	31.63	—
2,4-(C_1)$_2$*		12.59	211	4,6-(C_1)$_2$	17.49	21.00	279
2,6-(C_1)$_2$		12.79	212	4,5-(C_1)$_2$	17.85	—	—
3,4-(C_1)$_2$	15.51	13.85	227				
3,5-(C_1)$_2$	16.47	13.80	220				
2,3-(C_1)$_2$		12.12	218				

* Also for 2,5- isomer.

tion coefficient for benzene–water, t = temperature (°C), n_C-number of carbon atoms in the side-chain, s = standard error and n = number of data.

For alkylphenols (calculated from ref. 17): $v^{20} = 1.289 \cdot M - 37.36$, $M = 94$–150, $2s = 6.8$, $n = 9$; $\mathrm{d}v^t/\mathrm{d}t = 1.56 \cdot 10^{-3} \cdot M - 0.0777$, $t = 25$–200°C, $2s = 0.0132$.

In addition, literature data for the gas chromatographic relative retention times of alkylphenols on 60 phases were used; these data were partially summarized by Kharlamovich and Churkin[18].

RESULTS AND DISCUSSION

From the values of the solubility parameters calculated on the basis of heats of vaporization (Tables II and III), the tendencies related to the phenolic structure of the molecules can be seen. The addition of a hydroxyl group to the molecule increases the δ_T value, while the addition of a CH_2– group decreases it. Also, the influence of an alkyl chain in the *ortho* position relative to the hydroxyl group is considerably stronger than that of an alkyl chain in the *meta* or *para* position. For dibasic phenols, an increase in temperature causes a greater decrease in the δ_T value than that for monobasic phenols. This effect is connected with the lower $\mathrm{d}H_v^t/\mathrm{d}t$ value for the latter compounds.

The energy density of intermolecular interactions caused by dispersion forces and hydrogen bonds shows a trend towards lower values as the length of the alkyl chain increases. At the same time, the energy density of polar interactions increases.

These results are in accordance with the increase in molecular polarization caused by an increase in the alkyl chain length in 2-alkylresorcinols[20]. Considering this effect, it is necessary to note that the equation of Böttcher, derived for spherical

TABLE II

SOLUBILITY PARAMETERS FOR ALKYLPHENOLS, PhOHR

R	*20 °C*		*80 °C*				$-\frac{d\delta_T}{dt}\cdot 10^2$	T_C (°K)**
	δ_T	δ_T*	δ_T	δ_d	δ_p	δ_h		
—	12.0	12.6 (ref. 8)***	11.3	8.7	2.7	6.8	1.05	695
2-C_1	11.1	11.3 (ref. 10)	10.5	8.4	0.4	6.3	1.02	693
2-C_2	10.7	—	10.1	8.0	1.6	5.8	1.12	701
2-C_3	10.4	—	9.7	7.8	1.8	5.5	1.20	707
2-C_4	10.2	—	9.4	7.6	2.1	5.1	1.26	715
2-C_5	9.9	—	9.1	7.3	2.4	4.9	1.31	724
2-C_6	9.7	—	8.9	7.1	2.7	4.6	1.36	731
3- and 4-C_1	11.7	12.2 (ref. 8)	10.9	8.3	3.3	6.3	1.22	710/709
3- and 4-C_2	11.4	11.3 (ref. 10)	10.6	8.1	3.5	5.8	1.35	711/718
3- and 4-C_3	11.2	—	10.3	7.8	3.7	5.5	1.46	717/723
3- and 4-C_4	10.9	—	10.0	7.6	3.9	5.1	1.55	732/734
3- and 4-C_5	10.7	—	9.8	7.5	4.0	4.9	1.62	742/743
3- and 4-C_6	10.5	—	9.5	7.2	4.2	4.6	1.68	740/752
2,6-$(C_1)_2$	10.7	—	10.1	8.1	1.4	5.8	1.12	708
2,4-$(C_1)_2$	11.0§, §§	11.2 (ref. 10)	10.1	8.1	1.4	5.8	1.37	707
2,3-$(C_1)_2$	10.9§	11.2 (ref. 10)	10.1	8.1	1.4	5.8	1.41	717
3,5-$(C_1)_2$	11.6§	11.3 (ref. 10)	10.6	8.1	3.5	5.8	1.41	720
3,4-$(C_1)_2$	11.3§	11.3 (ref. 10)	10.6	8.1	3.4	5.8	1.08	730

* See text.

** In the absence of boiling point data, the values of $\Delta t_B(CH_2)$ for 2-R = 14.5° and for 3- and 4-R = 15.5° were used.

*** 11.4 according to ref. 3.

§ Without smoothing of ΔH_v values over n_C.

§§ Also for the 2,5- isomer.

molecules with a dipole at the centre and used by Hansen[2], when used with alkylphenols that have long alkyl chains, gives apparent δ_p^2 values that are up to 10 times lower than the actual values (taking into account the acentric location of the dipole).

The separation of δ_p^2 and δ_h^2 is very approximate and only a rough estimate can be made. Probably an examination of the absorbances of associated hydroxyl groups in the 2.75–3.20 μm range in the IR spectra would show the order of δ_h^2 values 2-<4-<5-alkylresorcinol, in accordance with their acidities[16].

The total solubility parameters calculated from the molar distribution coefficients between an apolar solvent and water (marked with asterisk in Tables II and III) are in fair agreement with those calculated from the heats of vaporization. In previous calculations for monobasic phenols, cited in Table II, the empirical value $\delta_h = 19.0$ was mostly used[8]. The specific interaction of alkylresorcinols with water is stronger than that of alkylphenols and therefore the δ_h values must be lower. Indeed, for the latter compounds in benzene–water, the value of $\delta_h = 18.0$ is suitable. From these data, one can conclude that the degree of association of phenols in the vapour phase is negligible.

The calculation of δ_x values for phenols by using eqn. 4 is impossible owing to the lack of data for most stationary phases. However, in order to establish the predicting power of this equation in this instance, by using the known δ_x values for alkylphenols, we have calculated the δ_S values for 60 stationary phases. Some of these re-

TABLE III

SOLUBILITY PARAMETERS FOR ALKYLRESORCINOLS, Ph-1,3-(OH)$_2$R

R	*20 °C*		*80 °C*				$-\frac{d\delta_T}{dt}\cdot 10^2$	T_C *(°K)***
	δ_T	δ_T*	δ_T	δ_d	δ_p	δ_h		
—	15.4	14.7***	14.4	9.0	5.5	9.8	1.65	804
2-C$_1$	13.6	13.8	12.7	8.6	2.8	8.9	1.55	775
2-C$_2$	13.3	13.5	12.3	8.3	3.8	8.3	1.69	777
2-C$_3$	13.0	13.2	11.9	8.1	4.0	7.7	1.80	779
2-C$_4$	12.8	13.0	11.7	7.9	4.6	7.3	1.89	783
2-C$_5$	12.6	12.9	11.4	7.6	4.9	6.9	1.97	787
2-C$_6$	12.5	12.8	11.2	7.4	5.2	6.6	2.04	792
2-C$_7$	12.4	12.6	11.1	7.2	5.6	6.4	2.10	798
2-C$_8$	12.2	—	11.0	7.0	5.9	6.1	2.15	804
4-C$_1$	13.9§	14.0	12.9	8.6	3.6	8.9	1.65	782
4-C$_2$	13.5	13.6	12.5	8.4	4.2	8.3	1.68	786
4-C$_3$	13.2	13.3	12.2	8.1	4.8	7.7	1.71	792
4-C$_4$	13.0	13.0	11.9	8.0	4.9	7.3	1.73	798
4-C$_5$	12.8	12.8	11.7	7.8	5.3	6.9	1.75	805
4-C$_6$	12.6	12.6	11.6	7.5	5.9	6.6	1.77	813
4-C$_7$	12.5	12.5	11.4	7.3	6.0	6.4	1.78	823
4-C$_8$	12.4	—	11.3	7.1	6.3	6.1	1.79	830
5-C$_1$	14.7§§	14.2	13.6	8.8	5.3	8.9	1.73	811
5-C$_2$	14.2	13.8	13.2	8.5	5.8	8.3	1.81	822
5-C$_3$	13.9	13.5	12.8	8.3	5.9	7.7	1.87	831
5-C$_4$	13.6	13.2	12.5	8.1	6.1	7.3	1.92	837
5-C$_5$	13.4	13.1	12.2	7.9	6.2	6.9	1.96	848
5-C$_6$	13.2	12.9	12.0	7.7	6.4	6.6	2.00	858
5-C$_7$	13.2	12.8	11.8	7.5	6.5	6.4	2.33	870
5-C$_8$	12.9	—	11.7	7.3	6.8	6.1	2.06	882

* See text.

** In the absence of boiling point data the values of $\Delta t_B(CH_2)$ for 2-R = 12°, 4-R = 14° and 5-R = 17° were used.

*** Calculated as 14.9 according to ref. 19.

§ Without smoothing of ΔH_v values over n_C for R = 4.6 — (C$_1$)$_2$ and 4.5 — (C$_1$)$_2$, 13.0 and 14.4, respectively.

§§ See §, for R = 2.5 – (C$_1$)$_2$, 5-C$_1$ – 2-C$_2$ and 5-C$_1$ – 2-C$_6$, 13.3, 12.7 and 12.4, respectively

sults are shown in Table IV. On 59 phases (sodium dodecylbenzenesulphonate being the only exception), the calculated δ_S values were distributed into two groups in accordance with the structure of the phenol. The values, calculated from the data for *meta*- and *para*-substituted alkylphenols, are as a rule higher than those calculated from the data for *ortho*-substituted alkylphenols. For example, the δ_S values obtained for glycerine are 13.6 ± 0.2 and 11.8 ± 0.3, respectively (at 160 °C). For this phase, some vapour pressure data are known[21,22] and from these data δ_S values of 13.6–15.4 were estimated at the same temperature.

For *ortho*-substituted alkylphenols, the relative retention times are higher than those calculated from the Rohrschneider equation (exceptionally, 2,6-dimethylphenol often behaves in the same manner as the 3,4- and 3,5- isomers). Sometimes, the relative retention is so high that eqn. 4 has an imaginary solution, particularly

TABLE IV

TOTAL SOLUBILITY PARAMETERS FOR SOME GROUPS OF LIQUID PHASES, CALCULATED BY USING EQN. 4 ON THE BASIS OF RETENTION DATA FOR ALKYLPHENOLS ($M \leqslant 122$)

Phenols	*Phase*	δ_T, *160 °C*	$d\delta_T/dt \cdot 10^2$	*n*	*Temperature range (°C)*
3-, 4-, 3,4- and 3,5-substituted phenols (δ_S')	Apiezon L (M,W)	11.05 ± 0.84	5.00 ± 0.7	41	135–200
	Phthalates	11.18 ± 0.48	5.13 ± 0.4	39	115–190
	Phosphates	11.91 ± 1.08	5.30 ± 0.4	45	110–200
	Sugars, alcohols	13.79 ± 0.80	3.67 ± 1.0	47	140–180
Ortho-substituted phenols (except 2,6-isomer (δ_S'')	Apiezon L (M,W)	7.73 ± 1.34	2.36 ± 1.1	40	135–200
	Phthalates	8.50 ± 1.02	2.84 ± 0.8	44	115–190
	Phosphates	9.47 ± 1.32	2.46 ± 0.6	48	110–200
	Sugars, alcohols	12.07 ± 0.90	1.94 ± 1.3	41	140–180

on apolar phases. Apparently, for *ortho*-substituted alkylphenols, those factors which are critical for obeying to the Rohrschneider equation (in particular the distribution in the stationary phase and the fugacity coefficient) are different from those of the standard compound (phenol). Probably, with 2,6-dimethylphenol mutual compensation of these factors takes place. It is noteworthy that such phenomena have also been noticed in extraction. For instance, for the distribution of 3-methylphenol in an apolar solvent–water system, a value of $\delta_w = 19.0$ is appropriate, but for the 2-isomer $\delta_w = 18.0$ is more suitable[8].

The interaction of sodium dodecylbenzenesulphonate with phenols is strong and the solute emerges from the column at a temperature considerably higher than its normal boiling point (methyl- and dimethylphenols at 260°C).

Thus, with a suitable standard substance, the Rohrschneider equation can be used for the estimation of the total solubility parameters (Table V) and also the heats of vaporization for compounds with any polarity.

It may also be possible for gas chromatography to be used for estimating the components of the total solubility parameter.

CONCLUSION

By means of saturated vapour pressures, distribution coefficients in an apolar solvent–water system and gas chromatographic retention data, values of the total solubility parameters for phenols have been obtained. However, the use of empirical solubility parameter values for water and suitable standard substances in these methods was necessary. The calculated values of the solubility parameters and their temperature dependance for about 50 phenols would be useful in order to choose the optimal operating conditions for their chromatographic and extractive separation.

TABLE V

COMPARISION OF TOTAL SOLUBILITY PARAMETER VALUES CALCULATED FOR SOME ALKYLPHENOLS BY USING EQNS. 1 AND 4 ON VARIOUS STATIONARY PHASES

Stationary phases: A, Apiezon L, $\delta'_s = 12.07$, $\delta''_s = 8.90$; B, Exythrit, $\delta'_s = 14.14$, $\delta''_s = 12.68$; C, dimenthyl phthalate, $\delta'_s = 12.27$, $\delta''_s = 9.35$; D, tri-2,4-xylenyl phosphate, $\delta'_s = 15.03$, $\delta''_s = 11.83$.

R	*150 °C*			*136 °C*		*120 °C*	
	Eqn. 1	*Eqn. 4, A*	*Eqn. 4, B*	*Eqn. 1*	*Eqn. 4, C*	*Eqn. 1*	*Eqn. 4, D*
2-C_1	9.7	9.82	9.78	9.9	9.95	10.1	10.17
3-C_1	10.1	10.07	10.06	10.2	10.22	10.4	10.42
4-C_1	10.1	10.07	10.04	10.2	10.20	10.4	10.42
2,3-$(C_1)_2$	9.3	9.34	9.37	9.4	9.47	9.6	9.61
2,4-$(C_1)_2$	9.3	9.29	9.28	9.4	9.40	9.6	9.61
2,5-$(C_1)_2$	9.3	9.27	9.27	9.4	9.42	9.6	9.61
2,6-$(C_1)_2$	9.3	9.25	9.10	9.4	9.24	9.6	9.58
3,4-$(C_1)_2$	9.6	9.68	9.70	9.8	9.84	10.0	10.04
3,5-$(C_1)_2$	9.6	9.63	9.63	9.8	9.81	10.0	10.04
2-C_2	9.3	9.24	9.24	9.4	9.38	—	—
3-C_2	9.6	9.63	9.62	9.8	9.80	—	—
4-C_2	—		9.62	9.8	9.78	—	—
5-C_2	—		9.26	-	—	—	—

REFERENCES

1 J. H. Hildebrand, J. M. Prausnitz and R. L. Scott, *Regular and Related Solutions*, Van Nostrand, Reinhold, New York, 1970, pp. 116–196 and 207–215.
2 C. M. Hansen, *J. Paint Technol.*, 39 (1967) 109 and 511.
3 L. Snyder, in J. J. Kirkland (Editor), *Modern Practice of Liquid Chromatography*, (in Russian). Mir, Moscow, 1974, pp. 97–122.
4 A. Hartkopf, *J. Chromatogr. Sci.*, 12 (1974) 113.
5 T. Wakabayashi, *Bull. Chem. Soc. Jap.*, 40 (1967) 2836.
6 J. Kojima, M. Yoshida and M. Tanaka, *J. Inorg. Nucl. Chem.*, 32 (1970) 987.
7 L. Rohrschneider, *J. Gas Chromatogr.*, 6 (1968) 5.
8 L. I. Melder and H. Tamvelius, *Izv. Akad. Nauk Est. SSR. Khim. Geol.*, 21 (1972) 204.
9 H. Burrell, *Interchem. Rev.*, 14 (1955) 31.
10 H. Tamvelius and L. I. Melder, *Tr. Tallin. Politekh. Inst.*, No. 356 (1974) 49.
11 J. G. Helpinstill and M. van Winkle, *Ind. Eng. Chem., Process Des. Develop.*, 7 (1968) 213.
12 G. C. Pimentel and A. L. McClellan, *The Hydrogen Bond*, (in Russian), Mir, Moscow, 1964, p. 194.
13 H. Stage, E. Müller and P. Faldix, *Erdöl Kohle*, 6 (1953) 375.
14 H. A. Kundel, Ü. E. Lille and N. Kaidash, *Trudy Tallin. Polytech. Inst.*, No. 392 (1975).
15 R. C. Reid and T. K. Sherwood, *The Properties of Gases and Liquids* (in Russian), Khimiya, Leningrad, 1971, pp. 111–162.
16 Ü. E. Lille, *Issledovaniya v Oblasti Alkilrezortsinov, Dok. Dissertation*, Tallinn, 1973, pp. 83 and 44.
17 H. Raudsepp, unpublished data.
18 G. D. Kharlamovich and Y. V. Churkin, *Fenoly*, Khimiya, Moscow, 1974, p. 52.
19 K. B. Sandell, *Monatsh. Chem.*, 89 (1958) 36.
20 B. Granzhan, Ü. Lille and L. Bitter, *Izv. Akad. Nauk Est. SSR. Khim. Geol.*, 22 (1973) 103.
21 *Handbook of Chemistry and Physics*, Vol. 2, Chemical Rubber Publishing Co., Cleveland, Ohio, 37th ed., p. 2185.
22 J. L. Knunjants (Editor), *Kratkaya Khimitcheskaya Entsiklopediya*, Sovetskaya Entsiklopediya, Moscow, 1961, p. 972.

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RETENTION OF HYDROCARBONS ON GAS CHROMATOGRAPHIC SORBENTS CONTAINING AN ORGANO-CLAY*

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SUMMARY

The equations describing retention on a combined sorbent containing a solid adsorbent and a stationary liquid phase (SLP) have been obtained on the basis of a theory of retention developed earlier. Experimental methods have been proposed for estimating the relative roles of dissolution and adsorption for combined sorbents based on organo-clays. The distribution coefficients of C_6–C_{10} hydrocarbons between a carrier gas and an SLP layer, SLP surfaces, a solid support and organo-vermiculite, and SLP–solid support and SLP–organo-vermiculite interfaces have been determined.

The selectivity of adsorption in the separation of the pairs benzene–cyclohexane and *m*-xylene–*p*-xylene has been studied for sorbents containing pure and impregnated organo-vermiculites. It has been shown that the selectivity depends on the polarity of the SLP.

INTRODUCTION

The use of combined sorbents containing a solid adsorbent, a stationary liquid phase (SLP) and a solid support increases the possible applications of gas chromatography as selectivity can be controlled simply by varying the proportions of the constituents of the combined sorbent. However, it is difficult to choose the most suitable proportions as changes in sorbent characteristics are not additive. It is necessary to take into consideration modifications of the adsorbent by the SLP and changes in sorptive properties of SLP thin layers effected by the solid support[1].

Combined sorbents of the above type, containing an organo-clay and an SLP, are widely used in gas chromatography for the separation of aromatic isomers[2–4]. However, until recently no research had been devoted to the retention mechanism and no quantitative estimation of the roles of adsorption and dissolution had been made. In addition to Bentone 34, studied as early as 1957[5], more selective organo-clays have been proposed, particularly those based on vermiculite[6,7]. Gas chromatographic in-

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vestigations with various organo-clays have shown that the vermiculite derivatives based on vermiculite from a deposit in the Soviet Union (Kovdor) have improved the selectivity (compared with commonly used bentones) towards aromatic hydrocarbons in general and also some of their isomers[8].

In the light of the above earlier work, it was considered of interest to investigate the retention mechanism on a combined sorbent containing a highly selective organo-vermiculite.

RETENTION ON SORBENTS CONTAINING AN ORGANO-CLAY AND A STATIONARY LIQUID PHASE ON A SOLID SUPPORT

The general theory of retention on adsorption-active solids impregnated with SLPs has been discussed earlier[9,10]. The equation describing the net retention volume as a sum of retention volumes on sorbent constituents for a sorbent based on an organo-clay and an SLP is

$$V = k_{GS}s_{GS} + k_{GA}s_{GA} + k_{GL}s_{GL} + k_{S}s_{LS} + k_{A}s_{LA} + k_{L}v_{L} \tag{1}$$

where V is the net retention volume, k_{GS} and k_{GA} are the distribution coefficients of a substance between the carrier gas and the uncoated surfaces of the solid support and the organo-clay, respectively, k_S and k_A are the distribution coefficients between the carrier gas and the surfaces of the solid support and the organo-clay coated with SLP, respectively, k_{GL} is the distribution coefficient between the carrier gas and the gas–SLP interface, k_L is the distribution coefficient between the carrier gas and an SLP layer, s_{GS}, s_{GA}, s_{GL}, s_{LS} and s_{LA} are the areas of the gas–solid support, gas–organo-clay, gas–SLP, SLP–solid support and SLP–organo-clay interfaces, respectively, and v_L is the SLP volume.

Consideration of the cases that can be realized in practice simplifies eqn. 1 (Table I).

For practical purposes, it is convenient to use, firstly, the retention volumes calculated per unit mass of the solid support in the column ($V_N = V/m_S$), and secondly, the distribution coefficients calculated per unit mass of the sorbent constituents for characterizing sorptive processes:

$$K_{GS} = k_{GS}\sigma_S,\ K_{GA} = k_{GA}\sigma_A \text{ and } K_L = k_L/d \tag{2}$$

where σ_S and σ_A are the specific surface areas of the solid support and the organo-clay,

TABLE I

VARIATION OF EQN. 1 FOR DIFFERENT SORBENTS

Sorbent	*Variant of eqn. 1*
Organo-clay on solid support	$V = k_{GS}s_{GS} + k_{GA}s_{GA}$
SLP on solid support*	$V = k_{GL}s_{GL} + k_{S}s_{LS} + k_{L}v_{L}$
Organo-clay and SLP on solid support*	$V = k_{GL}s_{GL} + k_{S}s_{LS} + k_{A}s_{LA} + k_{L}v_{L}$

* SLP coats solid support and organo-clay completely.

respectively, and d is the specific gravity of the SLP. Thus, for example, for sorbents with pure and impregnated organo-clays, the following equations are valid:

$$V_N = K_{GS} + K_{GA}P_A \tag{3}$$

$$V_N = \varkappa + K_A P_A + K_L P_L \tag{4}$$

where $P_A = m_A/m_S$, $P_L = m_L/m_S$, m_A, m_L and m_S are the masses of the organo-clay, SLP and solid support, respectively, in the column, and $\varkappa = K_{GL}\cdot(s_{GL}/m_S) + K_S\cdot(s_{LS}/m_S)$ is the contribution of adsorption on the gas–SLP and SLP–solid support interfaces.

In order to characterize the sorbent selectivity, use is made of the relative retention volumes. In the case of a "monophase" sorbent, this value is considered as the ratio of the distribution coefficients of two compounds. If, for example, in a sorbent that contains only an organo-clay, adsorption on a solid support is negligibly small ($K_{GS} \ll K_{GA}P_A$), then the relative retention volume, in accordance with eqn. 3, is

$$V_{rel.} = K_{GA,\,rel.} = K'_{GA}/K''_{GA} \tag{5}$$

In order to study selectivity caused by different processes in a sorbent that contains an organo-clay and an SLP, it is advisable to determine the distribution coefficients and to calculate their ratio. Thus, for adsorption on an SLP–organo-clay interface, we have:

$$K_{A,\,rel.} = K'_A/K''_A \tag{6}$$

Comparison of selective adsorption on the surface of a pure organo-clay and an organo-clay impregnated with SLP is of special interest.

The distribution coefficients can be determined under the condition that they are constant, *i.e.*, when P_A and P_L are varied over the range where they affect mainly one process. Thus, for example, in order to study dissolution, the amount of SLP must be sufficiently large for complete coating of the sorbent surface and the formation of a fairly thick layer to occur. If these conditions are satisfied, the dependences of the retention volumes on P_A and P_L are linear functions of the type $V_N = A_i + B_i P_{(A,L)}$. When P_L is a variable at constant $P_A = \bar{P}_A$, we obtain, in accordance with eqn. 4, the relationships between the linear dependence parameters and the distribution coefficients:

$$A = \varkappa + K_A\bar{P}_A \text{ and } B = K_L \tag{7}$$

Experimental confirmation of the linearity of these functions would support the validity of the chosen model of the sorbent structure and determination of their parameters would serve as a basis for determining the distribution coefficients.

It should be noted that the distribution coefficients may depend on the concentration of a substance in the carrier gas if the sorption isotherms are non-linear. The possibility of determining partial retention volumes and using them in plotting the sorption isotherms on the SLP–organo-clay interface has been shown earlier[11]. If the concentration in a carrier gas is small, the sorption isotherm for aromatic hydrocarbons on impregnated and non-impregnated organo-clays is described satisfactorily by an equation of the Langmuir type. This was confirmed by chromatographic and static methods[11]. In this instance, the dependence of the net retention volume, V_{GA}, on the gas–adsorbent interface on concentration, c, in the carrier gas is expressed by the equation

$$V_{GA} = K_{GA}m_A = a_m b/(1 + bc)^2 \tag{8}$$

where a_m and b are the constants of the Langmuir equation. The equation for the partial retention volume on the SLP–adsorbent interface is similar to eqn. 8. It is evident that in order for invariance of the retention volumes to be attained, experiments must be conducted at a small dose rate ($c \approx 0$), *i.e.*, when $V_{GA} \approx a_m b$.

EXPERIMENTAL

Apparatus

An LKhM-7A gas chromatograph (Mosneftekip, Moscow, U.S.S.R.) with a katharometer and a 3 m × 0.4 cm I.D. column was used. The detector and evaporator temperatures were 125° and 260°, respectively. Helium was used as the carrier gas at a flow-rate of 30–43 cm^3/min.

Compounds

Cyclohexane, *n*-nonane, *n*-decane, benzene, toluene, ethylbenzene, *o*-, *m*- and *p*-xylene and cumene were used with a dose rate of 0.1 μl.

Organo-clays

Dimethylbenzylalkylammonium (DMBA) and dimethyldioctadecylammonium (DMODA) derivatives of vermiculite from Kovdor, U.S.S.R., and DMODA vermiculite from South Africa were used. DMBA and DMODA vermiculites were obtained by cation exchange between vermiculite and an aqueous solution of DMBA or DMODA chloride according to the procedure described by Taramasso and Veniale[6,7].

Stationary liquid phases

Dinonyl phthalate (DNP), dibutyl tetrachlorophthalate (DBT), mineral oil and diisooctyl sebacate (DIS) were used.

Solid support

The solid support was Chromosorb P (60–80 mesh), silanized.

The sorbent was prepared by stirring a weighed amount of the organo-clay with a benzene solution of the SLP for about 2 h. The suspension obtained was mixed with Chromosorb P and heated until the benzene had evaporated.

The net retention volume calculated per unit mass of solid support was obtained by the equation

$$V_N = j\Theta qt/m_S \tag{9}$$

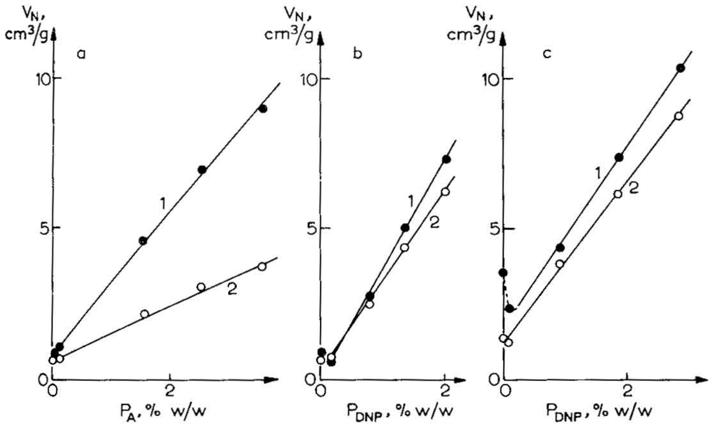

Fig. 1. Net retention volumes calculated per solid support mass as a function of DMBA vermiculite and DNP content at 84°. (a) P_{DNP} = 0; (b) P_A = 0; (c) $\bar{P}_A$ = 0.89 wt.%. 1, Ethylbenzene; 2, *n*-nonane.

where j is the compressibility correction factor, Θ is the product of the correction for the water vapour pressure in a soap flow meter and corrections for reducing to normal conditions; q is the flow-rate of the carrier gas, t is the corrected retention time and m_S is the mass of the solid support.

RESULTS AND DISCUSSION

The retention of hydrocarbons was studied on sorbents containing DMBA vermiculite and DNP or DBT at 84°.

The retention volumes calculated by eqn. 9 were used for plotting retention volume *versus* the content of organo-clay or SLP, as shown in Fig. 1. The dependence over the range of small values of P_{DNP} is shown by the dashed line in Fig. 1c. For cyclohexane and *n*-decane, the graphs were similar to that for *n*-nonane, and those for aromatic hydrocarbons were similar to that for ethylbenzene.

TABLE II

PARAMETERS A_i AND B_i AND CONFIDENCE LIMITS* FOR SORBENTS CONTAINING DMBA VERMICULITE (KOVDOR) AND DNP

Dependence	*Compounds*	$A_i \pm \Delta A_i$ (cm^3/g)	$B_i \pm \Delta B_i$ (cm^3/g)
		(K_{GS})	(K_{GA})
$V_N = A + BP_A$, $P_{DNP} = 0$	*n*-Nonane	0.60 ± 0.06	90 ± 5
	Ethylbenzene	1.00 ± 0.07	230 ± 20
		($\varkappa$)	(K_{DNP})
$V_N = A + BP_{DNP}$, $P_A = 0$	*n*-Nonane	0.3 ± 0.1	290 ± 7
	Ethylbenzene	0.2 ± 0.1	365 ± 10
			(K_{DNP})
$V_N = A + BP_{DNP}$, $\bar{P}_A = 0.89\%$	*n*-Nonane	1.4 ± 0.1	255 ± 5
	Ethylbenzene	1.4 ± 0.2	320 ± 10

[*] Calculated according to Doerffel[12].

TABLE III

HYDROCARBON DISTRIBUTION COEFFICIENTS FOR SORBENTS CONTAINING DMBA VERMICULITE AND DNP AT 84° (cm³/g)

Compound	$P_A = 0$, $P_{DNP} = 0$	$A + BP_A$, $P_{DNP} = 0$		$A + BP_{DNP}$, $P_A = 0$		$A + BP_{DNP}$, $\bar{p}_A = 0.89\%$	K_A*
	K_{GS}	K_{GS}	K_{GA}	$\varkappa$	K_{DNP}	K_{DNP}	
Cyclohexane	0.15	0.10	14	0.0	44	43	6
n-Nonane	0.75	0.60	90	0.3	290	255	30
n-Decane	1.30	1.20	210	0.6	600	565	60
Benzene	0.35	0.40	95	0.0	69	61	21
Toluene	0.35	0.45	150	0.2	165	140	28
Ethylbenzene	0.95	1.00	230	0.2	355	320	33
p-Xylene	0.80	1.10	240	0.2	365	350	35
m-Xylene	0.95	1.10	380	0.2	375	350	45
o-Xylene	0.95	1.70	360	0.3	445	440	55
Cumene	1.20	1.50	290	0.2	540	495	35

* K_A was determined separately.

The parameters of the dependences $V_N = A_i + B_iP_{(A,L)}$ were calculated by the least-squares method. The results and the precision are given in Table II, using *n*-nonane and ethylbenzene as examples. Table III shows the distribution coefficients for all of the hydrocarbons analyzed in the system DMBA vermiculite–DNP.

It can be seen from Table II that A_i and B_i were determined with satisfactory accuracy, except for the case when $\varkappa$ is small.

When sorbents that contain an organo-clay are used, the retention value may be considered to be the sum of contributions from the following processes: adsorption on the gas–solid support, gas–organo-clay, SLP–organo-clay, gas–SLP and SLP–solid support interfaces (in this work, the last two effects were not determined separately) and dissolution in the SLP layer. Such a conclusion follows from the significance of the distribution coefficients given in Table III.

The K_{GS} values determined on a solid support covered with an organo-clay and without an organo-clay are similar. This indicates that an organo-clay does not prevent adsorption on the surface of a solid support. The addition of an SLP to a solid support decreases the contribution of the adsorption to the retention value ($K_{GS} > \varkappa$). It thus follows that an organo-clay, unlike an SLP, does not modify a solid support and the application of adsorption-active solid supports, especially at a low organo-clay content, is inefficient.

An approximate calculation by eqns. 3 and 4, using the data from Table III, shows that the adsorption contributions K_{GS} and $\varkappa$ can be neglected when $P_A > 7$–10 wt.% and $P_L > 2$–5 wt.%.

The values of K_L determined in the presence of an organo-clay differ from those for an SLP without an organo-clay, indicating that the solubility in an SLP layer also changes in the presence of an organo-clay.

The role played by adsorption on an organo-clay surface decreases sharply upon addition of an SLP ($K_{GA} > K_A$). This decrease is different for different compounds: 3.1-fold for *n*-nonane, 4.6-fold for benzene and 8.4-fold for cumene.

The results for the DMBA vermiculite–DBT system confirm the above trends (with a few exceptions) and are not given in this paper.

On the basis of the results, we can conclude that the net retention volume calculated per solid support mass depends linearly on the organo-clay and SLP contents (over the range investigated). However, a change in the distribution coefficients in the packing constituents as a result of the mutual effects of the constituents must be taken into account for all of the dissolution and adsorption processes considered.

It should be noted that the distribution coefficients for gas–SLP (K_L) are higher than those for adsorption on organo-vermiculite (K_{GA} and K_A), which indicates that an alteration of the SLP content changes the retention volume more than an alteration of the organo-vermiculite content.

Let us now consider the role of the dissolution and adsorption processes not in terms of absolute retention values but of separation selectivity for the chosen pairs of compounds. As shown by White[5], organo-clays are selective for the separation of aromatic hydrocarbons from non-aromatic hydrocarbons and also for the separation of *meta-* from *para*-isomers of aromatic hydrocarbons. In order to characterize selectivity, it is advisable to choose pairs of compounds with similar boiling points. The role of specific effects is then more pronounced. Taramasso and Veniale[6,7] used the pairs benzene–cyclohexane and *m*-xylene–*p*-xylene to study the selectivity of organo-clays.

The characteristics of the retentions of cyclohexane, benzene and xylenes on sorbents containing organo-vermiculite and SLP were determined. The calculated ratios of the distribution coefficients for the above pairs of compounds on impregnated and non-impregnated organovermiculites ($K_{GA,\ rel.}$ and $K_{A,\ rel.}$) are given in Table IV.

TABLE IV

RATIO OF DISTRIBUTION (ADSORPTION) COEFFICIENTS FOR THE SYSTEMS GAS–ORGANO-VERMICULITE AND GAS–(SLP–ORGANO-VERMICULITE INTERFACE)*

Sorbent	*Temperature (°C)*	*Benzene–cyclohexane*	*m-Xylene–p-xylene*
DMBA vermiculite (Kovdor)	84	6.9	1.58
+ mineral oil	80	6.5	1.30
+ DIS	90	3.9	1.17
+ DNP	84	3.6	1.27
+ DBT	84	2.0	1.00
DMODA vermiculite (Kovdor)	91	9.6	1.75
+ DIS	98	—	1.29
DMODA vermiculite (South Africa)	91	9.0	1.72
+ DIS	59	10.0	1.50

* The SLPs are arranged in order of increasing polarity.

The results in Table IV show that the adsorption selectivity on the organo-clay surface decreases upon impregnation with SLP, the decrease being greater with an increase in the polarity of the SLP. A similar effect has been observed[4] for a sorbent containing Bentone 245 and mineral oil or DNP.

When a more polar SLP is used, such as dinitrochlorobenzene or triscyanethoxypropane, the organo-vermiculite selectivity decreases even more markedly, and when the amount of SLP is sufficient the separation on a sorbent is determined mainly by the chromatographic properties of the SLP.

From Table IV, it also follows that the higher the selectivity of adsorption on the initial organo-vermiculite, the higher is the selectivity for the SLP–organo-vermiculite interface (DIS was used as the SLP).

The results have shown that the selectivity of the combined sorbent can be changed by adding various organo-clays. As the sorbent selectivity can be regulated not only by a change in adsorptive properties of the organo-clay (the modifying effect of the SLP) but also by dissolution in an SLP layer, the desirable selectivity of the sorbent as a whole can be obtained by impregnation of an organo-clay either with large amount of an SLP of low polarity or with a small amount of an SLP of higher polarity. These conclusions are of important practical interest and, in our opinion, increase the possible applications of organo-clays.

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REFERENCES

1 J. Serpinet, *J. Chromatogr.*, 68 (1972) 9.
2 S. F. Spencer, *Anal. Chem.*, 35 (1963) 592.
3 J. V. Mortimer and P. L. Gent, *Nature (London)*, 197 (1963) 789.
4 M. S. Vigdergauz and O. G. Chabrova, *Neftekhimiya*, 5 (1965) 160.
5 D. White, *Nature (London)*, 179 (1957) 1075.
6 M. Taramasso and F. Veniale, *Contrib. Mineral. Petrol.*, 21 (1969) 53.
7 M. Taramasso, in K. I. Sakodynskii and S. A. Volkov (Editors), *Gas chromatography*, Vol. 10, NIITEKhIM, Moscow, 1969, p. 49.
8 V. G. Berezkin, V. S. Gavrichev, L. N. Kolomiets, A. A. Korolev, V. N. Lipavskii, N. S. Nikitina and V. S. Tatarinskii, *Gazovaya Khromatografiya v Neftekhimii (Gas Chromatography in Petroleum Chemistry)*, Nauka, Moscow, 1975.
9 V. G. Berezkin, V. P. Pahomov, V. S. Tatarinskii and V. M. Fateeva, *Dokl. Akad. Nauk SSSR*, 180 (1968) 1135.
10 V. G. Berezkin, *J. Chromatogr.*, 65 (1972) 227.
11 V. G. Berezkin and V. S. Gavrichev, *Zh. Fiz. Khim.*, 47 (1973) 2859.
12 K. Doerffel, *Statistik in der Analytischen Chemie*, Leuna-Merseburg, Leipzig, 1966.

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GAS CHROMATOGRAPHY OF NITROGEN- AND PHOSPHORUS-CONTAINING COMPOUNDS

A NEW, HIGH-SENSITIVITY, VARIABLE SELECTIVITY DETECTOR*

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SUMMARY

Gas chromatographic analysis of nitrogenous and phosphorated compounds is simplified by the use of thermionic detectors which respond selectively to these substances.

The new detector described here works on the principle of thermionic detection, showing high sensitivity and marked selectivity towards phosphorus and nitrogen containing molecules, and discriminating well between them. The selectivity factor between the two heteroatoms may be varied by changing the geometrical arrangement of the electrodes. The detector lends itself to numerous applications, one particularly interesting field being the determination of inorganic compounds containing nitrogen and phosphorus.

INTRODUCTION

In 1964 Karmen and Giuffrida[1–3] demonstrated that the conventional flame ionization detector (FID) shows much greater sensitivity to organic compounds containing phosphorus or halogens if the flame is doped with sodium salt vapours. A special sodium thermionic detector was built, in which a ring electrode, coated with molten sodium sulphate, was placed around the flame. These detectors, known as thermionic, alkali-flame, etc., were immediately put to use to determine phosphorated and chlorinated pesticide traces.

Later investigations into the selective behaviour of this detection system, depending on the type of alkali metal salts used, showed that the detector response could be enhanced towards other heteroatoms such as nitrogen, arsenic[4] and sulphur[5]. The chemico-physical mechanism by which this is achieved is still not altogether clear. However, from the point of view of gas chromatography applications, the thermionic detector is of great interest in solving analytical problems requiring maximum sensitivity and selectivity. The detector response is enhanced for organic phos-

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phorus- and nitrogen-containing substances, and is therefore extremely useful for both research and routine analysis. It may be applied meaningfully for the determination of pesticide residues, either phosphorated or nitrogenous, herbicides, or in the fields of toxicology, forensic medicine, pharmacology, etc., or wherever high sensitivity and selectivity are required and the active components are almost always nitrogenous.

The analysis of phosphorus-containing substances does not represent a big problem, since the thermionic detector is especially sensitive to this element; analysis of nitrogen compounds is slightly more complicated, because it calls for an optimisation of operative parameters, providing some difficulty in ensuring a controlled, repeatable, and specific response coupled to a high sensitivity.

The detection system is, however, simple, and is suitable for a broad range of applications, thus explaining the interest it has aroused over the last 10 years. Numerous detectors have been produced, with widely varying geometry, single or double flames, and different alkali metal salts. The commercially available thermionic detectors for phosphorus and nitrogen offer many original features, but not all are equipped to exploit fully this detection principle, and may suffer some analytical limitations.

THE NITROGEN–PHOSPHORUS SELECTIVE DETECTOR (NPSD)

Description

The detector is designed to ensure that all the thermionic detection operative parameters are independent and easily controlled. A schematic diagram of the NPSD is shown in Fig. 1. The alkali source, the heart of the thermionic detector, is a potas-

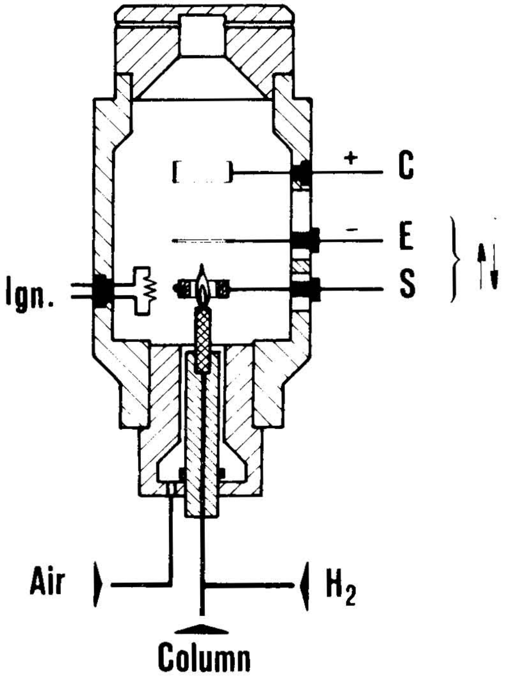


Fig. 1. Schematic diagram of the NPSD. C = Collector electrode; E = adjustable height polarization electrode; S = adjustable height alkali salt source.

sium chloride pellet. This particular salt has been chosen for the following reasons: it requires a low hydrogen flow-rate, suppresses the response to halogenated compounds, gives an enhanced response to phosphorus, and works equally well with nitrogenous compounds. The alkali source can be shifted over the flame to give the maximum ionization current, or lowered beneath the flame allowing typical FID operations. The polarization electrode is also movable and can be shifted vertically to alter the electric field in which the complex ions generated by the detection mechanism are collected, thus considerably increasing the analytical range of the detector. The hydrogen flow-rate is adjusted by the pressure reducers already mounted on the gas chromatograph.

Operation

The alkali source having been positioned to give maximum electrical conductivity of the flame, the standing current can be reduced as shown in Fig. 2 by adjusting the position of the polarization electrode. Detector sensitivities (signal-to-noise ratios) for the nitrogen and phosphorus operation modes are achieved from different standing current levels. In the right conditions, the charges producing the standing current cause a minimum of interference, and the ions formed can be collected "cleanly". The results of a trial with the NPSD appear to be in agreement with the mechanism suggested by Cremer[6] whereby complex ions of increased and different lifetimes are

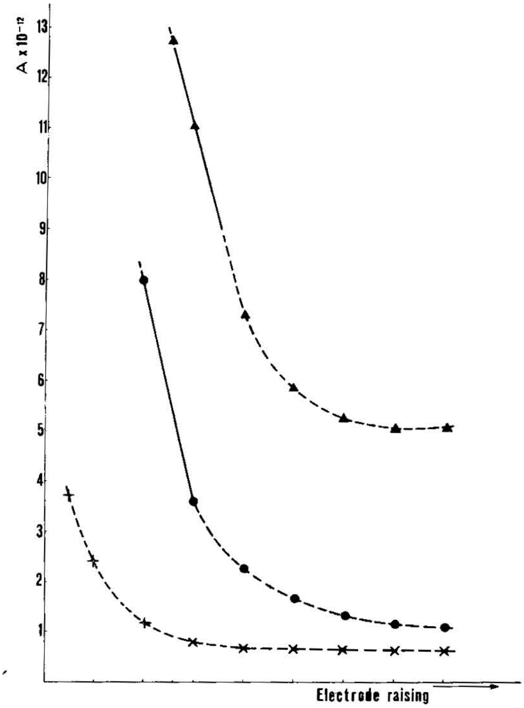


Fig. 2. Standing current patterns following upward displacement of the polarization electrode. Full line: optimum sensitivity range. Hydrogen flow-rate: ▲, 39 ml/min; ●, 36 ml/min; ×, 30 ml/min.

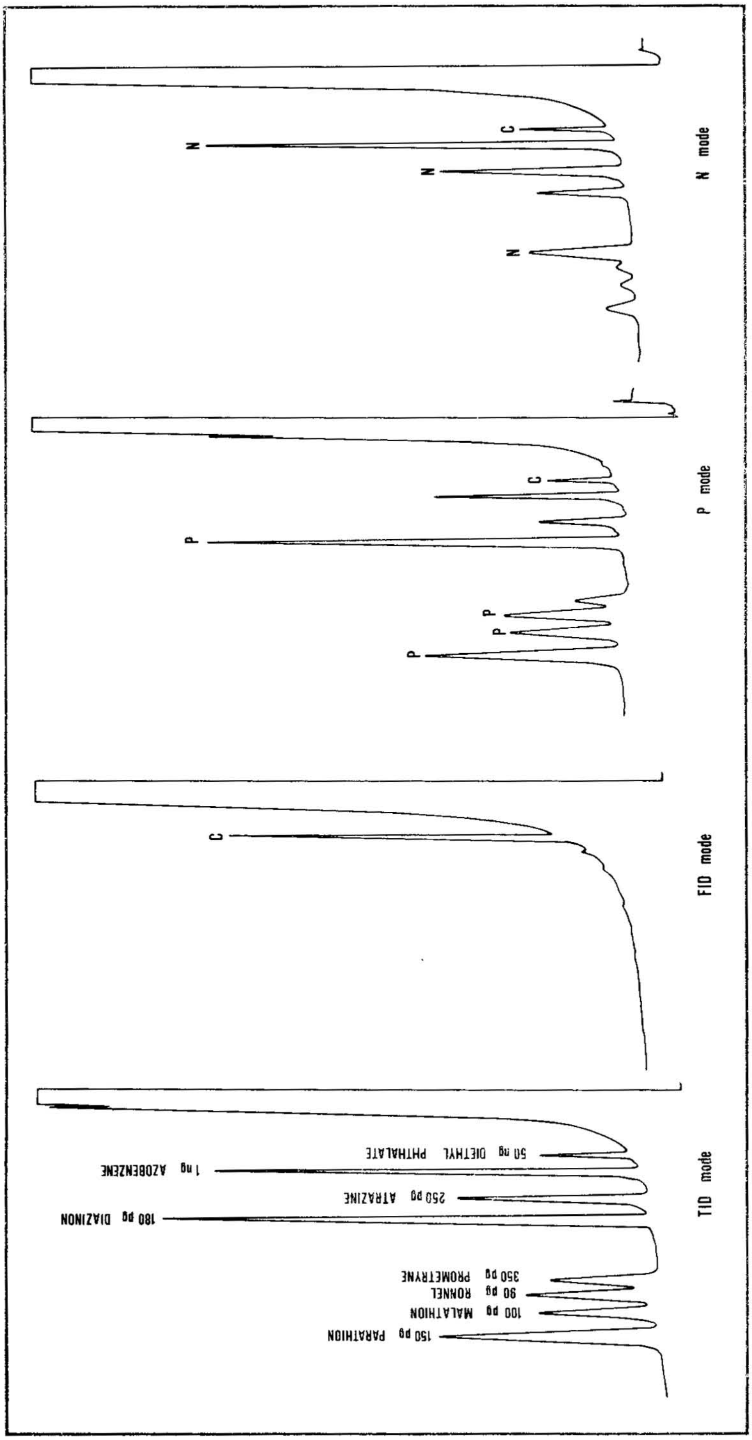


Fig. 3. Different NPSD responses to a standard solution containing nitrogen and phosphorus compounds and diethyl phthalate. Column: 2 m × 3.5 mm I.D., 5% OV-1 on 80–100 mesh Gas Chrom Q; column temperature: 210°; carrier gas: nitrogen, 50 ml/min.

formed by interaction between alkali ions and ionized or neutral radicals containing heteroatoms.

Altering the geometry of the electrical field[7] by shifting the polarization electrode, the charges can be collected more selectively either for phosphorus or for nitrogen. Moving the electrode towards the flame, and increasing the standing current, gives a better phosphorus/nitrogen (P/N) signal ratio, while if the electrode is moved away from the flame, the standing current drops and the P/N signal ratio improves in favour of nitrogen.

To underline the detector's ability to be selective for one or other of these two heteroatoms, a solution containing nitrogen and phosphorus compounds, and diethyl phthalate as organic reference substance, was analysed with the detector in four different modes. Fig. 3 shows the chromatograms obtained with a 2 m glass column of 5% OV-1 on Gas Chrom Q at 210° using nitrogen as carrier gas, and demonstrates the possibility of changing the selectivity ratio towards either nitrogenous or phosphorated compounds. To obtain the FID tracing, the potassium chloride pellet was lowered beneath the flame.

The optimum hydrogen flow-rate depends on the carrier gas type: using helium, the hydrogen flow-rate should be set at 25–30 ml/min, and at 35–40 ml/min when nitrogen is used (with the carrier gas flow-rate in the range 40–50 ml/min).

The detector behaves quite differently with nitrogen and helium. This effect appeares to be related to the different thermal conductivities of helium and nitrogen which changes the environmental conditions of the flame (temperature and ion concentration). Using helium, the selectivity ratio against carbon is about 100 times greater for phosphorus and 10 times greater for nitrogen; sensitivity, in terms of minimum detectable amount, is increased about ten-fold for phosphorated compounds while being slightly decreased for nitrogen-containing compounds (Fig. 4).

DETECTOR PERFORMANCE

Sensitivity and selectivity

Compared to the conventional FID, the NPSD offers improved sensitivity, ranging from 50 to 100 times for nitrogen substances, and from 500 to 1000 times for phosphorated substances, according to the amount of nitrogen and phosphorus in the molecule. Table I gives the detection limit for some nitrogen- and phosphorus-containing compounds.

It is to be noticed that the detector gives the best absolute sensitivity for phosphorated compounds: as shown in Fig. 5, parathion and malathion can be detected easily in quantities below 10^{-12} g. In view of this extreme sensitivity to phosphorus, analytical techniques might be developed involving the formation of phosphorated derivatives[8–10], allowing determination of traces of organic substances which would otherwise not be detectable. The selectivity of response to carbon varies from 10^3 for nitrogen compounds and 10^4 for phosphorated substances, the selectivity being moveable, as already described, towards phosphorus or nitrogen, just by raising or lowering the electrode over the flame.

Some properties of the NPSD

It is interesting to note that in case of substances containing both phosphorus

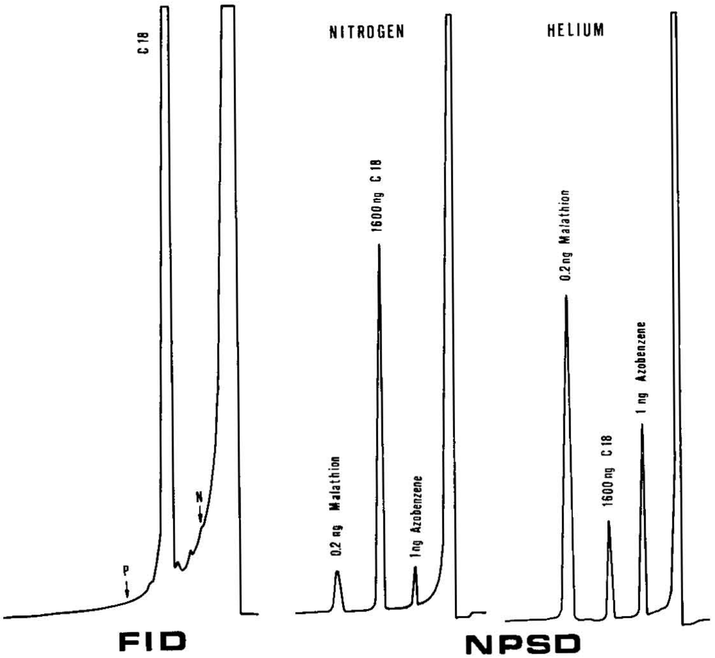


Fig. 4. Dependence of NPSD behaviour on carrier gas type (nitrogen and helium) compared with FID response.

TABLE I

DETECTION LIMIT FOR SOME NITROGEN- AND PHOSPHORUS-CONTAINING COMPOUNDS

Compound	*Detection limit (g/sec)*	*Per cent heteroatom*	*Detection limit to heteroatom*
Dimethylnitrosamine	7×10^{-13}	N = 37.82	2.6×10^{-13}
Caffeine	3×10^{-13}	N = 28.85	1×10^{-13}
Atrazine	1.5×10^{-13}	N = 32.58	5×10^{-14}
Prometryne	3×10^{-13}	N = 29.02	9×10^{-14}
Diazinon	1×10^{-14}	P = 10.18	1×10^{-15}
Malathion	2×10^{-14}	P = 9.38	2×10^{-15}
Parathion	1.5×10^{-14}	P = 10.64	1.6×10^{-15}

and nitrogen, such as diazinon (2N = 9.20%; 1P = 10.18%) the detector responds as if only phosphorus were in the molecule. The detector behaviour is similar when compounds containing only nitrogen and those containing only phosphorus emerge from the column with the same retention time.

Also, for substances containing phosphorus and chlorine, such as ronnel (3 Cl = 33.08%; 1P = 9.63%), the detector is phosphorus-sensitive. On the other hand, compounds containing only chlorine do not respond, or give negative peaks, if large amounts are present.

The presence of phosphorus is therefore sufficient to identify the molecule as phosphorated, regardless of whether other detectable heteroatoms are also present.

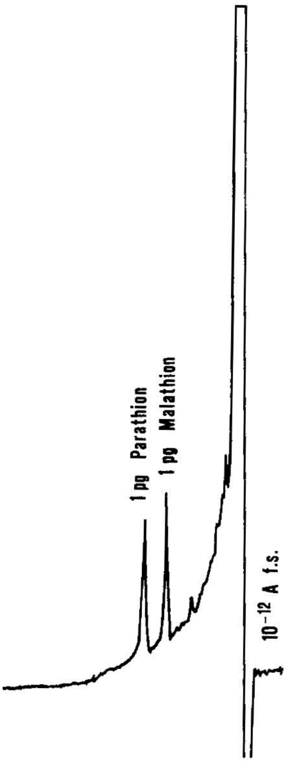


Fig. 5. High sensitivity determination of organophosphorus pesticides.

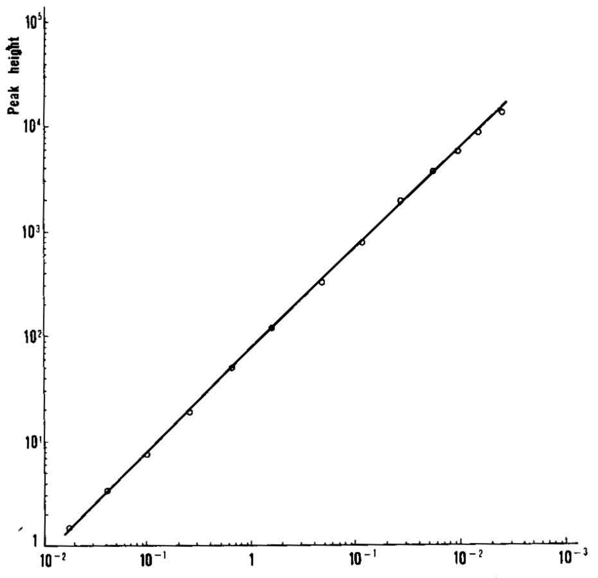


Fig. 6. NPSD response linearity for azobenzene.

Linearity

The NPSD gives a linear response over a wide range of concentration. Fig. 6 shows a linear plot for azobenzene to within 10^4. For phosphorated compounds, a similar degree of linearity is obtained.

Analytical applications

For the determination of pesticide residues in fruit, vegetables, soil, etc., phosphorated pesticides must be identified rapidly, together with any nitrogenous ones such as carbamates or triazine herbicides.

The NPSD is not only very sensitive to these substances, but is also useful in the identification of phosphorus or nitrogen peaks. The same detector operated as in the FID mode allows the identification of the organic co-extracts also present. Fig. 7 gives the gas chromatographic results for an extract of a culture medium treated with igran, a triazine herbicide. A 2 m column of 5% OV-1 was used, with a temperature program of 20°/min from 180 to 240°. Shifting the detector response to "N mode" and "P mode", and comparing the chromatograms obtained, at least four phosphorated pesticides were detected whose presence in the sample was not suspected. The FID tracing, obtained under the same conditions, shows peaks for organic co-extracts which the NPSD totally suppresses.

The high sensitivity of this detector, and its selective response reduces the "clean-up" needed before the GC analysis. The determination of the caffeine residue in decaffeinated coffee is an example. Fig. 8 shows the chromatogram of a water–

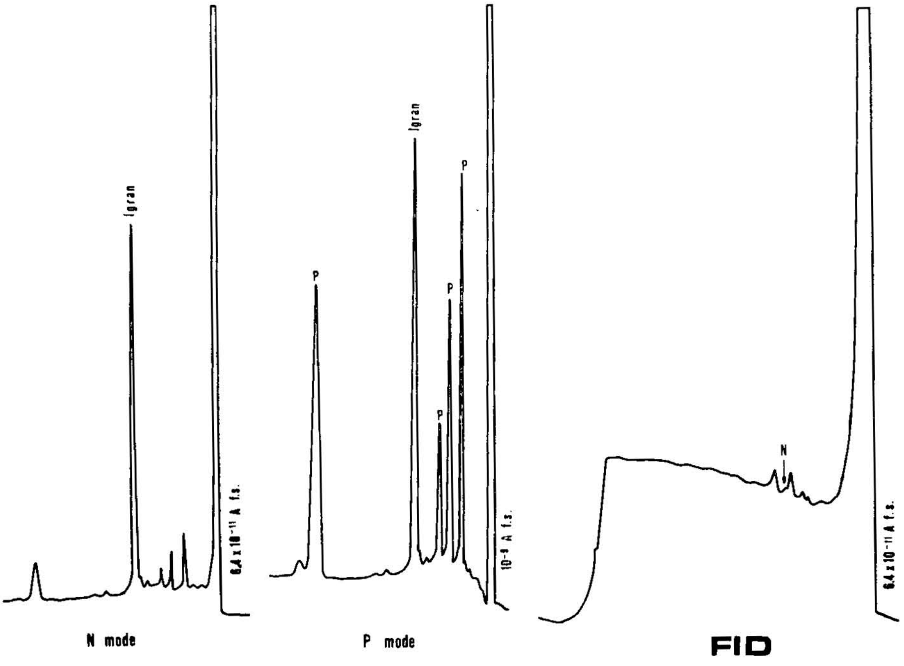


Fig. 7. Identification of a nitrogenous herbicide (igran) together with phosphorated and organic co-extracts ("P mode" and FID tracings).

acetone extract of coffee powder treated with magnesium oxide in comparison with the FID chromatogram, which here appears absolutely inadequate for quantitative purposes.

Using the NPSD and FID mounted in parallel at the column outlet, qualitative and quantitative results can be immediately obtained from biological extracts. The nitrogenous substances can be identified by simply comparing the two chromatograms, and determined quantitatively with a sensitivity roughly in agreement with the nitrogen content. Fig. 9 shows an example of a barbiturate (veronal) analysis extracted from organic material and chromatographed without any special "clean-up". A 2 m column of 3% OV-17 on 80–100 mesh Gas Chrom Q was used, with a temperature program of 5°/min from 160 to 280°. Compared to the FID, the NPSD chromatogram is extremely clean, and baseline drift due to the temperature program is almost totally absent.

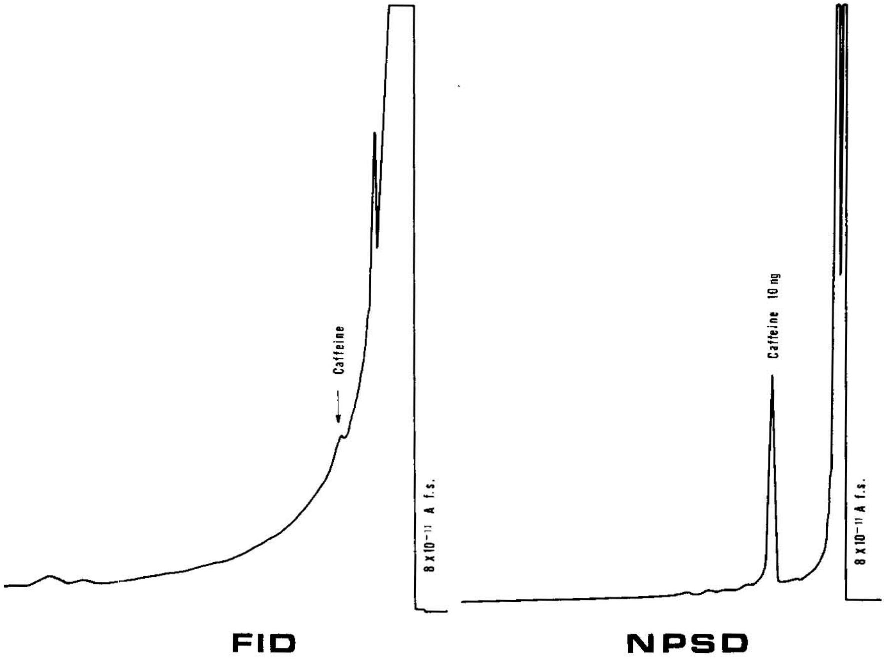


Fig. 8. Caffeine residue in a water–acetone extract of decaffeinated coffee chromatographed with NPSD and FID mounted in parallel.

The performance of the detector with inorganic compounds containing phosphorus of nitrogen has been also investigated. Fig. 10 shows traces of PCl_3 in diethyl ether, and Fig. 11 and 12 show the analysis of N_2O in nitrogen and NH_3 in an aqueous solution. The detector sensitivity towards these substances, which contain no ionizable carbon, is good —in any case much better than a thermal conductivity detector. This confirms that the detection mechanism is not the same as in a conventional flame ionization detector, where the response depends on the formation of ions as a result of carbon oxidation. It might be interesting to go into this question further, analysing different inorganic substances and assessing detector efficiency, both with regard to sensitivity and linearity of response.

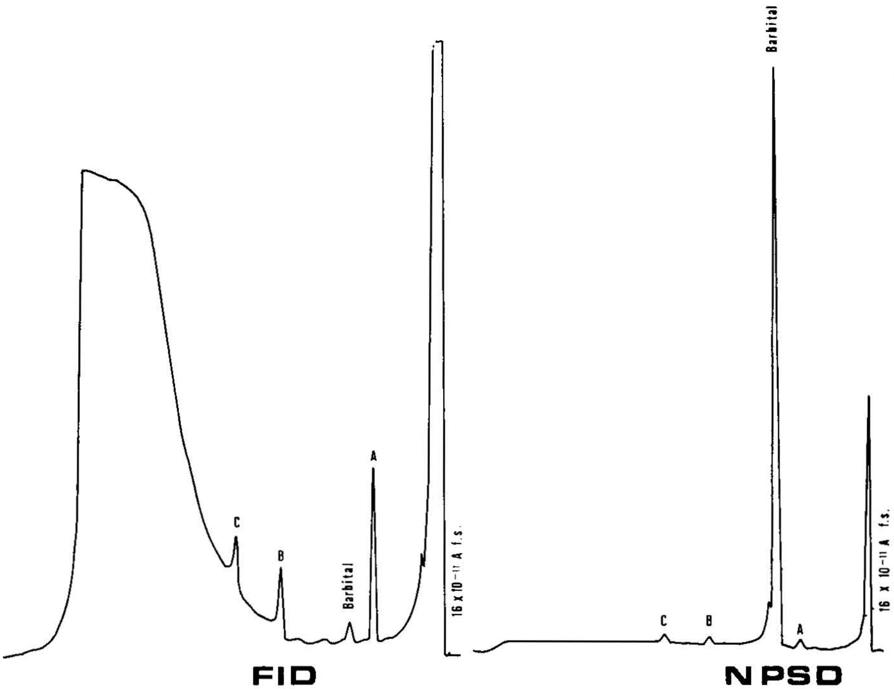


Fig. 9. Temperature programmed chromatograms (NPSD and FID) of a barbiturate extracted from organic material without any special clean-up. Column: 2 m × 3.5 mm I.D., 3% OV-17 on 80–100 mesh Gas Chrom Q; column temperature: 160–280°, 5°/min; carrier gas: helium, 45 ml/min.

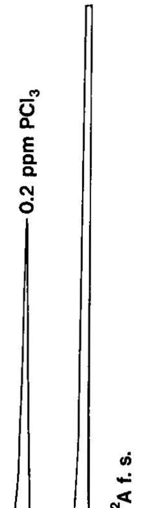
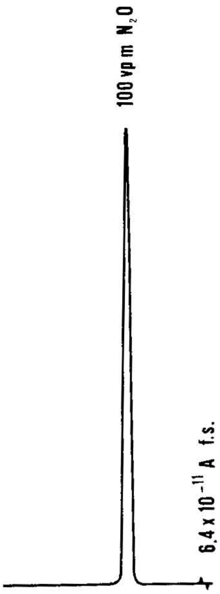


Fig. 10. Determination of PCl_3 in diethyl ether. Column: 2 m × 3.5 mm I.D., 7% DC 200 on 80–100 mesh Gas Chrom Q; column temperature: 60°; sample injected: 1 μl.



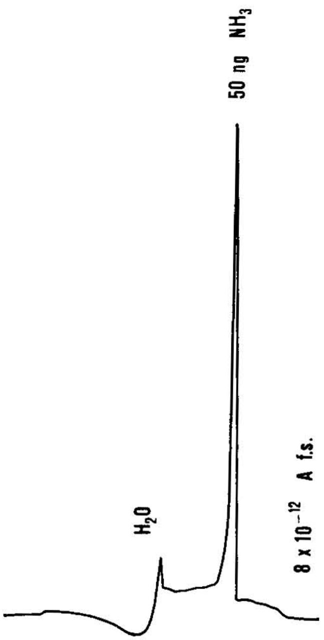


Fig. 11. Detection of N_2O in nitrogen. Column: 2 m × 3.5 mm I.D., 80–100 mesh Chromosorb 102; column temperature: 50°; sample injected: 2 ml.

Fig. 12. Determination of NH_3 in aqueous solution. Column: 2 m × 3.5 mm I.D., 80–100 mesh Chromosorb 103; column temperature: 120°.

CONCLUSIONS

The new nitrogen–phosphorus detector described in this paper has significant advantages in terms of sensitivity, selectivity and versatility. The detector's capability of distinguishing molecules containing nitrogen from those containing phosphorus, and detecting them in extremely small amounts, is expected to be useful in solving many analytical problems. The NPSD possibilities for detecting inorganic substances containing nitrogen or phosphorus are of particular interest, as they demonstrate the high versatility of the detector.

REFERENCES

1 A. Karmen and L. Giuffrida *Nature (London)* 201 (1964) 1204.
2 L. Giuffrida *J. Ass. Offic. Agr. Chem.* 47 (1964) 293.
3 A. Karmen *Anal. Chem.*, 36 (1964) 1461.
4 N. F. Ives and L. Giuffrida, *J. Ass. Offic. Anal. Chem.*, 50 (1967) 1.
5 M. Dressler and J. Janák, *J. Chromatogr. Sci.*, 7 (1969) 451.
6 E. Cremer, *J. Gas Chromatogr.*, 5 (1967) 329.
7 W. A. Aue, C. W. Gehrke, R. C. Tindle, D. L. Stalling and C. D. Ruyle, *J. Gas Chromatogr.*, 5 (1967) 381.
8 R. Vîlceanu and P. Schulz, *J. Chromatogr.*, 82 (1973) 279.
9 M. P. Heenan and N. K. McCallum, *J. Chromatogr. Sci.*, 12 (1974) 89.
10 W. Vogt, K. Jacob and M. Knedel, *J. Chromatogr. Sci.*, 12 (1974) 658.

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DUAL-WAVELENGTH POINT ZIG-ZAG SCANNING OF ZONES ON THIN-LAYER CHROMATOGRAMS AS A TOOL FOR QUANTITATIVE ASSAY

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SUMMARY

Dual-wavelength difference photometry has been applied to zones on chromatograms. A zone was scanned, in two dimensions alternately, by two very narrow wavelength-selected pulses of light; the difference in the logarithm of the reflectance measurements between the two wavelengths was converted electronically into an absorption coefficient proportional to concentration according to the Kubelka–Munk equations, and this converted value was integrated two-dimensionally across the whole zone area. For zones of various sizes, shapes and concentrations, the integrated value was proportional to the applied solute concentration. Additional compensation was provided for coloured or UV-absorbing backgrounds that could not be compensated for by the dual-wavelength difference-photometric procedure.

INTRODUCTION

Thin-layer chromatography is a modern analytical technique, which permits the rapid and clear separation of a mixture into its components. For quantitative assay, however, each component separated as a zone is eluted, usually to give a transparent extract, which is assayed by spectrophotometry. This is because spectrophotometry of transparent materials is a well established technique and because the proportionality between absorbance and concentration (Beer's law) is obeyed for transparent extracts. Direct measurement of transmission, reflection or fluorescence of zones *in situ* on chromatograms is more useful if the direct assay gives more sensitive and more accurate results for the concentration of substance in the zones; in addition, the tedious elution procedure can be omitted. The advantages of these types of *in situ* measurement were stressed by Jork[1], Frei *et al.*[2] and Touchstone *et al.*[3],

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and achievements in the early stages of development were reviewed by Bush[4] and by Boulton[5,6]. Despite these promising advantages, the technique of direct photometry has not become fully established because of several difficulties, which are briefly summarized below.

(1) Beer's law is not obeyed for coloured zones on translucent thin-layer materials. The Kubelka–Munk equation[7–9], which is much more intricate with respect to the relationship between concentration and attenuance [$-\log T$ (transmittance) or $-\log R$ (reflectance) as a measure of light attenuation by both absorption and scattering][10], is expected to be obeyed for such translucent materials. A useful approximation of the general Kubelka–Munk equation to simpler analytical functions was reported by Goldman and Goodall[11,12] and by Treiber[13], with discussions on the errors included in the approximation. The Kubelka–Munk equation for the reflectance of infinitely thick layers has been applied by Hezel[14] for direct photometry. On the other hand, more general equations (including fluorescence, as well as scattering and absorption) were derived by Goldman[15].

(2) In order to examine the applicability of these general or approximate equations to zones on chromatograms, the photometric readings must be accurate and precise. However, the reading of attenuance at a single wavelength when a chromatogram is scanned along a line (denoted as the x axis) fluctuates considerably because the background of the white adsorbent on the plate is not completely homogeneous. This interferes with precise measurements and precludes further analysis of data.

(3) A light beam passed through a slit is commonly used as incident radiation. Such a slit produces a beam that illuminates a long narrow area in a round or elliptical zone in which the sample is distributed non-uniformly. The light transmitted through, or reflected from, the long narrow area is therefore composed of light fluxes of different intensities from different local densities along the y axis; the photo-current from a detector placed behind or above the zone thus gives a reading of average intensity. To eliminate the effect of local variations in density, a much smaller uniform area should be measured, or else the sample should be applied as a band before development in order to obtain uniform density along the y axis. Further, variations in size and shape of the zone introduce additional errors in readings by one-dimensional slit-beam scanning.

The second difficulty arising from background fluctuation due to scattering was overcome by using difference photometry with alternate dual-wavelength light pulses. This technique, which was developed by Chance[16,17] for reducing the fluctuation of the reading with time during a biochemical reaction in a translucent sample, was applied by Salganicoff *et al.*[18] and by Shibata[19] to reduce fluctuation in reading while scanning a translucent material along a line. The fluctuation due to scattering changes at a light-absorbing wavelength (λ_2) was compensated for by subtracting the fluctuation at a different wavelength (λ_1) at which the separated chromogen exhibited no absorption but experienced the same scattering fluctuations. In this way a very weak spot was recorded as a distinct peak on a straight horizontal base-line in an expanded full scale of the order of 0.1 as absorbance difference. The great improvement in sensitivity and accuracy attainable by dual-wavelength photometry of chromatograms has been discussed theoretically by Boulton and Pollak[20–23]. The use of an opal-glass plate behind the sample further improved the result when scattering by the sample was dependent on wavelength[10,19], although such a diffuser was not necessary for thin-layer chromatograms, where the scattering was independent of wavelength. The precise measurements thus achieved made it possible to solve the remaining problems.

In the third problem, due to local density variation, a zone was scanned in two dimensions[21,24]. In the present experiment, the stage on which the chromatogram was mounted was moved in a zig-zag manner in order to scan the zone two-dimensionally with dual-wavelength light pulses having a very minute cross-section. The light reflected from the small area was measured to obtain a signal of $D = \log(1/R_2) - \log(1/R_1)$, in which R_1 and R_2 are the reflectances at λ_1 and λ_2, respectively. These wavelengths were so selected that λ_2 included the absorption maxima of the substance chromatographed, but λ_1 did not include absorption by the separated chromogen.

In the present study, the applicability of the general Kubelka–Munk equations was examined without approximation. To achieve this, an electronic device referred to in this paper as a "linearizer" was used. The Kubelka–Munk equations for R_1 and R_2 can be expressed as follows:

$$R_1 = \frac{S}{S+1} \tag{1}$$

$$R_2 = \frac{\sinh bS}{a \sinh bS + b \cosh bS} \tag{2}$$

$$D = \log(R_1/R_2) \tag{3}$$

where

$$K = kd \text{ and } S = sd$$

$$a = \frac{S+K}{S} \text{ and } b = (a^2 - 1)^{\frac{1}{2}}$$

d being the thickness of the thin layer, s the scattering coefficient per unit thickness, and k the absorption coefficient per unit thickness, which is proportional to the substance concentration.

The two curves in Fig. 1 are examples showing the relationship between D and K at $S = 3$ and 7, respectively. It was assumed in the calculation that the scattering coefficient was identical at the two wavelengths, and that the light-scattering layer did not absorb light. The linearizer is an electronic device that converts the signal of D for the minute uniform area into a signal proportional to K for a pre-set value of S according to the above forms of the Kubelka–Munk equations; similar conversion by means of a computer was made by Goldman and Goodall[12], and the theoretical basis for its electronic simulation was reported by Pollak and Boulton[25,26]. Recording of K during the zig-zag scanning of a zone in the present experiments gave many spikes, as shown by curve A in Fig. 2b. The value of K for the minute square area was integrated to obtain the area (denoted as Ky) of each spike, and the values of Ky for many spikes were summed to obtain the zone volume (denoted as Kxy; see curve B in Fig. 2b) with the K value taken as the height. The parameter S was so

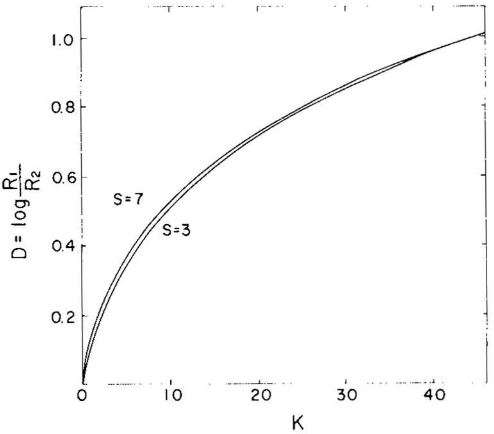
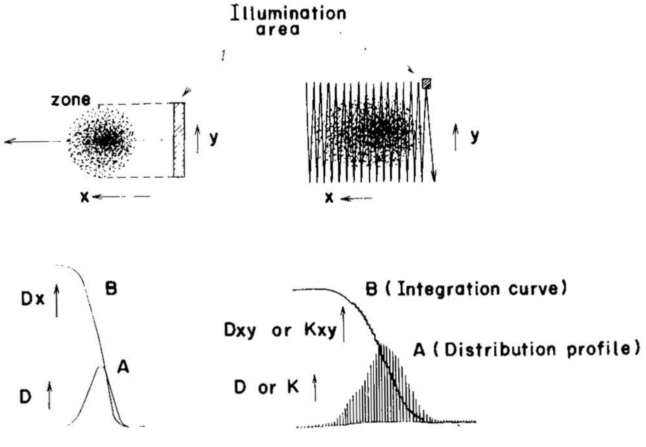


Fig. 1. Theoretical curves relating D and K calculated according to the Kubelka–Munk equations (see eqns. 1, 2 and 3 in the text) at values of $S = 3$ and 7.



(a) Slit-beam linear scanning **(b) Point zigzag scanning**

Fig. 2. Distribution profile and integration curve obtained by point zig-zag scanning (b) as compared with those obtained by slit-beam linear scanning (a).

varied as to obtain proportionality between Kxy and substance content. When the layer thickness (d) is constant, the value of K should be proportional to the substance concentration in the minute area, so that the value of Kxy integrated over the whole zone area should be proportional to the substance content in the zone. Two other methods of measurements were used for comparison; in one, the value of D for the minute area before the conversion was integrated to estimate the zone volume (Dxy) with the D value taken as the height, and in the second, the average reading of D for the long area in the zone of the slit-beam linear scanning system (Fig. 2a) integrated along the x axis to obtain a reading denoted as Dx for the whole zone[27].

The adsorbents for thin-layer chromatography sometimes contain impurities that run with the solvent front during chromatography and are distributed non-uniformly on the plate. Treatment with a spray reagent after chromatography often causes a similar non-uniform coloration of the background. Non-uniform background colour of UV absorption, which cannot be cancelled by dual-wavelength difference photometry because of its wavelength-dependent absorption, distorts the base-line. An example of such distortion by contamination near the solvent front is shown in Figs. 3a and 3b (in Fig. 3a, the pitch along the x axis in the point zig-zag scanning relative to the actual zone dimensions is much exaggerated for illustrative purposes). The fluctuation of the reading of D at the starting points of integration (dots in Figs. 3a and 3b) is due to such contamination. An electronic unit called a "background compensator" was developed to nullify the value of D at the starting points so as to obtain spikes on a straight base-line, as shown in Fig. 3c.

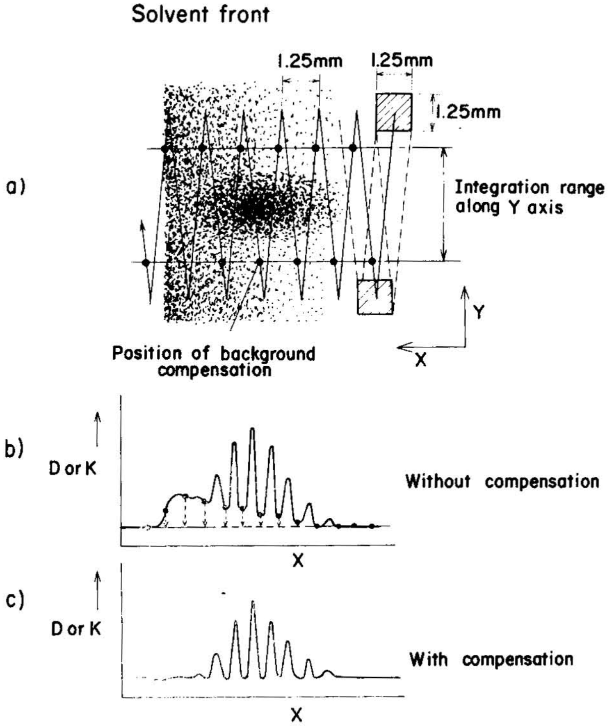


Fig. 3. Zig-zag scanning and compensation for contaminated background: (a) trace of the zig-zag scanning light beam across a zone near a contaminated solvent front; (b) distribution profile obtained without compensation; (c) distribution profile obtained with compensation. The hatched 1.25-mm-square blocks refer to the size and shape of the light beam cross-section, and the continuous and broken lines refer to the traces of the block centre and edges, respectively. The pitch of scanning was set to be identical with the dimension (1.25 mm) of the square block, so that the entire area including the sample zone was scanned exactly twice. The pitch along the x axis is exaggerated for illustrative purposes.

EXPERIMENTAL

Instrument and method

The dual-wavelength zig-zag scanner developed from the one described earlier[19] is illustrated schematically in Fig. 4. The optical systems are the same as before, except that two small grating monochromators were specially designed to illuminate a very small area vertically and alternately with two wavelength-separated light pulses. Part of the reflected light fell on a photomultiplier (Hamamatsu TV R446), and the alternate photo-currents for the two wavelengths were led to a logarithmic amplifier, background compensator, linearizer, integrator and, finally, recorder. For the two-dimensional scanning, the light beam was fixed, and the stage carrying the thin-layer plate was moved in a zig-zag manner. The area illuminated by the incident beam measured 1.25 × 1.25 mm (the pitch along the x axis on each scanning along the y axis was 1.25 mm), so that the beam scanned the entire area including the sample zone exactly twice (see Fig. 3a).

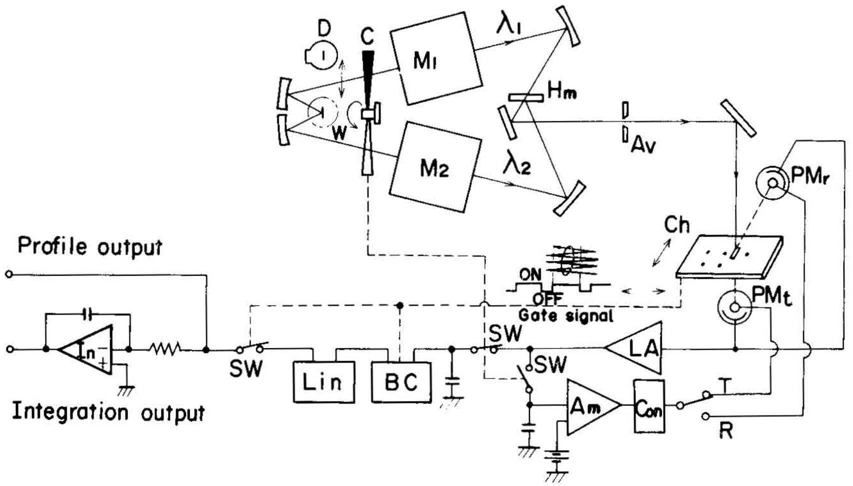


Fig. 4. Schematic diagram of a dual-wavelength zig-zag scanner. W = tungsten lamp; D = deuterium lamp; C = chopper; M_1, M_2 = monochromators; Hm = half mirror; Av = variable aperture; Ch = chromatogram; PMr, PMt = photomultipliers (PMr for reflectometry as applied in the present study); Con = d.c.–d.c. converter; Am = amplifier; LA = logarithmic amplifier; SW = gate switch; BC = background compensator; Lin = linearizer; In = analogue integrator.

The signal D was converted into a signal of K for a fixed value of S pre-set on the linearizer (the appropriate value of S was dependent on the adsorbent material, as will be demonstrated later). Five points were chosen at intervals on the theoretical curve (Fig. 1) for the appropriate value of S, and the five pairs of D and K values for these points were pre-set on the linearizer. The linearizer was so constructed that the output signal was proportional to the values of K corresponding to the signal of D on the curve passing through the five points and the origin. When the background was contaminated, the signal of D was passed through the background compensator before being led to the linearizer. The reading of D at each starting point of integration, which had been memorized in the compensator, was subtracted from the D values during scanning along the y axis, so that a profile on a straight zero-line (Fig. 3c) was obtained.

The K value was recorded with one of the dual pens on the recorder to obtain a distribution profile composed of many spikes, such as shown by curve A in Fig. 2b.

The signal of K from the linearizer was led to an integrator to obtain a signal of Kxy, which was recorded with another pen to obtain an integration curve such as shown by curve B in Fig. 2b. The plateau height of the integration curve was examined for its proportionality to the substance content in the zone. For comparison, the D values on the zig-zag scanning and the D values on the slit-beam linear scanning were integrated without the conversion to estimate the plateau heights, Dxy and Dx, respectively.

Materials for chromatography

The samples examined by the various techniques were caffeine, phenacetin and methyl yellow (Wako Junyaku, Osaka, Japan), erythrosine and Rose bengal (Tokyo Chemical Industry, Tokyo, Japan), various steroids (Applied Science Labs., State College, Pa., U.S.A.) and nicotinamide (Wako Junyaku). These compounds, and the solvents used for development, were of reagent grade. Plates pre-coated with silica gel, alumina or cellulose powder (layer thickness 0.25 mm; Merck, Darmstadt, G.F.R., or Wako Junyaku), or home-made plates coated with silica gel (thickness = 0.25 mm; Wako Junyaku) by means of a spreader (Mitsumi Kagaku Sangyo) were used for thin-layer chromatography. Paper chromatography was carried out on Toyo No. 51 paper.

RESULTS AND DISCUSSION

Effects of zone shape and size

The two-dimensional scanning was effective in obtaining reproducible readings for a zone of irregular shape. The data in Fig. 5 show an example obtained for a deformed elliptical zone of methyl yellow that had been developed on a layer of silica gel. The zone was scanned by dual-wavelength reflectometry in two ways (both one- and two-dimensionally), through its centre along three lines at different angles (A, B and C in Fig. 5). The linear scanning was made with a slit-beam with its length in cross-section adjusted to the width of the zone for each angle of scanning, and the two-dimensional scanning was made with a spot-beam with a minute square cross-section by moving the stage carrying the thin layer in a zig-zag manner. The values of D in these measurements were integrated over the whole area without being converted into K values. The distribution profiles and integration curves obtained by these measurement techniques are shown in the upper and lower parts, respectively, of Fig. 5. As expected, the point zig-zag scanning gave much better reproducibility. The integrated levels of Dxy obtained by the zig-zag scanning at the three angles agreed with each other, whereas the integrated levels of Dx obtained by slit-beam linear scanning at the three angles differed considerably.

This consistency in Dxy values measured at different angles for the same zone does not necessarily assure consistency of Dxy values for different zones containing the same amount of sample, but distributed differently. The D value for the minute area is not proportional to the substance content, so that a change in distribution may result in a change in the integrated Dxy value. Values of Dxy without the conversion (shown in Fig. 6 for three zones containing the same amount of methyl yellow) exhibit great variation; the Dxy level for the largest zone (C) is higher by 25% than the Dxy level for the smallest zone (B). The conversion into a K value

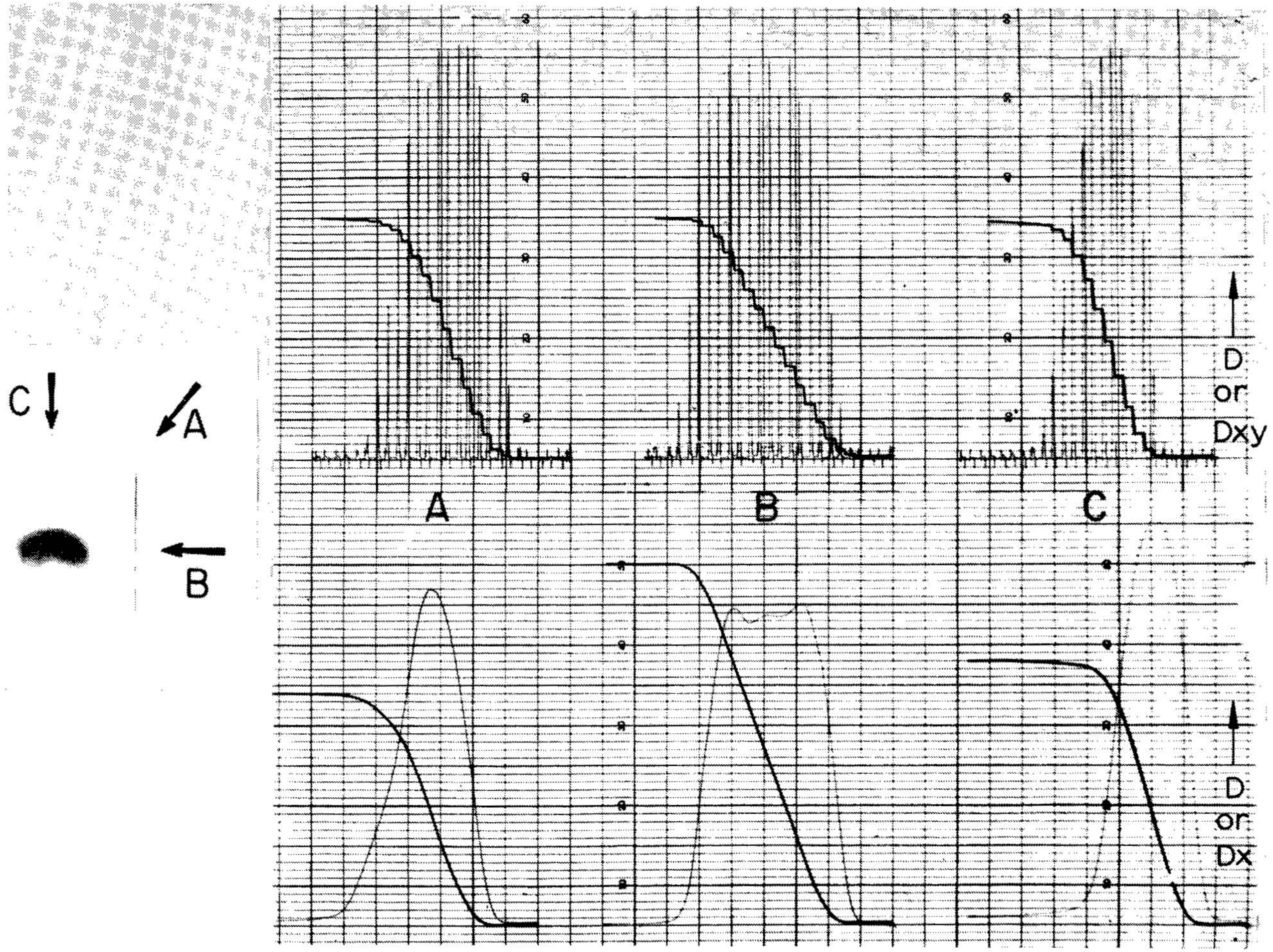


Fig. 5. Distribution profile in D and its integrated Dxy curve (upper figures) of an irregularly shaped zone of methyl yellow measured by the point zig-zag scanning method as compared with the profile and its integrated Dx curve (lower figures) for the same zone measured by slit-beam linear scanning. Scanning was by dual-wavelength reflectometry at 700 nm (λ_1) and 420 nm (λ_2) along three axes (A, B and C as shown on the left of the figure). The methyl yellow was separated on a silica gel plate (Merck F254), with methanol as developing solvent.

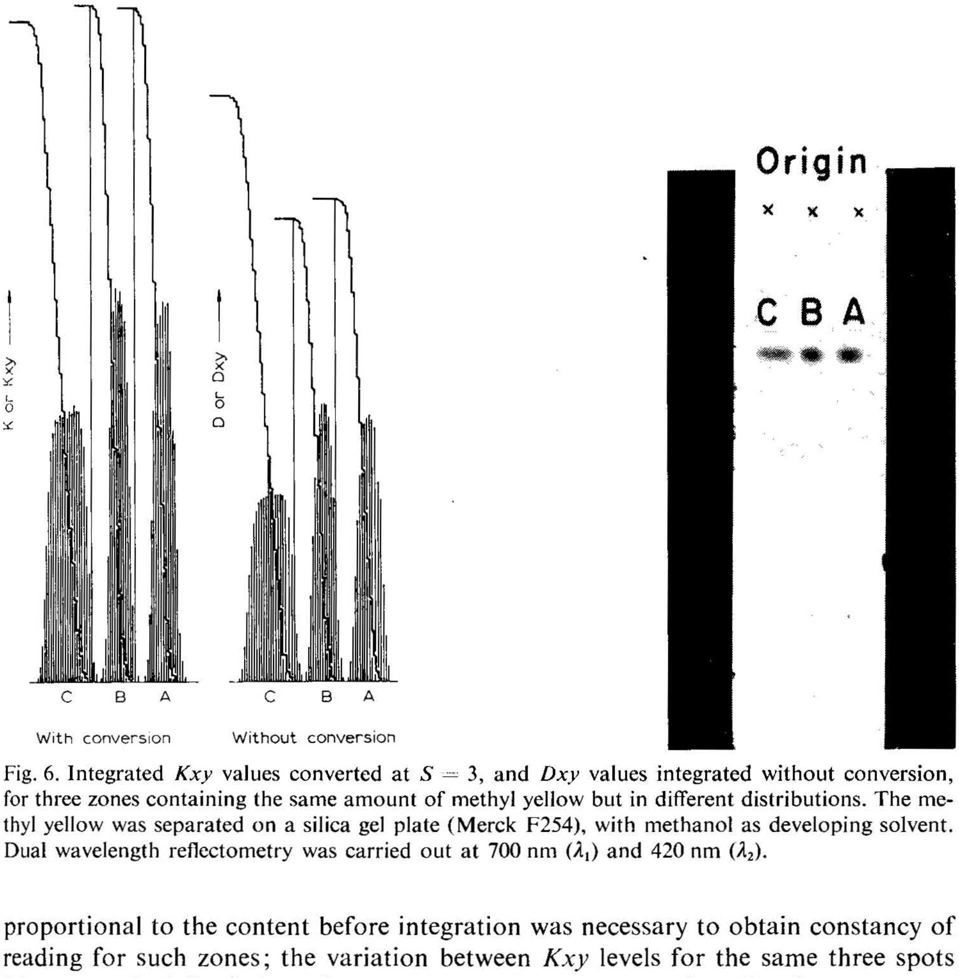


Fig. 6. Integrated *Kxy* values converted at $S = 3$, and *Dxy* values integrated without conversion, for three zones containing the same amount of methyl yellow but in different distributions. The methyl yellow was separated on a silica gel plate (Merck F254), with methanol as developing solvent. Dual wavelength reflectometry was carried out at 700 nm (λ_1) and 420 nm (λ_2).

proportional to the content before integration was necessary to obtain constancy of reading for such zones; the variation between *Kxy* levels for the same three spots (shown on the left of Fig. 6) is at most 2%. The constancy of reading for zones containing the same amount of sample thus achieved, together with the very low fluctuation in reading by dual-wavelength difference photometry, provided the basis for further precise analysis of data.

Dependency of Dx, Dxy and Kxy values on sample content

Fig. 7 shows the improvement in proportionality to the sample content that was achieved by zig-zag scanning of the spots in combination with conversion into *K* values. The data obtained by this combination technique for zones of caffeine (line A) and phenacetin (line C) developed on a silica gel plate (Merck F254) were compared with the data (curves B and D) for the same zones measured by slit-beam linear scanning without conversion. The conversion was made at $S = 3$, and the wavelengths for dual-wavelength reflectometry were 350 nm (λ_1) and 270 nm (λ_2); caffeine and phenacetin on silica gel show an absorption maximum ($\lambda_{\text{max.}}$) at 270 and 255 nm, respectively, and no absorption at 350 nm. The *Kxy* values obtained by integration of the *K* values ($S = 3$) measured by zig-zag scanning were proportional

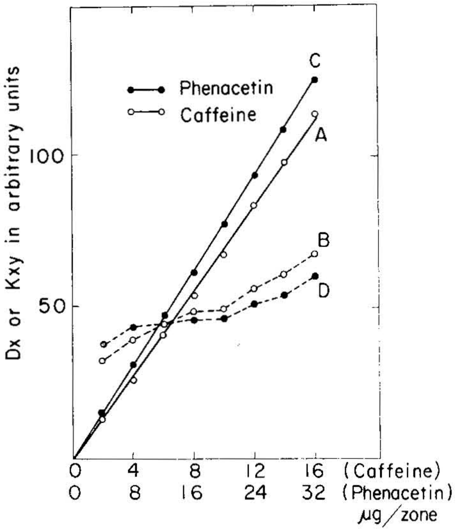


Fig. 7. Relationship between *Dx* value measured by slit-beam linear scanning, *Kxy* values ($S = 3$) measured by point zig-zag scanning and zone concentration. Open circles on line A and curve B are values of *Kxy* and *Dx*, respectively, for zones of caffeine; solid circles on line C and curve D are values of *Kxy* and *Dx*, respectively, for zones of phenacetin. These substances were separated in chloroform–acetone (6:1; v/v) on a Merck F254 silica gel plate and measured by dual-wavelength reflectometry at 350 nm (λ_1) and 270 nm (λ_2).

to the sample content, but the *Dx* values obtained by integration without conversion of the *D* values measured by linear scanning showed a non-linear relationship. Similar measurements were made for testosterone ($\lambda_{max.} = 250$ nm), progesterone ($\lambda_{max.} = 260$ nm), corticosterone ($\lambda_{max.} = 255$ nm), cortisone ($\lambda_{max.} = 250$ nm) and hydrocortisone ($\lambda_{max.} = 250$ nm) on a silica gel plate at $\lambda_1 = 350$ nm and $\lambda_2 = 250$ nm, and for food dyes such as Rose bengal ($\lambda_{max.} = 550$ nm) and erythrosine ($\lambda_{max.} = 530$ nm) on the same plate at $\lambda_1 = 700$ nm and $\lambda_2 = 545$ nm. The *Kxy* values at $S = 3$ for these samples also showed close proportionality to the sample contents, but the dependencies of the *Dx* values showed curves.

The data in Fig. 8 show the effect of the conversion of *D* into *K* for the two-dimensional measurements. Zones containing different amounts of caffeine separated on a Merck silica gel plate were measured by point zig-zag scanning with and without the conversion, followed by integration at wavelengths of 350 nm (λ_1) and 270 nm (λ_2). The *Kxy* values obtained with the conversion at $S = 3$ (solid circles on line A) indicate proportionality to caffeine content, while the *Dxy* values (curve B) obtained without conversion are not proportional to the content.

The value of *S* appropriate for obtaining proportionality was dependent on the chromatographic adsorbent. Zones of the same sample of caffeine, but on a different plate (Wako FM pre-coated), were measured by zig-zag scanning reflectometry at the same wavelengths (350 and 270 nm). Proportionality in this instance occurred with *Kxy* values converted at $S = 7$, but not with values converted at $S = 3$, as can be seen from line C and curve D, respectively, in Fig. 8. Similar experiments with caffeine on a home-made silica gel (Wako B-5UA) plate at $\lambda_1 = 350$ nm and

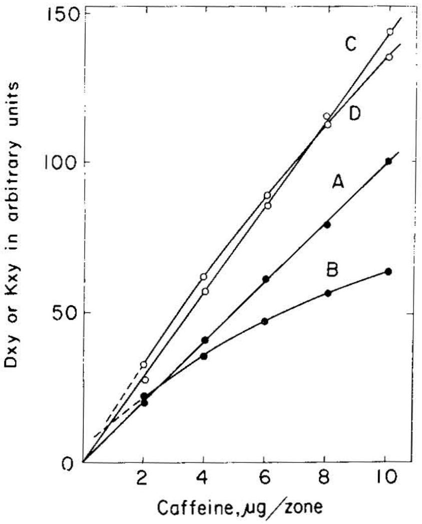


Fig. 8. Relationship between *Kxy* and *Dxy* values, with and without conversion, measured by point zig-zag scanning and caffeine concentration; *Kxy* values converted at $S = 3$ (line A) and *Dxy* values without conversion (curve B) for caffeine zones developed with chloroform–acetone (6:1) on a Merck F254 silica gel plate, and *Kxy* values converted at $S = 7$ (line C) and $S = 3$ (curve D) for caffeine zones developed with the same solvent mixture, but using a Wako FM plate. Measurements were made by dual-wavelength reflectometry at 350 nm (λ_1) and 270 nm (λ_2).

$\lambda_2 = 270$ or 285 nm, and with methyl yellow zones on a Merck cellulose plate at $\lambda_1 = 700$ nm and $\lambda_2 = 420$ nm, showed proportionality for *Kxy* values converted at $S = 3$. Fig. 9 shows data for methyl yellow zones ($\lambda_{\text{max.}} = 420$ nm) on Toyo No. 51 paper. In this instance, conversion at $S = 3$ gave *Kxy* values proportional to

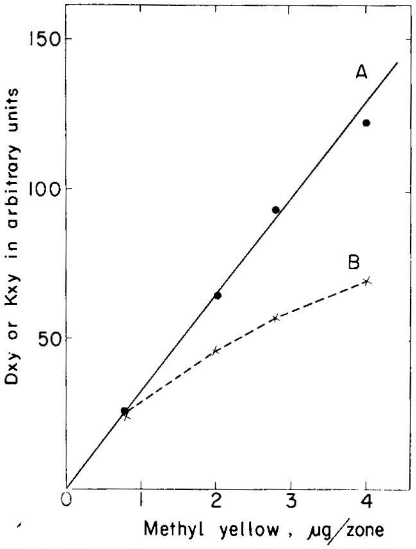


Fig. 9. *Kxy* values converted at $S = 3$ (line A) and *Dxy* values (curve B) measured by point zig-zag scanning for methyl yellow zones developed with chloroform on Toyo No. 51 paper; dual-wavelength reflectometry at 700 nm (λ_1) and 420 nm (λ_2).

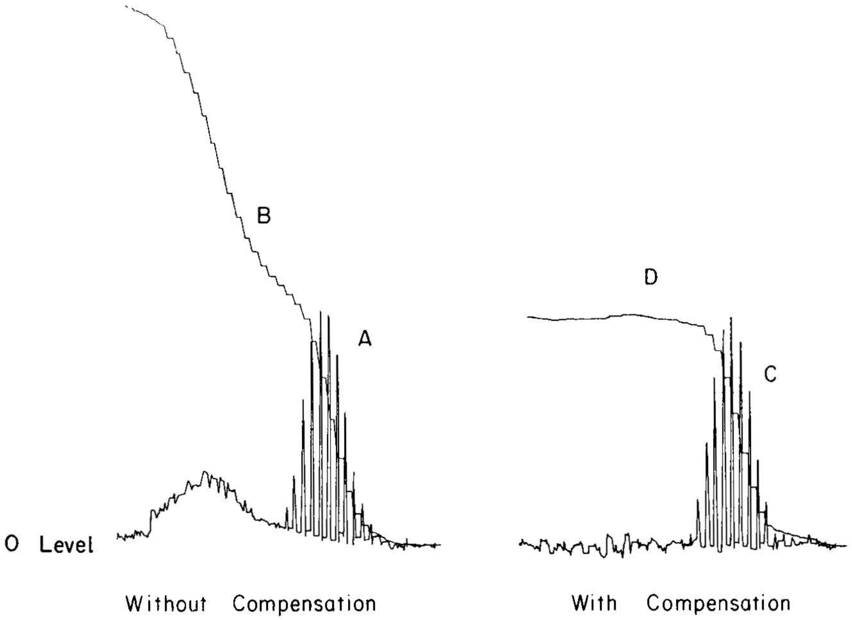


Fig. 10. Effect of background compensation in zig-zag scanning of a zone of nicotinamide on a contaminated background near the solvent front on a Merck silica gel plate; dual-wavelength reflectometry at 350 nm (λ_1) and 265 nm (λ_2). The nicotinamide was separated with methanol. Curves A and B are the K and Kxy signals, respectively, recorded without background compensation; curves C and D are the same signals recorded with compensation. The conversion into K was made at $S = 3$.

methyl yellow content (see line A). The Dxy values obtained by integration of D values without conversion are shown by curve B, which is bent at higher concentrations.

The two integration curves in Fig. 10 show the effect of background compensation observed for a zone of nicotinamide ($\lambda_{\text{max.}} = 265$ nm) developed on Merck silica gel. The integration curve (D) obtained with compensation shows stepwise increases in Kxy value to a plateau, whereas curve B (obtained without compensation) shows a further increase in Kxy value over the range of the plateau. This increase in Kxy value near the solvent front results from positive K values due to contamination around the zone, as can be seen from the distribution profile obtained without compensation.

All the data presented in this paper indicate the applicability of the general Kubelka–Munk equations for the measurement of absorbing zones separated on thin-layer chromatograms. This was demonstrated from the precise data obtained by the combined techniques of dual-wavelength difference reflectometry, two-dimensional zig-zag scanning and integration, conversion of D to K, and compensation for coloured background.

A similar investigation with transmission photometry as opposed to reflection photometry was also carried out, and will be reported elsewhere; the results may also be of value.

REFERENCES

1 H. Jork, *J. Chromatogr.*, 33 (1968) 297.
2 R. W. Frei, A. Kunz, G. Pataki, T. Prims and H. Zürcher, *Anal. Chim. Acta*, 49 (1970) 527.
3 J. C. Touchstone, S. S. Levin and T. Murawec, *Anal. Chem.*, 43 (1971) 858.
4 I. E. Bush, *Methods Biochem. Anal.*, 11 (1963) 149.
5 A. A. Boulton, in I. Smith (Editor), *Chromatographic and Electrophoretic Techniques*, Vol. I, Heinemann, London, 1969, p. 887.
6 A. A. Boulton, *Methods Biochem. Anal.*, 16 (1968) 328.
7 P. Kubelka and F. Munk, *Z. Tech. Phys.*, 12 (1931) 593.
8 P. Kubelka, *J. Opt. Soc. Amer.*, 38 (1948) 448.
9 A. Schuster, *Astrophys. J.*, 21 (1905) 1.
10 K. Shibata, *Methods Biochem. Anal.*, 7 (1959) 77.
11 J. Goldman and R. R. Goodall, *J. Chromatogr.*, 32 (1968) 24.
12 J. Goldman and R. R. Goodall, *J. Chromatogr.*, 40 (1969) 345.
13 L. R. Treiber, *J. Chromatogr.*, 100 (1974) 123.
14 U. Hezel, *Zeiss Information*, S50-741-J/73.9.10-SA, Carl Zeiss Oberkochen, 1973.
15 J. Goldman, *J. Chromatogr.*., 78 (1973) 7.
16 B. Chance, *Rev. Sci. Instrum.*, 22 (1951) 619.
17 B. Chance, *Methods Enzymol.*, 12 (1957) 273.
18 L. Salganicoff, M. Kraybill, D. Mayer and V. Legallais, *J. Chromatogr.*, 26 (1967) 434.
19 K. Shibata, *Biochim. Biophys. Acta*, 304 (1973) 249.
20 A. A. Boulton and V. Pollak, *J. Chromatogr.*, 45 (1969) 189.
21 V. Pollak and A. A. Boulton, *J. Chromatogr.*, 45 (1969) 200.
22 V. Pollak and A. A. Boulton, *J. Chromatogr.*, 50 (1970) 30.
23 V. Pollak, *J. Chromatogr.*, 77 (1973) 245.
24 J. Goldman and R. R. Goodall, *J. Chromatogr.*, 47 (1970) 386.
25 V. Pollak and A. A. Boulton, *J. Chromatogr.*, 50 (1970) 19.
26 V. Pollak and A. A. Boulton, *J. Chromatogr.*, 50 (1970) 39.
27 W. Schlemmer, *J. Chromatogr.*, 63 (1971) 121.

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EFFECT OF AQUEOUS CHLORINATION ON THE AROMATIC FRACTION OF DIESEL FUEL

ANALYSIS BY COMPUTER-ASSISTED GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

An investigation into the behaviour of aromatic hydrocarbons in chlorinated water is described. Aromatic fractions of diesel fuel were exposed to highly chlorinated water (100 mg/l free chlorine) for definite periods of time (1, 30 and 70 h), and the extracts were subjected to gas chromatographic–mass spectrometric analysis. Mass chromatograms of the various extracts, specific for aromatic hydrocarbons and possible chlorinated products, obtained by simple mathematical procedures, have been correlated with each other.

Conclusions are made on the reactivity of the various species: the rates of oxidation and chlorination are dependent on the number and orientation of the alkyl substituents. 1,3,5-Trimethylbenzene, a frequently encountered pollutant, shows a marked reactivity, its chlorination being almost complete after 1 h.

INTRODUCTION

Chlorine is widely used in water treatment as a disinfecting and oxidising agent[1]. In chlorination processes, organic constituents of water may be chlorinated to yield components which are of great concern in the consideration of water quality[2–4]. Details of the formation of various haloforms in chlorinated drinking waters have recently been published[5,6].

We have studied the possibility of chlorinated hydrocarbons originating from the chlorination of petroleum-derived compounds found in aqueous solutions. Such contamination of surface and drinking waters[7,8]. Substantial amounts of these materials are also normally present in domestic and industrial wastewater[9]. Petroleum hydrocarbons were chosen for this study because of their ubiquity and the bioresistant character of their chloro derivatives. Since aromatic hydrocarbons are more soluble in water and less resistant to chemical oxidation than aliphatic hydrocarbons[10], this study focuses on the aromatic fraction of diesel fuel.

Petroleum fractions are extremely complex mixtures which cannot be completely resolved into single components by high-resolution gas chromatography. We show that computer-assisted gas chromatography–mass spectrometry (GC–MS–COM) enables the specific detection of various groups of isomers and their chlorinated derivatives.

Chlorine disproportionates in water to produce hypochlorous and hydrochloric acids:

$$Cl_2 + H_2O \rightarrow HOCl + H^+ + Cl^-$$

Under conditions normally prevailing at water treatment plants, the equilibrium position of this reaction is on the side of the acids, and only at a very low pH is a substantial amount of elemental chlorine present. Hypochlorous acid is a weak acid with a pK value of 7.5 at 20°. The reactions of organic compounds in chlorinated water are therefore dependent on both the chlorine concentration and the pH.

The aim of this study is to identify those components of diesel fuel (or the equivalent No. 2 fuel oil) which tend to form chlorinated hydrocarbons. This is achieved by specific fingerprints obtained by GC–MS–COM. We report correlations between precursors and chlorinated products, and discuss the results in the light of the reactivities and reaction pathways of the various compound types.

EXPERIMENTAL

100 μl of a commercial diesel fuel were fractionated on a silica column (11 × 1.0 cm, Kieselgel-40 (Merck Darmstadt, G.F.R.), activated at 275° for seven h). After eluting the aliphatic fraction with 30 ml of *n*-pentane, the aromatic constituents were obtained by elution with 30 ml of methylene chloride. The organic solvents had been carefully redistilled, and the water purified in a quartz distillation apparatus.

The methylene chloride fraction was concentrated at room temperature to 1 ml. A 300-μl aliquot of the solution was added to one litre of water which had been buffered with 100 ml of a 0.1 *M* phosphate buffer solution. By adding Javelle water (Bender and Hobein, Zürich, Switzerland) a concentration of 100 mg/l of free chlorine ($HOCl + {}^-OCl$) and a pH of 7.2 was attained. The chlorine concentration was determined by iodometric titration[11]. Samples of one litre each were allowed to react at room temperature for 1, 30 and 70 h, respectively. After chlorination the samples were extracted three times with 20 ml of *n*-pentane. The combined extracts were dried over sodium sulphate (Merck, puriss, heated at 750° for 24 h), and concentrated at room temperature to 300 μl. A second oil solution was prepared without adding any chlorine in order to analyze the starting material.

The GC–MS–COM investigations were performed on a Finnigan Model 1015 D apparatus in combination with a Finnigan Model 6000 Interactive Data System. The operation of the quadruple-type mass spectrometer was controlled by the computer, which acquired, stored, and processed the resulting data.

The gas chromatograph was equipped with a Grob-type injector[12] and a glass capillary column (OV-101, 50 m × 0.32 mm) which had been purchased from H. and G. Jaeggi, Trogen, Switzerland. The end of the glass capillary column was coupled to the mass spectrometer by means of a platinum capillary (0.1 mm I.D.) leading directly to the ion source. A piece of glass capillary tubing, 2 cm long, was melted on to one end of the platinum capillary so that it could be connected to the column with PTFE shrink tubing. By quenching the platinum tubing, elution conditions comparable to normal GC operation could be achieved. This interfacing technique has been described in detail by Neuner-Jehle and co-workers[13,14]. The MS operating conditions were: electron energy 70 eV, emission current 350 μA, preamplifier sensitivity 10^{-7} A/V.

3 μl of the concentrated extracts were injected without stream splitting onto the column at ambient temperature (approx. 28°). After 30 sec the split valve was opened, allowing the septum and injection port to be purged at a flow-rate of 15 ml/min[15]. Subsequent to the elution of the solvent, the oven temperature was programmed to 200° at 3°/min, and the temperature of the GC–MS interface oven was held at 180°. Helium at 30 p.s.i. was used as carrier gas.

Mass spectra were scanned continuously and stored on a magnetic disc. The initial parts of the chromatograms were recorded over a mass range from 33 to 199 atomic mass units (a.m.u.) with an integration time of 8 msec per a.m.u. For the second part, a mass range from 33 to 240 a.m.u. was recorded with 7 msec integration time per a.m.u. The cut between the two parts was set after the elution of acenaphthene, which could be monitored on the real time display.

The Data System 6000 enabled the data to be processed by a very rapid and convenient direct interaction with the computer. Mass spectra or reconstructed chromatograms could be stored in two different memory regions and could be added together or subtracted from each other as required. Negative values obtained by subtraction were set equal to zero. This operational facility is provided mainly for background subtraction of mass spectra, but it can also be used for combining reconstructed chromatograms.

Total ion current (TIC) chromatograms were established by adding together all ion currents of each spectrum and plotting them *versus* the spectrum numbers. Mass chromatograms (MC) were reconstructed selecting the specified ion or ion group and plotting the ion intensities in the same manner. These latter chromatograms indicate specifically those compounds which contain the selected elements in their mass spectra and structures, respectively[16]. For example, two compounds which have different retention times and can be detected specifically in a MC of the respective molecular masses, can be studied in one single chromatogram by adding together the two particular MC's.

RESULTS AND DISCUSSION

Initially, a control sample of the aromatic fraction of diesel fuel was studied without chlorination to provide background information on the complex mixture, and to test the detection techniques. Fig. 1 shows the TIC chromatograms resulting

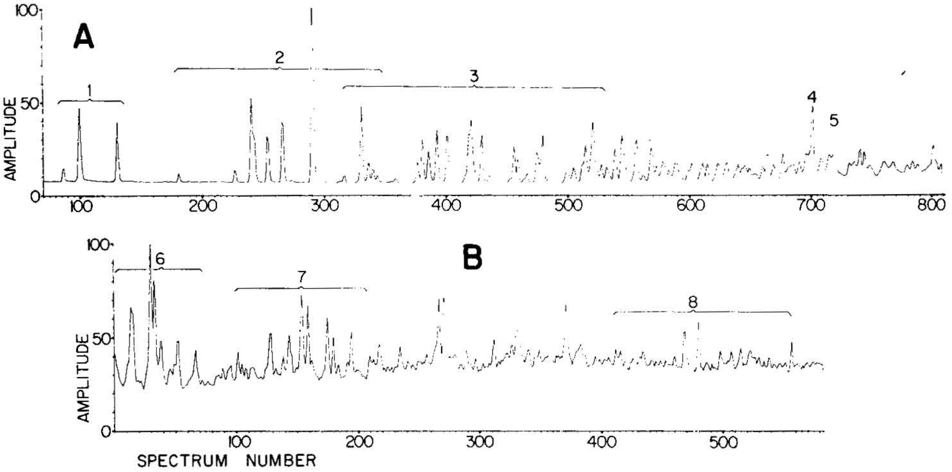

Fig. 1. Reconstructed total ion current chromatogram of the aromatic fraction of diesel fuel. A, Mass range = 33–199 a.m.u.; integration time: 8 msec/a.m.u. B, Mass range = 33–244 a.m.u.; integration time: 7 msec/a.m.u. 1 = C_2-benzenes; 2 = C_3-benzenes; 3 = C_4-benzenes; 4 = 2-methylnaphtahlene; 5 = 1-methylnaphthalene; 6 = C_2-naphthalenes; 7 = C_3-naphthalenes; 8 = phenanthrene, anthracene, C_1-phenanthrenes/anthracenes.

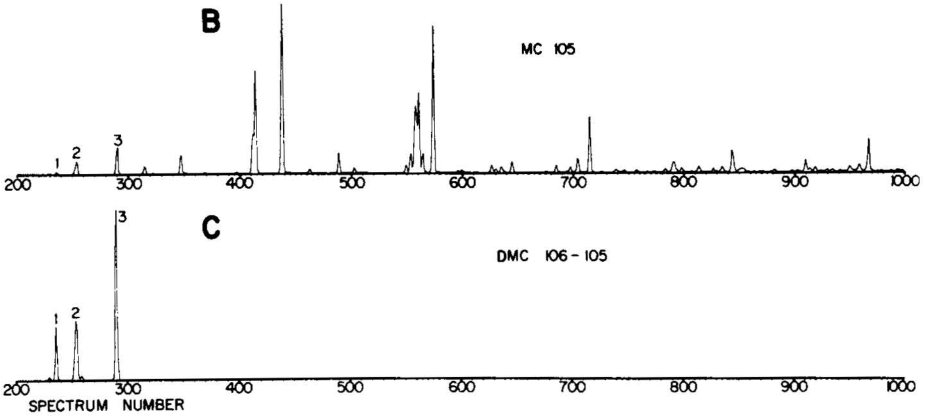

Fig. 2. Reconstructed mass chromatograms of the aromatic fraction of diesel fuel after 30 h chlorination. A = Mass chromatogram 106; B = mass chromatogram 105; C = difference mass chromatogram 106 − 105. 1 = Ethylbenzene; 2 = *m*/*p*-xylene; 3 = *o*-xylene.

from a GC–MS experiment. The chromatogram is split into two parts according to the storage in two different files of the disc memory. Because the highest peak of each part is used for normalization, the peak heights cannot be directly compared. The components in the second part are actually less abundant than the earlier eluting compounds. Most peaks contain more than one component, and many trace constituents are not detected. A large number of unresolved peaks gives rise to a chromatogram which cannot be reasonably interpreted. The ranges of the various compound types as indicated in Fig. 1 are based on MS evidence and on relative GC retention times.

The data resulting from the GC–MS experiment can be evaluated in a more sophisticated way using mass chromatographic techniques. Since most aromatic hydrocarbons show intense molecular ions in their mass spectra, they are well suited to specific detection by mass chromatography.

The mass chromatograms of several chlorinated mixtures were complicated; Fig. 2 exemplifies such a case and indicates how the disturbances can be removed. The MC for mass 106 (trace A) gives peaks for ethylbenzene, *o*/*m*-xylene (not resolved) and *p*-xylene. However, there are additional peaks which are eluted later than the C_2-benzenes*. The origin of this interference is demonstrated in trace B of Fig. 2, showing the MC for mass 105. In this chromatogram, the major peaks correspond to interference in trace A. This is due to the fact that compounds with ions of mass 105 have additional ions of mass 106 arising from the natural abundance of carbon-13 isotopes.

The selectivity is considerably improved by subtracting the MC for $M^+ - 1$ (MC 105, trace B) from the MC for M^+ (MC 106, trace A). In this way, only compounds having greater ion intensities of M^+ than of $M^+ - 1$ remain in the reconstructed chromatogram. Trace C of Fig. 2 demonstrates the efficiency of this subtraction procedure which eliminates the interfering peaks in the MC for the molecular ion of the C_2-benzenes.

In general, one can detect specifically a group of components which display in their mass spectra a greater intensity of ion A than ion B by subtracting MC (B^+) from MC (A^+). In this way all peaks which correspond to components having mass spectra with ion intensities $I(A^+) \leqslant I(B^+)$ are suppressed. If $I(A^+) > I(B^+)$, the difference mass chromatogram (DMC) reveals a peak with a height given by the intensity difference $I(A^+) - I(B^+)$. A similar technique was used to search for chlorinated hydrocarbons in the course of the impact of chlorine. The substitution by one or two chlorine atoms leads to an increase of the molecular mass by 34 or 68, respectively. The chlorinated products and their precursors could be represented in one single chromatogram by adding together the DMC for $[M^+ - (M^+ - 1)]$, $[(M^+ + 34) - (M^+ + 33)]$ and $[(M^+ + 68) - (M^+ + 67)]$. Figs. 3, 4 and 5 represent some of the resulting chromatograms for different types of compounds and varying reaction times.

Fig. 3 depicts the behaviour of C_3-benzenes. In the unchanged diesel fuel (reaction time 0, trace A), all eight possible isomeric C_3-benzenes were detected. Identification was based on their mass spectra and on co-injection gas chromatography of reference substances. Since acenaphthene (peak 16) also has a molecular mass of 154, this component also appears in the DMC (154 − 153). After a reaction time

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* C_n-benzene stands for benzenes with *n* additional aliphatic carbon atoms.

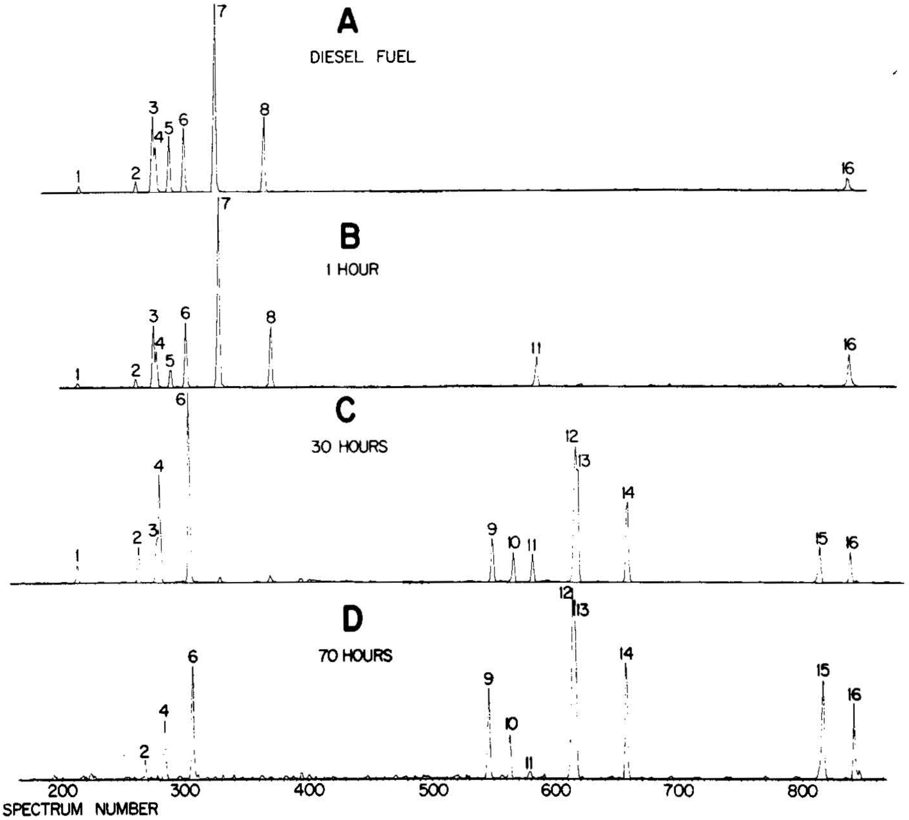

Fig. 3. Reconstructed mass chromatograms for the specific detection of C_3-benzenes and their chlorinated derivatives: MC (120 – 119) + (154 – 153) + (188 – 187). A, Diesel fuel before chlorination; B, 1 h chlorination; C, 30 h chlorination; D, 70 h chlorination. 1 = Isopropylbenzene; 2 = *n*-propylbenzene; 3 = 1-ethyl-4-methylbenzene; 4 = 1-ethyl-3-methylbenzene; 5 = mesitylene; 6 = 1-ethyl-2-methylbenzene; 7 = 1,2,4-trimethylbenzene; 8 = 1,2,3-trimethylbenzene; 9 = chloro-C_3-benzenes; 10 = chloro-C_3-benzenes; 11 = 2-chloromesitylene; 12 = chloro-C_3-benzenes; 13 = chloro-C_3-benzenes; 14 = chloro-C_3-benzenes; 15 = 2,4-dichloromesitylene; 16 = acenaphthene.

of 1 h the relative intensity of peak no. 5 (1,3,5-trimethylbenzene, mesitylene) has decreased to approximately 30% of its original value. This change can be correlated with the appearance of a chloro-C_3-benzene (peak no. 11). Peak no. 11 is then suspected to be a monochloromesitylene.

After 30 h of chlorination (trace C) the three isomeric trimethylbenzenes are no longer present, or only in trace quantities. A series of chlorinated C_3-benzenes with molecular masses of 154 (peaks 9–14) could be detected. It was not possible to determine their exact structures on the basis of their mass spectra. Peak 15 proved to be a dichloro-C_3-benzene. Only *n*-propyl-, 1-ethyl-3-methyl- and 1-ethyl-2-methylbenzenes (peaks 2, 4 and 6) remained in detectable amounts after a chlorination of 70 h. These components were obviously not oxidized; nor did they show any tendency to substitute chlorine. Peak no. 11 disappeared after 70 h reaction time, and we suspect that this chloromesitylene was chlorinated again yielding peak no. 15. Acenaphthene

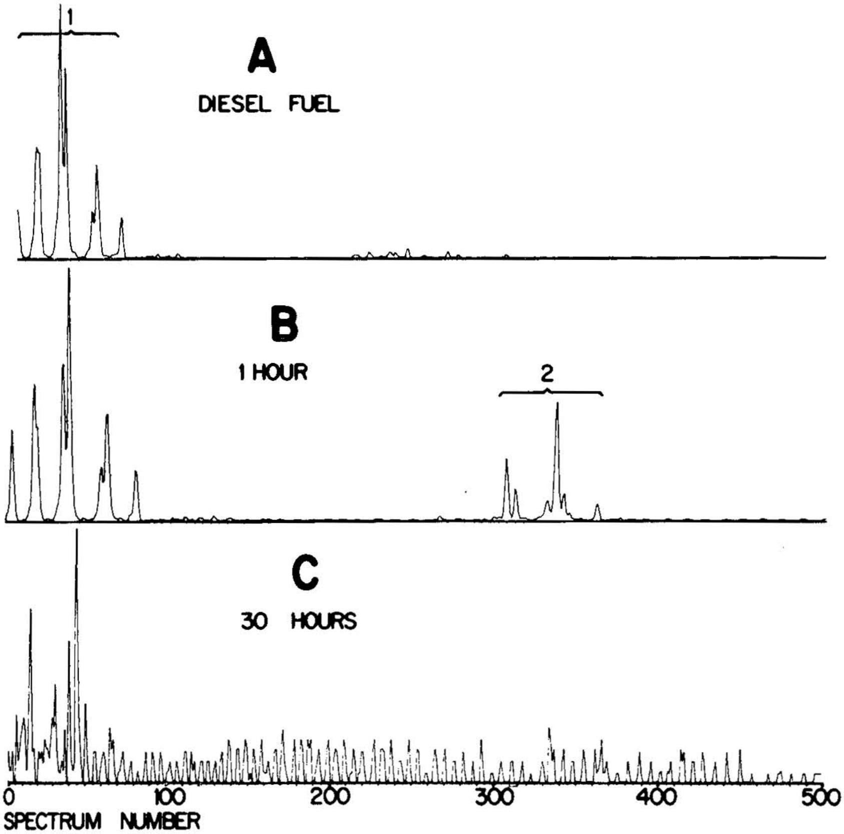

Fig. 4. Reconstructed mass chromatograms for the specific detection of C_2-naphthalenes and their chlorinated derivatives: MC(156 - 155) + (190 − 189) + (224 − 223). A, Diesel fuel without chlorination; B, 1 h chlorination; C, 30 h chlorination. 1 = C_2-naphthalenes; 2 = chloro-C_2-naphthalenes.

(peak no. 16) on the other hand proved to be very stable and did not change its concentration markedly. For an exact structure elucidation of peaks no. 11 and 15, mono- and dichloromesitylenes were prepared by chlorination of an aqueous solution of mesitylene. The aromatic positions of the chlorine atoms could then be derived from their proton magnetic resonance spectra.

Some of the isomeric C_2-naphthalenes (molecular mass 156) were chlorinated after 1 h reaction time. This group of chloro-compounds is readily recognized in trace B of Fig. 4. After 30 h of chlorination (trace C) these compounds had disappeared. Since no dichloro-products could be found, we assumed that an oxidative reaction had taken place. Some C_2-naphthalenes did survive the chlorination, but their identities could not be elucidated.

Fig. 5 shows the behaviour of naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, the methylindanes and tetralene. The effect of a 30-h chlorination produced a complete elimination of the three naphthalenes. No chlorinated products could be detected. Tetralene, 1-methylindane and 2-methylindane showed the highest stability. 4-Methylindane and 5-methylindane with methyl substituents on the aro-

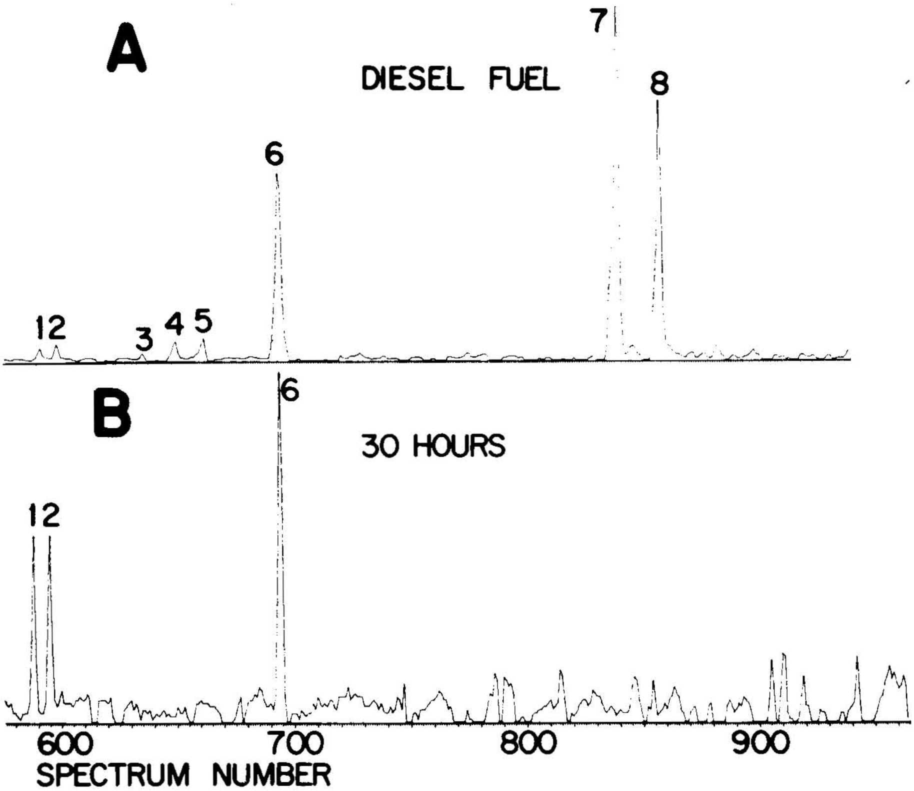

Fig. 5. Reconstructed mass chromatograms for the specific detection of naphthalene, tetralene, methylnaphthalenes and methylindanes: MC 128 + 142 + (132 − 131). A, Diesel fuel without chlorination; B, 30 h chlorination; 1 = 1-, 2-Methylindanes; 2 = 1-, 2-methylindanes; 3 = 4-, 5-methylindanes; 4 = 4-, 5-methylindanes; 5 = tetralene; 6 = naphthalene; 7 = 2-methylnaphthalene; 8 = 1-methylnaphthalene.

matic ring are degraded after the same reaction time. Phenanthrene and its methyl homologues were eliminated after 30 h of reaction, yielding no chlorinated products. Table I summarizes the results of the analyses.

In the reaction mixture a large number of interdependent reactions takes place, with the chlorination reaction competing with oxidative degradation. Because no intermediates of the oxidation were detected, we suppose the consecutive reaction steps of the oxidation to be fast. The distribution of the chlorinated products can be explained by an electrophilic aromatic substitution by chlorine. Alkyl substituents are activating and *o*/*p*-directing; if they are *m*-substituted to each other, the activating effect is enhanced.

1,3,5-Trimethylbenzene has shown the greatest chlorination rate among the C_3-benzenes, and we intend to use it as a tracer contaminant in further chlorination experiments. The fact that mainly monochlorinated products have been formed can be explained by the de-activating effect of chlorine substituents. Whether similar reactions take place under conditions present in water treatment will be further investigated.

TABLE I

AROMATIC HYDROCARBONS DETECTED IN DIESEL FUEL AND IN AQUEOUS SOLUTIONS OF DIESEL FUEL AFTER CHLORINATION

Diesel fuel

Benzenes: benzene, toluene, C_2-benzenes (*o*-, *m*-, *p*-xylol, ethylbenzene), C_3-benzenes (8 isomers), C_{4-10}-benzenes
Naphthalenes: naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, C_{2-8}-naphthalenes
Indanes: indane, C_1-indanes (4 isomers), C_{1-5}-indanes
Tetralenes: tetralene, C_{2-5}-tetralenes
Acenaphthene: biphenyl acenaphthene
Fluorenes: fluorene, C_{1-2}-fluorenes
Anthracenes: anthracene, C_{1-3}-anthracene
Phenanthrenes: phenanthrene, C_{1-3}-phenanthrenes, 4,5-methylenephenanthrene

1 h of chlorination

Chlorinated products: 2-chloromesitylene, chloro-C_{2-3}-naphthalene

30 h of chlorination

Chlorinated products: chloro-C_{2-3}-benzenes, 2,5-dichloromesitylene, chloro-C_1-indanes
Components eliminated: naphthalene, C_{1-4}-naphthalenes, C_1-indanes, phenanthrenes, anthracenes

70 h of chlorination

Components persisting after 70 h of chlorination: C_n-benzenes, chloro-C_n-benzenes, 2-chloromesitylene, dichloro-C_n-benzenes, acenaphthene

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REFERENCES

1 G. C. White, *Handbook of Chlorination*, Van Nostrand, New York, 1972.
2 R. H. Burtschell, A. A. Rosen, F. M. Middleton and M. B. Ettinger, *J. Amer. Water Works Ass.*, 51 (1959) 205.
3 J. A. Zillich, *J. Water Pollut. Contr. Fed.*, 44 (1972) 212.
4 E. L. Barnhart and G. R. Campbell, *Water Pollution Control Series*, Environmental Protection Agency, ORM Grant No. 12020 EXG, 1972.
5 J. J. Rook, *Water Treat. Exam.*, 23 (1974) 234.
6 T. A. Bellar, J. J. Lichtenberg and R. C. Kroner, *J. Amer. Water Works Ass.*, 66 (1974) 703.
7 K. Grob, K. Grob, Jr. and G. Grob, *J. Chromatogr.*, 106 (1975) 299.
8 K. Grob and G. Grob, *J. Chromatogr.*, 90 (1974) 303.
9 W. Giger, M. Reinhard and C. Schaffner, *Vom Wasser*, 43 (1974) 343.
10 W. J. Weber, *Physicochemical Processes for Water Quality Control*, Wiley, New York, 1972, p. 378.
11 G. C. White, *Handbook of Chlorination*, Van Nostrand, New York, 1972, p. 232.
12 K. Grob and G. Grob, *Chromatographia*, 5 (1972) 3.
13 N. Neuner-Jehle, F. Etzweiler and G. Zarske, *Chromatographia*, 6 (1973) 211.
14 F. Etzweiler and N. Neuner-Jehle, *Chromatographia*, 6 (1973) 503.
15 K. Grob and K. Grob, Jr., *J. Chromatogr.*, 94 (1974) 53.
16 W. H. McFadden, *Techniques of Combined Gas Chromatography/Mass Spectrometry*, Wiley, New York, 1973.

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MASS FRAGMENTOGRAPHIC DETERMINATION OF DIPHENYLHYDANTOIN AND ITS MAIN METABOLITE, 5-(4-HYDROXYPHENYL)-5-PHENYLHYDANTOIN, IN HUMAN PLASMA

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SUMMARY

A method is described for the mass fragmentographic determination of diphenylhydantoin and its main metabolite, 5-(4-hydroxyphenyl)-5-phenylhydantoin (4-OH-DPH), in human plasma as their dimethyl and trimethyl derivatives, respectively. The derivatives are formed by using the recently described extractive alkylation technique. Pentadeuterated 4-OH-DPH is used as the internal standard. Following acidic hydrolysis of the plasma sample, conjugated 4-OH-DPH and, indirectly, the dihydrodiol metabolite, 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin, are measured. Using 100-μl plasma samples, the lower limit of detection is about 10 ng/ml (0.03 nmole/ml).

INTRODUCTION

The major metabolite of diphenylhydantoin (DPH) in man and rats is 5-(4-hydroxyphenyl)-5-phenylhydantoin (4-OH-DPH)[1], which is found in the urine mainly conjugated with glucuronic acid[2]. In dogs, *meta*-hydroxylation to (3-OH-DPH) represents the major pathway[3].

The elimination of DPH is dose-dependent[4–6] and two hypotheses have been proposed in order to explain this dependence. The first is that the elimination is characterized by saturation or Michaelis–Menten kinetics[7], and the other is product inhibition of DHP metabolism by 4-OH-DPH[8,9]. The evaluation of these hypotheses in man requires the measurement of the plasma concentration of 4-OH-DPH.

A colorimetric method has been described by Dill *et al.*[10] but it is not sensitive enough to measure unconjugated 4-OH-DPH in plasma. The gas chromatographic (GC) measurement of 4-OH-DPH using derivatization with either trimethylsilyl groups[11] or flash methylation[12] has been described. When a flame ionization detector is used, these procedures are not sensitive enough to detect unconjugated 4-OH-DPH

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in plasma. Recently, Albert *et al.*[13] described a high-pressure liquid chromatographic procedure for separating and measuring 4-OH-DPH in plasma, but this method requires the administration of radioactively labelled DPH[14]. This paper describes the application of extractive alkylation[15] in the mass fragmentographic determination of DPH, 3-OH-DPH and 4-OH-DPH in plasma.

MATERIALS AND METHODS

Reference drugs

DPH, 4-OH-DPH, 3-OH-DPH, 5-(3,4-dihydroxyphenyl)-5-phenylhydantoin (3,4-diOH-DPH), 5-(4-hydroxy-3-methoxyphenyl)-5-phenylhydantoin ($3\text{-}CH_3O$,4-OH-DPH) and 5-(2,4-dihydroxyphenyl)-5-phenylhydantoin (2,4-diOH-DPH) were gifts from Parke, Davis & Co. (Detroit, Mich., U.S.A.). The internal standard, 5-(4-methylphenyl)-5-phenylhydantoin ($4\text{-}CH_3$-DPH), was purchased from Aldrich (Beerse, Belgium). The dihydrodiol metabolite of DPH [5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin] was isolated and purified from the urine of rats fed with 0.2% of DPH in the diet[16].

Chemicals

5,5′-[4-^{14}C]Diphenylhydantoin (50.5 mCi/mmole) was purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.) and 4-OH-[4-^{14}C]DPH was synthesized from potassium [^{14}C]cyanide according to the method of Henze and Isbell[17,18]. Tetrabutylammonium hydrogen sulphate (TBA-HSO_4) was purchased from Lab Kemi (Stockholm, Sweden). The TBA-HSO_4 was prepared as a 1 *M* solution in 0.2 *N* sodium hydroxide and extracted twice with methylene dichloride. The extracted TBA-HSO_4 solution resulted in a lower base-line in the chromatograms when analyzing on a gas chromatograph. Trimethylanilinium hydroxide (TMAH) was prepared as described by Brochmann-Hanssen and Oke[19]. The external standard, 9-bromophenanthrene, was purchased from Lab Kemi.

All other chemicals were of reagent grade.

Deuterated internal standards

5-(4-Hydroxy-3,5-dideuterophenyl)-5-phenylhydantoin (4-OH-DPH-d_2). 4-OH-DPH was stirred with an excess of bromine in chloroform at room temperature. This reaction was followed by gas chromatography of the trimethyl derivatives obtained by injecting a mixture of TMAH and a portion of the reaction mixture. The reaction was complete after 28 h and only 5-(4-hydroxy-3,5-dibromophenyl)-5-phenylhydantoin could be detected. Its identity was established by combined gas chromatography–mass spectrometry (GC–MS). The crude dibromo compound dissolved in methanol-O-d was stirred with deuterium gas in the presence of palladium on carbon. The exchange of bromine for deuterium was monitored by GC. After 6 h, the reaction was stopped, the catalyst was removed by filtration and the reaction mixture was evaporated to dryness. The residue, 4-OH-DPH-d_2, was characterized by GC–MS (Table I) and thin-layer chromatography (TLC).

5-(4-Hydroxyphenyl)-5-(2,3,4,5,6-pentadeuterophenyl)hydantoin (4-OH-DPH-d_5). Benzoic acid-d_5 was reacted with oxalyl chloride in benzene to give benzoyl chloride-d_5[20]. Condensation of benzoyl chloride-d_5 with anisole under Friedel–Crafts conditions produced 4-methoxybenzophenone-d_5[21], which, on prolonged heating with aluminium chloride, gave 4-hydroxybenzophenone-d_5[22]. 4-OH-DPH-d_5 was obtained when 4-hydroxybenzophenone-d_5 was subjected to reaction with ammonium carbonate and potassium cyanide in fused acetamide[17]. The product was characterized by GC–MS (Table I) and TLC.

Gas chromatography

A Varian 1400 gas chromatograph was used with a flame ionization detector (FID) and a 1.2 m × 1.4 mm glass column packed with 3% OV-17 on Gas-Chrom Q (80–100 mesh). The temperature of the injector was 290°, the column 225° and the detector 250°.

Mass spectrometry

An LKB 9000 combined gas chromatograph–mass spectrometer was used. The flash heater temperature was 270°. The glass column (1.2 m × 2 mm) contained 3% OV-17 on Gas-Chrom Q (80–100 mesh); the column temperature was either 250° or 270°. For mass fragmentography, a multiple ion detector (MID) was used. The following *m/e* settings were used for the methyl derivatives: 280 (DPH), 310 (3-OH-DPH and 4-OH-DPH), 312 (4-OH-DPH-d_2), 315 (4-OH-DPH-d_5), 340 (3,4-diOH-DPH, 2,4-diOH-DPH and 3-CH_3O,4-OH-DPH) and 342 (dihydrodiol-DPH). The ionization voltage was 20 eV.

Extraction procedure

Unconjugated 4-OH-DPH. The following procedure was developed for the measurement of unconjugated 4-OH-DPH in plasma by the mass fragmentographic method. In a small disposable glass tube, a 0.1-ml plasma sample was buffered with 0.05 ml of 3 *M* Tris–hydrochloric acid, pH 7.5, and 0.1 ml of internal standard (10 μg/ml of 4-OH-DPH-d_5)was added. The solution was extracted with 1.5 ml of methyl isobutyl ketone (water-saturated) for 10 min. After centrifugation, the organic phase was removed with a Pasteur pipette and transferred in to a tube containing 0.2 ml of 1 *N* sodium hydroxide solution. The tubes were shaken for 10 min and centrifuged. The organic phase was removed and discarded. To the aqueous phase, 0.05 ml of 1 *M* TBA-HSO_4 (in 0.2 *N* sodium hydroxide solution) was added, followed by 1.5 ml of methylene dichloride (containing 0.1 ml of methyl iodide). The tubes were shaken mechanically for 30 min. Following centrifugation, the lower, organic phase was carefully removed and transferred in to a clean, conical-tipped, test-tube. The solvent was removed either under a gentle flow of nitrogen with the tubes in a sand-bath (30–35°) or by placing the tubes in a hood overnight at room temperature. The residue was dissolved in 0.05 ml of methanol and 1–4 μl were injected into the LKB 9000 instrument for GC–MS.

Conjugated 4-OH-DPH. To a 0.05-ml plasma sample, 0.05 ml of internal standard (10 μg/ml of 4-OH-DPH-d_5) and 0.1 ml of 10 *N* hydrochloric acid were added. The tubes were covered with aluminium foil and heated in a water-bath at 95° for 60 min. After cooling, 0.1 ml of sodium hydroxide solution and 0.05 ml of 3 *M* Tris–hydrochloric acid, pH 7.5, were added. The pH of tubes at random was checked with litmus paper so as to ensure adequate adjustment of the pH. The samples were then processed as for unconjugated 4-OH-DPH.

RESULTS AND DISCUSSION

The overall scheme of the procedure used for the determination of DPH and its hydroxylated metabolites in plasma is given in Fig. 1. When methyl isobutyl ketone is used for the extraction of plasma buffered to pH 7.5 with Tris–hydrochloric acid, the recovery of [^{14}C]DPH and 4-OH-[^{14}C]DPH is greater than 99%. Following centrifugation, the organic phase is removed and added to 1 ml of 1 *N* sodium hydroxide and mixed. The recovery of DPH and 4-OH-DPH in the aqueous (sodium hydroxide) phase in this back-extraction step is greater than 99%. The volume and concentration of sodium hydroxide during back-extraction are critical.

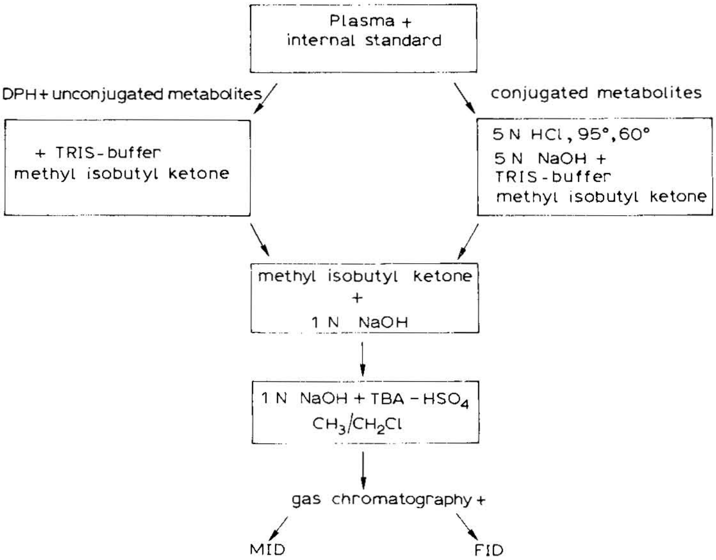

Fig. 1. Scheme for the general procedure.

Extractive alkylation

The extraction of anions from an aqueous alkaline phase as ion pairs with quaternary ammonium ions into an organic phase where the anions are alkylated[15] has been applied recently to analysis of a number of drugs[23–27]. The technique of extractive alkylation was adapted in order to determine the plasma concentration of DPH and its hydroxylated metabolites. The optimal conditions for extractive alkylation were studied by GC–FID. For these studies, DPH, 4-OH-DPH and 4-CH$_3$-DPH were used in a final volume of 1 ml. The concentration of sodium hydroxide and TBA-HSO$_4$ and the time of extraction were varied. The organic phase contained 100 μl of methyl iodide in 5 ml of methylene chloride. After the reaction was completed and the methylene chloride had been evaporated, the residue was dissolved in 50 μl of ethyl acetate containing 9-bromophenanthrene as an external standard. The peak heights were compared with the external standard and quantitated using [^{14}C]-DPH and 4-OH-[^{14}C]DPH. The influence of the concentration of TBA-HSO$_4$ and time on the extractive alkylation of 4-OH-DPH is shown in Fig. 2. The optimal conditions found for the extractive alkylation of 4-OH-DPH were 1 *N* sodium hy-

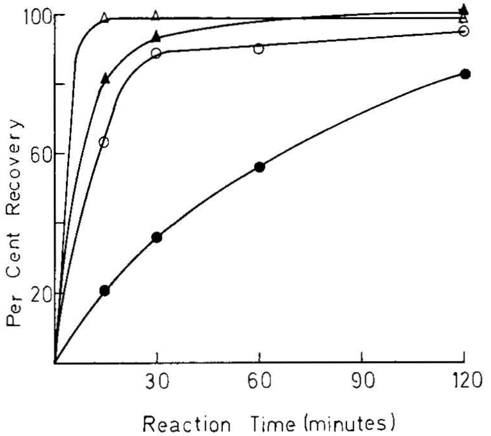

Fig. 2. Influence of concentration of tetrabutylammonium ion on the extractive alkylation of 4-OH-DPH. Concentration of 4-OH-DPH: 25 μg/ml in 1 *N* NaOH. Organic phase: 5 ml of methylene dichloride containing 100 μl of methyl iodide. Temperature: 23°. Concentration of tetrabutylammonium hydrogen sulphate: ●, 0.02; ○, 0.05; ▲, 0.10; △, 0.20 m*M*.

droxide in the presence of 0.2 *M* TBA-HSO_4 using an extraction time of 30 min. The concentration requirements for sodium hydroxide and TBA, and also the time required for extractive alkylation, were greater for 4-OH-DPH than for either DPH or 4-CH_3-DPH. Ethyl iodide could also be used as the alkylating agent, but under the above conditions complete extractive alkylation either required a reaction time of 120 min or the temperature had to be increased to 50° with shorter mixing times (Fig. 3).

The following DPH derivatives were successfully analyzed by extractive alkylation: DPH, 4-CH_3-DPH, 4-OH-DPH, 3-OH-DPH, 2,4-diOH-DPH, 3-CH_3O,4-

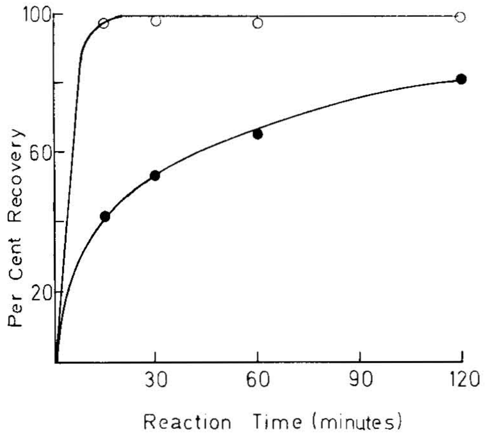

Fig. 3. Influence of reaction temperature on the extractive alkylation of 4-OH-DPH using ethyl iodide. Concentration of 4-OH-DPH: 25 μg/ml in NaOH and 0.2 m*M* tetrabutylammonium ion. Organic phase: 5 ml of methylene chloride containing 100 μl of ethyl iodide. Temperature: ●, 23°; ○, 50°.

OH-DPH, 4-OH, 4′-CH$_3$-DPH and 4,4′-diOH-DPH. The catechol metabolite, 3,4-diOH-DPH, was degraded under the alkaline conditions used and could not be measured. The dihydrodiol metabolite was not measured under the conditions described.

Standard curves for 4-OH-DPH prepared by using 1 ml of plasma samples and measured by GC–FID are linear from 0.5 to 10 μg/ml and from 10 to 100 μg/ml. The use of GC–FID is not sensitive enough to measure 4-OH-DPH at levels below 0.5 μg/ml without the appearance of interfering peaks.

Mass fragmentographic method

With the use of an MID, the extractive alkylation procedure produces reliable and reproducible results for unconjugated 4-OH-DPH in plasma. In a single day, it is possible to prepare, in duplicate, a standard curve for eight samples plus 40–50 samples from patients (a total of 116 tubes). Further, DPH and 3-OH-DPH can be determined in the same samples. A fragmentogram is shown in Fig. 4 for 3-OH-DPH and 4-OH-DPH added to drug-free control plasma and for a plasma sample from a patient administered DPH. With a column temperature of 270°, 3-OH-DPH and 4-OH-DPH can be separated and measured with injection of samples every 1.5 min. In order to detect DPH also, the temperature of the column can be decreased to 250°, permitting adequate separation of the three compounds. Standard curves for DPH determined by the general procedure are shown in Fig. 5a for concentrations from 0.02 to 0.2 μg/ml and 0.5 to 5 μg/ml. Standard curves for concentrations from 5 to

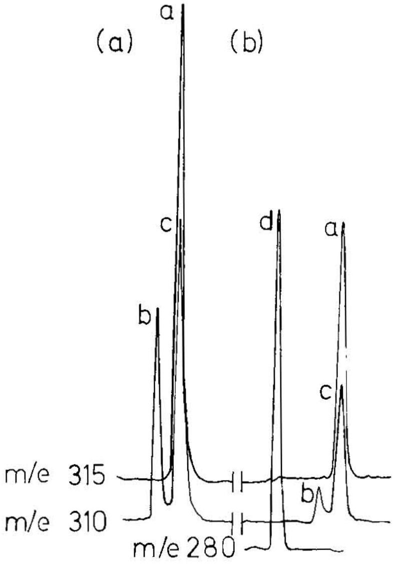

Fig. 4. Mass fragmentogram using 4-OH-DPH-d$_5$ (peak a) as internal standard. (a) Blank plasma with 0.125 μg of 3-OH-DPH/ml (peak b) and 0.25 μg of 4-OH-DPH/ml (peak c) added; (b) patients' plasma containing 6.6 μg/ml of DPH (peak d), 0.018 μg/ml of 3-OH-DPH (peak b) and 0.171 μg/ml of 4-OH-DPH (peak c).

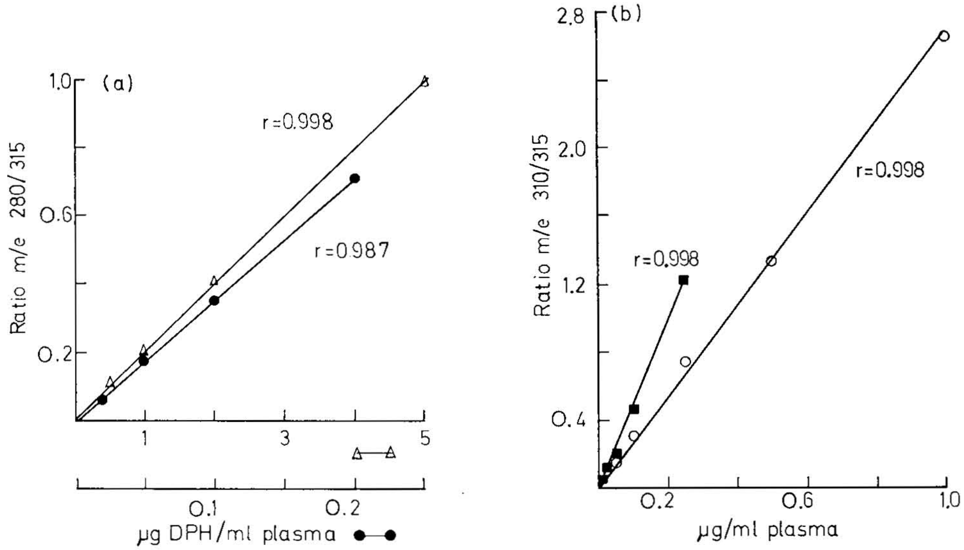

Fig. 5. (a) Standard curves obtained with DPH in human plasma at two concentration ranges. (b) Standard curves for 3-OH-DPH (■) and 4-OH-DPH (○) in human plasma. The internal standard was 4-OH-DPH-d_5, using the general procedure for mass fragmentography.

40 μg/ml are also linear. Fig. 5b shows the standard curve for 4-OH-DPH from 0.01 to 1 μg/ml in plasma and for 3-OH-DPH from 0.01 to 0.25 μg/ml in plasma. The lower limit for detection is about 0.01 μg/ml for DPH or its hydroxylated metabolites. Although the protocol outlines the procedure starting with 100 μl, plasma samples from newborns have been analyzed using 10 μl of capillary plasma[28].

TABLE I

COMPARISON OF MASS SPECTRA OF INTERNAL STANDARDS

4-OH-DPH-d_2		*4-OH-DPH-d_5*	
m/e	*% of base peak*	*m/e*	*% of base peak*
235*	100	233*	100
310	1.6	313	1.0
311	18.1	314	10.0
312	76.2	315	68.8
313	17.8	316	14.3
314	2.9	317	2.0

* Base peak.

Conjugated 4-OH-DPH and dihydrodiol-DPH

The concentration of conjugated 4-OH-DPH is calculated from measurements made after acid hydrolysis of the plasma sample. The present procedure does not measure dihydrodiol-DPH directly. However, with acid hydrolysis, the dihydrodiol metabolite of DPH forms equal amounts of 3-OH-DPH and 4-OH-DPH[16]. The measurement of 3-OH-DPH in the acid-hydrolyzed sample would then measure indirectly the dihydrodiol metabolite. In contrast to the dog, in man 3-OH-DPH does not appear to be a significant metabolite[3]. If 3-OH-DPH is assumed to be absent or an insignificant metabolite in man, then the above calculation should give an indirect measurement of the dihydrodiol metabolite. In support of this assumption, we have detected only small amounts (<20 ng/ml) of unconjugated 3-OH-DPH in the plasma of patients, except uremic patients, where we have observed concentrations of unconjugated 3-OH-DPH in plasma of 20–80 ng/ml. Further, the amount of 3-OH-DPH released during acid hydrolysis in most patients varied between 5–10% of the total 4-OH-DPH measured after acid hydrolysis. Thus, the following calculation was considered sufficiently accurate to determine conjugated 4-OH-DPH. Conjugated 4-OH-DPH was calculated by subtracting both the unconjugated 4-OH-DPH and the amount of 3-OH-DPH released by acid hydrolysis from the total 4-OH-DPH measured after acid hydrolysis. The quantitation of the dihydrodiol-DPH metabolite was calculated as twice the acid-hydrolyzed 3-OH-DPH. Assuming that dihydrodiol-DPH was not present but instead that 3-OH-DPH was conjugated, these calculations would give, at most, a 10% error in the value for conjugated 4-OH-DPH.

Internal standards

Originally, 4-OH-DPH-d_2 was used as the internal standard, but two problems were encountered. Firstly, the deuterium atoms were exchanged during the acid hydrolysis step used in the measurement of conjugated 4-OH-DPH, and thus the 4-OH-DPH-d_2 had to be added after hydrolysis. Secondly, small but significant variations in the day-to-day analysis of the same plasma were observed and occasionally duplicates would be markedly different and, although not evaluated, this effect was felt to be due to exchange of deuterium atoms during the handling of the samples.

As the internal standard 4-OH-DPH-d_5 can be added before acid hydrolysis because deuterium is not exchanged under these conditions, the variance noted in day-to-day runs or within a daily run were significantly decreased. The results in Table II show that a 2.4–6.4% variation was noted in six separate measurements on each of four plasmas using 4-OH-DPH-d_5, whereas the variation was 7.8–16.0% for 4-OH-DPH-d_2.

TABLE II

COMPARISON OF STANDARD DEVIATIONS WITH DIFFERENT INTERNAL STANDARDS

Results are means ± standard deviations for six determinations on plasma samples.

Patient	*4-OH-DPH-d_2 ($\mu g/ml$)*	*4-OH-DPH-d_5 ($\mu g/ml$)*
1	0.115 ± 0.009	$0.150 \pm 0.007^*$
2	0.319 ± 0.029	0.330 ± 0.021
3	0.165 ± 0.026	$0.135 \pm 0.005^*$
4	0.269 ± 0.043	$0.211 \pm 0.005^*$

* $p < 0.05$ compared with 4-OH-DPH-d_2 when analyzed by Student's *t*-test.

CONCLUSION

A sensitive and specific mass fragmentographic method has been developed for the detection of DPH, unconjugated 3-OH-DPH and 4-OH-DPH in 0.01–0.1-ml samples of plasma. The use of 4-OH-DPH-d_5 as an internal standard gives excellent reproducibility. After acidic hydrolysis of the original plasma sample, analysis by the method described permits the calculation of the concentration of conjugated 4-OH-DPH. In addition, the dihydrodiol-DPH metabolite can be determined indirectly from the 3-OH-DPH peak.

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REFERENCES

1 T. C. Butler, *J. Pharmacol. Exp. Ther.*, 119 (1957) 1.
2 E. W. Maynert, *J. Pharmacol. Exp. Ther.*, 130 (1960) 275.
3 A. I. Atkinson, J. MacGee, J. Strong, D. Garleiz and T. E. Gaffney, *Biochem. Pharmacol.*, 19 (1970) 2483.
4 K. Arnold and N. Gerber, *Clin. Pharmacol. Ther.*, 11 (1970) 121.
5 P. G. Dayton, S. A. Cucinell, M. Weiss and J. M. Perel, *J. Pharmacol. Exp. Ther.*, 158 (1967) 305.
6 H. Remmer, J. Hirschmann and I. Greiner, *Deut. Med. Wochenschr.*, 24 (1969) 1265.
7 N. Gerber and J. G. Wagner, *Res. Commun. Chem. Pathol. Pharmacol.*, 3 (1972) 455.
8 J. J. Ashley and G. Levy, *Res. Commun. Chem. Pathol. Pharmacol.*, 4 (1972) 297.
9 P. Borondy, T. Chang and A. J. Glazko, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 31 (1972) 582.
10 W. A. Dill, J. Baukema, T. Chang and A. J. Glazko, *Proc. Soc. Exp. Biol. Med.*, 137 (1971) 674.
11 T. Chang and A. J. Glazko, *J. Lab. Clin. Med.*, 75 (1970) 145.
12 A. Estas and P. A. Dumont, *J. Chromatogr.*, 82 (1973) 307.
13 K. S. Albert, M. R. Hallmark, M. E. Carroll and J. G. Wagner, *Res. Commun. Chem. Pathol. Pharmacol.*, 6 (1973) 845.
14 K. S. Albert, M. R. Hallmark, E. Sakmar, D. J. Weidler and J. G. Wagner, *Res. Commun. Chem. Pathol. Pharmacol.*, 9 (1974) 463.
15 A. Brändström and U. Junggren, *Acta Chem. Scand.*, 23 (1969) 2204.
16 T. Chang, A. Savory and A. J. Glazko, *Biochem. Biophys. Res. Commun.*, 38 (1970) 444.
17 H. R. Henze and A. F. Isbell, *J. Amer. Chem. Soc.*, 76 (1954) 4152.
18 B. Karlén, M. Garle, A. Rane, M. Gutova and G. Lindborg, *Eur. J. Clin. Pharmacol.*, 8 (1975) 359.
19 E. Brochmann-Hanssen and T. O. Oke, *J. Pharm. Sci.*, 58 (1969) 370.
20 R. Adams and L. H. Ulich, *J. Amer. Chem. Soc.*, 42 (1920) 599.
21 L. Gattermann, R. Ehrhardt and H. Maisch, *Chem. Ber.*, 23 (1890) 1204.
22 K. Tust and L. Gatterman, *Chem. Ber.*, 25 (1892) 3533.
23 H. Brötell, H. Ehrsson and O. Gyllenhaal, *J. Chromatogr.*, 78 (1973) 293.
24 H. Ehrsson, *Acta Pharm. Suecica*, 8 (1971) 113.
25 H. Ehrsson, *Anal. Chem.*, 46 (1974) 922.
26 H. Ehrsson and A. Tilly, *Anal. Lett.*, 6 (1973) 197.
27 M. Ervik and L. Gustavii, *Anal. Chem.*, 46 (1974) 39.
28 C. Hoppel, A. Rane and F. Sjöqvist, *Proceedings of a Symposium on Perinatal Pharmacology*, Raven Press, New York, in press.

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CHROM. 8660

GAS AND LIQUID CHROMATOGRAPHY ON SILOCHROM MODIFIED WITH A SURFACE FILM OF MELONE*

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SUMMARY

The chromatographic properties of the adsorbent obtained by polymerization of melamine previously adsorbed on the surface of a homogeneous macroporous adsorbent, Silochrom C-80, have been investigated. The product of polymerization is melone, which forms a film on the silica surface. The chromatographic properties of melone–Silochrom adsorbent were compared with the properties of pure Silochrom and the polymer Porapak-T. The melone–Silochrom adsorbent has a comparatively greater specificity. Melone film has an extremely high thermal stability (up to 600°) and is insoluble in common solvents. This makes melone–silica adsorbents suitable not only for gas chromatography but also for liquid chromatography. Examples of chromatographic separations on melone–Silochrom adsorbents of various mixtures (from gases to polynuclear aromatic compounds) are given.

INTRODUCTION

Of the silica adsorbents used in chromatography, airosilogels[1] (commercial name: Silochroms[2]) possess the most geometrical homogeneity. In particular, Silochrom C-80, with a high specific surface area ($s = 80$ m^2/g), has a narrow distribution of pore volumes, the mean effective pore diameter being *ca.* 500 Å. Consequently, Silochrom can be used not only as the adsorbent itself but also as an adsorbent–support for thin films of different organic substances. This permits us to obtain geometrically homogeneous adsorbents with chemically different surfaces. Silochrom has been used as an adsorbent–support of monolayers of molecules and macromolecules[3,4], and the chemical binding of polymer liquids with silica gel surfaces has also been described[5–8]. In this paper, a new adsorbent was studied, consisting of a melone polymer film synthesized from melamine directly on the surface of a macroporous adsorbent–support, Silochrom C-80. Melone is thermally stable up to 600°

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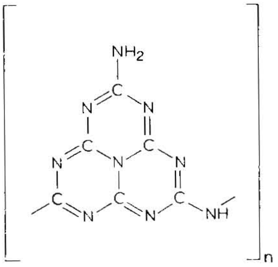
and is insoluble in water, acids and organic solvents. Therefore, adsorbents modified with a surface melone film can be used in both gas chromatography and liquid chromatography. Melone has the following structural unit:



It was assumed that a positive charge is localized on the central nitrogen atom[9]. Therefore, an adsorbent with a melone film on its surface is a specific adsorbent[10], but this specificity is influenced by interactions with the hydroxyl groups of the Silochrom surface.

EXPERIMENTAL

Melamine (20%, w/w, of Silochrom) is dissolved in boiling water and the required amount of Silochrom is added to the solution while vigorously stirring the mixture. Water is removed by evaporation, then the adsorbent is placed in a muffle furnace at 470° where the polymerization of adsorbed melamine into melone takes place immediately.

RESULTS AMD DISCUSSION

Fig. 1 shows that Silochrom with the melone surface film was more effective than pure Silochrom. For melone–Silochrom adsorbent, the curve is not only lower but at high u values it is flatter than the corresponding curve for pure Silochrom. This indicates that the mass exchange proceeds considerably more easily with the modified adsorbent. Probably, pores of smaller sizes being present to a small extent

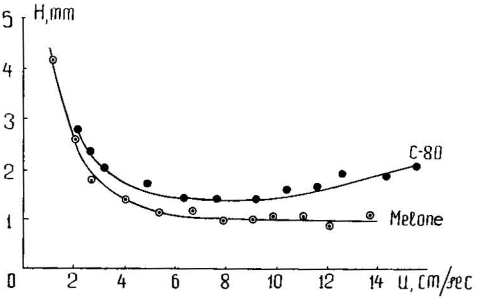


Fig. 1. Dependence of HETP (H) on gas flow-rate (u) for benzene at 150°. 1-m column with Silochrom C-80 covered with melone film, granule size 0.20–0.25 mm.

on the adsorbent–support were filled with melone and the adsorbent surface became geometrically more homogeneous. The decrease in specific surface area from 80 to 63 m^3/g supports this explanation.

In order to compare the chromatographic properties of melone–Silochrom adsorbent with those of pure Silochrom, the retention indices of a standard set of substances were measured and the relative polarity of the adsorbent was calculated in accordance with the Rohrschneider method[11]. It is clear from Table I that the relative polarity of the melone–Silochrom adsorbent is rather high, for benzene and ethanol being even higher than the relative polarity of pure Silochrom. Table II gives the values of retained volumes per unit surface area of melone–Silochrom adsorbent, $V_{A,1}$, at 150° for substances whose molecules belong to *A* and *B* groups in terms of their ability to undergo non-specific and specific intermolecular interactions, respectively[10].

TABLE I

KOVÁTS RETENTION INDICES AT 100° (I^{100}) OF SUBSTANCES ON SQUALANE, SILOCHROM C-80 AND MELONE FILM ON SILOCHROM C-80

Relative adsorbent polarity, $\Delta I = (I_{\text{adsorbent}} - I_{\text{squalane}})/100$.

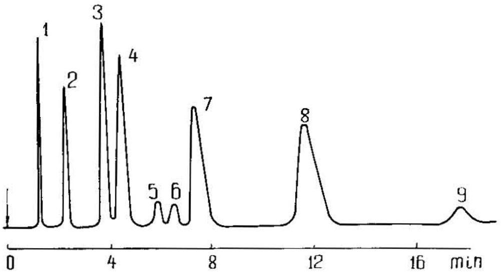
Substance	I^{100}			ΔI	
	Squalane	*Silochrom*	*Melone–Silochrom*	*Silochrom*	*Melone–Silochrom*
Benzene	649	663	680	0.14	0.31
Ethanol	384	918	948	5.34	5.64
Methyl ethyl ketone	531	1081	996	5.50	4.65
Nitromethane	457	780	797	3.23	3.40
Pyridine	695	1374	1158	6.79	4.63

TABLE II

RETENTION VOLUMES ($V_{A,1}$) PER UNIT SURFACE AREA OF SUBSTANCES AT 150° ON A COLUMN WITH MELONE FILM ON SILOCHROM C-80 ADSORBENT

Adsorbate	$V_{A,1}$ *(μm)*	*Adsorbate*	$V_{A,1}$ *(μm)*
n-Pentane	0.032	*n*-Propylbenzene*	0.690
n-Hexane	0.063	*n*-Butylbenzene*	1.425
n-Heptane	0.120	*o*-Xylene*	0.542
n-Octane	0.242	*m*-Xylene*	0.449
n-Nonane	0.411	*p*-Xylene*	0.457
n-Decane	0.792	Mesitylene*	0.887
Heptene-1*	0.157	Durene*	2.195
Cyclopentane	0.043	Acetonitrile*	0.284
Cyclohexene*	0.089	Thiophene*	0.104
Cyclohexane	0.066	Methyl propionate*	0.782
Benzene*	0.108	Methyl ethyl ketone*	0.938
Toluene*	0.241	Perfluorooctane	0.136
Ethylbenzene*	0.442	Perfluorobenzene*	0.120

* Belonging to *B* group (see text).



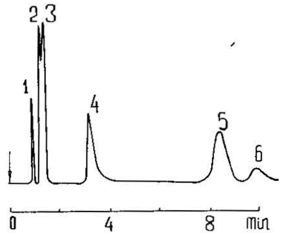


Fig. 2. Chromatogram of monoalkyl- and polymethylbenzenes on a 1.125-m column with melone–Silochrom adsorbent at 150°. Carrier gas: helium, flow-rate 24 ml/min. 1 = Benzene; 2 = toluene; 3 = ethylbenzene; 4 = *o*-xylene; 5 = isopropylbenzene; 6 = *n*-propylbenzene; 7 = mesitylene; 8 = *n*-butylbenzene; 9 = durene.

Fig. 3. Chromatogram of separation of different classes of substances with similar boiling points on a 1.125-m column with melone–Silochrom adsorbent at 150°. Carrier gas: helium, flow-rate 30 ml/min. 1 = Cyclohexane; 2 = thiophene; 3 = benzene; 4 = acetonitrile; 5 = methyl propionate; 6 = methyl ethyl ketone.

$V_{A,1}^{150^\circ}$ values for the molecules of these groups are not high and unsaturated compounds are retained more strongly than saturated compounds and fluorinated compounds more strongly than non-fluorinated compounds. These values illustrate the considerable contribution of specific interactions to the total energy of the adsorbate–melone–Silochrom interaction.

Fig. 2 shows the chromatogram of polymethylbenzenes and monoalkylbenzenes. Monoalkylbenzenes are retained much more weakly than the polymethylbenzenes, as occurs also on graphitized carbon black[12].

When separating mixtures of different classes of substances that have similar boiling points (Fig. 3), substances with an ether-oxygen and especially those with keto-groups are retained more strongly.

Table III gives a comparison of the differential molar changes of internal energy (heats of adsorption), $-\Delta U_1 = q_{V,1}$ (ref. 13), at small (zero) sample size (surface coverage) for several inorganic and hydrocarbon gases on the melone–Silochrom adsorbent and on Porapak-T. The results obtained show that the chromatographic properties of the melone–Silochrom adsorbent have considerable advantages over those of Porapak-T, *i.e.*, at substantially lower total energy values of adsorbate–adsorbent intermolecular interaction, stronger specific interactions take place with melone–Silochrom.

Melone–Silochrom adsorbent can be recommended for use in the separation and analysis of *A* and *B* group substances. Figs. 4 and 5 give the examples of chromatograms. Fig. 4 shows the chromatogram for the separation of C_1–C_4 hydrocarbons, including isomers. Fig. 5 shows the chromatogram of halothane, a new narcotic substance used in medicine. The percentage of impurity in it should not exceed 0.01 %. The asymmetric isomer of halothane is the most difficult to separate and to determine. The isomers and other impurities were well separated on a 2.5-m column with melone–Silochrom adsorbent at 80°.

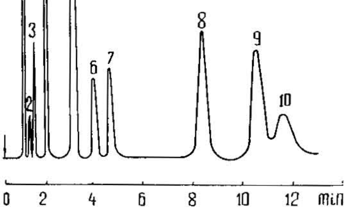
In principle, it is possible to produce melone–Silochrom adsorbents with specific surface areas from several to several hundred square metres per gram, because their specific surface areas are largely defined by the adsorbent–support surface,

TABLE III

ΔU_1 VALUES FOR SOME INORGANIC AND HYDROCARBON GASES ON MELONE FILM ON SILOCHROM C-80 AND PORAPAK-T

Adsorbate	*ΔU_1 (kJ/mole)*	
	Melone (21%, w/w) on Silochrom C-80 (s = 63 m^2/g; d = 500 Å)	*Porapak-T ($s \approx$ 250 m^2/g; $d \approx$ 90 Å)*
CO_2	23.1	25.1
H_2S	20.6	28.4
SO_2	27.6	38.0
N_2O	20.8	24.3
CH_4	9.6	17.7
C_2H_2	22.9	30.5
C_2H_4	16.7	24.7
C_2H_6	13.6	25.1
C_3H_6	25.2	32.6
C_3H_8	22.3	32.2
n-C_4H_8	31.5	36.4
i-C_4H_8	31.8	37.6
n-C_4H_{10}	28.8	36.4
i-C_4H_{10}	27.7	34.7

which is easy to change over a large range. Owing to their thermal stability up to 600° they can be used for the separation of the substances that have *A* and *B* group molecules, and from gases to high-boiling liquids and solids. Figs. 6 and 7 give examples of applications of melone–Silochrom adsorbent at high column temperatures.



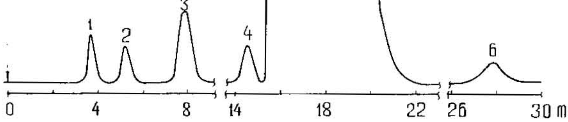
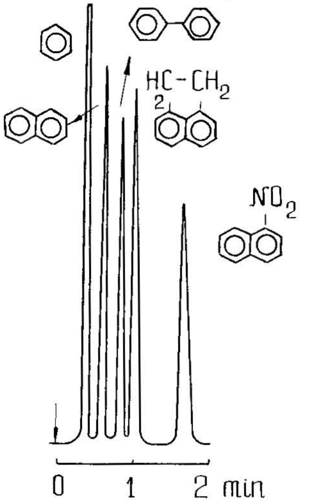
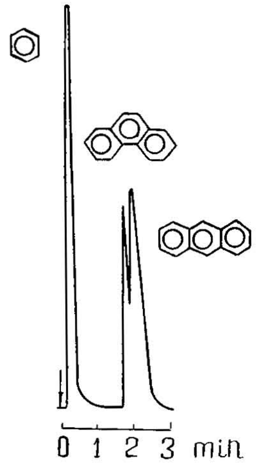


Fig. 4. Chromatogram of C_1–C_4 hydrocarbon gases on a 2-m column with melone–Silochrom adsorbent at 25°. Carrier gas: helium, flow-rate 35 ml/min. 1 = methane; 2 = ethane; 3 = ethylene; 4 = propane; 5 = propylene; 6 = isobutane; 7 = *n*-butane; 8 = isobutene; 9 = butene-1 + *trans*-butene-2; 10 = *cis*-butene-2.

Fig. 5. Chromatogram of halothane and its impurities on a 2.5-m column with melone–Silochrom adsorbent at 80°. Carrier gas: nitrogen, flow-rate 16 ml/min. 1 = 1,2,2-Trifluoro-1,1,2-trichloroethane; 2 = 1,2-dibromotetrafluoroethane; 3 = chlorallylene (internal standard); 4 = 1,1,2-trifluoro-2-chloro-1-bromoethane; 5 = 1,1,1-trifluoro-2-chloro-2-bromoethane (halothane); 6 = 1,1,1-trifluoro-1,2-dibromochloroethane.





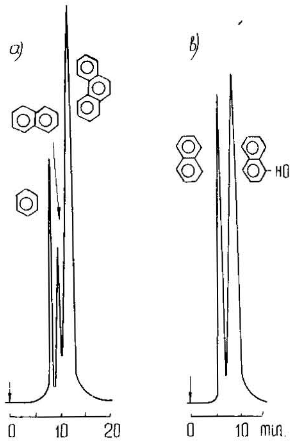


Fig. 6. Chromatogram of separation of benzene, naphthalene, diphenyl, acenaphthene and α-nitronaphthalene on a 1.125-m column with melone–Silochrom adsorbent at 320°. Carrier gas: helium, flow-rate 45 ml/min.

Fig. 7. Chromatogram of phenanthrene and anthracene on a 1.125-m column with melone–Silochrom adsorbent at 340°. Carrier gas: helium, flow-rate 45 cm/min.

Fig. 8. Example of application of melone–Silochrom adsorbent in liquid chromatography. Column, 0.5 m; temperature, 25°. (a) *n*-Hexane eluent, flow-rate 0.5 ml/min; (b) chloroform eluent, flow-rate 0.5 ml/min.

Fig. 8 shows that the melone–Silochrom adsorbent is also effective in liquid chromatography.

REFERENCES

1 N. K. Bebris, A. V. Kiselev and Yu. S. Nikitin, *Kolloidn. Zh.*, 29 (1967) 326.
2 N. K. Bebris, G. E. Zajceva, A. V. Kiselev, V. Ya. Mokeev, Yu. S. Nikitin and Ya. I. Yashin, *Chromatographia*, 3 (1971) 93.
3 A. V. Kiselev, N. V. Kovaleva and Yu. S. Nikitin, *J. Chromatogr.*, 58 (1971) 19.
4 E. N. Fuller, *Anal. Chem.*, 44 (1972) 1747.
5 J. N. Little, W. A. Dark, P. W. Farlinger and K. J. Bombaugh, *J. Chromatogr. Sci.*, 8 (1970) 647.
6 J. J. Kirkland and J. J. de Stefano, *J. Chromatogr. Sci.*, 8 (1970) 309.
7 J. Sebestian and J. Halash, *Chromatographia*, 7 (1975) 371.
8 J. J. de Stefano and J. J. Kirkland, *J. Chromatogr. Sci.*, 12 (1974) 337.
9 A. I. Finkelshtein and N. V. Spiridonova, *Usp. Khim.*, 22 (1964) 900.
10 A. V. Kiselev and Ya. I. Yashin, *Gas Adsorption Chromatography*, Plenum Press, New York, 1969.
11 L. Rohrschneider, *J. Chromatogr.*, 22 (1966) 6.
12 A. V. Kiselev, E. V. Kalashnikova, K. D. Shcherbakova and I. S. Zamanskaja, *Chromatographia*, 5 (1972) 278.
13 N. N. Avgul, A. V. Kiselev and D. P. Poshkus, *Adsorbcija Gasov i Parov na Odnorodnikh Poverkhnostjakh (Gas and Vapour Adsorption on Homogeneous Surfaces)*, Khimiya, Moscow, 1975.

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CHROM. 8656

DETERMINATION OF TRIMETHYLSILYL METHYLATED NUCLEIC ACID BASES IN URINE BY GAS–LIQUID CHROMATOGRAPHY*

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SUMMARY

A method for the determination of the urinary excretion level of methylated nucleic acid bases by gas–liquid chromatography (GLC) has been developed. A cleanup procedure prior to GLC analysis consisted of hydrolysis, filtration, charcoal adsorption, and anion exchange. Studies to determine optimum derivatization conditions for conversion of the methylated bases to their trimethylsilyl derivatives and to evaluate GLC parameters and columns to obtain the best separation were conducted. Application of the method was shown by determining the excretion levels of methylated bases in urine of normal controls and of patients with various types of malignancy.

INTRODUCTION

Biomedical interest in the methylated nucleic acid bases has been stimulated by reports of elevated levels of the methylated bases in malignant tumors[1–9], compared with normal control tissues[1,2,4,5,10]. These methylated bases, which are found as minor constituents of transfer ribonucleic acid (tRNA)[11–15] and to a lesser extent in ribosomal RNA[16], result from the modification of tRNA by specific tRNA methylases at the enzymatic level[17–20]. Abnormally high levels of tRNA methylase activity has been noted by Borek[12] in studies of thirty different malignant tumors compared with their corresponding normal tissues.

Although methylated nucleic acid bases have been shown to be normal excre-

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tion products in human urine as a result of the metabolic degradation of tRNA[15], Borek[12] noted elevated excretion levels for methylated bases by patients with malignant tumors. Weissmann *et al.*[9], Adams *et al.*[1], Fink *et al.*[4,5], and Park *et al.*[10], have shown that increased methylated base excretion occurs in patients with acute leukemia.

To establish if a relationship between the levels of methylated bases in urine and neoplastic growth exists and to determine if the measurement of these compounds might serve as either a diagnostic method or for monitoring the effects of treatments, an accurate, sensitive, and convenient method of analysis is necessary. Methods for the analysis of methylated bases in urine have been developed by Weissmann *et al.*[21], Adams *et al.*[1], and by Fink *et al.*[5] and consist of ion-exchange chromatography for sample cleanup, paper chromatography for separation, and ultraviolet (UV) spectroscopy for detection. A number of methylated bases in normal and cancer-patient urine have been detected and semi-quantitatively analyzed using these methods; however, these procedures lacked specificity and sensitivity, and were too time consuming for extensive clinical studies. High-pressure anion-exchange chromatography has been used by Waalkes *et al.*[22] for the analysis of minor nucleosides and bases in urine and gave quantitative results, but the method does not lend itself to the frequent rapid analyses for clinical studies.

Gas–liquid chromatographic (GLC) methods for the analysis of the major nucleic acid bases have been reported by Lakings and Gehrke[23,24], and the analysis of minor nucleosides by GLC has been developed by Chang *et al.*[25]. GLC, because of its sensitivity, specificity, and speed of analysis, presents a quantitative analytical tool with the ability of rapid, frequent analyses necessary for a study of the excretion levels of the methylated bases in urine. This investigation describes the development of such a procedure.

EXPERIMENTAL

Apparatus

A Model 402 biochemical gas chromatograph (F & M, Division of Hewlett-Packard, Avondale, Pa., U.S.A.) equipped with dual electrometers, dual hydrogen flame detectors, and an Electronix 16 dual pen strip chart recorder was used in this study.

For elevated temperature reactions, a sand-bath with a variable temperature control ($\pm 2^\circ$) was used.

Standard calibration solutions of the methylated bases, prepared from stock solutions, and samples were dried on a hot plate (75°) under a stream of pure nitrogen gas.

Glass ion-exchange columns (Fisher and Porter, Warminster, Pa., U.S.A.) used for sample cleanup were 5 × 150 mm with a 50-ml reservoir, 9 × 150 mm, and 15 × 150 mm.

Reagents

The methylated purine and pyrimidine bases (MeB) were obtained from Cyclo (Los Angeles, Calif., U.S.A.): 1-methyladenine (1-MeAde), 2-methyladenine (2-

MeAde), 1-methylhypoxanthine (1-MeHypo), N^2,N^2-dimethylguanine (N^2,N^2-DiMeGua), N^2-methylguanine (N^2-MeGua), 1-methylguanine (1-MeGua), and 7-methylguanine (7-MeGua); from Mann Research Labs. (New York, N.Y., U.S.A.): thymine (Thy), 5-methylcytosine (5-MeCyt), N^6,N^6-dimethyladenine (N^6,N^6-DiMeAde), and N^6-methyladenine (N^6-MeAde); and from Sigma (St. Louis, Mo., U.S.A.): N^6-(Δ^2-isopentenyl)adenine (N^6-Δ^2-IsoAde). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Regis (Chicago, Ill., U.S.A.) in PTFE-lined screw-cap vials. Acetonitrile and dichloroethane were of "nanograde" purity and were purchased from Mallinckrodt (St. Louis, Mo., U.S.A.) as were formic acid, acetic acid, and pyridine. The charcoal (80–100 mesh, Cat. No. 926043) was obtained from Regis. The AG 1 X 2 anion-exchange resin was purchased from Bio-Rad Labs. (Richmond, Calif., U.S.A.) The water was prepared by passing distilled water through a water demineralizer (Ion Exchange Products, Chicago, Ill., U.S.A.). The other reagents and materials used were of the highest purity available.

Chromatographic conditions

The chromatographic column and instrumental settings described earlier[26] were used in the initial part of this study. Also, the optimum silylating conditions and solvent developed for the major bases[26] were used. Initial studies showed that the methylated bases were silylated by BSTFA and were chromatographically stable. However, some MeB gave multiple peaks with the BSTFA–acetonitrile mixture, and complete separation was not obtained on the 1.0 m × 4 mm I.D. column (10% SE-30 on Supelcoport, 100–120 mesh).

To determine the optimum silylating conditions for the MeB that would give single reproducible peaks, an evaluation of acetonitrile, dichloroethane, hexane, and benzene as the silylation solvent at temperatures of 100° and 150° and for 15 and 30 min was conducted. To obtain the best separation of the MeB, the following columns were studied: 3, 5, and 10% SE-30; 3 and 5% OV-3; and 3 and 5% OV-7 on 100–120 mesh Supelcoport.

Cleanup studies

Previous reports[1,10] have shown that the excretion level of methylated bases in urine was quite low, *i.e.* 1–10 mg per 24-h urine volume. Because of this low excretion level and the presence of many urinary compounds that interfered with the GLC analysis of the MeB, a cleanup step was required. Many methods for sample cleanup were available and included liquid–liquid extraction, column chromatography, anion and cation exchange, and adsorption chromatography. Each of these methods was evaluated independently and in combination with the other procedures to develop a total cleanup method for the analysis of the MeB in urine that gave good recovery results and yet removed most of the interfering compounds from the urine sample.

Analysis of normal and cancer-patient urine

Normal 24-h urine specimens collected without preservative but refrigerated at all times were obtained from laboratory personnel and from the National Cancer Institute, NIH. Cancer-patient 24-h urine specimens were obtained from the National Cancer Institute, NIH. Aliquot samples were frozen and stored at −20° or lower. The normal and cancer subjects were maintained on a regular diet with restricted protein.

Beverages containing caffeine or theophylline were excluded the day before and during the collection period.

Calculation of the excretion levels of the methylated bases were as follows:

$$\mu\text{g MeB per aliquot} = \frac{H_{\text{MeB}}}{H_{\text{I.S.}}} \times \frac{\mu\text{g I.S.}}{RWR_{\text{MeB/I.S.}}}$$

$$RWR_{\text{MeB/I.S.}} = \frac{H_{\text{MeB}}}{H_{\text{I.S.}}} \times \frac{\mu\text{g I.S.}}{\mu\text{g MeB}}$$

where H_{MeB} and $H_{\text{I.S.}}$ = height of peak of methylated base and internal standard, respectively, and $RWR_{\text{MeB/I.S.}}$ = relative weight response of the methylated base to an internal standard.

To calculate the mg per 24-h urine volume excretion level of the methylated bases:

$$\text{mg MeB per 24-h urine} = \frac{\mu\text{g MeB per aliquot}}{\text{ml aliquot}} \times \frac{\text{24-h urine volume (ml)}}{1000\ (\mu\text{g/mg})}$$

The recovery of the MeB added to urine was determined by analyzing an aliquot of urine and calculating the milligram amount per 24-h urine of each methylated base. Another aliquot of the same sample was then "spiked" with a known amount of the MeB and analyzed.

RESULTS AND DISCUSSION

Chromatographic properties of the methylated bases

The determination of the optimum reaction conditions for converting the MeB to their volatile trimethylsilyl (TMS) derivatives consisted of experiments on silylation solvents and various reaction times and temperatures. Of the solvents evaluated, only dichloroethane and acetonitrile gave good symmetrical peaks for most of the MeB when silylated with BSTFA at 150° for 15 min. Double peaks for some MeB were obtained with acetonitrile as solvent; and dichloroethane, which gave single peaks for all the bases except 5-MeCyt, showed incomplete silylation. One base, 1-MeHypo, did not give a good chromatographic peak under any of the experimental conditions and was therefore not included in further studies. However, dichloroethane–acetonitrile (1:1) as solvent gave good single peaks for all the MeB except 5-MeCyt, which was a doublet.

The reproducibility of silylation and stability of the derivatives were determined by analyzing standard mixtures of the MeB at the optimum silylating conditions (BSTFA–acetonitrile–dichloroethane mixture, 2:1:1, v/v, heated in a closed tube at 150° for 15 min). Four independent samples were used to determine the reproducibility; the average relative molar response ($RMR_{\text{MeB/I.S.}}$), standard deviation (S.D.), and retention times are presented in Table I. One of the samples used in the reproducibility study was analyzed on the next three days to determine the stability of the derivatives, and these data are presented in Table II.

TABLE I

REPRODUCIBILITY OF DERIVATIZATION AND CHROMATOGRAPHY OF THE TMS METHYLATED BASES

Detector flame ionization detector. Silylation with BSTFA in dichloroethane–acetonitrile (2:1:1, v/v) at 150° for 15 min. Retention times on a 10% SE-30 on Supelcoport (100–120 mesh) column; initial temperature, 90°; programmed at a rate of 7.5°/min. $RMR_{MeB/I.S.}$: four independent determinations, relative molar response to acenaphthene as internal standard; $RMR_{MeB/I.S.}$ = [area MeB/moles MeB]/[area I.S./moles I.S.]. Standard deviation calculated from four independent determinations; average relative S.D. — 3.8%.

Compound	*Retention time*	$RMR_{MeB/I.S.}$	*S.D.*
N^6-MeAde	13.2	0.58	0.024
N^6N^6-DiMeAde	13.0	0.64	0.029
N^6-Δ^2-IsoAde	18.4	0.86	0.025
2-MeAde	16.4	0.57	0.008
N^2-MeGua	17.2	0.58	0.017
N^2,N^2-DiMeGua	18.0	0.83	0.020
7-MeGua	19.6	0.39	0.032
5-MeCyt	9.6–12.0	0.73*	0.024
Thy	6.8	0.63	0.025

* Two peaks were obtained for 5-MeCyt, each was integrated independently then added to obtain total area for *RMR* calculation.

In a later study on the optimum silylation of the five MeB (N^6,N^6-DiMeAde, N^6-MeAde, N^2-MeGua, N^2,N^2-DiMeGua, and 7-MeGua), it was found that the BSTFA–acetonitrile (1:1) mixture heated in a closed vial at 150° for 15 min gave single, reproducible peaks. The $RMR_{MeB/I.S.}$ values for these five bases were the same as shown in Table I. Further, the greater solubility of the MeB and the ease of handling a single solvent made acetonitrile the solvent of choice.

Once reproducible chromatographic peaks had been obtained for the TMS MeB, experiments were conducted to obtain a GLC column that would separate these

TABLE II

STABILITY OF TMS METHYLATED BASES

Samples used in reproducibility study. Detector, flame ionization detector. Samples silylated with BSTFA in dichloroethane–acetonitrile (2:1:1, v/v) at 150° for 15 min. $RMR_{MeB/I.S.}$ = relative molar response to acenaphthene as internal standard; $RMR_{MeB/I.S.}$ = [area MeB/moles MeB]/[area I.S./moles I.S.]. Silylated samples left at room temperature in a closed reaction vial between injections.

Compound	$RMR_{MeB/I.S.}$			
	After 1 day	*After 2 days*	*After 3 days*	*After 4 days*
N^6-MeAde	0.56	0.57	0.59	0.56
N^6N^6-DiMeAde	0.67	0.65	0.65	0.66
N^6-Δ^2-IsoAde	0.90	0.89	0.85	0.87
2-MeAde	0.56	0.57	0.59	0.60
N^2-MeGua	0.59	0.58	0.56	0.58
N^2,N^2-DiMeGua	0.84	0.84	0.81	0.78
7-MeGua	0.43	0.39	0.43	0.41
5-MeCyt	0.73	0.71	0.75	0.70
Thy	0.60	0.62	0.64	0.62

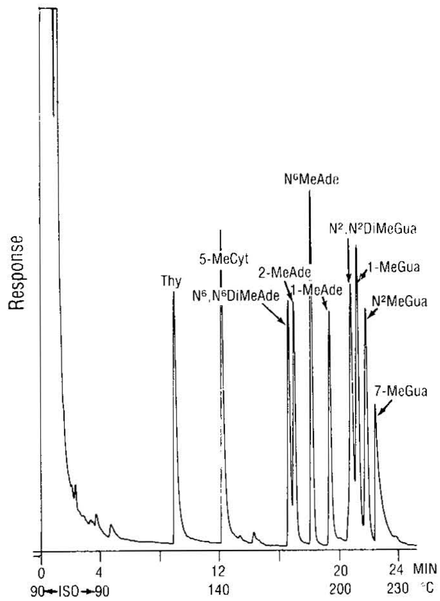

Fig. 1. GLC analysis of methylated bases in a standard mixture. Column: 5% OV-3 on 100–120 mesh Supelcoport, 1.0 m × 4 mm I.D. Sample: 100 μg of each MeB. Silylation with 200 μl BSTFA and 200 μl acetonitrile at 150° for 15 min. Chromatographic conditions: volume injected, 4 μl; initial temperature, 90°; initial hold, 4 min; program rate, 7.5°/min; final temperature, 260°; attenuation, 1.3×10^{-9} A.f.s.

compounds. A baseline separation of all the commercially available MeB was not considered essential in the early stages of this research as the MeB composition of urine had not previously been well defined. Of the three liquid phases investigated, only OV-3 gave separation of all the MeB, but not baseline separation. The best chromatograms were obtained with a 1.0 m × 4 mm I.D. glass column packed with 5% OV-3 on 100–120 mesh Supelcoport. A typical chromatogram of ten MeB derivatives is shown in Fig. 1.

Cleanup method development

Each of the cleanup methods evaluated, including liquid–liquid extraction, column chromatography, cation and anion exchange and adsorption chromatography, provided partial cleanup of urine for methylated base analysis. Liquid–liquid extractions removed many urinary compounds from the MeB, but urinary salts remained in the aqueous phase with the MeB. No organic solvents were found that would extract the MeB quantitatively from the urine samples. The MeB were not adsorbed by any of the column chromatographic materials evaluated and were thus separated from urinary compounds that were retained by the adsorbent. However, urinary salts also were not adsorbed and thus not separated from the MeB. Cation- and anion-exchange chromatography (CIE and AIE, respectively) did retain the MeB under proper conditions, but the high level of salts present in urine prevented the use of CIE and AIE as an initial cleanup step. Adsorption chromatography proved to be the most powerful cleanup method for MeB analysis in urine. The MeB were strongly adsorbed to charcoal, and the urinary salts and many organic compounds were removed from the sample by washing the charcoal with dilute acid or base and organic solvents. The cleanup method which provided the best sample cleanup with the lowest loss of MeB is presented below.

Cleanup method

Hydrolysis. (1) Place 4.0 ml of urine in a 16 × 75 mm Pyrex culture tube and make to 1 *N* in HCl by adding 1.0 ml of 5 *N* HCl. (2) Seal the tube tightly with a PTFE-lined screw cap and heat at 110° for 4.0 h. (3) Cool the sample and add 1.0 ml of 4.0 *N* KOH to partially neutralize the excess HCl.

Filtration. (1) Place the hydrolyzed sample in a 10-ml syringe with a 0.5-μm Solvinert filter (Millipore, Bedford, Mass., U.S.A.) and pass the sample slowly through the filter which retains the particulate matter present after sample hydrolysis. (2) Wash the hydrolysis tube and syringe with two 1-ml washes of 0.1 *N* HCl and add the washes to the previous filtrate.

Charcoal adsorption cleanup. (1) Place the filtered sample directly on a 70 × 5 mm charcoal column (80–100 mesh) and pass it through the column at a flow-rate (*ca.* 0.2 ml/min) slow enough to allow complete adsorption of the methylated bases. (2) Wash the charcoal column with 60 ml of de-ionized water, 30 ml of pyridine–95% ethanol (1:1), 50 ml of de-ionized water, and 25 ml of acetic acid at a flow-rate of *ca.* 1.0 ml/min. (3) Elute the methylated bases from the charcoal with 40 ml of formic acid–acetic acid (1:1) at a flow-rate of *ca.* 0.5 ml/min. (4) Dry the eluate on a hot plate (75°) under a stream of pure nitrogen gas.

Anion-exchange cleanup. (1) Dissolve the dried sample from the charcoal column in 2.0 ml of 0.1 *N* KOH and place it on a 70 × 9 mm AG 1 X 2 anion-exchange resin (acetate form) at a flow-rate of *ca.* 0.2 ml/min. (2) Wash the sample container with two 2-ml rinses of de-ionized water and place them on the resin after the sample is completely on the resin. (3) Wash the column with 100 ml of de-ionized water at a flow-rate of *ca.* 1.0 ml/min. (4) Elute the methylated bases from the resin with 40 ml of 0.02 *N* acetic acid at a flow-rate of *ca.* 0.5 ml/min and collect the eluate in a 50-ml beaker. (5) Add the internal standard (uracil) in dilute acetic acid (*ca.* 50 μg/1.0 ml) to the eluate.

Sample drying and silylation. (1) Concentrate the eluate from the AIE column to approximately 2.0 ml on a hot plate (75°) under a stream of pure nitrogen gas, then transfer it to a 4.5-ml culture tube with a PTFE-lined screw cap. (2) Rinse the beaker with two 1-ml portions of 0.02 *N* acetic acid and add to the culture tube. (3) Evaporate the sample to dryness on a hot plate (75°) under a stream of pure nitrogen. (4) To ensure complete dryness, add *ca.* 1 ml of dichloromethane and evaporate to azeotropically remove any remaining traces of water. (5) Silylate the dried sample with 0.2 ml of BSTFA and 0.2 ml of acetonitrile heated in a closed tube at 150° for 15 min. (6) Analyze the silylated sample on a 1.0 m × 4 mm I.D. 5% OV-3 on 100–120 mesh Supelcoport column.

An initial hydrolysis of the urine was found to be necessary to hydrolyze methylated nucleosides present in urine to the free bases and to breakdown many large molecules which allowed for easier sample cleanup. Filtration of the hydrolyzed urine prior to placing the sample on the charcoal column prevented the particulate matter from plugging the charcoal column and interfering with the cleanup method.

Analysis of normal and cancer-patient urine

Previous reports using ion-exchange chromatography, paper chromatography, and UV spectroscopy have shown that MeB are excreted in normal and in cancer-patient urine. Weissmann *et al.*[9] have reported a number of methylated purines in normal urine including 1-MeHypo at 0.4 mg per 24 h, 7-MeGua at 6.5 mg per 24 h, 8-OH-7-MeGua at 1.6 mg per 24 h, and N^2-MeGua at 0.5 mg per 24 h. Park *et al.*[10] reported MeB excretion levels for normal humans: 7-MeGua at 5.4 mg per 24 h, 8-OH-7-MeGua at 1.0 mg per 24 h, and N^2-MeGua at 0.4 mg per 24 h; and for leukemia patients: 7-MeGua at 8 mg per 24 h, 8-OH-7-MeGua at 4 mg per 24 h, and N^2-MeGua in quantities too small for quantitation. These data indicate an increase in the average excretion of MeB by leukemia patients as compared to normal humans.

Fink *et al.*[4,5] were able to detect 1-MeAde, N^6-MeAde, and various methylated nucleosides using a procedure similar to that of Weissmann. Also, Fink reported that the methylated nucleosides were excreted at higher levels than the free bases. Mirvish *et al.*[27] also found MeB in normal human urine at levels similar to those reported earlier. A study by Heirwegh *et al.*[6], and a later study by Waalkes *et al.*[28] showed that caffeinated beverages and high-purine diets did not affect the excretion of the MeB, but did increase total purine excretion.

The methylated base excretion levels of normal humans were determined by analyzing ten normal urines by the cleanup and GLC method outlined earlier. The excretion values obtained for the five bases detected (N^6,N^6-DiMeAde, N^6-MeAde, N^2N^2-DiMeGua, N^2-MeGua, and 7-MeGua) are presented in Table III. The average excretion and standard deviation of each base for the ten normals were calculated and ranged from 5.8 ± 2.4 mg per 24 h for N^6-MeAde to 1.1 ± 1.0 mg per 24 h for N^2-MeGua. These normal excretion values agree quite well with earlier reported values for these bases. A typical chromatogram for a normal urine sample is shown in Fig. 2.

TABLE III

ANALYSIS OF METHYLATED BASES IN NORMAL HUMAN URINE

mg MeB per 24-h urine = mg MeB per ml urine × 24-h urine volume (ml); mg MeB per ml urine = [area MeB × mg I.S.]/[area I.S. × $RWR_{MeB/I.S.}$]; $RWR_{MeB/I.S.}$ = [area MeB × mg I.S.]/[area I.S. × mg MeB].

Sample	*mg Methylated base per 24-h urine volume (ml)*				
	N^6,N^6-DiMeAde	*N^6-MeAde*	*N^2,N^2-DiMeGua*	*N^2-MeGua*	*7-MeGua*
1	trace	4.3	1.0	trace	5.4
2	1.4	2.8	0.5	trace	2.4
3	3.5	5.8	0.8	trace	3.1
4	3.5	5.8	0.8	3.5	7.1
5	trace	6.2	1.6	trace	6.1
6	2.1	5.6	impurity	trace	1.4
7	4.2	7.7	impurity	2.3	3.7
8	1.8	4.7	impurity	trace	3.7
9	4.4	10.6	2.0	3.3	7.7
10	3.5	5.8	1.2	1.1	5.2
Average ± S.D.	2.3 ± 1.0	5.8 ± 2.4	1.2 ± 0.5	1.1 ± 1.0	5.2 ± 2.1
Range	trace–4.4	2.8–10.6	0.5–2.0	trace–3.5	1.4–7.7

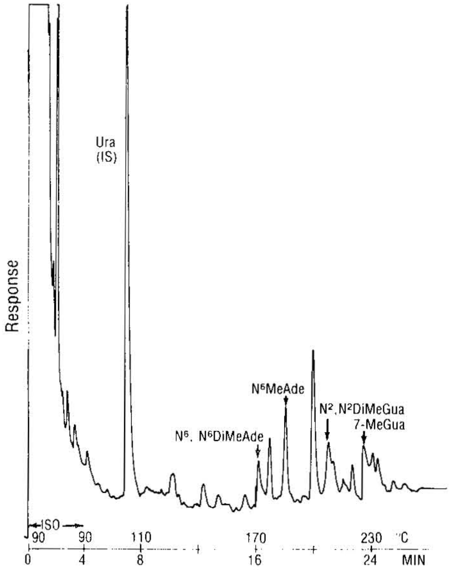

Fig. 2. GLC analysis of methylated bases in normal human urine. Sample: 4.0 ml. Attenuation: 6.4×10^{-10} A.f.s. Cleanup by hydrolysis, filtration, charcoal, and AIE. For further conditions, see the legend to Fig. 1.

To determine the excretion level of the methylated bases from cancer patients, fourteen cancer-patient urines were analyzed. Five different types of cancer were evaluated: three colon cancer, three breast cancer, three melanoma, three lung cancer, one kidney cancer, and one rectal cancer. The MeB excretion levels of the cancer patients were generally higher than the levels excreted by normal humans. The

TABLE IV

ANALYSIS OF METHYLATED BASES IN CANCER-PATIENT HUMAN URINE

Calculation of mg MeB per 24-h urine volume: see Table III.

Sample		*mg Methylated base per 24-h urine volume (ml)*				
		N^6,N^6-DiMeAde	*N^6-MeAde*	*N^2,N^2-DiMeGua*	*N^2-MeGua*	*7-MeGua*
Colon cancer	1	—	5.8	3.4	—	5.7
	2	5.9	10.7	3.7	5.2	12.9
	3	5.7	5.1	4.5	2.5	10.2
Breast cancer	1	-	5.0	4.0	—	4.9
	2	2.0	2.4	0.7	1.3	1.2
	3	9.6	6.6	4.3	2.5	8.8
Melanoma	1		14.8	5.2	—	9.8
	2	4.5	12.1	4.1	3.1	18.6
	3	5.5	5.3	1.0	1.0	9.5
Lung cancer	1			trace	—	trace
	2	1.4	2.3	1.6	1.7	6.2
	3	8.4	31.4	23.3	trace	9.1
Kidney cancer	1		24.6	3.1	—	—
Rectal cancer	1	—	10.0	7.4	—	6.2

TABLE V

COMPARISON OF EXCRETION LEVELS OF METHYLATED BASES FROM CANCER PATIENTS AND NORMAL SUBJECTS

mg MeB per 24-h urine = mg MeB per ml urine × 24-h volume urine (ml); mg MeB per ml urine = area MeB/area I.S. × mg I.S./$RWR_{MeB/I.S.}$.

Compound	*Average and range of mg MeB per 24-h urine excreted*	
	*Cancer patients**	*Normals***
N^6,N^6-DiMeAde	3.1 (trace- 9.6)	2.3 (trace- 4.4)
N^6-MeAde	9.7 (trace-31.4)	5.8 (2.8-10.6)
N^2,N^2-DiMeGua	4.7 (trace-23.3)	1.2 (0.5- 2.0)
N^2-MeGua	1.2 (trace- 5.2)	1.1 (trace- 3.5)
7-MeGua	7.4 (trace-18.6)	5.2 (1.4- 7.7)
Average total MeB	5.2	3.1

* Average for 14 different cancer patients.
** Average for 10 different normals.

amounts of MeB per 24-h urine volume for the cancer patients are presented in Table IV. Table V gives a comparison of the average amount of MeB excreted by cancer patients and normal humans. Chromatograms from a colon cancer- and melanoma-patient urine are shown in Figs. 3 and 4. The peak eluting between N^6,N^6-DiMeAde and N^6-MeAde has the same retention temperature as adenine, and guanine elutes with the same retention temperature as the peak between N^2,N^2-DiMeGua and N^2-MeGua.

The levels of methylated bases observed in normal and the increased amounts

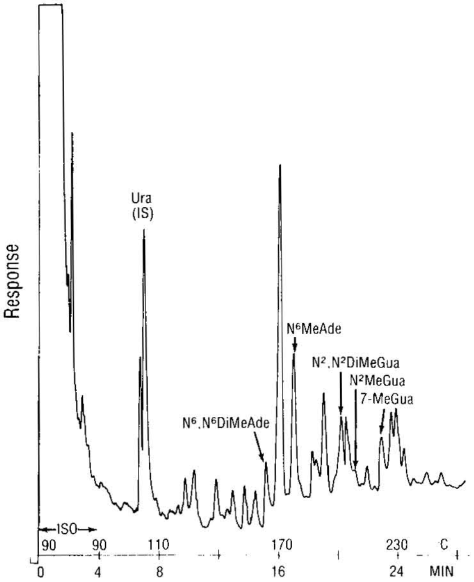

Fig. 3. GLC analysis of methylated bases in urine of a colon-cancer patient. Sample: 4.0 ml. Cleanup by hydrolysis, filtration, charcoal and AIE. For further conditions, see the legend to Fig. 1.

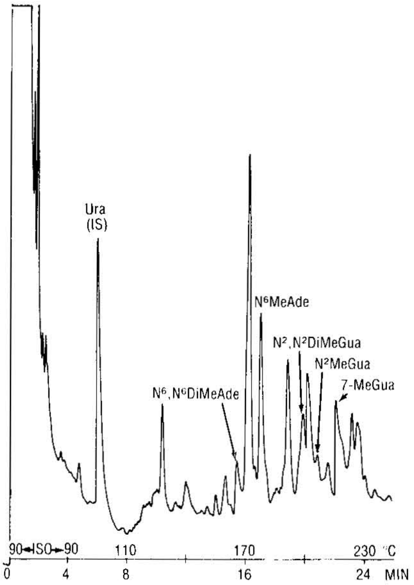

Fig. 4. GLC analysis of methylated bases in urine of a melanoma patient. Sample: 4.0 ml. Cleanup by hydrolysis, filtration, charcoal, and AIE. For further conditions, see the legend to Fig. 1.

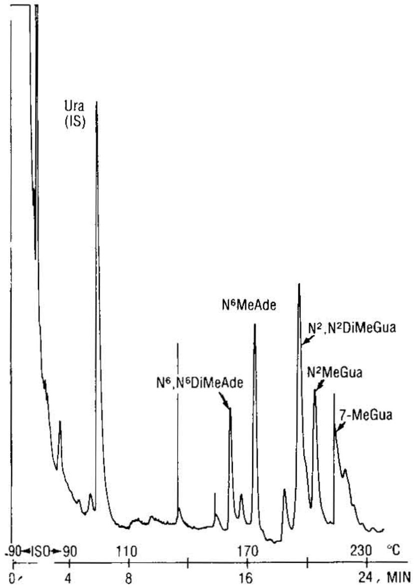

Fig. 5. GLC analysis of methylated bases in urine of a lung-cancer patient. Sample: 4.0 ml spiked with 50 μg of each MeB. Attenuation: 6.4×10^{-10} A.f.s. Cleanup by hydrolysis, filtration, charcoal, and AIE. For further conditions, see the legend to Fig. 1.

present in cancer patient urines may be due to the excretion of methylated nucleosides which are hydrolyzed to the free bases during the hydrolysis step in sample preparation. This is of particular importance for N^2,N^2-DiMeGua as analysis of the nucleoside, N^2,N^2-dimethylguanosine, has been reported in cancer patient and normal urine[22,25,28]. The corresponding nucleosides of the other methylated bases reported here have not been quantitatively analyzed in normal or cancer patient urine to date. Further research is necessary to determine whether the primary excretion product is the free base, nucleoside, or a combination of both.

The per cent recovery of the MeB through the analytical method was determined by analyzing "spiked" and "unspiked" aliquots of the same urine sample. A total of twelve recovery samples was analyzed, and the average per cent recoveries and ranges for the methylated bases were: N^6,N^6-DiMeAde, 49% (43–58%); N^6-MeAde, 53% (43–60%); N^2N^2-DiMeGua, 96% (87–109%); N^2-MeGua, 53% (43–65%); and 7-MeGua, 94% (90–109%); a chromatogram obtained from a lung cancer-patient urine plus the added MeB is shown in Fig. 5.

The low recovery obtained for the adenine derivatives and N^2-MeGua can be attributed to the pyridine–ethanol (95%) (1:1) wash of the charcoal column, which partially elutes these compounds. However, this wash was found to be essential in that it removed the methylated xanthines from the sample, and as stated earlier, xanthine and its methylated derivatives interfere with the chromatography of the methylated bases. With better control over the intake of caffeined beverages, this pyridine–ethanol (95%) wash would not be needed; and the recovery of the adenine derivatives and N^2-MeGua would be similar to that obtained for N^2,N^2-DiMeGua and 7-MeGua.

CONCLUSIONS

A GLC method for the analysis of methylated nucleic acid bases in human urine has been developed. The GLC and instrumental conditions of the methylated bases were first determined. The MeB were converted to their volatile TMS derivatives with BSTFA in acetonitrile as solvent by heating in a closed vial at 150° for 15 min. The derivatized bases were then analyzed on a chromatographic column of 5% OV-3 on Supelcoport (100–120 mesh).

Prior to the GLC analysis of MeB in urine, the sample must be cleaned to remove salts and interfering compounds. The cleanup method developed consisted of four steps: hydrolysis, filtration, charcoal adsorption, and AIE. The hydrolysis step converted any methylated nucleosides present to the free bases. The filtration step removed particulate matter present after hydrolysis which would interfere with further cleanup. Charcoal adsorption and AIE removed the salts and many other urinary compounds from the methylated bases and provided a relatively clean sample which could be analyzed by GLC.

Using this method, ten normal human and fourteen cancer-patient urines were analyzed. An increased excretion level for some methylated bases by cancer patients over normal humans was observed, and as noted in Table V, the overall average increase in excretion was about 70%. However, it is recognized that the number of samples analyzed was insufficient for definitive conclusions to be made. Whether this increased excretion is due to greater tRNA methylation by tRNA methylases and/or secondary to the more rapid turnover of the tumor tissue tRNA for the cancer patients has not been ascertained. Future studies will determine whether excretion levels of methylated bases may be useful as a means for monitoring tumor growth or in evaluating the response of patients with malignancy to treatment.

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REFERENCES

1 W. S. Adams, F. Davis and M. Nakatani, *Amer. J. Med.*, 28 (1960) 726.
2 B. M. Bollard, R. H. Culpan, H. Marks, H. McIlwain and M. Shepherd, *J. Ment. Sci.*, 106 (1960) 1250.
3 G. B. Chheda, A. Mittelman and J. T. Grace, *J. Pharm. Sci.*, 58 (1969) 75.
4 K. Fink and W. S. Adams, *Arch. Biochem. Biophys.*, 126 (1968) 27.
5 K. Fink, W. S. Adams, F. W. Davis and M. Nakatani, *Cancer Res.*, 23 (1963) 1824.
6 K. C. M. Heirwegh, C. Ramboer and J. Degroote, *Amer. J. Med.*, 42 (1968) 913.
7 L. R. Mandel, P. R. Srinivasan and E. Borek, *Nature (London)*, 209 (1966) 586.
8 E. S. McFarlane and G. J. Shaw, *Can. J. Microbiol.*, 14 (1968) 185.
9 B. Weissmann, P. A. Bromberg and A. B. Gutman, *J. Biol. Chem.*, 224 (1957) 423.
10 R. W. Park, J. F. Holland and A. Jenkins, *Cancer Res.*, 22 (1962) 469.
11 P. L. Bergquist and R. E. F. Mathews, *Biochem. J.*, 85 (1967) 305.
12 E. Borek, *Cancer Res.*, 31 (1971) 596.
13 D. B. Dunn, *Biochim. Biophys. Acta*, 34 (1959) 286.
14 R. H. Hall, *Biochemistry*, 4 (1965) 661.
15 G. L. Viale, A. F. Restelli and E. Viale, *Tumori*, 53 (1967) 533.
16 A. Dlugajczyk and J. J. Eiler, *Nature (London)*, 212 (1966) 611.
17 B. B. Biswas, M. Edmonds and R. Abrams, *Biochem. Biophys. Res. Commun.*, 6 (1961) 146.
18 S. Fujioka, R. C. Ting and R. C. Gallo, *Cancer Res.*, 31 (1971) 451.
19 D. H. Riddick and R. C. Gallo, *Cancer Res.*, 30 (1970) 2484.
20 O. K. Sharma and L. A. Loeb, *Biochem. Biophys. Res. Commun.*, 50 (1973) 172.
21 B. Weissmann, P. A. Bromberg and A. B. Gutman, *J. Biol. Chem.*, 224 (1957) 407.
22 T. P. Waalkes, S. R. Dinsmore and J. E. Mrochek, *J. Nat. Cancer Inst.*, 51 (1973) 271.
23 D. B. Lakings and C. W. Gehrke, *J. Chromatogr.*, 62 (1971) 347.
24 D. B. Lakings and C. W. Gehrke, *Clin. Chem.*, 18 (1972) 810.
25 S. Y. Chang, D. B. Lakings, R. W. Zumwalt, C. W. Gehrke and T. P. Waalkes, *J. Lab. Clin. Med.*, 83 (1974) 816.
26 C. W. Gehrke and D. B. Lakings, *J. Chromatogr.*, 61 (1971) 45.
27 S. S. Mirvish, J. Medalie, C. A. Linsell, E. Yousuf and S. Reyad, *Cancer*, 27 (1971) 736.
28 T. P. Waalkes, C. W. Gehrke, R. W. Zumwalt, S. Y. Chang, D. B. Lakings, D. C. Tormey, D. L. Ahmann and C. G. Moertel, *Cancer*, 36 (1975) 390.

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HIGH-RESOLUTION LIQUID CHROMATOGRAPHIC ANALYSIS OF METHYLATED PURINE AND PYRIMIDINE BASES IN TRANSFER RNA

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SUMMARY

Methylated and major purine and pyrimidine bases were separated and quantified by high-resolution liquid chromatography after hydrolyzing transfer ribonucleic acids (tRNAs). Separation was accomplished by eluting the hydrolyzed samples from an anion-exchange column with a concentration gradient of ammonium acetate at pH 9.2. Isolated samples of tRNA were hydrolyzed to the free bases with a trifluoroacetic acid–formic acid mixture at 200°. Detection limits of 100–200 ng/ml were measured for the methylated bases; analytical data are reported for ten methylated bases plus the four major bases of calf liver and rat liver tRNA.

INTRODUCTION

Major purine and pyrimidine bases, nucleosides, and nucleotides have been measured using a variety of analytical techniques including gas–liquid chromatography (GLC)[1–5], high-performance liquid chromatography (HPLC)[6–11], and thin-layer or paper chromatography[12–14]. Reliable results on the composition of the major purine and pyrimidine bases in nucleic acids have been obtained with many of these methods reported[1–4,6,11–14]. However, only the method by Randerath *et al.*[13–15] provides the necessary separation of the major and minor bases when a quantitative analysis is required for a minor nucleic acid constituent, such as a methylated base in tRNA. Certain minor nucleic acid constituents cannot be separated from the major

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[*] Operated for the Energy Research and Development Administration by Union Carbide Corporation.

bases by GLC, and the derivatizing agent can produce extraneous peaks on the chromatogram[16]. The HPLC methods were developed to provide rapid analysis of nucleic acid composition[6,7,10,11,17], and not for the separation and analysis of minor and methylated bases present in specific ribonucleic acids. Recent results by Sen and Ghosh[9] indicate that HPLC can be used to separate some major nucleosides from minor methylated nucleosides in tRNA, although quantification was not described.

Using radioactive labeling and two-dimensional paper chromatography, Munns *et al.*[12] and Randerath *et al.*[13–15] have developed methods for the analysis of methylated nucleic acid components, although not all the major and methylated components were completely separated. Radioactive methionine was used to label the methylated bases in the method by Munns *et al.*[12], providing results for *in vitro* studies; however, for *in vivo* experiments, dilution of the radioactive label makes the method impractical. Randerath *et al.*[13–15] developed a post-hydrolysis labeling technique which allows the analysis of any isolated tRNA. However, the analysis time is long, and sample manipulations require special care if satisfactory results are to be obtained.

The presence of methylated purine and pyrimidine bases in tRNA has been known and studied for some time. The methylation of the major bases by specific tRNA methylases occurs on the intact macromolecule. Increased tRNA methylases activity has been found in a variety of different tumors as summarized by Borek[18]. Reports on the increased urinary excretion of certain methylated nucleic acid derivatives by cancer patients[19–23] support the evidence for increased methylation of tRNA in cancer cells. Previous investigations by Berquist and Mathews[24] and particularly Viale *et al.*[25] have shown that tRNAs from tumor cells contain increased amounts of methylated bases compared to their normal tissue counterpart. However, other studies[17] have indicated little if any differences in methylated base content between tRNA in normal and related tumor tissues. Further analyses are needed, particularly of individual isoaccepting tRNAs, since minor variations may not be detected in a total population of tRNAs.

A reliable and practical method for the quantitative analysis of the methylated bases present in tRNA would provide further information on the extent of modification in the tRNA of neoplastic cells. This in turn would aid in understanding the effects of the increased tRNA methylase activity found in tumor cells and the greater urinary excretion of certain methylated nucleosides and bases by patients with malignant disease. This report describes a quantitative method applied to tRNA for the analysis of specific methylated bases that have been detected in the urine from patients with cancer.

MATERIAL AND METHODS

Apparatus

A Mark II UV Analyzer (Oak Ridge National Laboratory, Oak Ridge, Tenn., U.S.A.) equipped with a 150 × 0.455 cm I.D. column containing Aminex 27 (Bio-Rad Labs., Richmond, Calif., U.S.A.) anion-exchange resin, a two-chamber gradient generating system[26] and a programmable temperature control system was used for this study. The eluate from the chromatographic column was monitored at 254 and 280 nm by a duoMonitor (Laboratory Data Control, Riviera Beach, Fla., U.S.A.), and a Servo/Riter II (Texas Instruments, Houston, Tex., U.S.A.) dual-pen recorder was used to record the chromatograms.

A sand-bath with a variable temperature control (±2°) was used for hydrolysis of the transfer RNA samples.

Reaction vials described by Gehrke and Lakings[27] were made from Pyrex 9826 culture tubes (Corning, Corning, N.Y., U.S.A.).

Reagents

The major and methylated bases were obtained from the following sources. Calbiochem (Los Angeles, Calif., U.S.A.): adenine (Ade), cytosine (Cyt), guanine (Gua), uracil (Ura), 6-methyladenine (6-MeAde), 6-dimethyladenine (6-Me_2Ade), 5-methylcytosine (5-MeCyt), pseudouridine (Ψ), and thymine (Thy); Cyclo Chem. (Los Angeles, Calif., U.S.A.): 2-methyladenine (2-MeAde), 3-methylcytosine (3-MeCyt), 1-methylguanine (1-MeGua), 7-methylguanine (7-MeGua), 2-methylguanine (2-MeGua), 2-dimethylguanine (2-Me_2Gua), and 1-methylhypoxanthine (1-MeHypo); and Sigma (St. Louis, Mo., U.S.A.): 1-methyladenine (1-MeAde).

Calf liver tRNA was purchased from Sigma, rat liver tRNA was obtained from General Biochemicals (Chagrin Falls, Ohio, U.S.A.).

Formic acid (97–100%) and trifluoroacetic acid (TFA) were obtained from Matheson, Coleman and Bell (Norwood, Ohio, U.S.A.). Acetic acid and ammonium hydroxide used to prepare buffers were from J. T. Baker (Phillipsburg, N.J., U.S.A.).

The anion-exchange resin used for separation of the purine and pyrimidine bases was Aminex A-27 (a 12–15 μm particle size strong base anion-exchange resin of 8% nominal crosslinking) and was purchased from Bio-Rad Labs.

Sample preparation

Approximately 2.0 mg of tRNA was dissolved in 2.0 ml of water, and 400-μl and 75-μl aliquots were placed in a culture tube with a PTFE-lined screw cap. Hydrolysis of the tRNAs to the free bases was accomplished using the method of Lakings and Gehrke[3]. By this procedure, 400 μl of a 1:1 trifluoroacetic acid–formic acid mixture were added to the sample, the vial tightly sealed, and the contents heated at 200° for 1.5 h. The hydrolyzing agents were removed by volatilizing at 60° under a stream of nitrogen, the residual sample was dissolved in 1.0 ml of 0.1 *N* NaOH and placed on the liquid chromatograph.

RESULTS

Separation studies on the major and methylated bases

Initial data indicated that the best separation of the major and methylated bases would be accomplished by gradient elution at a basic pH. Singhal and Cohn[10] reported on the anion-exchange chromatographic parameters for the separation of nucleosides and showed partial base separation under these conditions (0.2 *M* ammonium acetate, pH 9.7). Using these conditions as a starting point, optimal conditions were sought for separation of the four major bases (Ade, Cyt, Gua, and Ura) and twelve methylated bases (1-MeAde, 2-MeAde, 6-MeAde, 6-Me_2Ade, 3-MeCyt, 5-MeCyt, 1-MeGua, 2-MeGua, 7-MeGua, 2-Me_2Gua, 1-MeHypo, and Thy) and to include pseudouridine at a major to minor base ratio of 10 to 1. Chromatographic

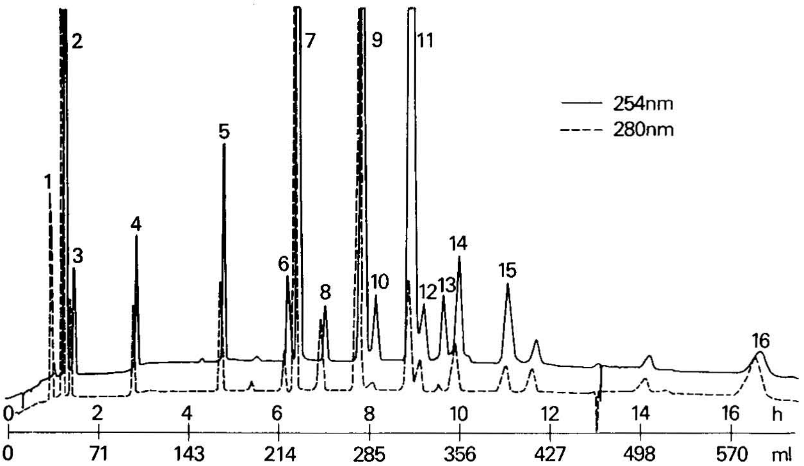

Fig. 1. Standard mixture of bases and methylated bases at a 10:1 ratio. Buffer of first chamber: 0.015 *M* ammonium acetate (pH 9.2); of second chamber: 1.2 *M* ammonium acetate (pH 9.2). Temperature: ambient for the first 1.75 h, then programmed to 60° in 1.5 h, and maintained a 60° for the remainder of the analysis. Flow-rate: 35.6 ml/h (3.65 ml/cm^2·min). UV output: 0.04 O.D. unit full scale. 1 = 3-MeCyt; 2 = Cyt; 3 = 5-MeCyt; 4 = 1-MeAde; 5 = 1-MeGua; 6 = Thy; 7 = Ura; 8 = 7-MeGua; 9 = Gua; 10 = 2-MeAde; 11 = Ade; 12 = 2-MeGua; 13 = 1-MeHypo; 14 = 2-Me$_2$Gua; 15 = 6-MeAde; 16 = 6-Me$_2$Ade.

TABLE I

METHYLATED AND MAJOR BASE STANDARD VALUES

Compound	*Retention volume* (ml)*	*R.S.D.***	*Peak height* (cm)*	*R.S.D.***
3-MeCyt	31.1	4.7	9.57***	6.6
Cyt	39.8	8.3	19.95	7.5
5-MeCyt	47.1	5.3	5.38	6.8
1-MeAde	97.2	4.8	7.34	4.9
1-MeGua	165.7	6.5	9.90	6.9
ψ	190.5	7.3	2.33	9.9
Thy	214.8	7.0	4.42	5.3
Ura	229.8	6.9	11.57	9.8
7-MeGua	244.6	6.1	3.58***	5.3
Gua	255.0	5.7	7.10	8.9
2-MeAde	268.6	9.4	3.18	5.9
Ade	300.0	7.0	9.71	5.3
2-MeGua	310.9	5.5	2.74	8.6
1-MeHypo	314.7	5.6	3.08	8.1
2-Me$_2$Gua	328.3	5.7	4.90	7.9
6-MeAde	380.8	6.0	2.88	7.3
6-Me$_2$Ade	619.1	5.2	1.85***	9.2

* Average of six independent analyses.

** Relative standard deviation.

*** 280-nm scale used for quantitation for 3-MeCyt, 7-MeGua, and 6-Me$_2$Ade.

conditions which yielded the best separation using the "Autograd" system[26] utilized a 0.015 *M* ammonium acetate (pH 9.2) in the first chamber and a 1.2 *M* ammonium acetate buffer (9.2) in the second chamber. The temperature was maintained at ambient room temperature for the first 1.75 h, then programmed to 60° in 1.5 h, and maintained at 60° for the remainder of the analysis. The flow-rate through the column was maintained between 34 and 36 ml/h (3.5–3.7 ml/$\text{cm}^2 \cdot$min). A typical chromatogram of the major and methylated bases at a 10:1 ratio is shown in Fig. 1. Table I gives the average elution volume of each base for six independent analyses and the relative standard deviation of the elution volume for each base.

Quantitation of the major and methylated bases separated by HPLC

The minimum detectable level (MDL) and the linearity of response over a range of concentration were evaluated since quantitative analysis of the methylated bases in tRNA was the primary goal. Standard stock solutions of four bases were analyzed at concentrations of 1–5 µg/ml for the methylated bases and 5–40 µg/ml for the major bases. Graphs of peak area *versus* concentration and peak height *versus* concentration were constructed and found to be linear for each compound over the concentration range of interest. Peak height and peak area gave similar results. Measurement of the width at half peak height was found to be difficult and a possible source of error in calculating peak area due to the narrow width of some peaks. Consequently, the peak height was chosen for quantitative calculation. Figs. 2 and 3 present the peak height data for the four major bases and for four selected methylated bases, respectively.

The quantitative analysis of the base composition of a tRNA was performed by using a simple proportion as the peak heights of the bases were linear over a wide concentration range.

$$\mu\text{g base in tRNA} = \mu\text{g base standard} \times \frac{\text{peak height base in tRNA}}{\text{peak height base standard}}$$

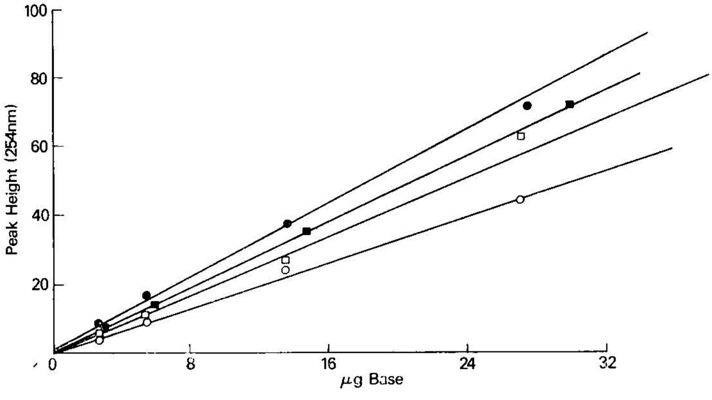

Fig. 2. Standard curves for major bases. Each point represents an average of three independent analyses. Chromatographic conditions were as given in Fig. 1. ●, Cyt; ■, Ura; □, Ade; ○, Gua.

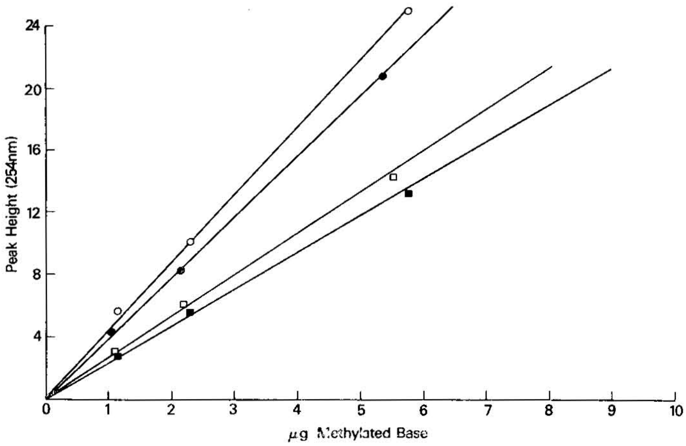

Fig. 3. Standard curves for four methylated bases. Each point represents an average of three independent analyses. Chromatographic conditions were as given in Fig. 1. Other methylated bases gave similar graphs. ○, Thy; ●, 6-MeAde; □, 2-Me$_2$Gua; ■, 7-MeGua.

Six independent analyses of a standard solution of methylated and major bases were made. The average peak height and relative standard deviation are presented in Table I.

The MDL for the methylated bases was determined by analyzing successively lower concentrations of selected methylated bases until a signal to noise ratio of 3:1 was obtained. The MDL for the methylated bases using this analytical system was found to be between 0.1 and 0.2 μg/ml.

Hydrolysis evaluation

The stability of the major bases during hydrolysis with a formic acid–TFA mixture at 200° for 1.5 h had been determined previously[3]. No loss or destruction of the major bases was found. To determine the effect of this hydrolysis method on the methylated bases, a standard mixture containing the twelve methylated bases and pseudouridine at a concentration of 2.5 μg/ml were carried through the entire hydrolysis and analytical procedure. Only two of the compounds were found to be affected by the hydrolysis conditions. Pseudouridine was completely degraded with no UV absorbing peaks appearing on the chromatogram, and about 15% of 1-MeAde partially isomerized to 6-MeAde. This change had been demonstrated previously by Munns *et al.*[12]. As a consequence, the pseudouridine level in tRNA could not be determined by this method; and measured amounts of 1-MeAde and 6-MeAde were combined and reported as one value. Table II shows the good stability of the methylated bases when carried through the hydrolysis step and analysis by liquid chromatography, with the exception of a small loss of 1-MeAde.

Several of the major and methylated nucleosides were also hydrolyzed and analyzed to determine whether complete hydrolysis to the free base had occurred. If the conditions for hydrolysis were carefully followed, total conversion of all the nucleosides to their corresponding base invariably resulted. However, incomplete hydrolysis occurred if the temperature fell below 200° or was maintained for less than

TABLE II

STABILITY OF THE METHYLATED BASES DURING HYDROLYSIS

Compound	*Peak height*[*]		*% Recovery*
	Unhydrolyzed[*]	*Hydrolyzed*[**]	
3-MeCyt	22.8[***]	22.7[***]	99.6
5-MeCyt	17.8	17.5	98.4
1-MeAde	17.3	14.4	83.2
1-MeGua	21.4	21.2	99.1
Thy	11.6	11.2	96.6
7-MeGua	6.6[***]	6.2[***]	93.9
2-MeAde	7.9	7.6	96.2
2-MeGua	6.6	6.0	90.9
1-MeHypo	8.5	8.4	98.8
2-Me_2Gua	8.4	8.1	96.4
6-MeAde	21.3	20.9	98.1
6-Me_2Ade	5.8[***]	5.6[***]	96.6

[*] Peak height in cm measured at 254 nm.

[**] Each value represents an average of three independent analyses for unhydrolyzed and hydrolyzed peak heights.

[***] Peak height measured at 280 nm for 3-MeCyt, 7-MeGua, and 6-Me_2Ade.

1.0 h. If the tRNA sample was incompletely degraded to its free bases, corresponding nucleosides and nucleotides could be detected. Absence of such peaks on the chromatograms provided a means whereby total hydrolysis of the tRNA sample could be verified.

TABLE III

COMPOSITION OF RAT LIVER AND CALF LIVER tRNA IN WEIGHT AND MOLE PERCENTAGES

Compound	*Rat liver tRNA*		*Calf liver tRNA*	
	w/w %[*]	*mole %*[**]	*w/w %*[*]	*mole %*[**]
3-MeCyt	0.04	0.14	0.02	0.05
Cyt	9.15	37.64	11.60	32.31
5-MeCyt	0.12	0.42	0.34	0.84
1-MeAde	0.03	0.10	0.05	0.11
1-MeGua	0.07	0.18	0.04	0.07
Thy	0.12	0.43	0.30	0.74
Ura	4.25	17.34	4.56	12.59
7-MeGua	0.06	0.15	0.05	0.09
Gua	7.75	23.38	17.64	36.13
Ade	5.55	18.71	7.32	16.78
2-MeGua	0.13	0.34	0.03	0.06
2-Me_2Gua	0.07	0.17	0.03	0.05
6-MeAde	0.33	0.99	0.09	0.19
6-MeAde + 1-MeAde[***]	0.45	1.09	0.14	0.30

[*] w/w % = weight methylated base/weight tRNA × 100.

[**] Mole % = moles methylated base/moles total bases × 100.

[***] 6-MeAde and 1-MeAde were combined due to isomerization of 1-MeAde to 6-MeAde.

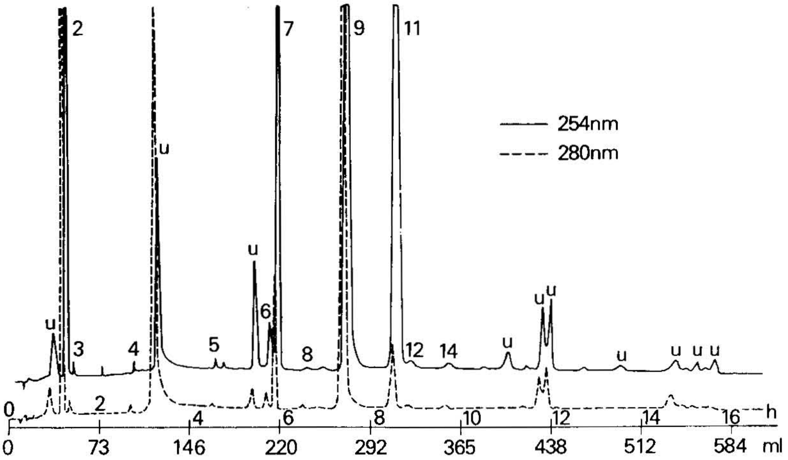

Fig. 4. HPLC analysis of 0.5 mg calf liver tRNA. For analysis conditions and peak identification, refer to Fig. 1. Flow-rate: 36.5 ml/h (3.75 ml/cm²·min). UV output: 0.08 O.D. unit full scale. U indicates unknown peaks on the chromatogram. Hydrolysis conditions were as given in text.

Analysis of selected tRNA samples

The amounts of the various methylated bases present in calf liver and rat liver tRNA were determined by the methods described earlier. A 2.0-mg sample of tRNA was dissolved in 2.0 ml water. Duplicate aliquot samples of 0.5 and 0.07 ml for calf liver tRNA (*ca.* 0.5 mg and 70 μg tRNA) and 0.25 and 0.05 ml for rat liver tRNA (*ca.* 0.25 mg and 50 μg tRNA) were analyzed. Analytical data for ten methylated and four major bases were determined and these data are presented in Table III.

Figs. 4 and 5 are chromatograms for the HPLC analysis of methylated bases

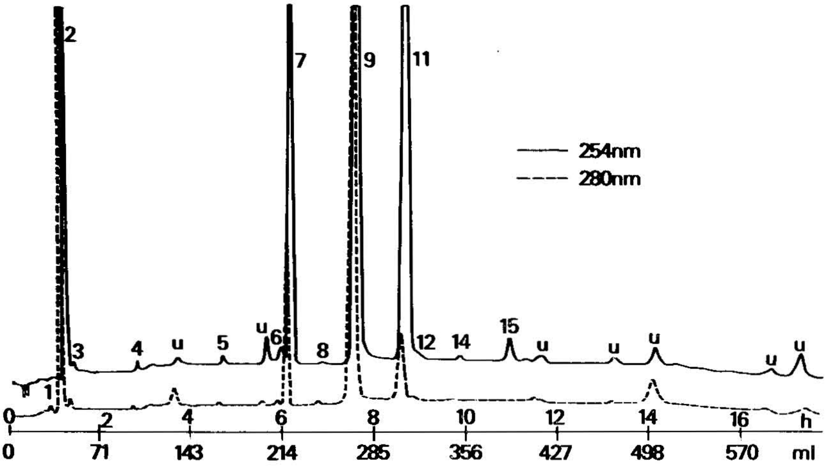

Fig. 5. HPLC analysis of 0.25 mg rat liver tRNA. For analysis conditions and peak identification, refer to Fig. 1. Flow-rate: 36.5 ml/h (3.75 ml/cm²·min). UV output: 0.04 O.D. unit full scale. U indicates unknown peaks on the chromatogram. Hydrolysis conditions were as given in text.

in calf liver and rat liver tRNA, respectively, and show the presence of a number of unidentified minor bases.

DISCUSSION

The quantitative analysis of the major and methylated base content of tRNAs found in normal and related tumor tissue provides information as to structural modifications and may provide clues as to possible functional or behavioral differences observed between normal and cancer cells. Since these macromolecules serve important roles in cell growth and its regulation, further study and understanding of the modified base content would be of distinct value. The method, as described in this report, had direct application for the separation and determination of the major and methylated purine and pyrimidine bases in tRNA. This procedure may be applicable to the quantitative measurement of those methylated purines and pyrimidines found as catabolic end products in the urine of normal subjects and cancer patients. No attempt was made to identify or measure the content of those structurally modified bases having substituents other than methyl groups. Further studies are necessary to determine if the analysis of other modified degradation products of nucleic acids could be performed using this procedure. This seems feasible with minor modifications in either the hydrolysis step or the liquid chromatographic separation.

The sensitivity of the method, found to be 100–200 ng methylated base per milliliter, could be improved by using a detector cell with a longer path length than the 3-mm path length used in this study. The analysis of the major and methylated bases on the same chromatogram is possible using an electronic integrator or a second recorder with a variable range. The analysis time may be shortened by changing the eluant concentrations, the column length, or the flow-rate. These parameters were not fully evaluated because the procedure as developed gave the required separations and the analysis time was not a limiting factor. Application of the method has been demonstrated using both rat and calf liver tRNA and has been shown to be sensitive and to provide reproducible results. Further studies are in progress to compare analytical results for tRNAs from tumor tissue with those from tRNAs obtained from normal tissue.

REFERENCES

1 T. Hashizume and Y. Sasaki, *Anal. Biochem.*, 24 (1968) 232.
2 D. B. Lakings and C. W. Gehrke, *J. Chromatogr.*, 62 (1971) 347.
3 D. Lakings and C. Gehrke, *Clin. Chem.*, 18 (1972) 810.
4 J. MacGee, *Anal. Biochem.*, 14 (1966) 305.
5 A. Patel, *Ph. D. Thesis*, University of Missouri, Columbia, Mo. 65201, 1972.
6 C. A. Burtis, *J. Chromatogr.*, 51 (1970) 183.
7 C. Horvath and S. R. Lipsky, *Anal. Chem.*, 41 (1969) 1227.
8 J. Kirkland, *J. Chromatogr. Sci.*, 8 (1970) 72.
9 G. Sen and H. Ghosh, *Anal. Biochem.*, 58 (1974) 578.
10 R. Singhal and W. Cohn, *Biochemistry*, 12 (1973) 1532.
11 M. Uziel, C. Koh and W. Cohn, *Anal. Biochem.*, 25 (1968) 77.
12 T. Munns, K. Podratz and P. Katzman, *Biochemistry*, 13 (1974) 4409.
13 E. Randerath, C-T. Yu and K. Randerath, *Anal. Biochem.*, 48 (1972) 172.
14 K. Randerath and E. Randerath, *Procedures Nucleic Acid Res.*, 2 (1971) 796.

15 E. Randerath, L. Chia, H. Morris and K. Randerath, *Biochim. Biophys. Acta*, 366 (1974) 159.
16 D. B. Lakings, C. W. Gehrke and T. P. Waalkes, 1973, unpublished observations.
17 R. Singhal and W. Cohn, *Anal. Biochem.*, 45 (1972) 585.
18 E. Borek, *Cancer Res.*, 31 (1971) 596.
19 S. Chang, D. Lakings, R. Zumwalt, C. Gehrke and T. Waalkes, *J. Clin. Med.*, 83 (1974) 816.
20 K. Fink and W. Adams, *Arch. Biochem. Biophys.*, 126 (1968) 27.
21 J. Mrochek, S. Dinsmore and T. Waalkes, *J. Nat. Cancer Inst.*, 53 (1974) 1553.
22 T. P. Waalkes, C. W. Gehrke, R. W. Zumwalt, S. Y. Chang, D. B. Lakings, D. C. Tormey, D. L. Ahmann and C. G. Moertel, *Cancer*, 36 (1975) 390.
23 B. Weissman, P. Bromberg and A. Gutman, *Biol. Chem.*, 224 (1957) 423.
24 P. L. Berquist and R. E. F. Mathews, *Biochem. J.*, 85 (1962) 305.
25 G. L. Viale, A. F. Restelli and E. Viale, *Tumori*, 53 (1967) 533.
26 D. D. Chilcote, C. D. Scott and J. W. W. Pitt, *J. Chromatogr.*, 75 (1973) 175.
27 C. W. Gehrke and D. B. Lakings, *J. Chromatogr.*, 61 (1971) 45.

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STUDIES ON THE INTERACTION BETWEEN SMALL MOLECULES BY GEL FILTRATION IN SEPHADEX LH-20

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SUMMARY

A method for the study of the interaction between small molecules by gel filtration is described, based on the difference in the retention of the reactants and product in Sephadex LH-20 gel. The interaction between the anionic detergent sodium dodecyl sulphate and methylene blue and tryptophan in acidic solution is described.

INTRODUCTION

A considerable amount of information is available on the interaction of small molecules with Sephadex gels of high density, *e.g.*, Sephadex G-10, G-25 and LH-20[1–6]. The interaction is determined by hydrogen bonding, π-electron or hydrophobic interaction, depending upon the gel and solutes involved[2–6].

Recently, we observed that N-acetyl esters of aromatic amino acids are retained (by hydrophobic interaction) in Sephadex LH-20 (hydroxypropyl derivative of Sephadex G-25) gel[6]. It was also inferred that the presence of charges in the aromatic amino acids interfered with their retention in the gel. N-acetylphenylalanine, which carries a negative charge at neutral pH, is not retained in the gel although at acidic pH, where no effective charge is present, the molecule is considerably retained.

Based on the above observations, an attempt has been made to study the interaction between small molecules by gel filtration. When a group containing a relatively large non-polar moiety with charge(s) attached to it interacts with a similar group but with opposite charge(s), a complex having a reduced or no effective charge would result. This complex would be retained more than the reactants in the gel. In reactions where non-polar moieties having similar types of charge interact, the resulting complex, having a more effective charge, would be eluted before any of the reactants.

In this paper, we report a study of the first type. A Sephadex LH-20 gel column is equilibrated with a solution of reactant, R_I, at a desired concentration. A small amount of a solution of the other reactant, R_{II}, in which the total concentration of R_I is equal to the concentration at which the gel column has been equilibrated, is then added to the column. If R_{II} interacts with R_I to form a complex, the sample in which R_{II} is equilibrated with R_I will be depleted with respect to R_I. As the mobility of the complex would be less than the mobility of R_I, the front depleted in R_I will move along the column faster than the complex. The resultant elution profile will show a trough below the base-line of R_I solution representing the depletion. This would be followed by the appearance of a peak of the complex enriched in R_I. From the areas of the troughs or crests of the complex resulting from different R_I concentrations with a constant R_{II} concentration, the binding parameters can be determined. This principle was used in order to study the interaction between the anionic detergent sodium dodecyl sulphate (SDS) and methylene blue (MB) and tryptophan.

MATERIALS AND METHODS

Sephadex LH-20 (Sigma, St. Louis, Mo., U.S.A.), L-tryptophan (Merck, Darmstadt, G.F.R.) and methylene blue (Stain Grade; BDH, Poole, Great Britain) were used. Both tryptophan and the dye showed a single elution peak when eluted in the gel. Sodium dodecyl sulphate was recrystallized twice from 50% ethanol. Columns of dimensions 11 × 1.8 and 12 × 2.1 cm with bed volumes (V_t) of 28 and 42 ml were used for the methylene blue and tryptophan studies, respectively. Distilled water was adjusted to pH 2.2 with hydrochloric acid (AnalaR).

The gel was soaked in the acidic solution (pH 2.2) for *ca.* 60 h before it was packed into the column. For equilibration by different concentrations of MB or tryptophan, the gel in the column was washed until the absorbance of portions of eluate from the gel was identical with the absorbance of the washing solvents.

A 1-ml volume of stock SDS was added to 1 ml of MB or tryptophan so that the concentration of the dye or amino acid was equal to its equilibration concentration in the gel column. Then 1 ml of the mixture was loaded into the column and 5-ml aliquots of eluate were collected, except around the trough, where the volume of each aliquot taken was 2 ml. The flow-rate was 25–30 ml/h. The concentration range for MB was $5 \cdot 10^{-6}$–$2.5 \cdot 10^{-5}$ *M* (SDS, $2.5 \cdot 10^{-3}$ *M*). The range of tryptophan concentration was $5.5 \cdot 10^{-5}$–$2.6 \cdot 10^{-4}$ *M* (SDS, $1 \cdot 10^{-2}$ *M*).

RESULTS

Elution profiles are shown in Fig. 1. The elution volumes of the dye and tryptophan were 85 and 78 ml, respectively. The lowest values of the troughs appeared at the positions of the elution peaks of the above two compounds within 5 ml. With MB, the visible depletion of its concentration, indicated by the movement of a zone having a low dye concentration, could be seen. This MB-depleted zone was followed by a dye-enriched (complex between the dye and detergent) front. The enriched front showed a gradual decrease in concentration (dissociation of the complex) as it moved through the length of the gel. By adjusting the flow-rate and volumes of aliquots collected, a separation of about 5–7 ml was obtained between the value when the trough reached equilibrium and the appearance of the complex. This procedure was followed for the tryptophan–SDS system also.

The number of moles of MB or tryptophan bound by SDS in the gel can be obtained from the procedure of Fairclough and Fruton[7]. The difference between the

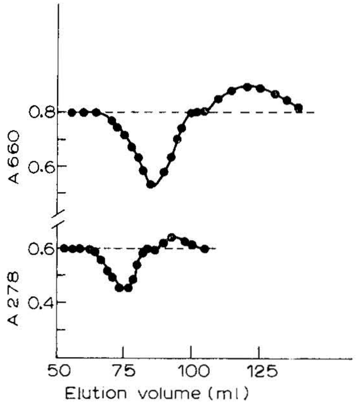

Fig. 1. Elution profile for measurements of the binding of MB (top) and tryptophan (bottom) with SDS at pH 2.2 and 25°. MB $1.1 \cdot 10^{-5}$ M, SDS $2.5 \cdot 10^{-6}$ M. Tryptophan $1.09 \cdot 10^{-4}$ M, SDS $1 \cdot 10^{-5}$ M.

base-line absorbance of each fraction constituting the trough was determined and used in the equation

$$\mu\text{moles bound} = \frac{\sum_i (\Delta A_i \times \text{ml}_i)}{\varepsilon \times 10^{-3}}$$

where ΔA_i is the difference between the base-line absorbance value and that of fraction i and ε is the molar absorptivity of either MB or tryptophan. The values of ε are $7.2 \cdot 10^4$ at 660 nm and $5.7 \cdot 10^3$ at 278 nm for MB and tryptophan, respectively.

The binding parameters, *viz.*, the number of binding site(s), n, and the association constant, k, were obtained from the double reciprocal plot of Klotz:

$$\frac{1}{\bar{\nu}} = \frac{1}{n} + \frac{1}{nkC}$$

where $\bar{\nu}$ is the number of moles of MB or tryptophan bound and C is the concentration of either MB or tryptophan with which the gel is equilibrated.

DISCUSSION

The first necessary criterion for the study of binding equilibria by gel filtration is that the minimum in the concentration in the depleted front (trough) should appear at the elution volume of the compound with which the column has been equilibrated. In the present study, the minima in the troughs appear within 5 ml of the elution volumes of both MB and tryptophan. The second criterion is that the appearance of the enriched front of the equilibrating solvent (complex) should not interfere with the measurements in the trough region. In this study, by adjusting the flow-rate and aliquots collected, a separation of 5–7 ml can be obtained between the value at which equilibrium of the depleted front is attained and the appearance of the complex-enriched front.

The double reciprocal plot in Fig. 2 shows a reasonably good linear relationship between $1/\bar{\nu}$ and $1/C$. The plot results in 1:1 stoichiometry and an association

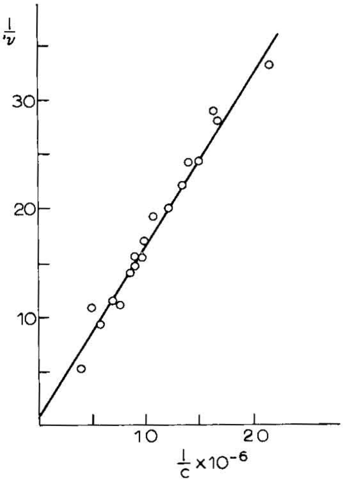

Fig. 2. Double reciprocal (Klotz) plot for the interaction between SDS and MB at pH 2.2 and 25°.

constant of $6.6 \cdot 10^4$ mole/l for the reaction between MB and SDS. The 1:1 stoichiometry indicates a simple metathetical reaction. The evidence of 1:1 complex formation for the above interaction is already available in the literature[8]. The association constant of the above reaction ($C_{16}H_{18}N_3S^+$ and $^-O_4SC_{12}H_{25}$) compares well with the association constant of $4.2 \cdot 10^4$ obtained from conductivity measurements for the reaction between the decyltrimethylammonium ion and azobenzene-4-sulphonate ($C_{13}H_{20}N^+$ and $^-O_3SC_{12}H_9$)[9].

Fig. 3 shows the double reciprocal plot for the tryptophan–SDS system. Of the various straight lines that can be drawn through the points, the one (shown in Fig. 3) resulting in an association constant of $2.8 \cdot 10^3$ and binding site (n) 1 represents most satisfactorily the binding isotherm (inserted in Fig. 3). These parameters describe the binding isotherm reasonably well in the region where the double reciprocal plot shows considerable scattering. The interaction between tryptophan and SDS can, therefore, be considered to be a simple metathetical reaction.

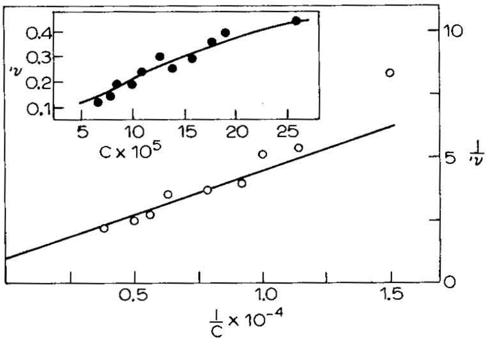

Fig. 3. Double reciprocal (Klotz) plot for the interaction between SDS and tryptophan at pH 2.2 and 25°. Insert: the line represents the binding isotherm between tryptophan and SDS with $n = 1$ and $k = 2.8 \cdot 10^3$ and the circles are experimental results.

The interaction between SDS and tyrosine and phenylalanine did not show any well defined trough by which studies such as those described above could be made. The complexes formed, probably having low association constants, dissociate rapidly while moving through the gel bed. This indicates that unless the association constant between the reactants are above certain value, the interaction between the molecules cannot be studied by the method described here.

REFERENCES

1 H. Determann, *Gel Chromatography*, Springer, Berlin, Heidelberg, New York, 2nd ed., 1969.
2 A. J. W. Brook and S. Housley, *J. Chromatogr.*, 42 (1969) 112.
3 C. A. Streuli, *J. Chromatogr.*, 56 (1971) 219 and 225.
4 J.-C. Janson, *J. Chromatogr.*, 28 (1967) 12.
5 H. Determann and K. Lampert, *J. Chromatogr.*, 69 (1972) 123.
6 V. Prakash and P. K. Nandi, *J. Chromatogr.*, 106 (1975) 23.
7 G. F. Fairclough, Jr., and J. S. Fruton, *Biochemistry*, 5 (1966) 673.
8 P. Mukerjee and K. J. Mysels, *J. Amer. Chem. Soc.*, 77 (1955) 2937.
9 A. Pacter and M. Danbrow, *Proc. Chem. Soc.*, (1962) 220.

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CHROM. 8612

GEL PERMEATION CHROMATOGRAPHY OF POLYMERS ON MACROPOROUS SILICAS*

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SUMMARY

The gel permeation chromatography and adsorption of polystyrenes from different solvents on macroporous silicas (Silochroms) have been investigated, and the adsorption data and chromatographic parameters have been analysed. The influence of the pore diameter on the internal diffusion coefficient macromolecules and the width of the chromatographic peak is shown. The range of applicability of Silochroms to chromatographic fractionation of polymers was determined.

INTRODUCTION

Under the conditions of gel permeation chromatography (GPC) polymer macromolecules are distributed according to the dimensions of their random statistical coils[1,2]. Non-swelling, rigid, porous adsorbents are sometimes to be preferred over swelling cross-linked organic gels (Sephadex, Sepharose). The accessibility of adsorbent pores to macromolecules of various sizes mainly determines the retention time of these molecules in a column. As was shown by Eltekov and co-workers[3–5], the accessibility of pore volume and internal surface, and the effective coefficients of diffusion of polystyrene macromolecules into the silica gel pores, depend substantially on the adsorbent pore sizes, the pore-size distribution and the temperature. Additionally, the chemical state of the surface of the column packing material has a significant influence in adsorption and chromatographic studies. Undesirable adsorption effects, which distort the character of the macromolecule distribution, may be encountered in GPC conditions on macroporous silicas. These effects can be eliminated by a chemical or adsorption modification of the silica surface[2,6–8].

This communication presents the results of polystyrene adsorption and GPC on a number of macroporous silicas of different pore sizes, and on chemically modified silica, to estimate the possibility of using these silicas in GPC of polymers, and to determine the correlation between adsorption data and chromatographic parameters.

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* Presented at the 5th Soviet–Italian Chromatography Symposium, Tallinn, April 22–25, 1975.

TABLE I

CHARACTERISTICS OF SILOCHROMS

Sample	*Mean pore diameter (Å)*	*Pore volume, V_s (cm³/g)*	*Specific surface, S (m²/g)*
C-1	400	1.1	112
C-2	500	1.3	86
C-3	500	1.2	80
C-4	1100	1.4	45
C-5	1600	1.2	24

EXPERIMENTAL

Materials

Macroporous silicas Silochroms obtained from Aerosil[10] were used as adsorbents and packing material in the chromatographic columns; their characteristics are listed in Table I. The mean diameter and pore volume V_s were determined using a mercury porosimeter. The specific surface areas S of the Silochrom samples were calculated by the BET method from low-temperature nitrogen adsorption data. The column packing was graded from 90 to 160 μm.

Sample C-3 was obtained from Silochrom C-2 by treatment of it with a 20% toluene solution of dimethyldichlorosilane for 48 h at 110°. The sample was then washed with toluene, and dried. Before the experiments, all samples were evacuated at 140° for 6 h.

Narrow fractions of polystyrene (PS) were used, *viz.* the standards of Waters Ass. (Milford, Mass., U.S.A.) with weight-average molecular weights M_w of 10,000, 20,400, 51,000, 97,000, 411,000, 498,000, 860,000, and 1.8×10^6 (polydispersity of PS standards, $M_w/M_n = 1.1$). Two polydisperse polymers, PS-1 and PS-2, with viscosity-average molecular weights $M_\eta = 160{,}000$ and $M_\eta = 14{,}000$, were also used. The molecular weights of these two polymers were determined at 20° in benzene using the Mark–Houwink equation with $K = 1.23 \times 10^{-4}$ and $a = 0.72$ (see ref. 9).

Carbon tetrachloride, benzene, toluene, ethylbenzene, and *n*-heptane were used as solvents.

Adsorption

Adsorption experiments were carried out under static conditions. 4 g of a PS solution of known concentration were poured into an adsorption ampoule with a 0.2-g portion of the dried adsorbent. Ampoules were sealed and stored until adsorption equilibrium was reached, and the equilibrium solution was analyzed by a differential refractometer–interferometer. The amount of adsorption was calculated from the relation[3]:

$$\Gamma = \frac{(C_0 - C)\, m_s}{mS} \tag{1}$$

where C_0 and C are concentrations of the polystyrene solution before and after adsorption, and m and m_s are the masses of adsorbent and solution.

Gel permeation chromatography

GPC experiments were carried out using an XG-1302 Special Construction Bureau Academy of Science (U.S.S.R.) liquid chromatograph with a differential refractometer as detector. The columns, 1200 mm × 8 mm I.D., were dry-packed with Silochrom. The solvent (toluene) flow-rate was 1 ml/min, and 1-ml polymer samples were introduced into the liquid chromatograph at a concentration of 5 mg/ml.

For the parameters of GPC we chose the distribution coefficient K, the height equivalent to a theoretical plate H, the relative height of a theoretical plate h, and the resolution R. Definitions of GPC parameters are listed in Table II.

RESULTS AND DISCUSSION

Fig. 1 shows the isotherms of PS-1 and PS-2 adsorption from dilute solution in carbon tetrachloride and ethylbenzene on Silochroms C-2 and C-3. The figure indicates that modification of the Silochrom surface sharply changes the character of adsorption of polystyrene macromolecules due to a reduction in both the specific and the non-specific (dispersion) interactions between the macromolecules with aromatic rings and the silica surface. The adsorption of polystyrene is changed from positive for hydroxylated Silochrom C-2 to negative for silanized Silochrom C-3. Adsorption of PS-1 and PS-2 from solution in ethylbenzene is negative, which can be attributed to the effect of specific interactions between electron donor molecules of ethylbenzene and protonized silanol groups of the Silochrom surface. The amount of adsorption of PS macromolecules from solution in carbon tetrachloride is about 1 mg/m^2. We assume these values of adsorption to indicate the adsorption of macromolecules parallel to the surface. According to this simple model, the thickness of the adsorbed layer of polystyrene is about 10–15 Å.

Gel permeation chromatography

Chromatograms of the PS standards and *n*-heptane are characterised by nar-

TABLE II

CHROMATOGRAPHY PARAMETERS

V_i is the elution volume of species i; V_0 is the void volume of a column (the elution volume of the macromolecules not penetrating into the pores); V_t is the total volume of a column (the elution volume of small molecules, *e.g.* *n*-heptane); W_i is the chromatogram base width of species i; L is the column length; H_0 is the height of the theoretical plate, as found for *n*-heptane.

Parameter	*Symbol*	*Definition*
Distribution coefficient	K	$K = \frac{V_i - V_0}{V_t - V_0}$
Height equivalent to a theoretical plate (HETP)	H	$H = L\left(\frac{W_i}{4\,V_i}\right)^2$
Relative value of height of theoretical plate	h	$h = \frac{H_i}{H_0}$
Resolution ($V_i > V_j$)	R	$R = \frac{2\,(V_i - V_j)}{(W_i + W_j)}$

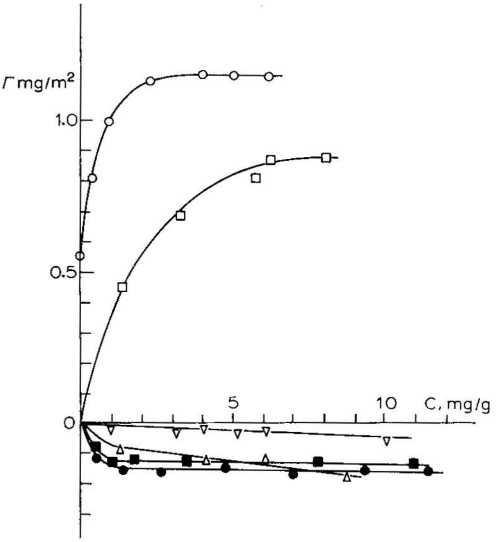

Fig. 1. Adsorption isotherms of PS-1 (○, △, ●) and PS-2 (□, ▽, ■) from solutions in carbon tetrachloride (○, □, △, ▽) and ethylbenzene (●, ■) on Silochroms C-2 (○, □, ●, ■) and C-3 (△, ▽).

row symmetrical peaks, but chromatographic peaks of the polydisperse samples PS-1 and PS-2 are wide and asymmetrical. Fig. 2 shows logarithmic dependences of the molecular weight of the polystyrene standards as a function of V_i for investigated Silochroms. All the curves display linear parts, almost identical in all cases, and the curves are shifted upwards along the log M_W axis in accordance with pore size (increasing from C-1 to C-5). The difference $V_t - V_0$ corresponds to the pore volume

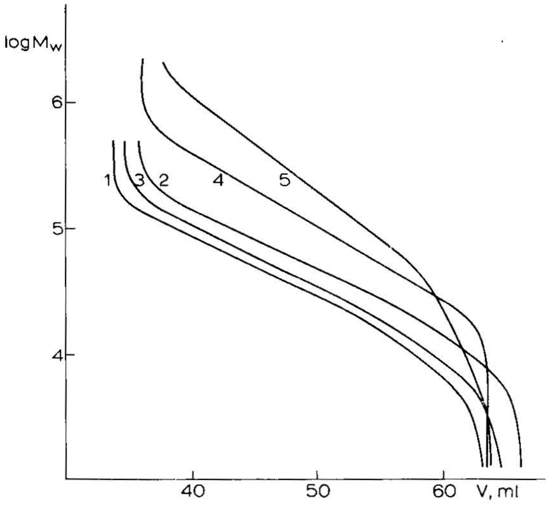

Fig. 2. Log M_W as a function of V_i on Silochroms. 1 = C-1; 2 = C-2; 3 = C-3; 4 = C-4; 5 = C-5.

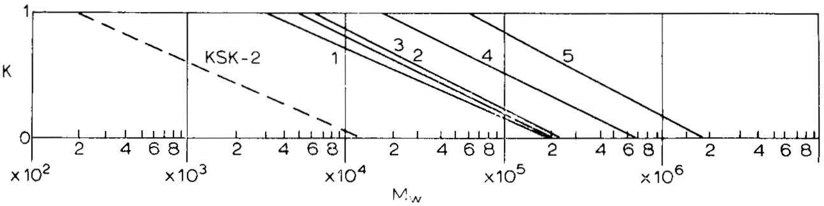

Fig. 3. K as a function of molecular weight of polystyrene for Silochroms. 1 = C-1; 2 = C-2; 3 = C-3; 4 = C-4; 5 = C-5. KSK-2 = silica gel with mean pore diameter of 140 Å used as standard.

of the packing material, and the values of these differences for the Silochroms are close to values V_s given in Table I. A column filled with a single Silochrom is appropriate for a certain range of molecular weights, as seen in Fig. 3 which gives plots of the distribution coefficient K as function of the molecular weight. Values of K for KSK-2 have been taken from ref. 2. A set of columns, filled with three or four Silochroms, can be used to perform fractionation of polydisperse polymers in a range of M from 10^2 to 10^6.

Fig. 4 shows the dependences of H as a function of log M for PS standards on the investigated silicas. The lowest values of H are observed for Silochrom C-3. The Silochrom modified by silane has a more uniform surface, and leads to a more dense packing of particles in the column due to a removal of electric charge from the surface. The presence of charges on the surface leads to loose packing through electrostatic repulsion between the particles. That the values of H are somewhat higher for samples C-4 and C-5 than for samples C-2 and C-1 is possibly due to their wider pores and associated loose packing of particles in the column. All plots of H as function log M are characterised by a maximum, which arises from a drastic reduction of accessible pores as the dimensions of macromolecule coil increase. The maxima for all species of polymer correspond to values of K in the range 0.1–0.2. If

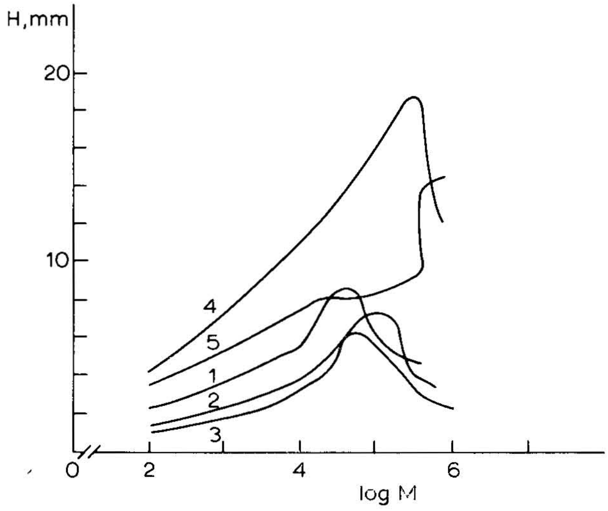

Fig. 4. Dependence of H on log M for narrow fractions of polystyrene on Silochroms. 1 = C-1; 2 = C-2; 3 = C-3; 4 = C-4; 5 = C-5.

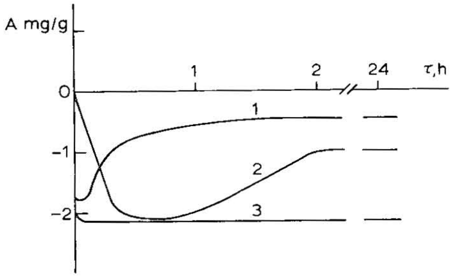

Fig. 5. Diffusion of PS macromolecules with $M_w = 10{,}000$ (1), 97,000 (2) and 498,000 (3) into pores of Silochrom C-2 at 20°. Solvent, toluene.

coil diameter and pore openings are close to each other, the diffusion coefficient is sharply reduced.

Fig. 5 shows the kinetics of adsorption of a narrow fraction of PS with M_w 10,000, 97,000 and 498,000 from solutions in toluene on sample C-2. The figure indicates that PS from solutions in toluene is adsorbed negatively as compared with solutions in ethylbenzene. At first, molecules of toluene quickly diffuse into the pores of the Silochrom particles, and only then do molecules of PS with M_w 10,000 and 97,000 penetrate displacing molecules of solvent from the pore space. The values of the D-coefficients for effective diffusion of macromolecules into pores may be estimated according to the equation of diffusion of adsorbent into spherical particles[11],

$$D = 0.049\, R^2 \tau_{0.5}^{-1} \qquad (2)$$

where R is the radius of Silochrom particle (0.02 cm) and $\tau_{0.5}$ is the time for achieving a value of adsorption equal to half of the equilibrium value.

Due to differences of the r-radii of gyration of macromolecules[12], the rate of diffusion of macromolecules PS with $M_w = 10{,}000$ ($r = 32$ Å) is higher ($D = 2.5 \cdot 10^{-8}$ cm^2/sec) than of PS with $M_w = 97{,}000$ ($r = 120$ Å, $D = 0.36 \cdot 10^{-8}$ cm^2/sec). Macromolecules of PS with $M_w = 498{,}000$ ($r = 300$ Å) can not penetrate into pores. However, they do not prevent the diffusion of toluene molecules into pores of Silochrom with $r = 500$ Å. The value of the diffusion coefficient for toluene calculated from kinetic data is equal to $D_T = 6 \cdot 10^{-7}$ cm^2/sec, as in the presence of macromolecules with $M_w = 97{,}000$ the diffusion of toluene molecules into pores of Silochrom C-2 is slowed down ($D_T = 3 \cdot 10^{-8}$ cm^2/sec).

Table III gives the values of h for n-heptane and the resolution R for two pairs of PS in columns filled with different adsorbents. The data demonstrate that a better separation within the pair of 20,400 and 97,000 PS is achieved on Silochroms with pore diameters from 400–600 Å. This is due to the fact that about 70% of all the pores are accessible to macromolecules of PS 20,400 and about 20% to PS 97,000. For Silochroms with pore dimensions in the range 800–1500 Å, the values of K for PS 20,400 and PS 97,000 are close to unity; the pores are almost identically accessible to both polymers, and hence the difference between the K values is less and resolution is poor. The second pair, PS 97,000–411,000 is separated almost identically on all Silochroms.

TABLE III

COMPARISON OF GPC PARAMETERS FOR VARIOUS SILOCHROMS

Sample	M_w	*h*	*R*
C-1	20.400	3.0	1.02
	97.000	2.7	0.53
	411.000	2.1	
C-2	20.400	2.7	0.94
	97.000	4.5	0.76
	411.000	2.2	
C-3	20.400	3.4	1.1
	97.200	4.7	0.59
	411.000	2.5	
C-4	20.400	3.0	0.43
	97.200	3.9	0.52
	411.000	4.6	
C-5	20.400	2.2	0.30
	97.200	2.2	0.48
	411.000	2.6	

The values of *h* are close to each other for all the Silochroms; however, as expected, the values are smaller for Silochroms with the maximum pores. This could be explained by the pores of Silochrom C-5 being almost identically accessible to molecules of toluene and macromolecules of PS, except PS with $M_w = 411{,}000$, for which *h* is higher, and the coefficient of effective diffusion smaller. The highest values of *h* for each Silochrom are observed in the case where the dimensions of the macromolecule coil and pore are closest; the smallest when the macromolecule coil easily penetrates into pores or does not penetrate at all.

Thus, the analysis of the values of *h* allows us to obtain valuable information on the correlation between the molecular weight of a polymer and the pore sizes of the packing material.

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REFERENCES

1 J. J. Kirkland, *Modern Practice of Liquid Chromatography*, Wiley-Interscience, New York, London, 1971.
2 L. A. Grigoryeva, T. I. Ryabinina and Yu. A. Eltekov, *Visokomolekulyarniye Soyedineniya*, 15A (1973) 238.
3 E. K. Bogacheva, A. V. Kiselev, Yu. S. Nikitin and Yu. A. Eltekov, *Visokomolekulyarniye Soyedineniya*, 10A (1968) 574.
4 Yu. A. Eltekov, in D. H. Everett (Editor), *Surface Area Determination*, Butterworths, London, 1970, p. 259.
5 E. K. Bogacheva and Yu. A. Eltekov, *Visokomolekulyarniye Soyedineniya*, 16A (1974) 617.
6 A. V. Kiselev, T. I. Ryabinina and Yu. A. Eltekov, *Dokl. Akad. Nauk S.S.S.R.*, 200 (1971) 1132.

7 Yu. A. Eltekov, A. V. Kiselev, T. D. Khokhlova and Yu. S. Nikitin, *Chromatographia*, 6 (1973) 187.
8 T. A. Romanova and Yu. A. Eltekov, *Kolloidn. Zh.*, 36 (1974) 905.
9 S. R. Rafikov, S. A. Pavlova and N. I. Tverdokhlebova, *Methods of Determining Molecular Weights and Polydispersity of High Molecular Compounds*, Izdat. Akad. Nauk, Moscow, 1963.
10 N. K. Bebris, A. V. Kiselev, V. Ya. Mokeev, Yu. S. Nikitin and Ya. I. Yashin, *Chromatographia*, 4 (1971) 92.
11 J. Crank, *The Mathematics of Diffusion*, Oxford University Press, Oxford, 1957.
12 G. V. Schulz and H. Baumann, *Macromol. Chem.*, 114 (1968) 122.

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CHROM. 8643

PYROLYSIS GAS CHROMATOGRAPHY AS AN AID TO THE IDENTIFICATION OF *PENICILLIUM* SPECIES

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(Received July 14th, 1975)

SUMMARY

Using pyrolysis gas chromatography it was possible to identify each of a series of eleven *Penicillium* species and the related species *Aspergillus niger* CMI 31821 and *Neurospora crassa* CMI 75723, based on relative peak heights and retention times of the most reproducible peaks in each pyrogram.

INTRODUCTION

The methods currently available for the detection and classification of microorganisms are time consuming and non-quantitative. There have been several attempts to apply physical methods to the identification of bacteria; in particular infrared spectrophotometric methods have been examined by Randall *et al.*[1] and Levine *et al.*[2,3], with limited success in obtaining reproducible results[4]. Partial identification is possible in the case of certain bacteria[5]. The related technique of attenuated total reflection has been examined and the differences between normal and diseased tissues can be demonstrated[6], however, as these spectra provide essentially the same information as transmittance spectra, it is unlikely to be any more successful for identification purposes.

Pyrolysis gas–liquid chromatography (GLC) gives characteristic and reproducible fragment patterns which can be used as fingerprints of the original samples. Reiner[7] applied this technique to bacteria, attempting to exploit the possible chemical differences between bacterial strains of similar antigenic or pathogenic character. He showed that each organism gave a unique pyrogram, although the profiles obtained for *Escherichia coli* and *Shigella boydii* were similar[8]. Subsequently it was shown that such programs could provide a basis for classification at subspecies level[9], in particular the *Salmonella*[10]. Oyama[11] and Oyama and Carle[12] agreed with these observations but showed that the media should be identical when considering different organisms.

The technique of pyrolysis GLC has also been used by Myers and Watson[13] in the diagnosis of viral and fungal diseases in plants and by Hall and Bennett[14] in the identification of cockroaches at species level. The coupling of a mass spectrometer

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to the pyrolysis GLC system has permitted an examination of the chemical structure of pyrolysis products and the potential of the technique for the detection of extra-terrestrial life has been examined by Simmonds[15] and Simmonds *et al.*[16].

Prior pyrolysis followed by silylation of the less volatile polar products allows separation and identification of algae and *E. coli* in sea-water[17]. Combined pyrolysis–mass spectroscopy has been applied by Anhalt and Fenselau for the characterisation of certain pathogenic bacteria[18].

The *Penicillia* are ubiquitous and can grow on a variety of organic substrates[19] and can be responsible for spoilage and economic loss. Several species are important

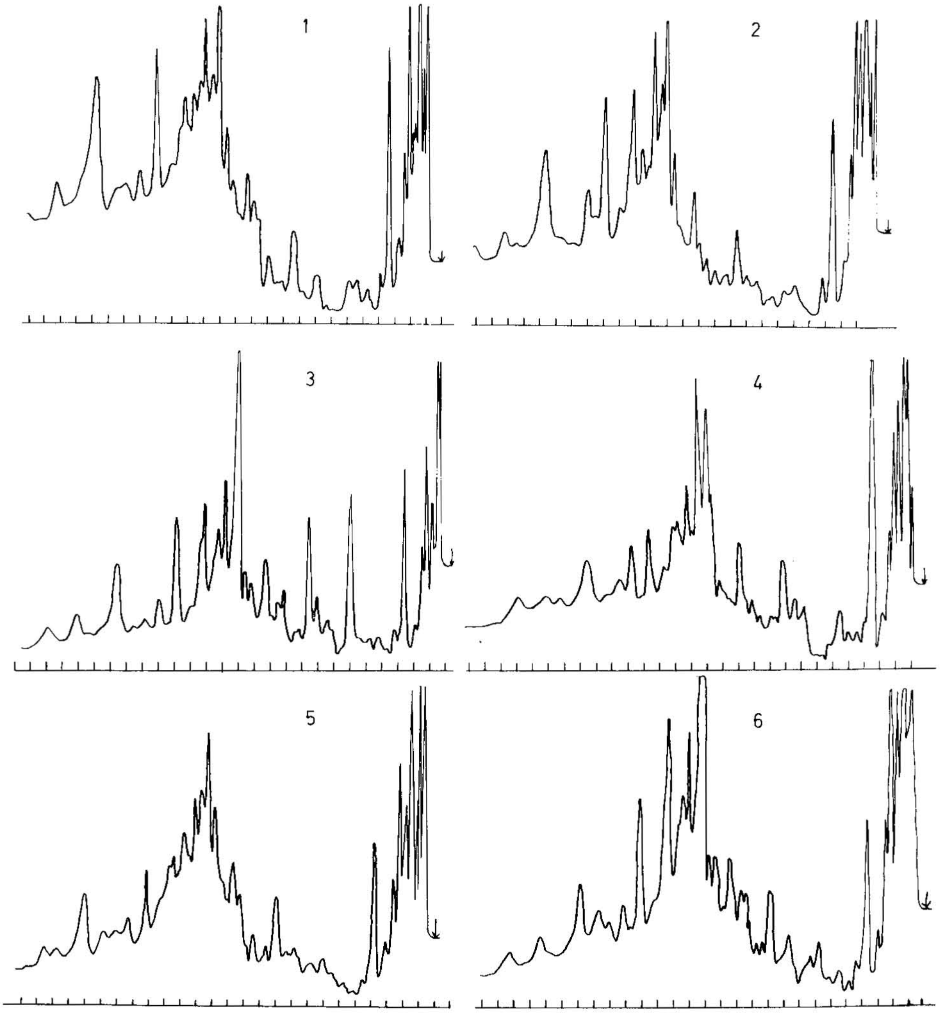

Fig. 1. Pyrograms for *Penicillium* and related species: (1) *P. chrysogenum*, CMI 26211; (2) *P. luteum*, A40; (3) *P. griseofulvum*, A41; (4) *P. lanosoviridae*, CMI 39818; (5) *P. charlesii*, CMI 40232; (6) *P. lavendulum*, CMI 40570; (7) *P. parvum*, CMI 40587; (8) *P. thomii*, CMI 40027; (9) *P. rubrum*, CMI 40036; (10) *P. patulum*, CMI 28808; (11) *P. pulvillorum*, CMI 29215; (12) *Aspergillus niger*, CMI 31821; (13) *Neurospora crassa*, CMI 75723.

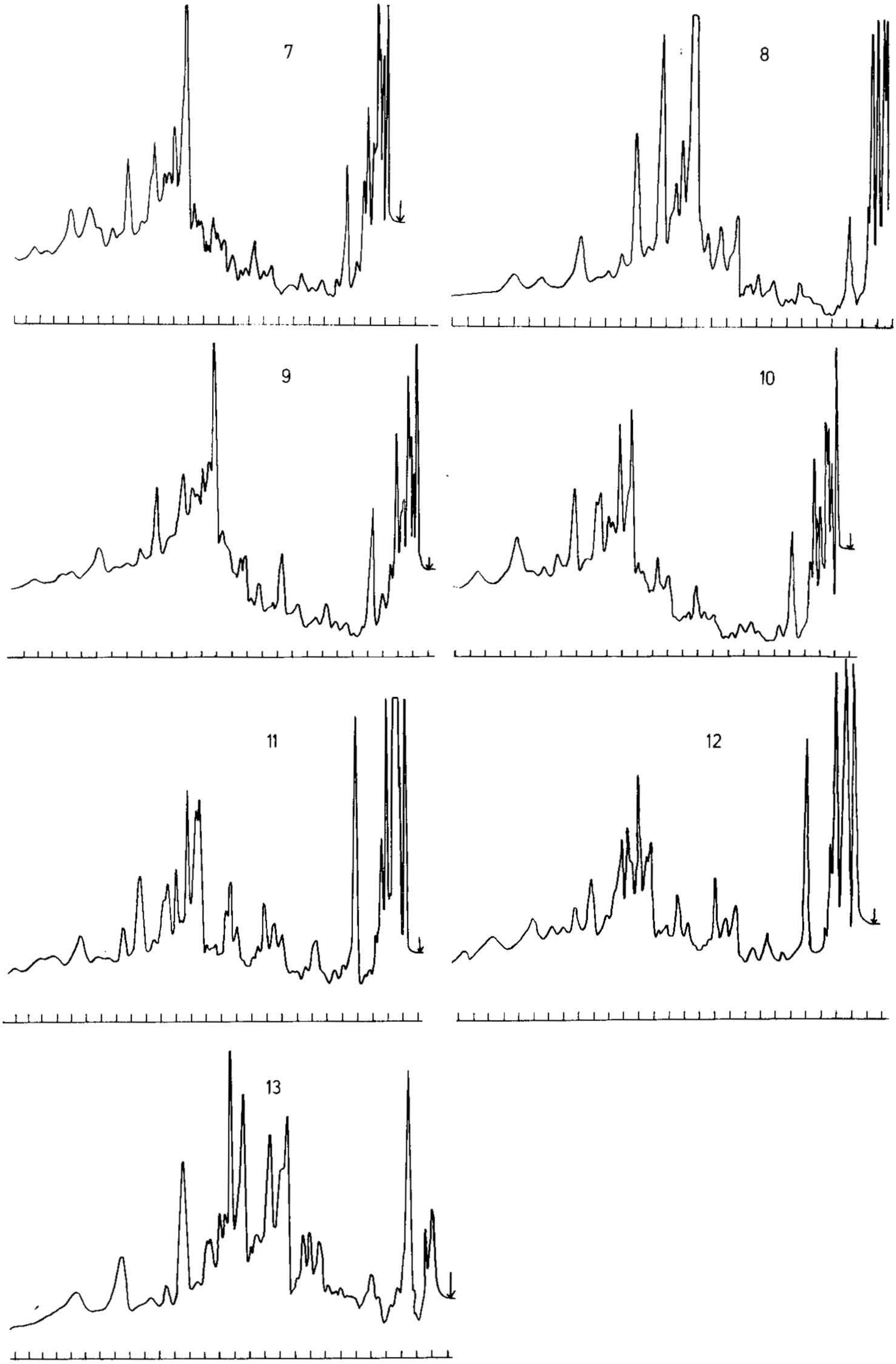

Fig. 1 (*continued*).

TABLE I

RELATIVE PEAK HEIGHTS OF PYROGRAMS OF *P. parvum*

Peak No.	*Relative peak height*						
	1	*2*	*3*	*4*	*5*	*6*	*Range*
1	158.8	122.5	134.3	141.2	160.6	129.8	38.1
2	13.7	10.3	14.6	8.7	9.4	11.5	5.9
3	15.7	13.3	10.8	13.2	10.6	14.2	5.1
4	5.8	7.5	3.8	5.9	8.5	7.6	4.7
5	21.5	23.3	29.2	19.6	27.5	25.2	9.6
6	10.8	11.6	13.2	16.5	9.5	15.2	7.0
7	19.6	25.0	27.3	18.2	24.5	19.8	9.1
8	6.9	8.3	10.2	12.4	9.7	6.5	5.9
9	47.0	50.0	55.5	47.2	51.2	48.1	8.5
10	8.8	15.0	15.8	7.6	9.3	12.1	8.2
11	5.5	5.0	5.6	7.8	9.9	4.9	5.0
12	23.5	31.6	32.1	20.2	24.1	30.1	11.9
13	29.4	35.0	37.2	29.9	38.6	31.3	9.2
14*	35.3	37.5	37.2	34.9	37.0	36.7	2.6
15	23.5	30.0	32.3	23.6	24.2	26.5	8.8
16	29.4	21.6	24.2	26.2	19.8	20.5	9.6
17	311.7	278.3	291.3	279.1	285.2	301.2	33.4
18*	78.4	76.6	77.5	76.8	78.5	76.9	1.9
19	13.7	6.6	12.5	8.1	11.7	9.7	7.1
20	17.6	21.6	22.3	15.6	18.1	19.3	6.7
21*	92.1	88.3	91.1	89.8	88.7	89.7	3.8
22*	100.0	100.0	100.0	100.0	100.0	100.0	0.0
23	18.6	25.0	20.0	15.2	26.1	21.1	10.9
24	11.7	8.3	6.2	12.2	11.0	8.4	6.0
25	32.3	18.3	30.0	19.3	25.2	31.3	14.0
26	43.1	51.6	43.2	57.1	47.2	45.1	14.0
27	3.9	5.8	4.0	5.9	6.2	4.3	2.3
28	10.2	14.3	11.2	12.3	15.1	14.0	4.9
29*	15.3	15.8	15.7	16.1	16.0	15.4	0.8

* Most reproducible peaks.

TABLE II

RELATIVE PEAK HEIGHTS AND AREAS FOR THE MOST REPRODUCIBLE PEAKS IN PYROGRAMS OF *P. parvum*

	Height					*Area*				
	14	*18*	*21*	*22*	*29*	*14*	*18*	*21*	*22*	*29*
	35.3	78.4	92.1	100	15.3	34.6	75.2	91.1	100	16.8
	37.5	76.6	88.3	100	15.8	35.2	75.7	89.4	100	17.3
	37.2	77.5	91.1	100	15.7	37.0	75.0	92.2	100	17.4
	34.9	76.8	89.8	100	16.1	35.5	76.2	90.9	100	16.8
	37.0	78.5	88.7	100	16.0	36.1	75.3	90.7	100	16.9
	36.7	76.9	89.7	100	15.4	35.7	74.9	94.7	100	16.8
Average	36.4	77.3	89.9		15.7	35.6	75.5	91.6		16.9
% Variation	3.6	1.2	2.1		2.5	3.4	0.9	2.9		1.8

agents in food production and in industrial fermentations. The taxonomy of the genus is very complex[20] and because of the paucity of obvious taxonomic characters and the arbitary nature of many of the groupings, the genus remains taxonomically difficult even to the expert[21]. It was considered that the use of pyrolysis GLC could aid in rapid and reproducible identification of this genus and this idea is examined herein.

EXPERIMENTAL

Preparation of samples

The *Penicillium* species studied were: (1) *P. chrysogenum*, CMI 26211, (2) *P. luteum*, A40, (3) *P. griseofulvum*, A41, (4) *P. lanosoviridae*, CMI 39818, (5) *P. charlesii*, CMI 40232, (6) *P. lavendulum* CMI 40570, (7) *P. parvum*, CMI 40587, (8) *P. thomii*, CMI 40027, (9) *P. rubrum*, CMI 40036, (10) *P. patulum*, CMI 28808, and (11) *P. pulvillorum*, CMI 29215. For comparative purposes (12) *Aspergillus niger*, CMI 31821, and (13) *Neurospora crassa*, CMI 75723, were also used.

To remove any possibility of contamination with complex materials, the

TABLE III

RETENTION DATA OF THE COMPONENTS DERIVED FROM *P. parvum*

Peak No.	*Retention time (min)*						
	1	*2*	*3*	*4*	*5*	*6*	*Average*
1	7.5	7.6	7.5	7.8	7.5	7.6	7.6
2	8.4	8.5	8.4	8.5	8.5	8.5	8.5
3	10.4	10.5	10.4	10.5	10.3	10.4	10.4
4	11.5	11.6	11.4	11.4	11.5	11.5	11.5
5	13.0	13.2	13.1	13.3	13.2	13.1	13.2
6	14.3	14.4	14.2	14.5	14.1	14.3	14.3
7	17.0	17.2	17.0	17.4	17.0	17.2	17.1
8	17.8	17.6	17.7	17.9	17.8	17.6	17.7
9	19.2	19.4	19.0	19.4	19.5	19.3	19.3
10	20.3	20.4	20.4	20.5	20.2	20.2	20.3
11	21.2	21.2	21.4	21.5	21.2	21.3	21.3
12	22.2	22.3	22.4	22.5	22.3	22.4	22.4
13	23.2	23.4	23.1	23.5	23.1	23.3	23.4
14	24.6	24.6	24.5	24.9	24.8	24.8	24.7
15	26.5	26.5	26.6	26.8	26.7	26.7	26.6
16	27.3	27.5	27.2	27.5	27.2	27.3	27.3
17	28.2	28.4	28.2	28.5	28.2	28.3	28.3
18	29.9	29.8	30.0	30.0	29.7	29.7	29.7
19	30.5	30.3	30.3	30.2	30.1	30.3	30.3
20	31.2	31.0	31.0	31.4	31.3	31.2	31.2
21	32.5	32.5	32.5	32.5	32.6	32.5	32.5
22	36.0	36.2	36.2	36.4	36.1	36.2	36.2
23	38.2	38.4	38.4	38.2	38.2	38.2	38.3
24	39.9	39.5	39.5	40.0	39.7	40.0	39.8
25	41.4	41.3	41.3	41.3	41.3	41.4	41.3
26	43.7	43.6	43.6	43.8	43.6	43.6	43.6
27	47.2	47.0	47.0	47.2	47.3	47.5	47.2
28	48.6	48.8	48.8	48.7	48.9	48.9	48.7
29	52.5	52.4	52.4	52.1	52.4	52.5	52.4

organisms were grown on a synthetic medium containing: K_2HPO_4, 1 g/l; KCl, 0.5 g/l; $MgSO_4 \cdot 7H_2O$, 0.5 g/l; $FeSO_4$, 0.01 g/l; vitamin-free casamin acids (Difco), 1 g/l, and sucrose, 20 g/l. When solidified media were required Oxoid agar No. 3, 15 g/l, was added. The pH of all media was adjusted to 5.5 prior to use.

The organisms were grown for fourteen days in liquid culture at 25° to obtain mature mycelium, for three days to give young mycelium and for thirty days for production of spores. The spores of *P. chrysogenum* were readily obtained from the surface of cultures grown on solidified media. It proved easier to obtain spores from the other species from liquid culture, by removing the mycelium by filtration, resuspending in water and disrupting the mycelium in a blender. The mycelial strands could then be almost completely removed by filtration through non-adsorbent cotton wool. The filtrate was then subjected to 5 min vibration at 15 kHz. The suspension was then centrifuged to obtain the spores. Microscopic examination revealed the absence of mycelium.

All samples of mycelium and spores were harvested by centrifugation at 4000 *g*, at 4° for 10 min, washed twice with distilled water and dried by lyophilisation.

Apparatus and conditions

Pyrolysis investigations were carried out on 50–80 μg samples of the fungi using a Pye 104 series (Model 64) chromatograph with a P.V.4080 pyrolysis module. As the samples were of a powdery nature the coil method of Thompson[22], in a 2-mm quartz tube, was used, a new ferromagnetic wire and quartz tube being used for each sample. The optimum conditions for analysis, determined in preliminary studies were: A pyrolysis time of 10 sec at 770° was employed throughout. Chromatography was carried out using 10 ft. × 6 mm O.D. dual glass columns packed with 10% Carbowax 20M on 80–100 mesh Phase Separations A.B.S. (Queensferry, Great Britain). The temperature was programmed to increase at a linear rate of 4°/min from 60–140°,

TABLE IV

RELATIVE HEIGHTS OF REPRODUCIBLE PEAKS AND THEIR RETENTION TIMES (min)

No.	*Relative peak height*																						
	7–7.9	*8–8.9*	*9–9.9*	*10–10.9*	*11–11.9*	*12–12.9*	*13–13.9*	*14–14.9*	*15–15.9*	*16–16.9*	*17–17.9*	*18–18.9*	*19–19.9*	*20–20.9*	*21–21.9*	*22–22.9*	*23–23.9*	*24–24.9*	*25–25.9*	*26–26.9*	*27–27.9*	*28–28.9*	*29–29.9*
1													40								37		
2												8	54	9									
3		17																					
4						13			37				13										
5																							
6											21									31	21		
7																		36					76
8											9.5							34					
9					16	11												32					
10								6											42		15		
11								12						13									
12								14			83												
13															29		125		74		11		

initial hold 10 min, final hold infinity. The detector temperature was 250°. The air pressure was 50 lb./sq.in.; the hydrogen pressure, 25 lb./sq.in. and the carrier gas (nitrogen) pressure, 50 lb./sq.in. The nitrogen flow-rate was 40 ml/min. The recorder was set at 1 mV f.s.d.; attenuation, various. The chart speed was 5 mm/min.

RESULTS

Preliminary pyrograms obtained with mature mycelia showed variation in relative peak heights for each species studied. In an attempt to eliminate this variation the three basic components of the fungal colonies —the mature mycelia and young mycelia and the spores— were separated and individually studied. The pyrograms for young mycelia and for spores showed greater variation than for mature mycelia, which were then solely studied but in more detail.

The difficulty in controlling the sample size in replicate experiments made it necessary to use relative rather than absolute values of peak heights and areas. The peak heights and areas were calculated relative to a well resolved peak which appeared in the pyrograms at a retention time of 36 min for all the species studied. No significant difference was found between relative peak height or area measurements (estimated by planimeter) so that peak heights were used after the initial studies were completed. The peak heights in each case were measured above a base line contructed by linear interpolation between valleys.

Typical pyrograms for each species studied are shown in Fig. 1.

Each mature mycelium was examined in six replicates. Results showing relative peak heights for *P. parvum* are shown in Table I, relative peak areas and peak heights for the peaks with the most reproducible heights/areas are given in Table II and the retention data in Table III.

The relative peak heights for the most reproducible peaks for the series of species examined is given in Table IV.

30–30.9	*31–31.9*	*32–32.9*	*33–33.9*	*34–34.9*	*35–35.9*	*36–36.9*	*37–37.9*	*38–38.9*	*39–39.9*	*40–40.9*	*41–41.9*	*42–42.9*	*43–43.9*	*44–44.9*	*45–45.9*	*46–46.9*	*47–47.9*	*48–48.9*	*50–50.9*	*51–51.9*	*52–52.9*	*53–53.9*	*54–54.9*	*55–Inf.*
						100																		
						100																		
						100		23					63					21						
						100							61											
80	55					100		29		90														
						100												15						
		90				100															16			
						100																		
48	28					100															7			
	13					100		23		12														
						100																		
225						100				10														
						100																		

DISCUSSION

The taxonomy of the important genus, *Penicillium*, is extremely complex[20]. Most species exhibit shades of green which change as the colonies age and, in addition, vary with cultural conditions. Their fruiting (spore bearing) structures are small and disintegrate with age, the asexual spores are among the smallest in true fungi and only rarely show distinctive features. Sexual states, if occurring at all, may require months to reach fruition. The most definitive work is by Raper and Thom[19], but because of the paucity of obvious taxonomic characters and the arbitrary nature of many of the manual's groupings the genus remains taxonomically difficult[21].

Such problems suggest the need for alternative approaches to classification and species identification. Hence a chemotaxonomic approach which has been useful in identification of other materials of biological origin was considered.

The data given in Table IV show that it is possible to clearly distinguish between eleven *Penicillia* and the two related species *Aspergillus niger* CMI 31821 and *Neurospora crassa* CMI 75723 by pyrolysis GLC using mature lyophillised mycelia. Each pyrogram contained reproducible peaks, amongst the more variable ones, with characteristic relative heights and retention times.

The existence of the reproducible peaks in relatively non-reproducible pyrograms was an interesting feature of the results. This effect is in contrast to those obtained with bacteria where a high degree of reproducibility of the pyrograms was reported[7-9]. This feature might not be unexpected on the basis of the varied metabolic activities going on in different areas of the mycelium, *e.g.* the production of secondary metabolites causes the chemical composition to vary depending on the "physiological age" of the mycelial strand and the age of the culture[23]. Although many workers have emphasised the importance of the "physiological age"[24-26], very little cytochemical differentiation has been reported on the mycelia of fungi.

For identification purposes mature mycelia were found to be necessary, as pyrograms containing young and mature mycelia showed great variability in relative peak heights.

For spores a greater uniformity in chemical composition would be expected. However, pyrograms for individual species showed too great a variability to be used for identification purposes. This effect might be due to physiological age; the only feasible method of spore separation does not discriminate on age.

Work is currently being carried out in the identification of *Aspergillus* species and to examine the chemical origin of the reproducible peaks within a species.

REFERENCES

1 H. M. Randall, D. W. Smith, A. C. Colm and W. J. Nungester, *Amer. Rev. Tuberc.*, 63 (1951) 373.
2 S. Levine, H. J. R. Stevenson and R. H. Bordner, *Science*, 118 (1953) 141.
3 S. Levine, H. J. R. Stevenson, L. A. Chambers and B. A. Kenner, *J. Bacteriol.*, 65 (1953) 10.
4 J. W. Riddle, P. W. Kabler, B. A. Kenner, R. H. Bordner, S. W. Rockwood and H. J. R. Stevenson, *J. Bacteriol.*, 72 (1956) 593.
5 G. Chatot, J. P. Murgat, J. F. Grollier and R. Fontages, *Methodes Phys. Anal.*, 4 (1968) 279.
6 F. S. Parker and R. Ans, *Anal. Biochem.*, 18 (1967) 414.
7 E. Reiner, *Nature (London)*, 206 (1965) 1272.
8 E. Reiner and W. H. Erwing, *Nature (London)*, 217 (1968) 191.

9 E. Reiner, *J. Gas Chromatogr.*, 5 (1967) 65.
10 E. Reiner, J. J. Hicks, M. M. Ball and W. J. Martin, *Anal. Chem.*, 44 (1972) 1058.
11 I. Oyama, *Nature (London)*, 200 (1963) 1058.
12 I. Oyama and G. C. Carle, *J. Gas Chromatogr.*, 5 (1967) 151.
13 A. Myers and L. Watson, *Nature (London)*, 223 (1969) 964.
14 R. C. Hall and G. W. Bennett, *J. Chromatogr. Sci.*, 11 (1973) 439.
15 P. G. Simmonds, *Appl. Microbiol.*, 20 (1970) 567.
16 P. G. Simmonds, G. P. Schulman and C. H. Stembridge, *J. Chromatogr. Sci.*, 7 (1969) 36.
17 J. B. Derenbach and M. Ehrhardt, *J. Chromatogr.*, 105 (1975) 339.
18 J. P. Anhalt and C. Fenselau, *Anal. Chem.*, 47 (1975) 219.
19 K. B. Raper and C. Thom, *A Manual of Penicillia*, Williams and Wilkins, Baltimore, 1949.
20 G. Smith, *An Introduction to Industrial Mycology*, Edward Arnold, London, 6th ed., 1969, p. 172.
21 J. I. Pitt, *Mycologia*, 65 (1973) 1135.
22 W. C. Thompson, *Lab. Pract.*, 18 (1969) 1074.
23 G. Ainsworth and A. Sussman, *The Fungi*, Vol. II, Academic Press, London, 1966, p. 474.
24 A. A. Eddy and D. H. Williamson, *Nature (London)*, 179 (1957) 1252.
25 G. C. Madonza and J. R. Villanueva, *Nature (London)*, 195 (1962) 1326.
26 G. Shihla, F. Schlenk and J. L. Dainko, *J. Bacteriol.*, 82 (1961) 808.

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TYPES OF ADSORBENT AND RELATIONSHIPS BETWEEN R_M VALUES OF A SUBSTANCE AND THE COMPOSITION OF THE MOBILE PHASE IN THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Relationships between R_M values of substances and the adsorption data for the components of the mobile phase and its composition were investigated, using silica gel G and aluminium oxide G as adsorbents. The substances chromatographed were quinoline derivatives, fluorenone and carbazole. The mobile phases were benzene–chloroform and benzene–acetone mixtures.

The results showed good agreement between the theoretical and experimental relationships $R_{M_{1,2}} = \mathrm{f}(\varphi_1)$ (where φ_1 is the composition of the mobile phase) on the two adsorbents. The results indicate that it may be possible to extend the interpretation of the chromatographic process and to predict the optimal conditions for the separation of mixtures on different types of adsorbents to a greater extent than has been permitted so far.

INTRODUCTION

The wide application of thin-layer chromatography (TLC) in the direct control of chemical processes in industry and in the laboratory necessitates investigations that would permit the rapid and accurate prediction of the optimal separation conditions and the correct interpretation of the chromatographic process. So far, the most interesting theoretical investigations in this field have been those by Snyder[1–3], Turina *et al.*[4], Soczewiński and co-workers[5–7] and Ościk and co-workers[8–11].

The theory deduced by Ościk and co-workers, based on the thermodynamics of the process of adsorption from solution, permits a relationship to be derived between certain thermodynamic values and chromatographic parameters. For this reason, this theory was chosen as the starting point in the present investigations.

The equation that results from this theory, which has been discussed earlier[10–16], permits the R_M values of the chromatographed substances to be related simply to the adsorption data of the components of the liquid mobile phase and to the composition of the phase:

$$R_{M_{1,2}} = \varphi_1 \Delta R_{M_{1,2}} + (\varphi_1^s - \varphi_1)(\Delta R_{M_{1,2}} + A_z) + R_{M_2} + Y \qquad (1)$$

where R_M is related to the R_F value by the equation

$$R_M = \log\left(\frac{1 - R_F}{R_F}\right) \tag{2}$$

$R_{M_{1,2}}$ represents the R_M value of a substance z obtained by using a two-component solvent mixture consisting of solvents 1 and 2; R_{M_1} and R_{M_2} represents the R_M values of the substance z obtained when using pure solvents 1 and 2, respectively, as the mobile phase, so that

$$\Delta R_{M_{1,2}} = R_{M_1} - R_{M_2} \tag{3}$$

φ_1 represents the volume fraction of solvent 1 in the two-component solvent mixture in the mobile phase and φ_1^s represents the volume fraction of solvent 1 of the two-component solvent mixture in the surface phase. Hence the difference $\varphi_1^s - \varphi_1$ expresses the adsorption of the components of the binary mobile phase, and can be determined experimentally from the adsorption isotherms of the mobile phase components. By defining the distribution function of the components of the mobile phase as

$$K_1 = \frac{\varphi_1^s (1 - \varphi_1)}{\varphi_1 (1 - \varphi_1^s)} \tag{4}$$

we obtain

$$\varphi_1^s - \varphi_1 = \frac{\varphi_1 (K_1 - 1)(1 - \varphi_1)}{1 + (K_1 - 1)\varphi_1} \tag{5}$$

When using ideal or conformal (regular) mixtures as the mobile phase, it can be assumed that

$$-\log K_1 = \Delta R_{M_{1,2}} \tag{6}$$

It is then possible to estimate the distribution function, K_1, from the chromatographic data of the substance using pure solvents as the mobile phase. Thus, from eqn. 5 it is possible to calculate the difference $\varphi_1^s - \varphi_1$ for each component of the mobile phase, without using adsorption isotherm data.

A_z in eqn. 1 represents $\log k_{1,2}^{\infty}$, where $k_{1,2}^{\infty}$ is the hypothetical partition coefficient of substance z between components 1 and 2 of the binary solvent mixture. This coefficient represents the interaction between the molecules of substance z and those of the components of the solvent mixture (mobile phase).

Y is a value that depends on the type of substance being chromatographed and on the mobile phase components. For ideal mobile phases it is zero. For mobile phases that represent conformal (regular) solutions, Y is small for each mobile phase composition and can be disregarded.

A detailed discussion and the means of using the above equations were given in earlier papers[10-15]. The agreement between theoretical values estimated by means of this equation and experimental values was demonstrated for systems of mixed ideal or conformal solvents used as mobile phases. All measurements were carried out on silica gel as adsorbent.

Generalization of the above theoretical considerations will be possible after extending the investigation to adsorbents that differ considerably in their properties. Of course, when using liquid mobile phases that are not ideal solutions, values such as the distribution function K_1 and the partition coefficient $k_{1,2}^{\infty}$ in a given case cannot possibly have the same physical meaning as that for ideal systems. Earlier considerations[14,15] permit the assumption that quantitative analysis of the parameters in eqn. 1 will also allow the analysis of a chromatographic process by using solvents that have properties different from those of ideal solutions.

The mechanism of adsorption in chromatography on aluminium oxide differed from that observed on silica gel. Aluminium oxide is the most widely used adsorbent, apart from silica gel, for the chromatographic analysis of petroleum products[17]. As a result of structural differences between aluminium oxide and silica gel[18], alcohols, for example, may be weaker eluents on silica gel than on aluminium oxide in comparison with other solvents.

The above considerations prompted investigations aimed at comparing the theoretical and experimental relationships between the R_M values of a substance and the composition of the mobile phase on two different adsorbents, *viz.*, aluminium oxide and silica gel.

EXPERIMENTAL

Experimental functions $R_{M_{1,2}} = f(\varphi_1)$ were obtained by measuring the R_F values of substances (Table I) by ascending TLC. Silica gel G and aluminium oxide G for TLC (activity I) (Merck, Darmstadt, G.F.R.) were used as adsorbents. The model substances chromatographed were quinoline, isoquinoline, 2-methylquinoline, 4-methylquinoline and fluorenone (class B) and carbazole (class AB) according to the classification of Pimentel and McClellan[19], each having electron-donor properties. Benzene–chloroform (N + A) and benzene–acetone (N + B) mixtures were used as mobile phases. The solvent systems are relatively simple, especially when the polar component is non-associated. The influence of the composition of the mobile phase on the adsorption of the chromatographed substance will depend to some extent on the solvation constant in this phase, and the actual situation in such a chromatographic system may be different from that in a known model system when theoretical parameters are predicted. The above liquid mobile phase systems are those most often used in adsorption chromatography, which is why they were chosen for these experiments.

Chromatography was carried out in the usual way, as described in detail elsewhere[12,13,15,20,21]. The results are presented as graphs of $R_{M_{1,2}} = f(\varphi_1)$ obtained theoretically on the basis of the eqn. 1 (solid and broken lines) and experimentally (points).

The values of the distribution function K_1, characteristic of the adsorption of the components of the liquid mobile phase, were found by assuming that $-\log K_1 = \Delta R_{M_{1,2}}$. This value permitted, in turn, the difference $\varphi_1^s - \varphi_1$ to be found[12–15]. Suitable values of $A_z = \log k_{1,2}^{\infty}$, were chosen for the calculated function $R_{M_{1,2}} = f(\varphi_1)$ (ref. 10). The values of A_z give an idea of the molecular interactions between the molecules of substance z and those of the components of the solvents.

TABLE I

R_F VALUES OF SOLUTES CHROMATOGRAPHED ON ALUMINIUM OXIDE AND SILICA GEL

Mobile phases: benzene–chloroform and benzene–acetone.

Solute	*C_6H_6–$CHCl_3$*			*C_6H_6–$(CH_3)_2CO$*		
	Vol. fraction of $CHCl_3$	*R_F*		*Vol. fraction of $(CH_3)_2CO$*	*R_F*	
		Al_2O_3	*SiO_2*		*Al_2O_3*	*SiO_2*
Quinoline	0.0	0.30	0.03	0.0	0.30	0.03
	0.1	0.34	0.04	0.1	0.69	0.19
	0.3	0.38	0.06	0.3	0.87	0.58
	0.5	0.40	0.07	0.5	0.91	0.69
	0.7	0.50	0.07	0.7	0 91	0 75
	0.9	0.52	0.07	0.9	0.91	0.75
	1.0	0.56	0.07	1.0	0.91	0.70
Isoquinoline	0.0	0.14	0.01	0.0	0.14	0.01
	0.1	0.20	0.04	0.1	0.14	0.01
	0.3	0.28	0.07	0.3	0.74	0.52
	0.5	0.33	0.08	0.5	0.79	0.65
	0.7	0.32	0.09	0.7	0.80	0.71
	0.9	0.43	0.08	0.9	0.78	0.71
	1.0	0.37	0 08	1.0	0.74	0.67
2-Methylquinoline	0.0	0.32	0.02	0.0	0.32	0.02
	0.1	0.42	0.04	0.1	0.69	0.22
	0.3	0.46	0.07	0.3	0.87	0.62
	0.5	0.53	0.07	0.5	0.89	0.76
	0.7	0.56	0.08	0.7	0.90	0.84
	0.9	0.57	0.09	0.9	0.86	0.81
	1.0	0.61	0.09	1.0	0.79	0.76
4-Methylquinoline	0.0	0.16	0.02	0.0	0.16	0.02
	0 1	0.28	0.04	0.1	0.63	0.17
	0.3	0.35	0.04	0.3	0.83	0.50
	0.5	0.40	0.07	0.5	0.88	0.59
	0.7	0.42	0.05	0.7	0.85	0.68
	0.9	0.38	0.05	0.9	0.84	0.69
	1.0	0.50	0.05	1.0	0.79	0.68
Fluorenone	0.0	0.59	0.28	0.0	0.59	0.28
	0.1	0.73	0.38	0.1	0.66	0.43
	0.3	0 81	0.48	0.3	0.78	0.65
	0.5	0.75	0.54	0.5	0.85	0.77
	0.7	0.75	0.57	0.7	0.90	0.88
	0.9	0.80	0.59	0.9	0.93	0.91
	1.0	0.91	0.59	1.0	0.93	0.93
Carbazole	0,0	0.60	0.48	0.0	0.60	0.48
	0,1	0.70	0.51	0.1	0.66	0.65
	0.3	0.77	0.59	0.3	0.77	0.82
	0.5	0.78	0.62	0.5	0.84	0.88
	0.7	0.79	0.62	0.7	0.85	0.93
	0.9	0.77	0.68	0.9	0.88	0.94
	1.0	0.76	0.68	1.0	0.87	0.95

TABLE II

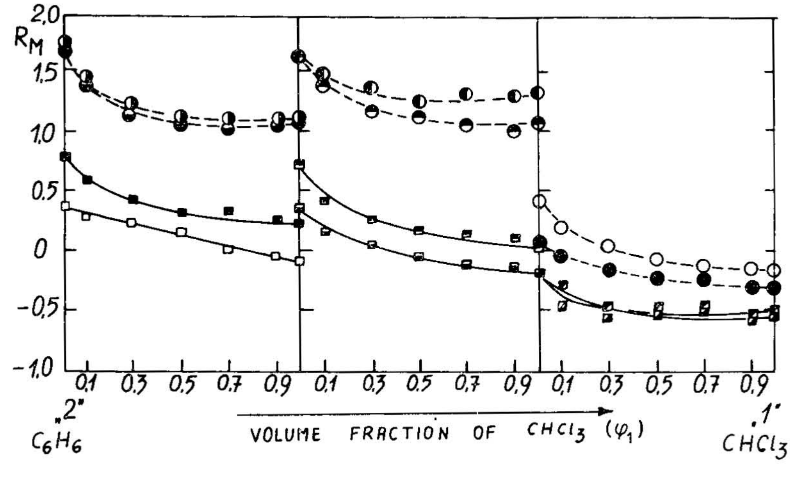
VALUES OF A_z AND K_1 OF SOLUTES CHROMATOGRAPHED ON ALUMINIUM OXIDE AND SILICA GEL AS ADSORBENTS

Mobile phases: benzene–chloroform and benzene–acetone.

Solute	C_6H_6–$CHCl_3$				C_6H_6–$(CH_3)_2CO$			
	A_z		K_1		A_z		K_1	
	Al_2O_3	SiO_2	Al_2O_3	SiO_2	$AlsO_3$	SiO_2	Al_2O_3	SiO_2
Quinoline	+0.62	−0.43	+3.3	+3.5	−0.23	0.0	+16.0	+81.22
Isoquinoline	−0.21	−0.56			−0.50	+0.51		
2-Methylquinoline	−0.06	0.92			−0.25	−0.04		
4-Methylquinoline	−0.26	−0.54			−0.67	+0.29		
Fluorenone	−1.32	−0.16			0.0	+1.23		
Carbazole	−1.88	+0.18			−0.69	+0.87		

RESULTS AND DISCUSSION

The experimental results show two cases, when the mixed mobile phase consists of an active solvent of either A or B type plus an N-type solvent. In N + B systems, solvation effects may also be observed, while the interaction of acetone with the adsorbent surface (silica) may result in complexes with surface hydroxyl groups[22].



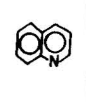
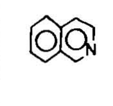
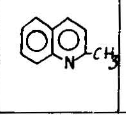
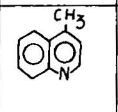
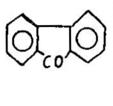
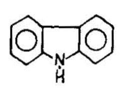
Al_2O_3	□	■	⬓	⬒	◩	◪
SiO_2	◑	◒	◓	◐	○	●
SOLUTE						

Fig. 1. Theoretical (solid and broken lines) and experimental (points) $R_{M_{1,2}} = f(\varphi_1)$ relationships for quinoline, isoquinoline, 2-methylquinoline, 4-methylquinoline, fluorenone and carbazole obtained on aluminium oxide (squares) and silica gel (circles). Mobile phase: benzene–chloroform.

In agreement with Snyder[23], for molecular complexes in the liquid phase between molecules of a solvent and molecules of a chromatographed substance, the energy of interaction will not be determined by the adsorption energy of the substance but by the adsorption energy of the complex formed.

Soczewiński and Matysik[24] reported that quinoline derivatives used as model substances do not form hydrogen bonds with ketone (acetone) molecules. However, this does not mean that in the systems examined interactions of another type might not occur.

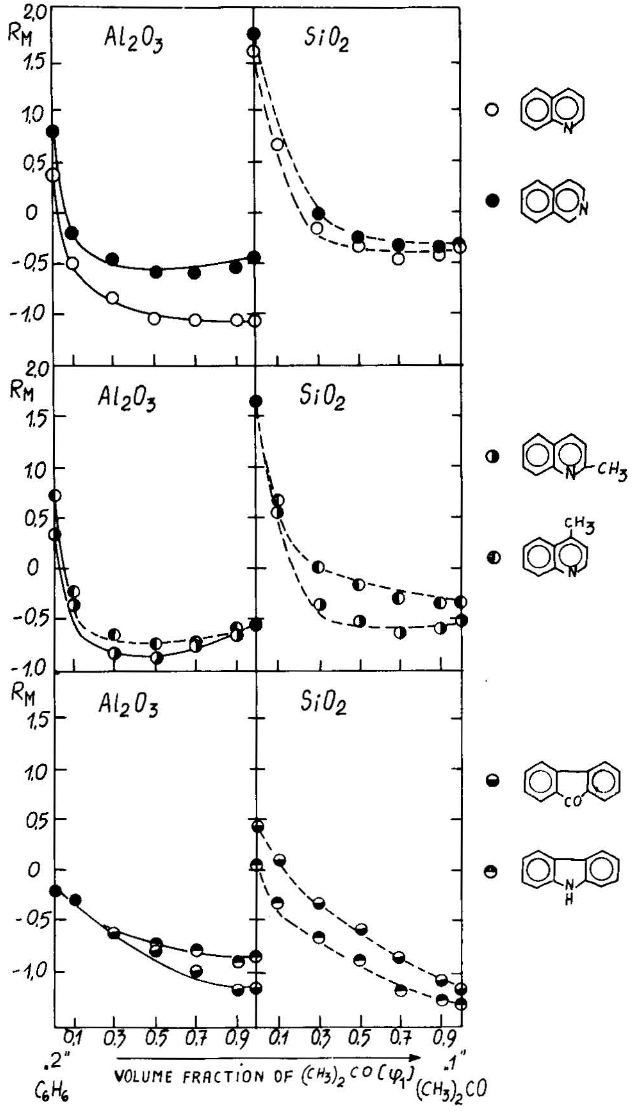


Fig. 2. Theoretical (solid and broken lines) and experimental (points) $R_{M1,2} = f(\varphi_1)$ relationships for quinoline, isoquinoline, 2-methylquinoline, 4-methylquinoline, fluorenone and carbazole obtained on aluminium oxide and silica gel. Mobile phase: benzene–acetone.

In the methyl-substituted quinolines examined, mesomeric effects can also occur, although it seems that steric effects prevail with 2-methylquinoline. This is shown by the different values of A_z for this substance (Table II) in comparison with those for other quinoline derivatives. The negative values of A_z observed for the substances examined illustrate the stronger interactions of the substances with the less active component of the mobile phase. It is interesting that these values do not depend on the type of adsorbent.

For the benzene–chloroform mobile phase, the values of the adsorption equilibrium constant (distribution function) are very close on both adsorbents (K_1 = 3.3–3.5), while for the benzene–acetone mobile phase the values differ widely (16.0 on aluminium oxide and 81.22 on silica gel).

Fig. 1 shows the relationships $R_{M_{1,2}} = f(\varphi_1)$ for the quinoline derivatives and fluorenone and carbazole obtained on the two adsorbents with benzene–chloroform as the mobile phase. There is a fairly good agreement between the experimental and theoretical relationships on both adsorbents. For the quinoline derivatives, both the theoretical and experimental relationships differ largely in the R_M values of the substances examined. However, the shapes of the curves are similar.

A comparison of the $R_{M_{1,2}} = f(\varphi_1)$ relationships and the values of K_1 on the adsorbents used indicates a close similarity between the adsorption processes undergone by the molecules of the mobile phase in the two chromatographic systems. Similar shapes of the theoretical and experimental $R_{M_{1,2}} = f(\varphi_1)$ functions were observed when using fluorenone and carbazole.

A different shape of the theoretical and experimental $R_{M_{1,2}} = f(\varphi_1)$ relationships can be seen in Fig. 2, which shows that the functions differ on the two adsorbents with regard to both R_M values and shape. The benzene–acetone mobile phase shows the presence of more complex and stronger molecular interactions, the change in the molecular interactions being indicated by the very different $\Delta R_{M_{1,2}}$ values for the substances.

The theoretical and experimental results indicate the possibility of extending the use of the equation to a larger number of adsorbents. It is hoped that eqn. 1 may be used for the prediction of optimal separation conditions of mixtures of substances and for the interpretation of the chromatographic process to a greater extent than that suggested by current investigations.

REFERENCES

1 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York 1968.
2 L. R. Snyder and D. L. Saunders, *J. Chromatogr.*, 44 (1969) 1.
3 L. R. Snyder, *Advan. Chromatogr.*, 4 (1967) 3.
4 S. Turina, M. Trbojević and M. Kaštelan-Macow, *Anal. Chem.*, 46 (1974) 988.
5 E. Soczewiński and M. Ciszewska, *J. Chromatogr.*, 96 (1974) 163.
6 E. Soczewiński and W. Gołkiewicz, *Chromatographia*, 5 (1972) 431; 5 (1972) 594; 6 (1973) 269.
7 E. Soczewiński and H. Szumiło, *J. Chromatogr.*, 81 (1973) 99.
8 J. Ościk, *Rocz. Chem.*, 31 (1957) 621; 34 (1960) 745.
9 J. Ościk, *Przem. Chem.*, 40 (1965) 279; 46 (1967) 593; 44 (1965) 129.
10 J. Ościk and J. K. Różyło, *Chromatographia*, 4 (1971) 516.
11 J. Ościk and G. Chojnacka, *J. Chromatogr.*, 93 (1974) 167.
12 J. K. Różyło, *J. Chromatogr.*, 93 (1974) 177.
13 J. K. Różyło, *Chem. Anal. (Warsaw)*, 19 (1974) 1167.

14 J. K. Różyło, *Chromatographia*, 8 (1975) 390.
15 J. K. Różyło, *Chem. Anal. (Warsaw)*, 20 (1975) 489.
16 E. Soczewiński, W. Gołkiewicz and H. Szumiło, *J. Chromatogr.*, 45 (1969) 1.
17 L. R. Snyder, *J. Chromatogr.*, 5 (1961) 430; 6 (1961) 22; 8 (1962) 178.
18 L. R. Snyder, *J. Chromatogr.*, 25 (1966) 274.
19 G. C. Pimentel and A. L. McClellan, *The Hydrogen Bond*, Freeman, San Francisco, 1961.
20 A. Waksmundzki and J. K. Różyło, *J. Chromatogr.*, 33 (1968) 90; 33 (1968) 96.
21 J. K. Różyło, *Wiad. Chem.*, 4 (1968) 301.
22 J. Ościk, R. Nasuto and S. Ząbek, *Folia Soc. Sci. Lublin., Sect. C*, Vol. 7/8 (1967/68) 49.
23 L. R. Snyder, in C. N. Reilley (Editor), *Advances in Analytical Chemistry and Instrumentation*, Vol. 3, Interscience, New York, 1964, p. 251.
24 E. Soczewiński and G. Matysik, *VIth International Symposium on Chromatography and Electrophoresis, Brussels, 1970*, Presses Académiques Européennes, Brussels, 1971, p. 203.

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COMPARISON OF THEORETICAL AND EXPERIMENTAL R_M VALUES IN THIN-LAYER ADSORPTION CHROMATOGRAPHY ON SILICA GEL ADSORBENTS OF DIFFERENT POROSITY

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SUMMARY

The agreement between theoretical and experimental $R_{M_{1,2}}$ values obtained for some aromatic compounds by thin-layer adsorption chromatography has been examined in relation to the pore diameter (60–1000 Å) of the silica gel adsorbents used. The greatest changes in the parameters considered take place when the pore diameter does not exceed 200 Å, above this value the changes are slight. Fairly good agreement between values calculated from the theoretical relation $R_{M_{1,2}} = f(\varphi_1)$ and experimental $R_{M_{1,2}}$ values has been obtained for all of the adsorbents examined.

INTRODUCTION

Mathematical models which take into account the properties of chromatographic systems are useful in predicting optimum separation conditions[1–10]. Theoretical relations for mixed-solvent systems give best agreement with experimental results if the solvents form ideal solutions, *i.e.*, those without selective molecular interactions[5,6,11–14], otherwise complications may occur[4,8,15]. Ościk[4,5,16–18] derived such a relation based on the thermodynamic theory of adsorption:

$$R_{M_{1,2}} = \varphi_1 \Delta R_{M_{1,2}} + (\varphi_1^s - \varphi_1)(\Delta R_{M_{1,2}} + A_z) + R_{M_2} + Y \qquad (1)$$

The definition and method of calculation of the quantities in eqn. 1 can be found in the preceding[19] and earlier[5,11,12,14] papers. The equation has been found to be applicable even for non-ideal (interacting) solvent systems.

Many papers stress the role of the adsorbent in thin-layer adsorption chromatography (TLAC)[3,11–15,20–22]. The porosity of the adsorbent is among the factors which affect its interaction with solutes and solvents and thus the chromatographic mobility of compounds.

EXPERIMENTAL

Experimental R_F values (Table I) obtained by TLAC served as a basis for calculating R_M values on four silica gel adsorbents of a different pore diameter (E. Merck, Darmstadt, G.F.R.). The mobile phase was carbon tetrachloride–chloroform (N + A)[23]. The lack of hydrogen-bond interactions is a characteristic of this system. The compounds investigated were 1-naphthol and 2-naphthol (class AB, according to the classification of Pimentel and McClellan[23]), quinoline (class B), 8-hydroxyquinoline (class AB), 8-methylquinoline (class B), fluorenone (class B) and carbazole (class AB). The chromatographic process was carried out by the ascending technique as described previously[11,12,24,25]. The spots were detected with ultraviolet (UV) light or with iodine vapour.

The results of the measurements are given as graphs of the theoretical relation $R_{M_{1,2}} = \mathrm{f}(\varphi_1)$ calculated from eqn. 1 (solid and broken lines) and as the experimental points.

Values of K_1[11–15], which are characteristics of the adsorption of the components of the mobile phase and are required for finding the difference $\varphi_1^s - \varphi_1$, were obtained by assuming that $-\log K_1 = \Delta R_{M_{1,2}}$. The values of K_1 and $\Delta R_{M_{1,2}}$ are given on the graphs as functions of the pore diameters of the adsorbents, *d*, *i.e.* as $K_1 = \mathrm{f}(d)$ and $\Delta R_{M_{1,2}} = \mathrm{f}(d)$. The quantity $A_z = \mathrm{f}(d)$ is also given.

RESULTS AND DISCUSSION

The solvent mixture, carbon tetrachloride–chloroform, used in this study as the mobile phase, can be included in systems in which interactions of the hydrogen-bond type are absent[26]. Soczewiński and Szumiło[8,27] suggested that such systems are the most suitable for theoretical considerations because of the small extent of molecular interaction between their components. These systems have no ability for association. In some cases the effect of the composition of the solvent mixture on the "chromatographic adsorption coefficient" is proportional to the solvation equilibria present in the mobile phase[27] and the actual situation is different from that in an ideal model system.

In a series of earlier papers[11–15], the possibility of interpretation and prediction of optimum conditions for the chromatographic process was examined on adsorbents of different specific surface area and using eqn. 1. In some cases satisfactory results were obtained, but in others deviations occurred between theoretical and experimental $R_{M_{1,2}}$ values. The deviations were difficult to explain. They may be caused by a molecular-sieve effect or by failure to account correctly for the microporous structure of the adsorbent. In the present study, silica gel adsorbents with a different porosity, ranging from 60 to 1000 Å in diameter, were used. Other studies have been made of the properties of adsorbents in relation to porosity[2,3] and the number of hydroxyl groups present.

Fig. 1 shows the theoretical and experimental values of $R_{M_{1,2}}$ for TLAC of 1- and 2-naphthols. A fairly good agreement between these values was obtained for all four adsorbents (L,M,N,and O). The values of R_{M_1}, R_{M_2} and $R_{M_{1,2}}$ for the naphthols change with the porosity of the adsorbent. A similar agreement between the theoretical and experimental values was obtained for 8-aminoquinoline and 8-hydroxyquinoline (Fig. 2). It is interesting that the extent of separation of the examined sub-

TABLE I

EXPERIMENTAL R_F VALUES OBTAINED BY TLAC ON SILICA GEL ADSORBENTS OF DIFFERENT POROSITY (*d*)

Mobile phase, carbon tetrachloride–chloroform.

Compound	*Vol. fraction of chloroform*	*Adsorbent*			
		L (*d* = 60 *Å*)	*M* (*d* = 200 *Å*)	*N* (*d* = 500 *Å*)	*O* (*d* = 1000 *Å*)
1-Naphthol	0	0.03	0.09	0.12	0.22
	0.1	0.07	0.21	0.23	0.40
	0.3	0.12	0.36	0.40	0.61
	0.5	0.18	0.45	0.52	0.71
	0.7	0.26	0.50	0.58	0.75
	0.9	0.33	0.53	0.61	0.71
	1.0	0.32	0.50	0.60	0.66
2-Naphthol	0	0.02	0.05	0.08	0.14
	0.1	0.04	0.14	0.18	0.31
	0.3	0.08	0.25	0.32	0.52
	0.5	0.14	0.35	0.44	0.65
	0.7	0.20	0.43	0.51	0.69
	0.9	0.27	0.49	0.54	0.65
	1.0	0.26	0.46	0.54	0.57
Quinoline	0	0.02	0.05	0.10	0.10
	0.1	0.04	0.17	0.18	0.20
	0.3	0.11	0.29	0.27	0.37
	0.5	0.17	0.38	0.37	0.50
	0.7	0.21	0.46	0.46	0.56
	0.9	0.27	0.54	0.51	0.56
	1.0	0.24	0.44	0.52	0.54
8-Hydroxyquinoline	0	0.04	0.09	0.15	0.24
	0.1	0.15	0.31	0.35	0.56
	0.3	0.20	0.41	0.56	0.72
	0.5	0.31	0.48	0.64	0.77
	0.7	0.35	0.54	0.66	0.80
	0.9	0.41	0.55	0.64	0.77
	1.0	0.39	0.51	0.58	0.69
8-Methylquinoline	0	0.03	0.08	0.10	0.18
	0.1	0.12	0.31	0.27	0.44
	0.3	0.18	0.42	0.47	0.66
	0.5	0.28	0.50	0.68	0.75
	0.7	0.35	0.57	0.62	0.78
	0.9	0.40	0.59	0.61	0.74
	1.0	0.39	0.56	0.56	0.66
Carbazole	0	0.11	0.22	0.29	0.39
	0.1	0.27	0.45	0.47	0.62
	0.3	0.38	0.57	0.61	0.77
	0.5	0.52	0.64	0.68	0.81
	0.7	0.58	0.68	0.69	0.82
	0.9	0.62	0.70	0.68	0.78
	1.0	0.61	0.69	0.63	0.69
Fluorenone	0	0.12	0.26	0.33	0.42
	0.1	0.30	0.53	0.50	0.62
	0.3	0.41	0.62	0.65	0.77
	0.5	0.54	0.68	0.71	0.82
	0.7	0.61	0.70	0.74	0.84
	0.9	0.64	0.73	0.77	0.82
	1.0	0.63	0.73	0.74	0.79

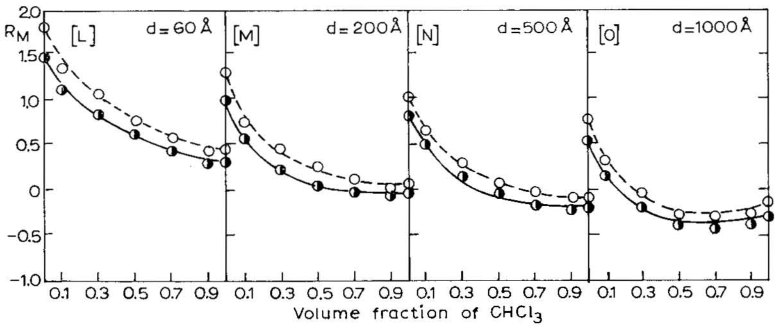


Fig. 1. Theoretical (solid and broken lines) and experimental values of $R_{M_{1,2}}$ obtained by TLAC for 1-naphthol (◑) and 2-naphthol (○) Silica gel adsorbent (pore diameter, *d* in Å): L (60); M (200); N (500) and O (1000). Mobile phase, carbon tetrachloride–chloroform.

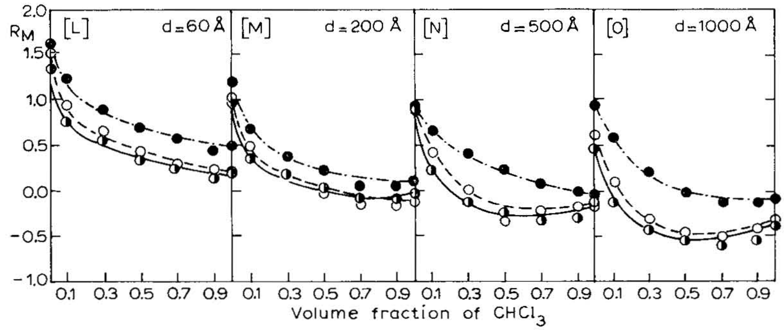


Fig. 2. Theoretical (solid and broken lines) and experimental values of $R_{M_{1,2}}$ obtained by TLAC for quinoline (●), 8-hydroxyquinoline (◑) and 8-methylquinoline (○). For details, see Fig. 1.

stances changes with the variation in porosity of the adsorbent. The different shape of the functions of 8-hydroxyquinoline and 8-methylquinoline can be explained in terms of the presence of steric effects. Good agreement between the theoretical and experimental values of $R_{M_{1,2}}$ was also obtained for fluorenone and carbazole (Fig. 3). The tendency to formation of a minimum in the graph for the adsorbent with widest pores is interesting.

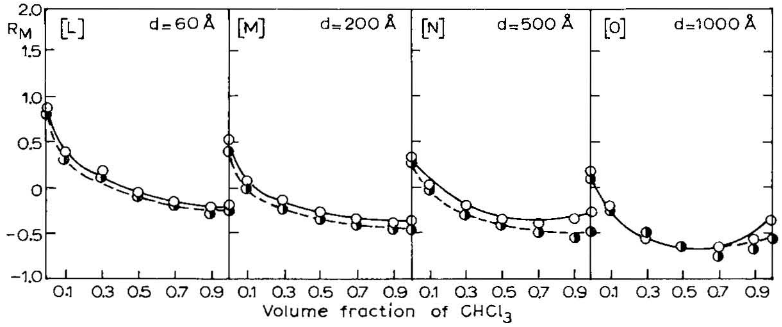


Fig. 3. Theoretical (solid and broken lines) and experimental values of $R_{M_{1,2}}$ obtained by TLAC for fluorenone (◑) and carbazole (○). For details, see Fig. 1.

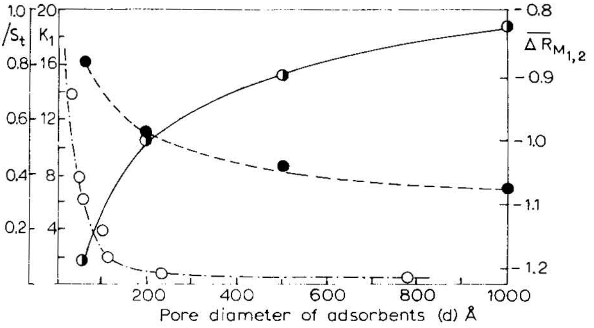
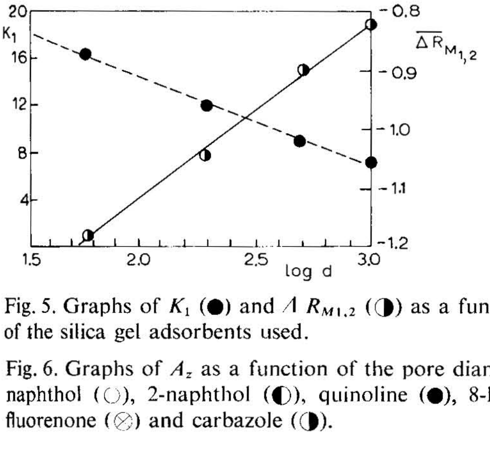


Fig. 4. Graphs of K_1 (●), $\Delta R_{M_{1,2}}$ (◑) and S_r/S_t (○) as a function of the pore diameter (*d*) of the silica gel adsorbents used.

A comparison of the values of K_1 with the porosity of the adsorbent provides information about the adsorption process in the present systems. It can be seen from Fig. 4 that for pore diameters of 60–200 Å (adsorbents L and M) a large change in K_1 occurs. For pore diameters greater than 200 Å (adsorbents N and O), the function $K_1 = f(d)$ is approximately linear and only changes slightly. According to Snyder and Ward[3], the initial large change in K_1 is due to a correspondingly large change in the number of reactive hydroxyl groups as compared with the total number of hydroxyl groups on the surface of the silica gel (S_r/S_t). The functions $K_1 = f(\log d)$ and $\Delta R_{M_{1,2}} = f(\log d)$ are presented in Fig. 5.



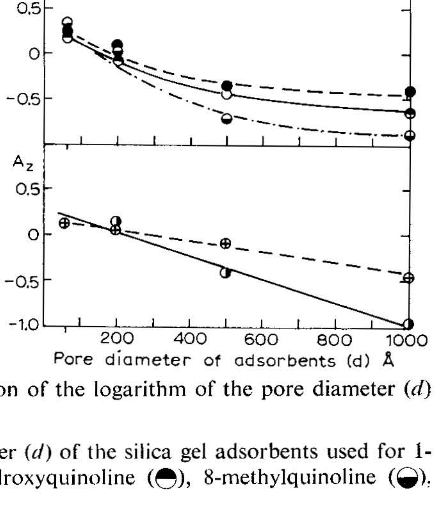


Fig. 5. Graphs of K_1 (●) and $\Delta R_{M_{1,2}}$ (◑) as a function of the logarithm of the pore diameter (*d*) of the silica gel adsorbents used.

Fig. 6. Graphs of A_z as a function of the pore diameter (*d*) of the silica gel adsorbents used for 1-naphthol (○), 2-naphthol (◐), quinoline (●), 8-hydroxyquinoline (◓), 8-methylquinoline (◒), fluorenone (⊗) and carbazole (◑).

The values of $A_z = \log k_{1,2}^{\infty}$ provide an indication of the interactions between molecules of the chromatograhped substances and the solvent. Graphs of this function against the pore diameter of the adsorbent are given in Fig. 6. It can be seen that for pore diameters greater than 200 Å the values of A_z are negative. Thus the porosity of the adsorbent causes deviations from the ideal behaviour accepted in deriving eqn. 1.

In conclusion, the greatest changes in the parameters of eqn. 1 take place when the pore diameter of adsorbent is less than 200 Å. The agreement between the theoretical and experimental values of $R_{M_{1,2}}$ obtained on silica gel adsorbents with pore diameter 60–1000 Å is fairly good and permits an extension of eqn. 1.

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REFERENCES

1 J. C. Giddings, *Anal. Chem.*, 37 (1965) 60.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968.
3 L. R. Snyder and J. W. Ward, *J. Phys. Chem.*, 70 (1966) 3941.
4 J. Ościk, *Fizicheskaya adsorbtsiya iz mnogokomponentnykh faz*, Nauka, Moscow, 1972.
5 J. Ościk and J. K. Różyło, *Chromatographia*, 4 (1971) 516.
6 J. Ościk and G. Chojnacka, *J. Chromatogr.*, 93 (1974) 167.
7 E. Soczewiński and M. Ciszewska, *J. Chromatogr.*, 96 (1974) 163.
8 E. Soczewiński and H. Szumiło, *J. Chromatogr.*, 81 (1973) 99.
9 E. Soczewiński and W. Gołkiewicz, *Chromatographia*, 5 (1972) 431; 5 (1972) 594; 6 (1973) 269.
10 S. Turina, M. Trbojević and M. Kaštelan-Macow, *Anal. Chem.*, 46 (1974) 988.
11 J. K. Różyło, *J. Chromatogr.*, 93 (1974) 177.
12 J. K. Różyło, *Chem. Anal. (Warsaw)*, 19 (1974) 1167.
13 J. K. Różyło, *Summaries of IV Polish Analytical Conference*, P.W.N., Warsaw, 1974, p. 119.
14 J. K. Różyło, *Chromatographia*, 8 (1975) 390.
15 J. K. Różyło, *Chem. Anal. (Warsaw)*, 20 (1975) 489.
16 J. Ościk, *Przem. Chem.*, 40 (1965) 279; 44 (1965) 129; 46 (1967) 593.
17 J. Ościk, *Bull. Soc. Acad. Polon. Sci. Ser. Chim.*, 9 (1961) 29.
18 J. Ościk, *Roczniki Chem.*, 31 (1957) 621; 34 (1960) 745.
19 J. K. Różyło, *J. Chromatogr.*, 116 (1976) 117.
20 M. P. Volynec, *Tonkoslonaja khromatografiya v neorganicheskom analize*, Nauka, Moscow, 1974.
21 A. A. Lurie, *Sorbenty i khromatograficheskie nositeli*, Nauka, Moscow, 1972.
22 F. Geiss, *Die Parameter der Dünnschichtchromatographie*, Vieweg, Braunschweig, 1972.
23 G. C. Pimentel and A. L. McClellan, *The Hydrogen Bond*, Freeman, San Francisco, 1961.
24 A. Waksmundzki and J. K. Różyło, *J. Chromatogr.*, 33 (1968) 90.
25 A. Waksmundzki, J. K. Różyło and A. Ścisłowicz, *J. Chromatogr.*, 46 (1970) 204.
26 J. Ościk and R. Nasuto, *Folia Soc. Scient. Lublinensis, Sect. C*, 7/8 (1967–1968) 61.
27 E. Soczewiński and H. Szumiło, *J. Chromatogr.*, 94 (1974) 229.

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CHROMATOGRAPHIC BEHAVIOUR OF ALKALOIDS ON THIN LAYERS OF ANION AND CATION EXCHANGERS

I. AG 1-X4 AND CELLEX D

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SUMMARY

The chromatographic behaviour of 48 alkaloids has been studied on Bio-Rad AG 1-X4, Cellex D and microcrystalline cellulose, eluting with solutions of different pH but constant ionic strength (0.5). Many interesting separations were effected on both AG 1-X4 and Cellex D layers.

The influence of pH on the chromatographic behaviour of alkaloids has been quantitatively studied and an equation was used that expresses the behaviour of the alkaloids on both AG 1-X4 (AcO^-) and microcrystalline cellulose layers. The non-applicability of this equation to Cellex D layers is discussed.

INTRODUCTION

The chromatographic characteristics of alkaloids on ion exchangers have been studied by column chromatography[1–7] and, to a lesser extent, with modified papers or papers impregnated with ion exchangers[8–12]. Most work, however, has concerned a small number of compounds and an accurate study of the parameters that determine the retention process (pH and ionic strength of the eluent, acid–base characteristics of the compounds) has not been made.

Owing to the lack of studies on thin layers of ion exchangers, we deemed it useful to study methodically the chromatographic behaviour of many alkaloids in order to find the best conditions for their separation. This first paper concerns anion exchangers with polystyrene (Bio-Rad AG 1-X4) and cellulose (Cellex D) matrices. The results achieved with these two exchangers are compared with those obtained on microcrystalline cellulose.

EXPERIMENTAL

The 48 alkaloids studied were dissolved in ethanol, with the exception of theobromine, which was dissolved in 2 *M* hydrochloric acid. The amounts of each alkaloid on the layer (relative to 1 μl of solution) are reported in Table I. A freshly prepared solution of ergotamine must be used in order to prevent the appearance on the layer of spots (with a yellow fluorescence) other than that (with a blue fluorescence) peculiar to the pure compound.

Of the 48 alkaloids, some (morphine, spermine, spermidine, cevadine, veratrine, protoveratrine A and ephedrine) cannot be detected on AG 1-X4 thin layers and they are therefore not reported in Table I.

Preparation of the layers

Layers of AG 1-X4 in the acetate form (Bio-Rad Labs., Richmond, Calif., U.S.A.) were prepared by mixing 3 g of the exchanger with 9 g of microcrystalline cellulose in 50 ml of water. The AG 1-X4 (AcO^-) was obtained by treating the commercial product (200–400 mesh) in the chloride form with 0.5 *M* sodium acetate solution until the complete disappearance of the chloride ion, then rinsing with water and methanol and drying at room temperature.

DEAE-cellulose (Cellex D) layers were prepared by dissolving 6 g of the exchanger in 50 ml of water. The DEAE-cellulose (Bio-Rad Labs.) was treated, before use, with 0.5 *M* acetate buffer, rinsed with water and methanol and dried at room temperature. Microcrystalline cellulose (E. Merck, Darmstadt, G.F.R.) layers were prepared by dissolving 12 g of the exchanger in 50 ml of water.

Detection

Many alkaloids, when observed under UV light (254 nm), give rise to black spots on a bright background or to fluorescence. Some compounds are fluorescent only in the protonated form; for this reason the layers, after elution and drying, were sprayed with 6 *M* acetic acid solution.

With Dragendorff reagent, modified according to Vágújfalvi[13], many alkaloids can be detected on AG 1-X4 and microcrystalline cellulose layers that have previously been sprayed with the acetic acid solution. This reagent, however, does not give good results on Cellex D layers. Ajmaline was detected by spraying the layer with a saturated solution of ceric sulphate in 60% sulphuric acid. The migration distance was 11 cm unless otherwise stated. The measurements were carried out at 25 ± 0.5°.

RESULTS AND DISCUSSION

AG 1-X4 (AcO^-)

In Table I are reported the R_F values of 41 alkaloids on AG 1-X4 (AcO^-) layers on elution with solutions of different pH values but constant ionic strength ($\mu = 0.5$). Table I also indicates the amounts of each alkaloid deposited on the layer. It is interesting to note that these amounts are generally smaller than those on papers impregnated with inorganic ion exchangers[9]. Alkaloids were classified in order of increasing R_F values on elution with 0.5 *M* ammonium acetate solution.

The affinities of most alkaloids towards the exchanger increase as the pH is increased. Such an effect, which has already been discussed for primary aromatic amines[14], is correlated with the stronger retention of the layer towards the alkaloids in the free base form.

The behaviour of caffeine and colchicine supports such an assumption; in fact, the R_F values of these compounds, which are in the free base form over the whole pH range studied, do not change with changes in pH.

TABLE I

R_F VALUES OF ALKALOIDS ON AG 1-X4 (AcO^-) THIN LAYERS

Alkaloid	*Eluent*				*Amount (μg)*
	1 M NH_3 + 0.5 M CH_3COONa	*0.5 M NH_3 + 0.5 M CH_3COONH_4*	*0.5 M CH_3COONH_4*	*0.5 M acetate buffer*	
Narceine	0.00	0.00	0.00	0.00	0.25
Ergocristine	0.00	0.00	0.00	0.05	0.4
Ergotamine	0.00	0.00	0.00	0.06	0.4
Papaverine	0.01	0.01	0.02	0.46	0.4
Jbogaine	0.02	0.02	0.03	0.36	0.4
Berberine hydrochloride	0.03	0.04	0.04	0.03	0.01
Reserpine	0.00	0.00	0.04	0.19	0.5
Boldine	0.02	0.02	0.07	0.25	0.4
Ergonovine	0.02	0.02	0.08	0.21	0.5
Narcotine	0.10	0.10	0.22	0.72	2.0
Hydrastine	0.21	0.21	0.36	0.73	0.8
Aminophylline	0.03	0.03	0.38	0.53	8.0
Theophylline	0.03	0.03	0.38	0.54	8.0
Colchicine	0.40	0.40	0.42	0.45	4.0
Yohimbine hydrochloride	0.07	0.07	0.44	0.58	4.0
Quinine	0.08	0.10	0.50	0.65	0.3
Quinidine sulphate	0.13	0.15	0.54	0.67	0.3
Brucine	0.23	0.26	0.66	0.70	6.0
Theobromine	0.41	0.52	0.68	0.68	8.0
Caffeine	0.69	0.69	0.68	0.68	8.0
Cinchonine hydrochloride	0.00	0.00	0.71	0.74	1.0
Cinchonidine	0.16	0.20	0.71	0.74	1.0
Strychnine	e.s.*	e.s.	0.77	0.79	6.0
Ajmaline	0.35	0.36	0.78	0.79	2.0
Lobeline hydrochloride	0.00	0.12	0.82	0.83	5.0
Tubocurarine	0.74	0.79	0.85	0.85	12.0
Cocaine	0.25	0.42	0.90	0.91	5.0
Atropine	0.61	0.88	0.91	0.92	10.0
Hyoscyamine	0.62	0.89	0.91	0.92	10.0
Eucatropine hydrochloride	0.62	0.89	0.91	0.92	10.0
Emetine hydrochloride	0.12	0.21	0.91	0.93	5.0
Ethylmorphine	0.41	0.47	0.91	0.94	8.0
Eserine sulphate (1)	0.28	0.50	0.93	0.94	3.0
(2)	0.54				
Homatropine	0.61	0.90	0.93	0.95	10.0
Scopolamine hydrochloride	0.68	0.72	0.93	0.95	7.0
Arecoline hydrochloride	0.79	0.88	0.94	0.95	10.0
Hyoscyne	0.93	0.96	0.96	0.95	10.0
Scopoline	0.90	0.96	0.98	0.96	7.0
Sparteine sulphate	0.94	0.97	0.98	0.97	10.0
Tropine	0.96	0.97	0.98	0.97	10.0
Prostigmine	0.96	0.97	0.98	0.97	10.0

* e.s. = elongated spot.

Theobromine and theophylline apparently behave anomalously as their R_F values increase as the pH decreases, notwithstanding the fact that their basic characteristics are similar to those of caffeine ($pK_a < 1$ for all three compounds). In order to explain such a result, it must be pointed out that, contrary to caffeine, the other two compounds may give rise to anionic species that are retained by the exchanger through an anion-exchange process.

The different chromatographic behaviour of theobromine and theophylline may be explained on the basis of their different acidic characteristics; theophylline, in fact, ($pK_{a2} = 8.6$)[15] is retained more strongly than theobromine ($pK_{a2} = 10.0$)[15].

The influence of ionic strength on the chromatographic behaviour of the alkaloids is more marked at low pH values of the eluent, that is, when the alkaloids are prevalently in the protonated form. As the ionic strength is decreased, an increase in the R_F values is observed. Similar behaviour has been found with phenols on polystyrene-based cation exchangers[16]; for these compounds, the greatest influence of the ionic strength is observed when they are in the deprotonated form.

As regards the relationship between the chromatographic behaviour of the alkaloids and their structure, it is difficult to compare compounds with different structures. When such a comparison is possible, it is found that the introduction into the molecule of one or more methoxyl groups results in a stronger retention by the exchanger. Some typical examples are the greater retentions of narcotine, brucine, quinine and quinidine in comparison with those of hydrastine, strychnine, cinchonine and cinchonidine, respectively.

Some alkaloids listed in Table I are also noticeably retained when eluting with 0.5 *M* acetate buffer. For these compounds, a remarkable increase in the R_F values can be achieved by eluting with 1 *M* acetic acid or adding ethanol to the aqueous solutions given in Table I. The increase observed with 1 *M* acetic acid may be attributed mainly to the decrease in the ionic strength of the eluent. With the addition of ethanol, more compact spots are observed, but there is a levelling of the R_F values overall at ethanol concentrations above 50% (v/v). An exception is narceine, which always remains at the starting point; such behaviour may be correlated with the presence in the molecule of a carboxylic group, which gives rise to an anion-exchange process with the exchanger.

The behaviour of eserine with 0.5 *M* sodium acetate and 1 *M* ammonia solution is very interesting from an analytical point of view. In fact, as indicated by the data in Table I, with this eluent eserine gives two spots, the one with higher R_F being red-violet and the other giving a yellow fluorescence under UV light after spraying with acetic acid solution. The presence of the red-violet spot can be used for the qualitative detection of this alkaloid, as such a test is selective and sensitive (3 μg).

Analytical application. Among the separations foreseeable on the basis of the R_F values reported in Table I, the following have been effected: cinchonine and cinchonidine (migration distance = 14 cm; 0.5 *M* sodium acetate + 1 *M* ammonia); tropine and atropine (0.5 *M* sodium acetate + 1 *M* ammonia); caffeine, theophylline and theobromine (0.5 *M* sodium acetate + 1 *M* ammonia); cocaine, scopoline and scopolamine (0.5 *M* sodium acetate + 1 *M* ammonia or 0.5 *M* ammonium acetate + 0.5 *M* ammonia); eserine and prostigmine (0.5 *M* ammonium acetate + 0.5 *M* ammonia); quinine (or quinidine) and cinchonine (or cinchonidine) (0.5 *M* ammonium acetate).

The separation reported in Fig. 1, with 1 *M* acetic acid as eluent, includes many alkaloids and cannot be effected with the eluents listed in Table I.

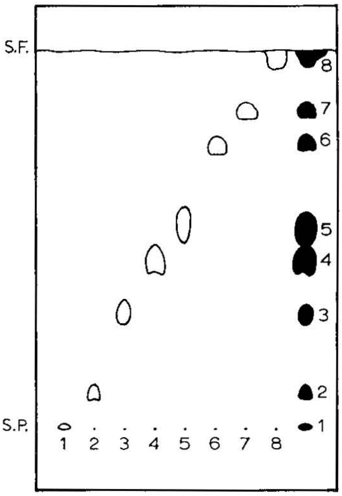

Fig. 1. Thin-layer chromatogram of alkaloids on AG 1-X4 (AcO$^-$). Eluent, 1 *M* acetic acid. Migration distance, 12.5 cm. Spots: 1, narceine (red); 2, berberine hydrochloride; 3, ergonovine; 4, reserpine; 5, ergocristine; 6, jbogaine; 7, quinine; 8, cocaine. Black spots: mixtures. Yellow fluorescence: berberine, reserpine and jbogaine. Blue fluorescence: ergonovine, ergocristine and quinine. Cocaine gives an orange colour with Dragendorff reagent.

Cellex D and microcrystalline cellulose

Table II gives the R_F values of 28 alkaloids on Cellex D layers with the same eluents as those used on AG 1-X4 (AcO$^-$). On this exchanger, fewer alkaloids are reported than on AG 1-X4, because Dragendorff reagent cannot be used as the detection agent.

The alkaloids are listed in order of increasing R_F values on elution with 0.5 *M* ammonium acetate + 0.5 *M* ammonia.

Table II also reports the chromatographic characteristics of the alkaloids on microcrystalline cellulose. On this exchanger also the alkaloids that react with Dragendorff reagent can be detected. Such compounds have R_F values $\geqslant 0.7$ with all of the eluents listed in Table II. The amounts of each alkaloid on Cellex D and microcrystalline cellulose layers are generally lower than those on AG 1-X4 (AcO$^-$).

From the comparison between the chromatographic behaviour of the alkaloids on Cellex D and microcrystalline cellulose, some interesting conclusions can be drawn:

(1) Among the alkaloids that are not protonated in the pH range studied, colchicine, theophylline and aminophylline show a chromatographic behaviour on Cellex D at changing pH values that is different from that on microcrystalline cellulose, while caffeine and theobromine exhibit high R_F values, which are independent of the pH, on both exchangers. The behaviour of colchicine on Cellex D may be ascribed to the progressive protonation of the functional group of the exchanger as the pH of the eluent is decreased, while that of theophylline and theobromine can also be correlated with the anion-exchange process between them and the exchanger with both 0.5 *M* sodium acetate + 1 *M* ammonia and 0.5 *M* ammonium acetate + 0.5 *M* ammonia as eluent.

(2) The alkaloids that are protonated in the pH range studied generally exhibit

TABLE II

R_F VALUES OF ALKALOIDS ON CELLEX D AND MICROCRYSTALLINE CELLULOSE (mC) THIN LAYERS

Alkaloid	*Eluent*							
	1 M NH_3 + 0.5 M CH_3COONa		*0.5 M NH_3 + 0.5 M CH_3COONH_4*		*0.5 M CH_3COONH_4*		*0.5 M acetate buffer*	
	Cellex D	*mC*	*Cellex D*	*mC*	*Cellex D*	*mC*	*Cellex D*	*mC*
Narceine	0.00	0.06	0.00	0.02	0.00	0.04	0.00	0.08
Reserpine	0.00	0.00	0.00	0.00	0.09	0.05	0.41	e.s.*
Ergocristine	e.s.	e.s.	0.03	0.01	0.06	0.04	0.41	0.23
Ergotamine	e.s.	e.s.	0.12	e.s.	0.17	0.06	0.51	0.31
Boldine	0.33	0.65	0.16	0.30	0.32	0.31	0.49	0.35
Jbogaine	0.14	0.08	0.18	0.08	0.24	0.12	0.84	0.49
Papaverine	0.12	0.32	0.20	0.32	0.37	0.40	0.89	0.82
Berberine hydrochloride	0.16	0.00	0.29	0.00	0.36	0.03	0.49	0.05
Ergonovine	0.30	0.30	0.35	0.31	0.49	0.24	0.65	0.24
Yohimbine hydrochloride	0.35	0.39	0.42	0.45	0.77	0.57	0.86	0.65
Narcotine	0.39	0.50	0.45	0.51	0.72	0.56	0.94	0.83
Aminophylline	0.47	0.63	0.48	0.63	0.81	0.64	0.85	0.68
Theophylline	0.48	0.67	0.49	0.65	0.83	0.66	0.88	0.70
Emetine hydrochloride	0.26	e.s.	0.49	e.s.	0.95	e.s.	0.95	0.93
Brucine	0.42	0.50	0.55	0.52	0.86	0.67	0.90	0.69
Quinidine sulphate	0.37	0.47	0.55	0.49	0.80	0.64	0.89	0.73
Quinine	0.38	0.50	0.56	0.54	0.81	0.67	0.89	0.74
Lobeline hydrochloride (1)	0.00	0.00	0.56	0.61	0.95	0.86	0.95	0.85
(2)	e.s.	e.s.						
Hydrastine	0.53	0.64	0.64	0.66	0.82	0.70	0.94	0.83
Strychnine	0.52	0.63	0.65	0.64	0.87	0.75	0.91	0.79
Cinchonine hydrochloride (1)	0.00	0.00	0.70	0.66	0.92	0.79	0.94	0.83
(2)	e.s.	e.s.						
Cinchonidine	0.51	0.65	0.70	0.66	0.92	0.79	0.94	0.83
Ajmaline	0.63	0.71	0.73	0.74	0.91	0.79	0.95	0.88
Colchicine	0.58	0.87	0.75	0.86	0.84	0.86	0.88	0.88
Ethylmorphine	n.d.**	0.82	0.80	0.84	0.95	0.95	0.95	0.95
Theobromine	0.83	0.73	0.83	0.73	0.88	0.80	0.89	0.80
Caffeine	0.84	0.81	0.88	0.80	0.89	0.81	0.89	0.83
Eserine sulphate	0.81	0.89	0.90	0.89	0.95	0.95	0.95	0.95

* e.s. = elongated spot.

** n.d. = not determined.

an increase in the R_F values on both layers as the pH of the eluent is decreased. Such an increase in R_F is more marked on Cellex D than on microcrystalline cellulose, and the results confirm the influence on the R_F value both of the protonation of the alkaloid and of the progressive protonation of the functional group of Cellex D.

It should be noted that narceine and berberine are strongly retained by microcrystalline cellulose, independently of the pH of the eluent. On both exchangers, the

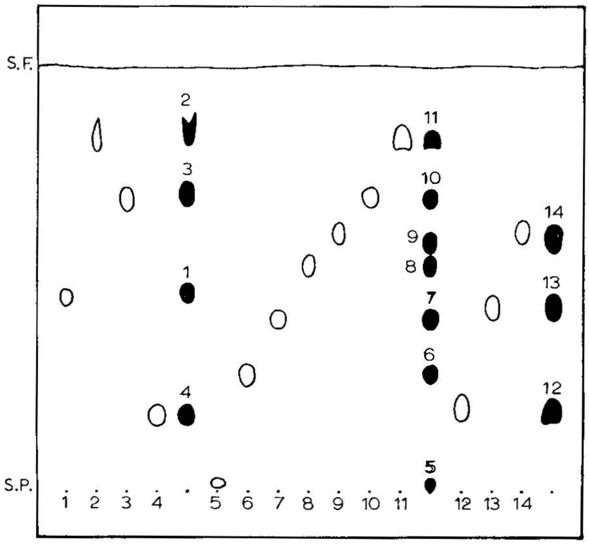

Fig. 2. Thin-layer chromatogram of alkaloids on Cellex D. Eluent, 0.5 *M* ammonia + 0.5 *M* ammonium acetate. Migration distance, 14 cm. Spots: 1, theophylline; 2, caffeine; 3, ajmaline; 4, jbogaine; 5, narceine (red); 6, berberine hydrochloride; 7, yohimbine hydrochloride; 8, brucine; 9, strychnine; 10, colchicine; 11, eserine sulphate; 12, papaverine; 13, narcotine; 14, hydrastine. Black spots: mixtures. Yellow fluorescence: berberine, jbogaine and papaverine. Yellow-green fluorescence: narcotine and hydrastine. Blue fluorescence: yohimbine and eserine. Caffeine, theophylline, brucine, strychnine and colchicine are visible in UV light as dark spots.

chromatographic behaviour of boldine is peculiar because, as the pH changes, its R_F value first decreases and then increases. On eluting with 0.5 *M* sodium acetate + 1 *M* ammonia, eserine, contrary to its behaviour on AG 1-X4 (AcO^-), does not give double spots on either Cellex D or microcrystalline cellulose layers.

Analytical application. Many separations can be effected on Cellex D layers. Most of these separations can also be obtained on microcrystalline cellulose, as it is predictable on the basis of the R_F values. Fig. 2 shows three separations on Cellex D with 0.5 *M* ammonium acetate + 0.5 *M* ammonia as eluent.

The separation of narceine, berberine, yohimbine, brucine, strychnine, colchicine and eserine cannot be effected on microcrystalline cellulose.

R_F versus pH graphs

In order to study quantitatively the influence of pH on the chromatographic characteristics of the alkaloids, we used the following relationship:

$$\left(\frac{1}{R_F} - 1\right) = \left(\frac{1}{R_{F\text{alk}}} - 1\right)\frac{K_a}{K_a + [H^+]} + \left(\frac{1}{R_{F\text{ac}}} - 1\right)\frac{[H^+]}{K_a + [H^+]} \quad (1)$$

which has been employed for phenols on Dowex 50-X4 (Na^+)[16], where K_a is the dissociation constant of the protonated amine group and $R_{F\text{ac}}$ and $R_{F\text{alk}}$ are the R_F values of the protonated and the free base form of the alkaloids, respectively.

AG 1-X4 (AcO^-). Fig. 3 shows the R_F *versus* pH curves obtained by applying

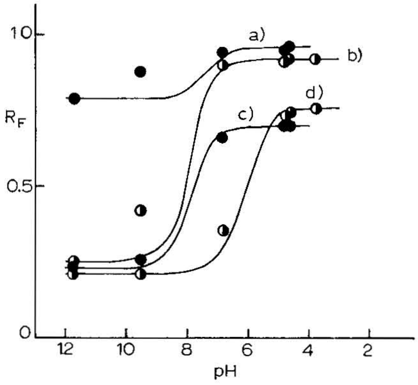

Fig. 3. R_F *versus* pH plots for alkaloids on AG 1-X4 (AcO^-) thin layers. (a) Arecoline; (b) cocaine; (c) brucine; (d) hydrastine.

eqn. 1, where the $R_{F\text{ac}}$ and $R_{F\text{alk}}$ values are those obtained on eluting with a solution at pH = 4.60 or 3.75 ($R_{F\text{ac}}$) and pH = 11.70 ($R_{F\text{alk}}$), indicated in Table III.

The good agreement between the experimental R_F values and those which can be drawn from the theoretical curves at the same pH values (see Fig. 3) supports the validity of eqn. 1 also in the case of anion exchangers. The R_F values obtained with ammonium acetate + ammonia (pH = 9.50) are an exception; such values (overall in the case of curves a and b in Fig. 3) are noticeably higher than those calculated on the basis of the pH of the eluent. Such differences between the experimental and theoretical values are correlated with the different pH values on the layer compared that of the eluent. In fact, with the method described previously[17], we have measured the pH on the layer and found values between 8.8 and 7.0 from the origin up to the solvent front. It should be noted that the two R_F values at pH = 9.50 for curves a

TABLE III

R_F VALUES OF ALKALOIDS ON AG 1-X4 (AcO^-) THIN LAYERS OBTAINED WITH ELUENTS AT DIFFERENT pH VALUES

1 *M* ammonia + 0.5 *M* sodium acetate (pH = 11.70); 0.5 *M* ammonia + 0.5 *M* ammonium acetate (pH = 9.50); 0.5 *M* ammonium acetate (pH = 6.80); 0.5 *M* acetic acid + 0.5 *M* sodium acetate (pH = 4.75); 0.25 *M* monochloroacetic acid + 0.5 *M* sodium acetate (pH = 4.60); 0.5 *M* monochloroacetic acid + 0.5 *M* sodium acetate (pH = 3.75).

*Arecoline**		*Cocaine***		*Brucine****		*Hydrastine*§	
pH	R_F	*pH*	R_F	*pH*	R_F	*pH*	R_F
11.70	0.79	11.70	0.25	11.70	0.23	11.70	0.21
9.50	0.88	9.50	0.42	9.50	0.26	9.50	0.21
6.80	0.94	6.80	0.90	6.80	0.66	6.80	0.36
4.75	0.95	4.75	0.91	4.75	0.70	4.75	0.73
4.60	0.96	4.60	0.92	4.60	0.70	4.60	0.74
		3.75	0.92			3.75	0.76

* pK_a = 7.41 at 17.5° (ref. 15).
** pK_a = 8.39 at 24° (ref. 15).
*** pK_a = 8.28 at 25° (ref. 15).
§ pK_a = 6.63 at 15° (ref. 15).

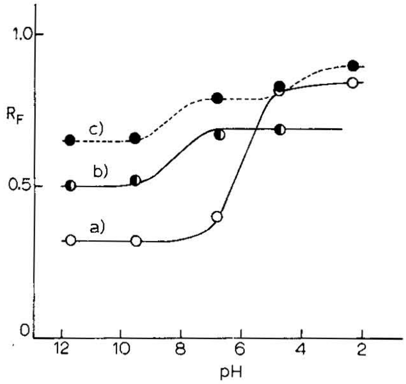

Fig. 4. R_F *versus* pH plots for alkaloids on microcrystalline cellulose thin layers. (a) Papaverine; (b) brucine; (c) cinchonidine.

and b in Fig. 3 correspond to pH values on the layer of 7.3 (curve a) and 8.9 (curve b), in good agreement with the pH values determined experimentally on the layer at distances corresponding to the R_F values of these two compounds.

As regards the possible application of eqn. 1 when using anion exchangers and particularly AG 1-X4 (AcO^-) layers, it must be pointed out that many alkaloids in the free base form are strongly retained by this exchanger ($R_F \leqslant 0.02$), and for this reason the $1/R_{F\mathrm{alk}}$ value is not reliable. Other alkaloids, on the other hand, give rise to elongated spots (*e.g.*, strychnine) and therefore eqn. 1 cannot be applied.

Cellex D. On this exchanger, eqn. 1 can be applied only in very few instances, which cannot be predicted, as the retention of the alkaloids does not depend only on the proportions of the protonated and free base forms of the alkaloid, but also on the progressive protonation of the functional group of the exchanger as the pH is decreased.

TABLE IV

R_F VALUES OF ALKALOIDS ON MICROCRYSTALLINE CELLULOSE THIN LAYERS OBTAINED WITH ELUENTS AT DIFFERENT pH VALUES

1 *M* ammonia + 0.5 *M* sodium acetate (pH = 11.70); 0.5 *M* ammonia + 0.5 *M* ammonium acetate (pH = 9.50); 0.5 *M* ammonium acetate (pH = 6.80); 0.5 *M* acetic acid + 0.5 *M* sodium acetate (pH = 4.75); 1 *M* acetic acid + 0.5 *M* sodium chloride (pH = 2.30).

*Papaverine**		*Brucine***		*Cinchonidine****	
pH	R_F	*pH*	R_F	*pH*	R_F
11.70	0.32 ($R_{F\mathrm{alk}}$)	11.70	0.50 ($R_{F\mathrm{alk}}$)	11.70	0.65 ($R_{F\mathrm{alk}}$)
9.50	0.32	9.50	0.52	9.50	0.66
6.80	0.40	6.80	0.66	6.80	0.79 ($R_{F\mathrm{ac}_1}$)§
4.75	0.82	4.75	0.69 ($R_{F\mathrm{ac}}$)	4.75	0.83
2.30	0.84 ($R_{F\mathrm{ac}}$)			2.30	0.90 ($R_{F\mathrm{ac}_2}$)§§

* $pK_a = 6.40$ at 25° (ref. 15).

** $pK_a = 8.28$ at 25° (ref. 15).

*** $pK_{a_1} = 8.40$ and $pK_{a_2} = 4.17$ at 15° (ref. 15).

§ R_F value of monoprotonated form of cinchonidine.

§§ R_F value of diprotonated form of cinchonidine.

Microcrystalline cellulose. Fig. 4 shows the R_F *versus* pH curves for papaverine, brucine and cinchonidine, obtained by applying eqn. 1 on the basis of the $R_{F\text{ac}}$ and $R_{F\text{alk}}$ values reported in Table IV.

With respect to the other curves, that of cinchonidine (curve c) exhibits two inflection points, which correspond to the protonation of its two basic groups ($\text{p}K_{a_1} = 8.40$ and $\text{p}K_{a_2} = 4.17$).

With respect to the curves relating to AG 1-X4 layers (Fig. 3), the better agreement between the experimental and theoretical points owing to the disappearance of the pH gradient on the layer should be noted. Such an occurrence is a further confirmation of the assumption that we made in order to explain the differences between theoretical and experimental points on AG 1-X4 (AcO^-) layers and of the applicability of eqn. 1 not only on strong cation[16] and anion exchangers but, in general, on every type of resin whose retention is not affected by the pH of the eluent, as with Cellex D.

REFERENCES

1 H. Watanabe, *Bunseki Kagaku (Jap. Anal.)*, 10 (1961) 271; 11 (1962) 233.
2 H. Watanabe, *Nippon Kagaku Zasshi*, 83 (1962) 54.
3 W. Kamp, *Pharm. Weekbl.*, 92 (1957) 1; 94 (1959) 521; 99 (1964) 1092.
4 J. R. Broich, M. M. de Mayo and L. A. dal Cortivo, *J. Chromatogr.*, 33 (1968) 526.
5 C. McMartin, P. Simpson and N. Thorpe, *J. Chromatogr.*, 43 (1969) 72.
6 J. H. Knox and J. Jurand, *J. Chromatogr.*, 82 (1973) 398; 87 (1973) 95.
7 E. Murgia and H. F. Walton, *J. Chromatogr.*, 104 (1975) 417.
8 E. Soczewiński and M. Rojowska, *J. Chromatogr.*, 27 (1967) 206.
9 I. D. Coussio, G. B. Marini-Bettòlo and V. Moscatelli, *J. Chromatogr.*, 11 (1963) 238.
10 W. Manikowski, *Ann. Acad. Med. Lodz*, 9 (1967) 227.
11 H. V. Street, *Clin. Chim. Acta*, 7 (1962) 226.
12 Z. I. El-Darowj and Z. M. Mobarak, *Pharmazie*, 29 (1974) 391.
13 D. Vágújfalvi, *Planta Med.*, 8 (1960) 34.
14 L. Lepri, P. G. Desideri and V. Coas, *J. Chromatogr.*, 90 (1974) 331.
15 D. D. Perrin, *Dissociation Constants of Organic Bases in Aqueous Solutions*, Butterworths, London, 1965.
16 L. Lepri, P. G. Desideri, M. Landini and G. Tanturli, *J. Chromatogr.*, 109 (1975) 365.
17 D. Cozzi, P. G. Desideri and L. Lepri, *J. Chromatogr.*, 42 (1969) 532.

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SYSTEMATIC ANALYSIS OF MYCOTOXINS BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The analysis of 37 mycotoxins and other fungal metabolites by thin-layer chromatography is described. Their R_F values in eight solvent systems, colour reactions after chemical detection and detection under UV light are presented. From the R_F values, "chromatographic spectra" which can be used as one of the characteristics for the characterization of unknown mycotoxins were constructed.

INTRODUCTION

Recently, increasing attention has been paid to the analysis of mycotoxins, of which over 100 are known at present, and screening methods for mycotoxins in contaminated products have been sought. Eppley[1] described a thin-layer chromatography (TLC) screening method for zearalenone, aflatoxin and ochratoxin. Steyn[2] developed a chromatographic separation and detection of eleven mycotoxins on thin layers. Fishbein and Falk[3] elaborated TLC procedures for five mycotoxins (aflatoxins, ochratoxins, aspertoxin, O-methylsterigmatocystin and sterigmatocystin) and some other fungal metabolites. The chromatography of eighteen mycotoxins in two different systems on thin layers and a combination of detection under UV light with spraying with *p*-anisaldehyde was applied by Scott *et al.*[4] in the screening of fungal extracts. Stoloff *et al.*[5] described selective extraction methods for aflatoxins, ochratoxins, zearalenone, sterigmatocystin and patulin and characterized these mycotoxins by TLC. The method was applied to different substrates and detection limits sufficient for the screening of mycotoxins were determined.

Analytical procedures for aflatoxins are best worked out in comparison with those for other mycotoxins. Jones[6] gave a survey of qualitative and quantitative TLC methods for aflatoxins. The most recommended material for TLC is silica gel.

The use of high-pressure liquid chromatography for the separation of aflatoxins[7] and gas chromatography for altenuene[8], patulin[9] and penicillic acid[10] has been described. However, TLC, owing to its simplicity, is the most frequently used chromatographic technique for mycotoxins.

In this paper, a systematic analysis of 37 mycotoxins and 6 other fungal metabolites by TLC in eight solvent systems combined with physical and chemical detection methods is described. Examples of the application of these procedures to the isolation and characterization of both known and unknown mycotoxins by use of chromatographic bioautography are described in the next paper[11].

MATERIALS AND METHODS

Mycotoxins and other fungal metabolites

We used the following mycotoxins and fungal secondary metabolites (lowercase letters in parentheses refer to the sources given below): 4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone (a), aflatoxin B_1 (b, c), aflatoxin B_2 (d), aflatoxin G_1 (c), aflatoxin G_2 (d), aflatoxin M_1 (c), altenuene (e), alternariol (e), alternariol monomethyl ether (AME) (e), aspergillic acid (r), citreoviridin (f), citrinin (r), curvularin (g), cyanein (r), cytochalasin A (h), cytochalasin B (h), cytochalasin D (h), diacetoxyscirpenol (i), N,N′-dibenzylethylenediamine (DBED) (e), fusaric acid (r), gliotoxin (r), griseofulvin (r), helvolic acid (r), kojic acid (r), luteoskyrin (i), monorden (k), mycophenolic acid (r), nivalenol (i), ochratoxin A (f, l, m), ochratoxin B (d), ochratoxin C (d), patulin (n), penicillic acid (n), rubratoxin B (l), rugulosin (r), secalonic acid (f), sterigmatocystin (f, i), terreic acid (r), T-2 toxin (a, m, o), trichothecin (r), vermiculin (p), viridicatum toxin (f) and zearalenone (d).

The sources of the metabolites were as follows: (a) Dr. S. G. Yates, U.S. Department of Agriculture, Northern Regional Research Laboratories, Peoria, Ill., U.S.A.; (b) Serva Feinbiochemica, Heidelberg, G.F.R.; (c) Dr. L. Shoetwell, U.S. Department of Agriculture, Northern Research Laboratories, Peoria, Ill., U.S.A.; (d) Dr. R. M. Eppley, Division of Food Chemistry and Technology, Washington, D.C., U.S.A.; (e) Dr. R. W. Pero, National Institute of Environmental Health Sciences, Research Triangle Park, N.C., U.S.A.; (f) Dr. P. S. Steyn, National Chemistry Research Laboratories, Pretoria, South Africa; (g) Dr. Richard, School of Chemical Research, Canberra, Australia; (h) Dr. Ch. Tamm, Institut für organische Chemie der Universität, Basle, Switzerland; (i) Dr. P. M. Scott, Food and Nutrition Division, Department of National Health, Ottawa, Ontario, Canada; (k) Dr. S. Wilkinson, Wellcome Research Laboratories, Beckenham, Great Britain; (l) Calbiochem, London, Great Britain; (m) Dr. R. D. Wyatt, Department of Poultry Sciences, North Carolina State University, N.C., U.S.A.; (n) Dr. H. J. Mintzlaff, Institut für Bakteriologie und Histologie, Kulmbach, G.F.R.; (o) Dr. H. R. Burmeister, ARS Culture Collection Investigations Fermentation Laboratory, 1815 North University Street, Peoria, Ill., U.S.A.; (p) Dr. J. Fuska, this Department; and (r) this Department.

Preparation of sample solutions

Stock solutions of fungal metabolites were prepared mostly at a concentration of 1 mg/ml. The numbers in parentheses below express the actual amounts of substances in micrograms applied on the chromatograms. The following solvents were used for the preparation of solutions: acetone for altenuene (5), alternariol (2), AME (2) and terreic acid (5); benzene for griseofulvin (5); benzene–acetonitrile (98:2) for aflatoxins B_1 (2), B_2 (0.025), G_1 (2) and G_2 (0.025); chloroform for aflatoxin M_1 (0.01), 4-acetamido-4-hydroxy-2-butenoic acid-γ-lactone (5), aspergillic acid (10), citreoviridin (5), cyanein (5), diacetoxyscirpenol (5), fusaric acid (50), gliotoxin (5), helvolic acid (5), luteoskyrin (2), mycophenolic acid (5), ochratoxin A (2), ochratoxin B (0.05), ochratoxin C (0.2), patulin (10), penicillic acid (2), rubratoxin B (10) sterigmatocystin (5), T-2 toxin (5), trichothecin (2), vermiculin (5) and zearalenone (2); chloroform–methanol (1:1) for secalonic acid (1) and viridicatum toxin (1); ethanol for nivalenol (5) and DBED (5); ethyl acetate for kojic acid (5) and rugulosin (2); and methanol for citrinin (0.5) curvularin (5), cytochalasins A (5), B (5) and D (5) and monorden (5).

Preparation of thin layers

Thin layers of silica gel G (Merck, Darmstadt, G.F.R.) of thickness 0.25 mm were prepared on glass plates (20 × 20 cm) employing an applicator. Silica gel for the application was prepared as given in ref. 12. When the plates were dry they were activated for 2 h at 110°. Before use, the plates were cleaned by developing them in the system that was subsequently used for their development with samples. After evaporating the solvents, the plates were stored for 24 h in a desiccator with dilute sulphuric acid (sp.gr. 1.440). The relative humidity in the desiccator was about 40%.

Pre-coated thin layers. Silufol sheets (Kavalier, Votice, Czechoslovakia) (20 × 20 cm) were cleaned and stored in the same manner as the glass plates with silica gel G.

Solvent systems.

A: benzene–methanol–acetic acid (24:2:1)[4].
B: toluene–ethyl acetate–90% formic acid (6:3:1)[4].
C: benzene–ethanol (95:5)[8].
D: chloroform–methanol (4:1).
E: chloroform–methyl isobutyl ketone (4:1)[2].
F: chloroform–acetone (9:1)[5].
G: chloroform–acetic acid–diethyl ether (17:1:3)[5].
H: *n*-butanol–acetic acid–water (4:1:4) (upper layer).

All solvents were purified before use by procedures described by Keil[13].

Method of application and development

After removal from the desiccator, the thin layer or the Silufol sheet was covered with a glass plate, except the area of the "origin", in order to prevent the absorption of moisture from the air. Thus, a relatively even moisture content of all of the plates was reached regardless of the moisture content of the ambient air[14]. The substances to be analyzed were applied to the plates from the stock solutions with micropipettes under orange light so that the resulting amounts were as given above. The spots were dried during application with a flow of cold air. The plates were developed in the dark in developing tanks (Zeiss, Jena, G.D.R.) saturated with solvent vapour. Each substance was chromatographed in two series in all eight solvent systems both on plates coated with silica gel G and on Silufol sheets. When the front of the system reached a height of about 10 cm above the origin, the development was interrupted, the chromatogram was dried in air and then detection was carried out.

TABLE I

$R_F \times 100$ VALUES OF MYCOTOXINS ON CLEANED SILICA GEL G PLATES AND CLEANED SILUFOL SHEETS IN 8 SOLVENT SYSTEMS AND RESULTS OF PHYSICAL AND CHEMICAL DETECTION

Colours: a = beige; b = blue; c = blue-grey; d = blue-green; e = brown-violet; f = dark pink; g = dark spot; h = dark yellow; i = grey; k = green-blue; l = grey-brown; m = green; n = green-grey; o = orange; p = pale beige; r = pale blue; s = pale brown; t = pale grey; u = pale green; v = pale pink; x = pale red; y = pale yellow; z = pink; α = red; β = violet; γ = yellow; δ = yellow-green; ε = yellow-orange. $^{+}$ = Tail; $^{++}$ = elongated spot. Fractions $\frac{12.0}{6.0}$ etc.: the numerators represent $R_F \times 100$ values on cleaned silica gel G plates and the denominators represent $R_F \times 100$ values on cleaned Silufol sheets.

$R_F \times 100$ *values on* $\frac{\textit{silica gel G}}{\textit{Silufol}}$

A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
$\frac{12.0}{6.0}$	$\frac{11.5}{10.0}$	$\frac{2.5}{3.0}$	$\frac{57.0}{41.0}$	$\frac{5.0}{9.0}$	$\frac{8.0}{3.0}$	$\frac{7.0}{8.5}$	$\frac{55.0}{43.0}$
$\frac{28.0^{+}}{28.0^{+}}$	$\frac{31.5^{+}}{20.5^{+}}$	$\frac{19.5^{+}}{13.0^{+}}$	$\frac{80.5^{+}}{68.5^{+}}$	$\frac{22.5^{+}}{26.0}$	$\frac{32.5^{+}}{32.0}$	$\frac{31.5^{+}}{34.5^{+}}$	$\frac{61.5^{+}}{60.5^{+}}$
$\frac{30.0}{29.0}$	$\frac{18.0}{15.5}$	$\frac{14.0}{12.5}$	$\frac{80.0}{70.0}$	$\frac{9.0}{17.5}$	$\frac{33.0}{22.0}$	$\frac{20.0}{29.0}$	$\frac{61.0}{55.0}$
$\frac{19.0^{+}}{26.0^{+}}$	$\frac{19.0^{+}}{16.0^{+}}$	$\frac{13.5^{+}}{11.0^{+}}$	$\frac{84.5^{+}}{69.0^{+}}$	$\frac{12.0^{+}}{17.0}$	$\frac{25.0^{+}}{22.0}$	$\frac{20.0^{+}}{26.0^{+}}$	$\frac{50.0^{+}}{55.0^{+}}$
$\frac{22.5}{24.5}$	$\frac{8.0}{11.0}$	$\frac{8.0}{9.0}$	$\frac{78.0}{68.5}$	$\frac{4.5}{11.0}$	$\frac{17.0}{18.5}$	$\frac{11.0}{20.5}$	$\frac{51.5}{48.0}$
$\frac{21.5}{}$	$\frac{15.5}{}$	$\frac{10.0}{}$	$\frac{78.5}{}$	$\frac{0.0}{}$	$\frac{7.5}{}$	$\frac{6.5}{}$	$\frac{64.0}{}$
$\frac{24.0}{19.5}$	$\frac{33.0}{19.5}$	$\frac{7.0}{5.0}$	$\frac{66.0}{51.0}$	$\frac{3.5}{3.0}$	$\frac{6.5}{4.0}$	$\frac{12.0}{10.0}$	$\frac{83.0}{74.0}$
$\frac{30.5}{23.0^{+}}$	$\frac{65.5}{42.5}$	$\frac{17.0}{9.0}$	$\frac{83.0}{56.5}$	$\frac{32.0}{23.0}$	$\frac{28.5}{13.0}$	$\frac{58.0}{37.0}$	$\frac{95.0}{89.0}$
$\frac{57.5}{31.0}$	$\frac{72.5}{52.0}$	$\frac{33.0}{23.0}$	$\frac{88.0}{70.0}$	$\frac{67.0}{54.0}$	$\frac{56.5}{36.0}$	$\frac{73.5}{58.0}$	$\frac{93.0}{87.0^{+}}$
$\frac{67\text{–}80^{++}}{66.0^{+}}$	$\frac{62\text{–}82^{++}}{64.0}$	$\frac{35\text{–}57^{++}}{29\text{–}61^{++}}$	$\frac{74\text{–}94^{++}}{88.5}$	$\frac{4\text{–}21^{++}}{70.0^{+}}$	$\frac{33\text{–}71^{++}}{69.0^{+}}$	$\frac{65\text{–}86^{++}}{74.5^{+}}$	$\frac{92.0^{++}}{95.5}$

Mycotoxin	*Detection*				
	Prior to chemical detection	*Chemical detection*		*After chemical detection*	
		p-Anisaldehyde	*$FeCl_3$*	*p-Anisaldehyde (UV, 366 nm)*	*$FeCl_3$ (UV, 366 nm)*
4-Acetamido-4-hydroxy-2-butenoic acid-γ-lactone	—	t	—	y	—
Aflatoxin B_1	b	m	—	o	b
Aflatoxin B_2	m	b	—	m	m
Aflatoxin G_1	b	—	—	v-o	b
Aflatoxin G_2	m	—	—	k	m
Aflatoxin M_1	b	—	—	z-o	r
Altenuene	z	e	β	m	r
Alternariol	b	s	s	b	b
AME	b	s	l	b	r
Aspergillic acid	g	o	γ	g	g

(Continued on p. 146)

TABLE I *(continued)*

$R_F \times 100$ *values on* *silica gel G* / *Silufol*

A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
2–25^{++}	0–48^{++}	0–5^{++}	0–24^{++}	0–13^{++}	0–20^{++}	37.0^{+}	72.0^{+}
21.0^{+}	0–24^{++}	9.0	49.0^{+}	0–15^{++}	16.0	9–27^{++}	45.0
25.5^{+}	30.0^{+}	9.5^{+}	80.0^{+}	1.0^{+}	9.5^{+}	8.0^{+}	86.0^{+}
23.5^{+}	23.0^{+}	6.0^{+}	60.5^{+}	1.5	5.5^{+}	10.0^{+}	79.0^{+}
18.0	27.0	5.0	64.5	0.0	0.0	2.5	90.0
18.0	22.0	5.5	54.0	0.0	0.0	4.5	78.0
39.0	30.5	19.5	90.0	14.5	34.5	27.5	82.0
28.5	22.5	16.0	83.0	18.0	21.5	33.5	73.0
31.0	27–44^{++}	2.0	31.0	0.0	0.0	21.0	69.0
23.5	11.5	0–11^{++}	70.0	0–12^{++}	0–14^{++}	12.5	58.0
17.0	7.0	11.0	53.0	6.5	8.5	11.0	57.0
50.0	42.5	31.5	85.0	18.5	32.0	30.5	81.0
34.0	41.5	24.5	80.0	22.5	23.0	43.5	88.0
40.5	46.0	19.0	91.0	24.0	38.0	43.0	66.5
28.0	24.0	20.5	71.0	28.5	34.5	41.0	71.5
52.5	53.0	28.0	89.0	15.0	29.0	50.0	93.5
34.5	39.5^{+}	24.5^{+}	83.5	28.0	23.0	44.0	0–100^{++}
12.5	15.0	2.5	52.0	0.0	2.0	6.0	65.0
4.0	5.0	0.0	31.0^{+}	0.0	0.0	1.5	44.0^{+}
0–45^{++}	60.0^{+}	0–12^{++}	0–46^{++}	0–17^{++}	0–15^{++}	0–38^{++}	0–100^{++}
0–13^{++}	0–12^{++}	1.5	62.0	0.0	0.0	0.0	94.0
7.0	5.0	0.0	50.0	0.0	0.0	0.0	67.5
7.0	0.0	0.0	36.5	0.0	0.0	0.0	50.0
52.0	59.5	34.5^{+}	79.0	0–11^{++}	0–23^{++}	56.5	95.0
28.5^{+}	42.0	22.5^{+}	71.0^{+}	30.5^{+}	28.0^{+}	47.0^{+}	86.5

Mycotoxin	*Detection*				
	Prior to chemical detection	*Chemical detection*		*After chemical detection*	
		p-Anisaldehyde	*FeCl$_3$*	*p-Anisaldehyde (UV, 366 nm)*	*FeCl$_3$ (UV, 366 nm)*
Citrinin	γ	—	γ	u	γ
Citreoviridin	γ	i	γ	γ	γ
Cyanein	—	c	—	g	—
Diacetoxyscirpenol	—	β	—	ε	—
N,N′-dibenzylethylene-diamine	—	γ	p	u	—
Fusaric acid	y	—	—	y	—
Gliotoxin	—	r	—	g	—
Griseofulvin	b	a	—	γ	b
Helvolic acid	γ	b	—	v	—
Kojic acid	u	p	f	u	g
Luteoskyrin	h	o	h	o	—
Nivalenol	—	t	—	i	—
Ochratoxin A	d	—	f	m	m

(Continued on p. 148)

TABLE I *(continued)*

$R_F \times 100$ *values on* $\frac{\textit{silica gel G}}{\textit{Silufol}}$

A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
41.0	46.0	12.0$^+$	65.0	0.0	2.0	33.5	79.0
80.0	72.0	75.0	91.0	53.0$^+$	73.5	86.0	87.0
34.0	37.0	22.0	72.0	20.5	25.0	30.0	78.0
24.0	27.0	14.0	61.5	19.5	17.0	24.0	70.5
32.0	43.0	18.5	76.0	18.5	22.5	31.0	88.5
27.5	31.5	14.5	66.5	17.5	15.5	26.5	76.0
0.0$^+$	0.0$^+$	0.0$^+$	34.0$^+$	0.0$^+$	0.0$^+$	0.0$^+$	84.0$^+$
0.0	0.0	0.0	28.0	0.0	0.0	0.0	88.0
0–34^{++}	51.5$^+$	1.5	0–38^{++}	0–4^{++}	0–5^{++}	27.5$^+$	92.0$^+$
28.0$^+$	37.0$^+$	16.0$^+$	62.5$^+$	13.5$^+$	16.0$^+$	27.5$^+$	87.0$^+$
45.0$^+$	55.5	0.0	54.0$^+$	0.0	5.0	20–37^{++}	72.0$^+$
28.0	32.5	0–9^{++}	68.5	0–6^{++}	0–11^{++}	12–35^{++}	85.0$^+$
67.5	71.0	59.0	93.0	71.5	74.0	78.0	84.5
51.0	49.5	46.0	81.5	80.5	56.5	73.0	83.0
55.0	66.0	55.0	79.0$^+$	51.0	49.0	63.0	90.5
31.0	43.5	29.5	62.5	51.5	39.5	44.0	81.0
43.5	32.0	16.5	92.0	16.0	38.5	38.5	81.0
34.5	46.0	26.5	80.0	36.5	33.5	47.0	88.5
68.5	55.5	43.0	92.0	63.0	73.5	69.5	81.5
52.0	43.0	52.0	83.0	64.0	55.0	64.0	83.0
0–12^{++}	0–41^{++}	0.0	0–12^{++}	0.0	0.0	0–17^{++}	57.5$^+$
28.5$^+$	0–37^{++}	14.0$^+$	61.0$^+$	14.5$^+$	20.0$^+$	0–24^{++}	75–100^{++}
56.5	58.5	40.0	88.5	61.5	61.5	64.0	84.0
32.0	50.5	29.0	81.0	50.0	39.5	51.5	84.5
57.0	65.5	29.5	81.5	29.0	42.5	71.5	87.0
33.0	42.0	22.0	77.0	27.0	27.0	44.0	93.0

Mycotoxin	*Detection*				
	Prior to chemical detection	*Chemical detection*		*After chemical detection*	
		p-Anisaldehyde	*FeCl$_3$*	*p-Anisaldehyde (UV, 366 nm)*	*FeCl$_3$ (UV, 366 nm)*
Ochratoxin B	b	—	—	b	—
Ochratoxin C	k	—	—	k	—
Patulin	γ	β	a	δ	—
Penicillic acid	—	β	—	α	—
Rubratoxin B	o	a	—	o	—
Rugulosin	γ	γ	y	γ	—
Secalonic acid	o	l	a	g	—
Sterigmatocystin	α	b	i	α	x
Terreic acid	γ	β	γ	γ	g
T-2 toxin	b	β	—	γ	—
Trichothecin	—	a	—	m	—
Viridicatum toxin	o	—	γ	o	—
Zearalenone	b	n	—	g	u
Mycophenolic acid	b	i	l	r	r

Detection reagents

(1) A solution of *p*-anisaldehyde, consisting of a mixture of 0.5 ml of *p*-anisaldehyde, 85 ml of methanol, 10 ml of glacial acetic acid and 5 ml of concentrated sulphuric acid was prepared just prior to use[4].

(2) A 1% solution of iron(III) chloride in ethanol[2].

Detection

The developed silica gel G plates were detected under UV light (UVIS, Desaga, Heidelberg, G.F.R.) at a wavelength of 366 nm and the Silufol sheets at wavelengths of 366 and 254 nm. One series of chromatograms was detected by spraying with *p*-anisaldehyde reagent[4]. The silica gel G plates were then heated for 15–20 min at 130° and the Silufol sheets for 15–20 min at 60° (Silufol sheets are bonded with starch and become black at higher temperatures).

The second series of chromatograms was detected by spraying with the 1% solution of iron(III) chloride in ethanol[2]. After spraying with the detection reagents, the chromatograms were checked again under UV light (as prior to the chemical detection).

RESULTS

When comparing R_F values on uncleaned silica gel G plates (plates not developed in the corresponding system but containing only a standard moisture content being placed in a desiccator) with R_F values on cleaned silica gel G plates and on cleaned Silufol sheets in parallel experiments, the highest variations in the R_F values were found for the uncleaned silica gel G plates, lower variations for the cleaned silica gel G plates and the lowest variations for the cleaned Silufol sheets. The silica gel G plates and Silufol sheets were developed prior to application of the sample as otherwise in some systems (especially systems A and D) a fluorescent strip appeared in one third or in the middle of the developed plate. This fluorescent strip stopped some spots at its front and thus the R_F values were distorted.

From the parallel experiments on cleaned silica gel G plates and Silufol sheets, average R_F values were calculated. In Table I, these average R_F values and results with all detection methods are given.

The R_F values of individual substances were plotted against the solvent systems and characteristic "chromatographic spectra" were thus obtained for each metabolite under defined conditions. Examples of the chromatographic spectra obtained on cleaned Silufol sheets for aflatoxin B_1, AME, kojic acid and trichothecin are shown in Fig. 1. These metabolites differ in their chemical structures and chromatographic spectra.

From the fungal secondary metabolites, macrolide antibiotics with known cytotoxicity[15–21] were also analyzed by the method described. The R_F values on previously developed silica gel G plates and Silufol sheets and the results of the physical and chemical detection of these metabolites are given in Table II.

DISCUSSION

Chromatographic systematic analyses of various compounds are known.

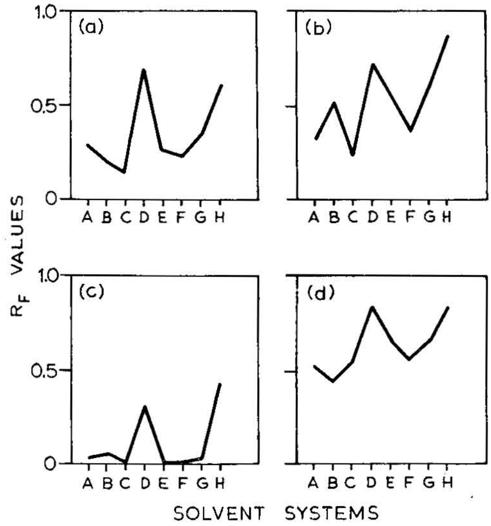

Fig. 1. Chromatographic spectra of secondary metabolites on cleaned Silufol sheets. (a) Aflatoxin B_1, (b) AME, (c) kojic acid, and (d) trichothecin.

Reio[22] described a paper chromatographic "reference system" for about 450 phenol derivates, mould metabolites and related compounds and for about 270 mono-, di- and trihydric phenol derivatives and various compounds of biochemical interest[23]. He developed the compounds in six solvent systems, and ten detection reagents and ultraviolet light were used for their detection. The R_F values of each compound were recorded on documentation reference cards in a form of "chromatographic spectrum" and the results of the detection were designated by a numerical index. Van Sumere *et al.*[24] obtained R_F values and described the detection of 93 phenolic substances and coumarins on thin layers using two solvent systems. They pointed out the advantages of using steamed plates in order to obtain a better separation of substances with similar polarities. A systematic analysis of antibiotics by paper chromatography has been described[25]. In the first stage, antibiotics were analysed simultaneously in four principal solvent systems and classified according to the results into 5 classes and 14 subclasses. Subsequently, supplementary systems were used and "summarized chromatograms" for 62 antibiotics were obtained. A systematic analysis of antibiotics by TLC was described by Aszalos *et al.*[26], in which 84 antibiotics were classified into four main classes according to their R_F values in three solvent systems. By the further application of 11 solvent systems, they obtained 15 sub-groups. For the routine identification of lichen-forming fungi by TLC, Culberson and Kristinsson[27] developed a systematic analysis of 104 products in three solvent systems. Spots of unknown substances were divided into eight classes according to the R_F values relative to the R_F values of two standard lichen substances. They recorded their results on cards in a similar manner to Reio. They consider that the advantage of using the R_F values for reference substances to which the R_F values of unknown substances are related, in comparison with the absolute R_F values of unknown compounds, consists in a smaller variation of the relative R_F values as a result of changes in the conditions of

TABLE II

R_F × 100 VALUES OF MACROLIDES ON CLEANED SILICA GEL G PLATES AND CLEANED SILUFOL SHEETS IN 8 SOLVENT SYSTEMS AND RESULTS OF PHYSICAL AND CHEMICAL DETECTION

Notes as in Table I.

R_F × 100 values on silica gel G / Silufol								*Macrolide*	*Detection*				
									Prior to chemical detection (UV, 366 nm)	*Chemical detection*		*After chemical detection*	
A	*B*	*C*	*D*	*E*	*F*	*G*	*H*			*p-Anisaldehyde*	*FeCl₃*	*p-Anisaldehyde (UV, 366 nm)*	*FeCl₃ (UV, 366 nm)*
23.0	53.0	15.0	72.0	18.0	16.0	31.0	98.0	Curvularin	—	z	—	m	—
26.0	36.5	10.0	63.5	12.0	11.0	22.5	94.0						
18.0	27.0	5.0	64.5	0.0	0.0	2.5	90.0	Cyanein	—	c	—	g	—
18.0	22.0	5.5	54.0	0.0	0.0	4.5	78.0						
41.0	57.5	25.5	85.0	17.0	33.5	46.5	99.5	Cytochalasin A	—	p	—	b	—
34.0	42.5	23.5	83.0	20.0	31.0	44.0	95.0						
28.5	46.0	13.5	78.0	8.0	16.0	24.0	98.5	Cytochalasin B	—	β	—	b	—
34.5	34.5	13.5	74.5	7.0	14.0	26.0	94.5						
19.5	24.5	10.0	75.5	0.0	8.0	14.5	90.0	Cytochalasin D	—	a	—	o	—
32.0	27.0	10.5	74.5	4.0	9.0	20.0	85.0						
39.5	55.5	34.5	76.0	46.0	42.5	53.0	97.5	Monorden	—	i	—	γ	—
35.0	44.0	22.0	77.0	33.5	31.5	42.5	94.5						
25.0	17.0	19.0	77.5	10.0	25.0	20.0	63.0	Vermiculin	—	s	—	γ	—
35.0	16.5	20.5	78.0	10.0	19.0	22.0	50.0						
56.5	58.5	40.0	88.5	61.5	61.5	64.0	84.0	Zearalenone	b	n	—	g	u

the environment compared with the absolute R_F values. Different aspects of the systematic analysis of substances, especially those of pharmaceutical interest, by paper and thin-layer chromatography were discussed by Macek[28].

We investigated 43 mycotoxins and other fungal secondary metabolites by means of our systematic TLC analysis in eight solvent systems. From the results, a characteristic chromatographic spectrum for each substance was constructed (Fig. 1) which is used as one of the primary criteria when comparing unknown mycotoxins isolated from different materials.

The solvent systems chosen have different polarities. Systems of low polarity are E, C, and F, those of medium polarity are G, A and B and the most polar are D and H. Systems that give a large scatter of R_F values for different mycotoxins are advantageous. The greatest scatter of R_F values is obtained with the systems of medium polarity.

On comparing the average R_F values for all of the substances analyzed on cleaned silica gel G plates and cleaned Silufol sheets, the latter were found to give lower R_F values.

Table I includes metabolites which gave "tails" in certain systems. Other metabolites moved in the form of elongated spots in some systems. The numerical limits given in the table indicate the beginning and the end of these elongated spots. The greatest problem occurred in the chromatography of rubratoxin B[29], which gave long tails along the whole length of the silica gel G plates when systems B and D were used. The sample of rubratoxin was probably not homogeneous as in system D, for example, it was separated into eight spots on Silufol sheets.

In Table I, the results of chemical and physical detection are indicated by letters. When evaluating the colour of a spot after chemical detection, it is sometimes difficult to define the colour (*e.g.*, light brown, beige, grey-brown, etc.). Similar problems are encountered in the exact description of fluorescence in physical detection (*e.g.* light yellow, yellow-orange, etc.). With Silufol sheets, the spots were checked prior to the chemical detection under UV light at 366 nm (longwave UV light) and 254 nm (shortwave UV light). In Table I, results obtained under longwave UV light are given. Under shortwave UV light, the spots fluoresced at the same wavelength but with lower intensity and luminance.

When comparing the chromatographic spectra of the individual metabolites and their chemical structures, we can draw the following conclusions. Aflatoxins have similar chromatographic spectra but when sterigmatocystin with a related chemical structure (coumarin) is added to this group, its resulting chromatographic spectrum is different. Similarly, when comparing metabolites from the scirpens (diacetoxyscirpenol, T-2 toxin and trichothecin), their chromatographic spectra are also differentiated.

Our aim, however, was not to investigate the relationships between the chemical structures and chromatographic spectra of fungal metabolites. The significance of this systematic analysis is in the use of chromatographic spectra as one of the fundamental characteristics which, together with other data (melting point, molecular weight, mass and infrared spectra, etc.), helps to identify unknown mycotoxins. By means of the chromatographic spectrum of an unknown substance, it is possible to carry out a preliminary identification of the metabolite obtained simply and rapidly, without expensive equipment, by comparing it with the known chromatographic spectra, or to eliminate the known metabolites from the unknown. We expect to use this system in the screening of mycotoxins, where it can be combined with the bioautographic detection of chromatograms employing an undemanding laboratory model, *Artemia salina* larvae[11]. The bioautographic detection of mycotoxins on thin-layer chromatograms is suitable especially at the stage where the unknown mycotoxins are available only in the form of crude extracts.

REFERENCES

1 R. M. Eppley, *J. Ass. Offic. Anal. Chem.*, 51 (1968) 74.
2 P. S. Steyn, *J. Chromatogr.*, 45 (1969) 473.
3 L. Fishbein and H. L. Falk, *Chromatogr. Rev.*, 12 (1970) 42.
4 P. M. Scott, J. W. Lawrence and W. van Walbeek, *Appl. Microbiol.*, 20 (1970) 839.
5 L. Stoloff, L. Nesheim, L. Yin, J. V. Rodricks, M. Stack and A. D. Campbell, *J. Ass. Offic. Anal. Chem.*, 54 (1971) 91.
6 B. D. Jones, *Tropical Products Institute Report 56/62*, Tropical Products Institute, London, 1972.
7 R. C. Garner, *J. Chromatogr.*, 103 (1975) 186.
8 R. W. Pero, R. G. Owens and D. Harwan, *Anal. Biochem.*, 43 (1971) 80.
9 A. E. Pohland, K. Sanders and C. W. Thrope, *J. Ass. Offic. Anal. Chem.*, 53 (1970) 692.
10 J. P. Snow, G. B. Lucas, D. Harvan, R. W. Pero and R. G. Owens, *Appl. Microbiol.*, 24 (1972) 34.
11 Z. Ďuračková, V. Betina and P. Nemec, *J. Chromatogr.*, 116 (1976) 155.
12 "Changes in Official Methods of Analysis", *J. Ass. Offic. Anal. Chem.*, 49 (1966) 229.
13 B. Keil, *Laboratorní Technika Organické Chémie*, Nakladatelství ČSAV, Prague, 1963.
14 K. Chmel, personal communication.
15 W. B. Turner, *Fungal Metabolites*, Academic Press, London, 1971.
16 S. B. Garter, *Nature (London)*, 213 (1967) 261.
17 V. Betina, P. Nemec, J. Dobiáš and Z. Baráth, *Folia Microbiol.*, 7 (1962) 353.
18 D. Bačíková, V. Betina and P. Nemec, *Naturwissenschaften*, 51 (1964) 445.
19 D. Bačíkova, V. Betina and P. Nemec, *Nature (London)*, 206 (1965) 1371.
20 J. Fuska, L. P. Ivanickaja, K. Horáková and I. Kuhr, *Antibiotics*, 27 (1974) 141.
21 J. Fuska, L. P. Ivanickaja, L. V. Makucho and L. J. Volkova, *Antibiotiki*, 19 (1974) 890.
22 L. Reio, *J. Chromatogr.*, 1 (1958) 338.
23 L. Reio, *J. Chromatogr.*, 4 (1960) 458.
24 C. F. van Sumere, G. Wolf, H. Teuchy and J. Kint, *J. Chromatogr.*, 20 (1965) 48.
25 V. Betina, *J. Chromatogr.*, 15 (1964) 379.
26 A. Aszalos, S. Davis and D. Frost, *J. Chromatogr.*, 37 (1968) 487.
27 Ch. F. Culberson and H.-D. Kristinsson, *J. Chromatogr.*, 46 (1970) 85.
28 K. Macek, in K. Macek (Editor), *Pharmaceutical Applications of Thin-Layer and Paper Chromatography*, Elsevier, Amsterdam, 1972, p. 116.
29 B. J. Wilson and R. D. Harbison, *J. Amer. Vet. Med. Ass.*, 163 (1973) 1274.

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BIOAUTOGRAPHIC DETECTION OF MYCOTOXINS ON THIN-LAYER CHROMATOGRAMS

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SUMMARY

A method for the bioautographic detection of mycotoxins on thin-layer chromatograms by using *Artemia salina* larvae is described. The method was tested on standard samples of mycotoxins (aflatoxin B_1, kojic acid and sterigmatocystin) and on the extracts from toxicogenic fungi isolated from different sources.

INTRODUCTION

Bioautography is used in the chromatography of biologically active substances, especially antibiotics. A recent critical review of the application of bioautography as a special detection method in the paper and thin-layer chromatography of antibiotics[1] indicated the wide range of biological systems used for bioautography: animal viruses, bacteriophages, bacteria, fungi, protozoa, algae and animal cells.

In the study of the toxicity of mycotoxins, *Artemia salina* larvae[2] have been shown to be a suitable model organism. A method for the bioautographic detection of mycotoxins on chromatograms has been developed that is suitable for the initial stages of the screening of mycotoxins when they are available only in crude extracts, either from pure cultures of fungi or contaminated food, fodder and raw material for foodstuffs as well as in instances when physical and especially chemical detection cannot be applied to chromatograms. The bioautographic detection is followed in our study of mycotoxins by a systematic analysis by thin-layer chromatography[3], which, together with other methods, helps to identify the known mycotoxins and to detect unknown mycotoxins.

MATERIALS AND METHODS

Test organism

Artemia salina larvae are grown from commercially available eggs in Frank's medium[4]. To 750 ml of tap water (total hardness of which is 8–14°, German scale) two teaspoonfuls (*ca.* 12.0 g) of sodium chloride (non-iodinated) are added and the pH of the medium is adjusted to 7.4 with 1 *N* potassium hydroxide solution. One teaspoonful (*ca.* 0.2 g) of eggs of *A. salina* is added to the medium, which is incubated at 28° while subjecting it to intensive aeration. Larvae appear within 18–24 h. The medium is made up daily to the original volume with distilled water.

Mycotoxins

We used aflatoxin B_1 (Dr. L. Shoetwell, U.S. Department of Agriculture, Northern Research Laboratories, Peoria, Ill., U.S.A.), kojic acid (this Department) and sterigmatocystin (Dr. P. S. Steyn, National Chemistry Research Laboratories, Pretoria, South Africa). Standard solutions of mycotoxins were prepared at a concentration of 1 mg/ml in the following solvents: benzene–acetonitrile (98:2) for aflatoxin B_1, ethyl acetate for kojic acid and chloroform for sterigmatocystin.

Solvent systems

Systems A, B, C, D, E, F, G and H from ref. 3 (this issue, p. 143) were used.

Chromatography of the standards of mycotoxins

Pre-coated thin layers on Silufol sheets (Kavalier, Votice, Czechoslovakia) (20 × 20 cm) were used; they were cleaned prior to chromatography by development in the system in which the sheets were subsequently developed with samples. From our set of systems for the systematic analysis of mycotoxins[3] the following were used: B, toluene–ethyl acetate–90% formic acid (6:3:1); and D, chloroform–methanol (4:1).

On a developed sheet, 10 μl of standard solutions of mycotoxins ($\equiv$ 10 μg of substance) were applied and developed in system D to a height of 10 cm. Simultaneously, Silufol sheets without standards were developed in systems B and D.

Bioautographic detection of mycotoxins on chromatograms

From the developed chromatograms on Silufol sheets, 1.5 × 10-cm strips were cut out vertically, and identified as follows:

strip a: a sheet developed in system D;

strip b: a sheet developed in system D (cleaning) and with a sample of sterigmatocystin developed again in this system;

strip c: a sheet cleaned in system D and with a sample of aflatoxin developed again in system D;

strip d: a sheet cleaned in system D and with a sample of kojic acid developed again in system D;

strip e: a sheet developed twice in system D (without sample);

strip f: a sheet developed once in system B (without sample).

These strips were cut into 1.5 × 1.5-cm squares and adsorbent from the squares was scraped into separate test-tubes. Into each test-tube, 1 ml of the suspension of *Artemia salina* larvae was added and thoroughly stirred, and from each tube 0.2-ml portions were pipetted into four porcelain wells. On average, 20–40 larvae were present in each well. The wells were checked at zero time with an SM XX stereoscopic microscope (Zeiss, Jena, G.D.R.) at a magnification 1.6 × 6.3 for the presence of dead larvae. The wells were covered with micro-scale cover slips in order to prevent evaporation of the solutions. Incubation was carried out at 20–22° and after 16 h the dead larvae in each well were counted under the stereoscopic microscope. The remaining living larvae were then killed by adding a few drops of chloroform and all larvae were counted again. On the white background of the well, orange larvae were clearly visible and could be distinctly discerned from brown ball-like eggs. The percentage mortality for all samples was calculated[2].

Cultivation of fungi

The use of the bioautographic detection of unknown mycotoxins in thin-layer chromatography was tested on crude extracts of secondary metabolites from 33 fungi isolated from various foodstuffs. From well sporulated cultures on slant agars (PCA medium: 200 g of potatoes, 100 g of carrots, 20 g of agar and 1000 ml of water), a spore suspension was prepared. Roux flasks of volume 2 l, containing 200 ml of YES medium[5], were inoculated with this suspension and cultivated under static conditions at 28° for 7–21 days.

Extraction

Mycelium of the grown culture and medium were separated by filtration. Mycelium was extracted twice with 50 ml of chloroform, filtered and the combined chloroform extracts were dried over anhydrous calcium chloride and evaporated to dryness under reduced pressure at 50°. The residues were dissolved in 2-ml volumes of chloroform. From the filtrate of the medium, 85 ml were taken and the pH was adjusted to 3.4 with dilute sulphuric acid (1:10). The filtrate was extracted with 50 ml of chloroform by shaking in a separating funnel and the aqueous layer was extracted again with 35 ml of chloroform in a separating funnel. The combined chloroform extracts were dried over anhydrous calcium chloride and the solvent was evaporated under reduced pressure at 50°. The residues were dissolved in 2-ml volumes of chloroform.

Testing the toxicity of extracts to A. salina

Into separate test-tubes, 100 μl of concentrated chloroform extracts from mycelium and from the medium and from the original filtrate of the medium were pipetted, allowed to evaporate in a thermostat at 60° and, when cooled, 25 μl of dimethyl sulphoxide (DMSO) were added to each, thus dissolving the residues. Into the control test-tubes, only 25 μl of DMSO were added. Into each test-tube, 1 ml of larval suspension was added and then the method of testing the standards of mycotoxins was followed. From the results obtained, the percentage mortality[2] was calculated for the samples investigated.

Chromatography of extracts and bioautographic detection

On Silufol sheets, already developed, 10–100 μl (depending on the percentage mortality) of chosen extracts from mycelium or medium filtrate were applied. The sheets with samples were developed in the system[3] in which the separation of the sample of extract was the best. From Silufol sheets, vertical strips with samples 1.5 cm wide were cut out along the whole chromatogram. These strips were cut into segments of 1 cm regardless of the position of the spots and the adsorbent was removed from them into separate test-tubes. For each strip, a control was also prepared; the control contained adsorbent from the square above the front of the chromatogram.

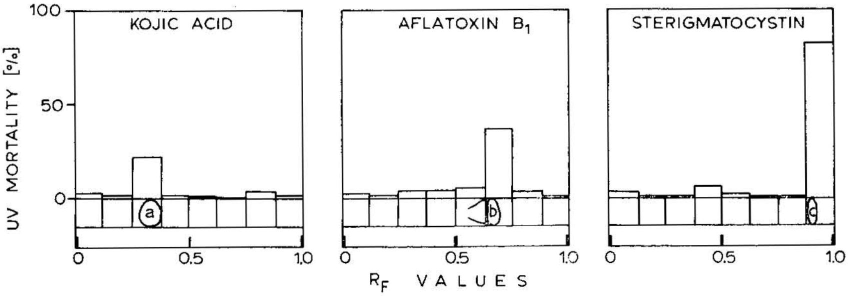

Fig. 1. Bioautographic detection of standard mycotoxins on Silufol sheets in system D. a, Dark yellow spot; b, blue-fluorescing spot; c, red-fluorescing spot.

Into each test-tube with adsorbent, 1 ml of larval suspension was added and then the method for bioautography of the standards of mycotoxins was followed.

Isolation of unknown active substances from the extract

For the isolation of an active substance, the extract of filtrate of culture No. S-656 (Aspergillus sp.) was chosen. On the starting point of a plate with an unbound layer of silica gel L, 100–160 μm (Lachema, Brno, Czechoslovakia), 250 μl of concentrated chloroform extract obtained from the medium after cultivation of culture S-656 were applied; the plate was developed in system D three times. Fluorescent spots were detected under UV light and the adsorbent from these areas was collected by means of vacuum, eluted with methanol and evaporated to dryness. The residues were dissolved in methanol and a portion of them was tested for toxicity to *A. salina* larvae. The other portion of the residues of individual spots was applied on a Silufol sheet and developed in system D in order to determine the homogeneity of eluates from individual spots. The whole process of the isolation of active substances was repeated on an unbound layer of silica gel L with a larger volume of extract (1.7 ml).

RESULTS

Bioautographic detection of the standards of mycotoxins

Average percentage mortalities for strips from Silufol sheets a, b, c, d, e and f for all squares without mycotoxins ranged from 0 to 9.0%. The average mortality

TABLE I

TOXICITY OF EXTRACTS FROM SELECTED FUNGI TO LARVAE OF *Artemia salina*

Culture	*Taxonomic classification*	*Mortality of larvae (%)*		
		Extract of mycelium	*Extract of medium filtrate*	*Medium filtrate*
S-649	Trichothecium sp.	100.0	100.0	63.8
S-656	Penicillium sp.	100.0	95.1	13.5
S-658	Aspergillus sp.	79.2	77.0	58.6
S-662	*Penicillium camemberti*	17.5	50.0	70.5
S-664	*Penicillium roqueforti*	27.0	74.0	2.6
S-669	*Aspergillus repens*	41.0	65.0	

for a square with sterigmatocystin ($R_F = 0.91$ in system D) was 81%, that for a square with aflatoxin ($R_F = 0.65$ in system D) 36% and that for a square with kojic acid ($R_F = 0.33$ in system D) 21% (Fig. 1).

Testing the extracts of fungi

The results of testing some extracts from mycelia and medium filtrates after cultivation of fungi, investigated bioautographically, are given in Table I.

Bioautographic detection of metabolites in the extracts from fungi

According to the percentage mortality in crude extracts of fungi, chosen ex-

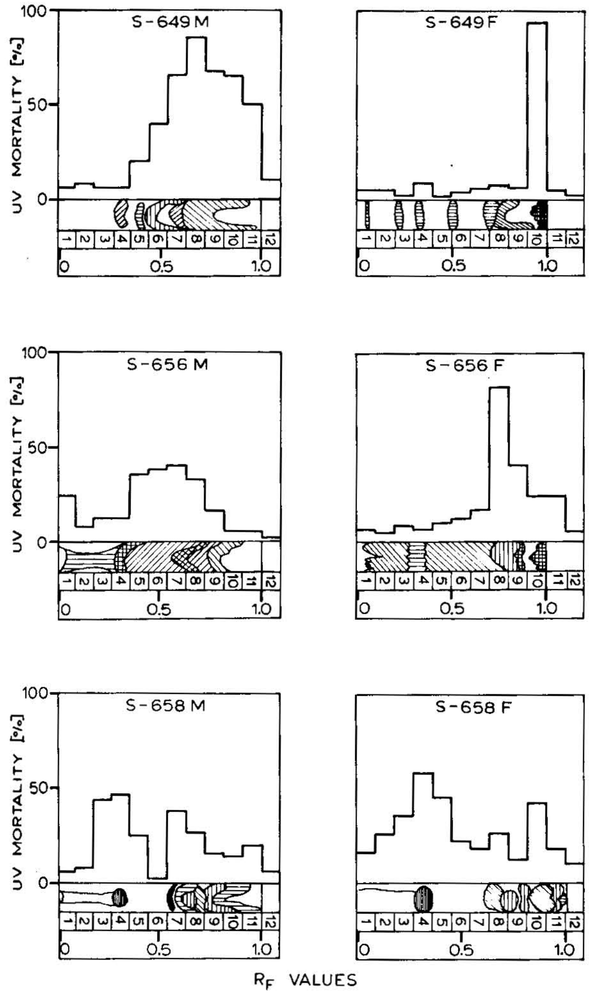

Fig. 2. Evaluation of bioautographic detection in cultures S-649, S-656 and S-658. All chromatograms were developed in system D. Applications on chromatograms: S-649, extract of mycelium 10 μl and extract of medium filtrate 20 μl; S-656, extract of mycelium 60 μl and extract of medium filtrate 60 μl; S-658, extract of mycelium 20 μl and extract of medium filtrate 25 μl. M = extract from mycelium; F = extract from medium filtrate.

tracts were examined by chromatography on Silufol sheets and detected bioautographically. The chromatographic results and the results of bioautographic detection are shown in Fig. 2. On the basis of a chromatographic comparison of the extracts of filtrate and mycelium (in system D) with the standards of aflatoxin B_1 ($R_F = 0.67$) and kojic acid ($R_F = 0.33$), it is considered that with culture No. S-658 kojic acid and aflatoxins are produced.

Isolation of active substances from a chromatogram

Residues of substances isolated from individual spots on the chromatogram on an unbound layer of silica gel were tested with *A. salina* and the percentage mortality showed that the active substance on the chromatogram was in the region of blue-green and dark green fluorescence (see Fig. 2). Adsorbents from these regions were combined, eluted with methanol and evaporated to dryness. The residues were dissolved in order to determine the toxic effect on *A. salina*. The remaining solution of active substances in methanol was applied on a Silufol sheet along the whole sheet (20 cm) and developed in system D. There were five different fluorescent strips which appeared under UV light. These were removed from the sheet, eluted and evaporated to dryness. From individual spots, the activity of the eluate towards *A. salina* was tested. The active ones were as follows: a pink-fluorescing substance (51% mortality), a green-fluorescing substance (59% mortality) and, closely above it, a yellow substance (85% mortality). These substances were analyzed by the chromatographic systematic analysis[3]. From the results, chromatographic spectra for all three substances were constructed (Fig. 3); these spectra were compared with those of 37 mycotoxins[3] and it was found that the chromatographic spectra of our three substances, produced by culture No. S-656, were different. We are still working on the identification of the unknown metabolites.

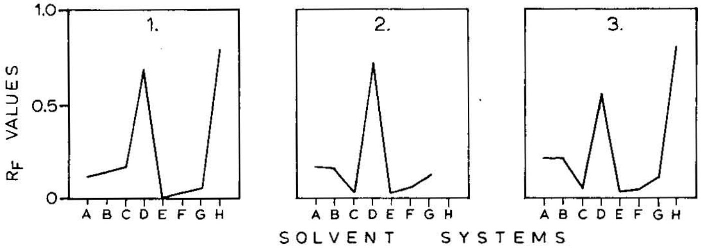

Fig. 3. Chromatographic spectra of isolated substances active towards *A. salina* from culture S-656. 1, Green-fluorescing substance; 2, yellow substance on the chromatogram; 3, pink-fluorescing substance.

DISCUSSION

Bioautographic detection is used mostly in the study of antimicrobial substances. The simple cultivation of microorganisms provides possibilities for determining the positions of antimicrobial substances on chromatograms. When studying mycotoxins by employing higher animals (mouse, rat, chicken, etc.), a similar detection would not be possible.

In our method of bioautographic detection, the sensitivity of *A. salina* larvae were tested on standard samples of mycotoxins[2]. The advantage of using this model organism for the bioautographic detection is the simplicity of cultivation of the larvae.

For testing the method for the detection of standard mycotoxins, one neutral (D) and one acidic (B) system were used for the development of chromatograms on Silufol sheets. The average percentage natural mortality of larvae in the detection of adsorbent from control squares from Silufol sheets is lower with the neutral system (0–6.7%) than with the acidic system B (0–9.0%).

When employing the acidic system (B), the pH of the medium is not substantially influenced after pouring the adsorbent from the squares cut from chromatograms with larvae cultivation medium.

The possibility of detecting mycotoxins by inserting the whole square from a Silufol sheet into a test-tube (or by cutting it into smaller squares) with larvae suspension was examined. This process did not prove to be suitable as the sharp edges of the aluminium foil damaged mechanically the larvae which had already stopped moving at zero time of testing.

Two types of cultures for testing the extracts for the content of mycotoxins were chosen: (1) fungi of random contaminated foodstuffs and raw materials for foods were isolated; (2) cultures used in the production of some foodstuffs were tested. Wei *et al.*[6] isolated a mycotoxin PR-toxin from the culture *Penicillium roqueforti*. Their culture was isolated, however, from contaminated fodder but, as shown by our results on the toxicity towards *A. salina* of extracts from *P. roqueforti* (S-664) used in the production of cheese, it is necessary to investigate the potential toxigenicity of fungi used in the foodstuffs industry, as the presence of mycotoxins in products can also be caused by used toxicogenic fungi.

The above method for the bioautographic detection of mycotoxins on thin layers by using *Artemia salina* larvae could be applied in screening mycotoxins in contaminated foodstuffs, raw materials for food and fodders owing to its simplicity and reproducibility.

REFERENCES

1 V. Betina, *J. Chromatogr.*, 78 (1973) 41.

2 Z. Ďurackova, V. Betina, B. Horníková and P. Nemec, *Zentralbl., Bakteriol., Parasitenk., Infektionskr. Hyg., Abt. 2*, in press.

3 Z. Ďuračková, V. Betina and P. Nemec, *J. Chromatogr.*, 116 (1976) 141.

4 S. Frank, personal communication.

5 N. D. Davis, N. L. Diener and D. W. Eldrige, *Appl. Microbiol.*, 14 (1966) 378.

6 R. D. Wei, P. E. Still, E. B. Smalley, H. K. Schoes and F. M. Strong, *Appl. Microbiol.*, 25 (1973) 111.

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CHROM. 8641

METHOD FOR THE MEASUREMENT OF PLASMA DEHYDROEPIANDROSTERONE BY GAS–LIQUID CHROMATOGRAPHY WITH ELECTRON CAPTURE DETECTION

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SUMMARY

A method is described for the measurement of plasma dehydroepiandrosterone, as the iodomethyldimethylsilyl ether derivative, by gas–liquid chromatography and electron capture detection using the relatively new and highly stable stationary phase Dexsil 300. Preliminary purification of the plasma extract was required and alumina column chromatography was utilised, both before and after derivatization of the steroid extract. Specificity, precision, sensitivity and accuracy were all satisfactory. The method was used to study the relationship between age and the level of plasma dehydroepiandrosterone in a group of normal women. A significant negative correlation was observed.

INTRODUCTION

A method for the measurement of testosterone in human plasma was described by Thomas[1] in which the practicability of the use of gas–liquid chromatography (GLC) techniques for the measurement of the very small quantities of steroid hormones in plasma was discussed. A particular advantage, namely the potential of the method for the analysis of other steroids within the same plasma extract, was also cited. It has been possible, with slight modification of the chromatography of the iodomethyldimethylsilyl ether derivatives on alumina, to measure free dehydroepiandrosterone (DHEA) also. The working time of the method has been reduced by the use of smaller alumina columns.

EXPERIMENTAL

Materials

Blood was obtained from normal women between 16.00 and 20.00 h. The heparinized plasma was separated and stored at −20°.

Neutral alumina (Woelm, Eschwege, G.F.R.) was partially deactivated by equilibration with water (9%, w/w). [^{3}H]DHEA was obtained from the Radiochemical Centre (Amersham, Great Britain) (specific activity: 10–25 Ci/mmole).

Reagents were as described by Thomas[1]. All glassware was washed successively with hot methanolic Decon, water and ethanol. Chromic acid was used instead of the Decon mixture for every fifth wash.

GLC was carried out on a Pye 104, Model 74 gas chromatograph fitted with a 7.5 mCi [^{63}Ni]electron capture detector. The detector was used in the pulse mode (500-μsec interval) and the detector oven was set at 300°. The column (1.5 m × 4 mm) was all glass and fitted to the detector with a standard glass-to-metal connector. The column was packed with Gas-Chrom Q (100–120 mesh) and coated with 1.5% Dexsil 300 (Analabs, North Haven, Conn., U.S.A.). Prior to packing, any coated support smaller than 100 mesh was removed by sieving. Before use the column was conditioned for a minimum of 24 h at 300° with a slow flow of nitrogen at approximately 25 ml/min. The operating temperature of the column was 250–260° with a gas flow-rate of 100 ml/min. When not in use the column was kept at 150° and the gas flow-rate reduced to 25 ml/min. Under these conditions the useful life of the column was at least six months.

Radioactive counting was performed on a Nuclear Chicago, Mark II, liquid scintillation counter. The counting fluid (10 ml) was 2.5 l toluene containing 11 g Permablend 111 (Packard, Downers Grove, Ill., U.S.A.).

Method

[^{3}H]DHEA (30,000 dpm in toluene, 10 μl) was added to plasma (3 ml), followed by 1 *N* NaOH (three drops). The mixture was left at room temperature for 15 min. The steroids were extracted with ether (2 × 5 ml). The ether extracts were combined and washed with water (3 × 1 ml) and taken to dryness under a stream of nitrogen at 60°. (This was the standard technique for drying used throughout the method.)

Hexane (2 ml) and 70% aqueous methanol (2 ml) were added to the dried extract. After vigorous shaking the two layers were separated by centrifugation (1000 *g*, 5 min). The upper hexane layer was discarded. The partition was repeated using more hexane (1 ml). This step removed less polar compounds including progesterone. The remaining methanolic extract was dried.

This extract was redissolved in benzene (0.5 ml) and subjected to alumina chromatography. A slurry of alumina (1.5 g) was prepared in benzene. The column was formed in a pasteur pipette with a drawn-out end lightly plugged with silanised glass wool. The extract was applied to the column: 0.8% ethanol in benzene (5 ml) was used for a pre-wash and was discarded. DHEA was eluted in a further 5 ml of 0.8% ethanol in benzene.

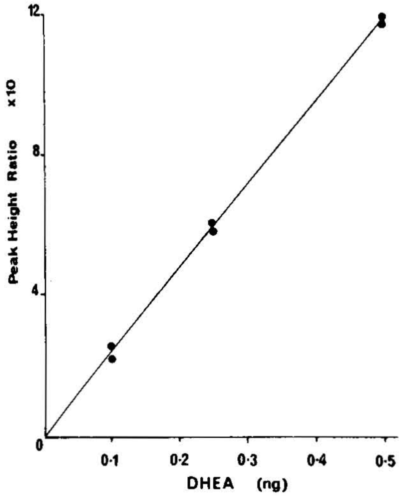
The fraction thus obtained was dried and converted to the iodomethyldimethylsilyl ether (IDMSE) derivatives[1]. These derivatives were dissolved in cyclohexane (0.5 ml) and subjected to further alumina chromatography. Chromatography was performed as before with the following exceptions: The slurry was prepared in cyclohexane; the sample was loaded in cyclohexane; the pre-wash was with a 1% acetone in cyclohexane mixture (5 ml); the derivatives were eluted in 1% acetone in cyclohexane (5 ml).

5α-Androstene-3β,17β-diol-IDMSE (20 ng) was added to the column eluate as internal standard for the GLC analysis. The mixture was dried and re-dissolved in hexane (0.1 ml). An aliquot (1/10) was taken for the determination of radioactivity. From the amount of radioactivity, correction was made for procedural losses. Further fractions (1/20) were taken for GLC analysis. The concentration of DHEA in plasma was calculated as follows:

$$\text{DHEA (ng/100 ml plasma)} = \frac{\text{ng in DHEA peak} \times 10^4}{\begin{array}{c}\text{equivalent amount of plasma injected}\\ \text{(ml)} \times \%\ \text{recovery } [^3\text{H}]\text{DHEA}\end{array}}.$$

RESULTS

Calibration curves of the ratio of the peak height of DHEA-IDMSE to the peak height of the internal standard (1 ng) against the quantity of DHEA were both linear and reproducible over the range 0.1–0.5 ng of DHEA (Fig. 1). A standard curve was run with each batch of analyses. A typical chromatogram of a plasma extract from a normal woman is shown in Fig. 2.



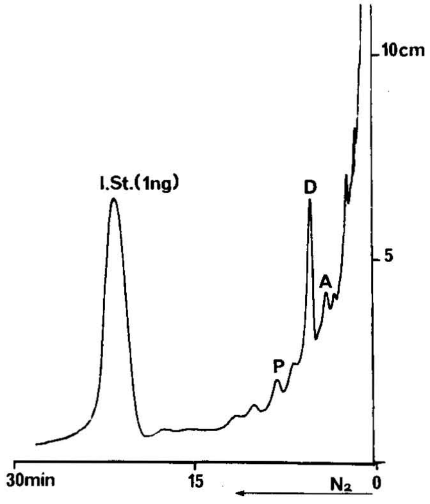


Fig. 1. Calibration curve of dehydroepiandrosterone-IDMSE using 5α-androstene-3β,17β-diol-IDMSE as internal standard.

Fig. 2. Gas chromatogram of the DHEA-IDMSE fraction from female plasma (3 ml). D = DHEA; A = androsterone; P = pregnenolone; I.St. = internal standard. Relative retention times: A = 0.165, D = 0.242, P = 0.367, I.St. = 1.00 (21.4 min).

Precision and sensitivity were calculated using the difference between duplicate analyses[2] and are shown in Table I.

Accuracy was determined by analysing normal saline samples to which a known amount of DHEA had been added, over the range 200–1000 ng/100 ml. The mean recovery from 24 analyses was 103.7 % with a standard deviation of 16.7 % (range 80.4–134.3 %). Recoveries were also determined after the addition of DHEA (500–1000 ng/100 ml) to plasma samples. A pool of plasma was repeatedly assayed.

TABLE I

PRECISION AND SENSITIVITY OF THE DETERMINATION OF DHEA IN PLASMA

No. of duplicates	*DHEA (ng/100 ml) (mean ± S.D.)*	*Precision (%)*	*Sensitivity DHEA (ng/100 ml)*
19	531 ± 36.9	± 7	56

The mean and standard deviation of the DHEA concentration were 740 ± 70 ng/100 ml ($n = 6$). The mean and standard deviation of the recovery were 104.7 ± 20.2% ($n = 9$, range 75–129%).

The overall recovery of radioactivity through the method was 42.6 ± 9%. This figure was determined from the recoveries obtained in the assay of 23 female-plasma samples.

Plasma was assayed in a group of 23 normal women. The mean and standard deviation were 539.4 ± 161 ng/100 ml. The age range was 30–75 years (mean and S.D., 48.6 ± 12.4 years). The plasma DHEA concentration was negatively correlated with advancing age (Fig. 3). This correlation was significant ($P < 0.01$).

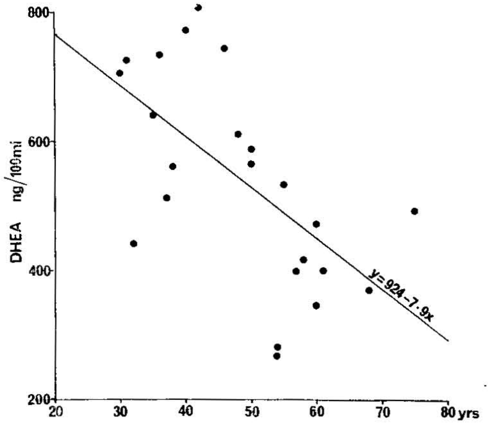


Fig. 3. Relationship of age and plasma DHEA in a group of normal women.

The specificity of the method was examined by the incorporation of a high-resolution paper chromatography step[3] into the method before the first alumina column stage. A series of nine plasma samples was processed in this manner and the results were compared with a duplicate series assayed in the normal way. The mean and standard deviation of the result were 439.0 ± 90.0 ng/100 ml, compared with that of 441.3 ± 90.7 ng/100 ml with the addition of paper chromatography to the method. No significant difference was found on application of a paired Student's t test to the comparisons ($t = -0.18$).

DISCUSSION

The precision and sensitivity of the method were found to be satisfactory. The accuracy as determined by analysis using normal saline as base was good but more variability was found when plasma was used. However, this is to be expected since the latter procedure requires the calculation of the difference between two measurements, each with an associated error, while the former requires only one measurement. The reliability of the method is comparable to other published procedures[4–7].

The use of 5α-androstene-3β,17β-diol as internal standard was justified in that any endogenous steroid in the plasma extract would be removed by chromatography on the alumina columns. Also, no interference has been found in that position on the gas chromatograms.

The specificity of the method was satisfactory in that a paper partition chromatography step incorporated into the method did not alter the result. The purification procedures of the method do impart some additional evidence of specificity. Through the method the compound has to behave as its radioisotope both before and after derivatization. Whilst this does not provide complete evidence that the DHEA peak on the gas chromatogram is homogeneous, further evidence may be inferred from the results of other workers, who, by using different techniques, obtained similar results. For instance, Gandy and Peterson[6] obtained values of 480 ± 320 ng/100 ml (range 140–1250) in a group of normal women (age range 20–40 years), using a double isotope derivative technique; Nieschlag *et al.*[5] obtained values of 405 ± 216 ng/100 ml (range 153–730) using radioimmuno-assay, and Rosenfeld[7], by a competitive binding assay, obtained 421 ± 303 ng/100 ml (range 137–1261).

Dexsil 300 has proved an excellent phase both in terms of its low background noise and stability. To achieve this certain precautions were found necessary, namely the removal of oxygen and water from the carrier gas. This was done by incorporating an oxygen scrubber in the gas line (Oxisorb; Supelco, Bellefonte, Pa., U.S.A.). In some instances, when the ECD standing current was lower than required, the use of a nitrogen purge gas at 25 ml/min was found beneficial. Difficulties with high background noise have been encountered with the prolonged use of other phases. The high temperature required to analyse steroids is probably the major cause of this.

An advantage of GLC methods over others is the potential for measuring a number of steroids within the same sample. The method described here allows the estimation of DHEA but the fraction eluted from the second alumina column contains other steroids such as androsterone and pregnenolone (although measurements of these steroids have not been evaluated). Also, if the column is further eluted with a mixture of acetone–benzene–cyclohexane (1:50:49), testosterone may be estimated. However, if the measurement of DHEA alone is required, it is possible, with the great sensitivity of the electron capture detector to the IDMSE derivative, to use as little as 0.5 ml of plasma for the analysis.

REFERENCES

1 B. S. Thomas, *J. Chromatogr.*, 56 (1971) 37.
2 J. B. Brown, R. D. Bulbrook and F. C. Greenwood, *J. Endocrinol.*, 16 (1957) 41.
3 B. S. Thomas and R. D. Bulbrook, *J. Chromatogr.*, 14 (1966) 28.
4 C. M. Andre and V. H. T. James, *Clin. Chim. Acta*, 43 (1973) 295.
5 E. Nieschlag, D. H. Loriaux and M. B. Lipsett, *Steroids*, 19 (1972) 669.
6 H. M. Gandy and R. E. Peterson, *J. Clin. Endocrinol.*, 28 (1968) 949.
7 R. L. Rosenfield, *Steroids*, 17 (1971) 689.

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CHROM. 8677

STUDY OF THE OXIDATION PRODUCTS OF PHENOLSILICON COMPOUNDS BY MOLECULAR LIQUID CHROMATOGRAPHY*

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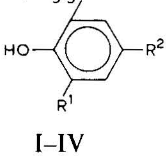
(Received August 5th, 1975)

SUMMARY

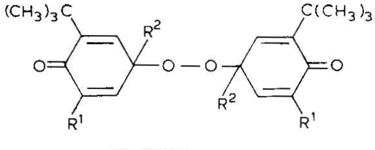
Molecular liquid chromatography was applied to the separation and analysis of thermally unstable organosilicon peroxides. The optimal conditions for the separation of the oxidation products of phenolsilicon compounds were determined and the influence of the amount of β,β'-oxydipropionitrile deposited on silica gel C-3 on the separation of these compounds was studied. By this method, bis(1-trimethylsilyl-3,5-di-*tert.*-butyl-2,5-cyclohexadien-4-one)peroxide was isolated preparatively. The degree of purity of fractions was determined by molecular liquid chromatography and IR spectroscopy.

INTRODUCTION

It was assumed that the oxidation of phenolsilicon compounds (I–III), in a similar manner to their alkyl-substituted analogue (IV), yields 4,4′-biscyclohexadienone peroxides (V–VIII), which can be used as a source of polymerization-initiating radicals.



I–IV



V–VIII

I, V: $R^1 = Si(CH_3)_3$; $R^2 = C(CH_3)_3$; II, VI: $R^1 = Si(CH_3)_2C_6H_5$; $R^2 = C(CH_3)_3$; III, VII: $R^1 = Si(CH_3)_3$; $R^2 = Si(CH_3)_3$; IV, VIII: $R^1 = C(CH_3)_3$; $R^2 = C(CH_3)_3$.

In order to study the mechanism of the thermal decomposition of peroxides of this type and to examine their interactions with different classes of compounds, one must have pure samples. Therefore, it is important to have suitable methods for the

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* Presented at the 5th Soviet–Italian Chromatography Symposium, Tallinn, April 22–25, 1975.

analysis and purification of 4,4′-biscyclohexadienone peroxides. Preliminary investigations showed that liquid chromatography is the most suitable technique for the separation and analysis of thermally unstable peroxides that decompose comparatively easily. In the literature, there is no evidence of application of this method to the study of the oxidation products of phenolsilicon compounds.

EXPERIMENTAL

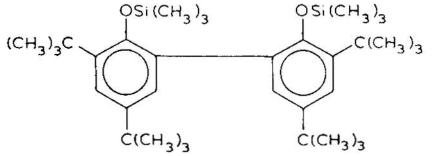
All investigations were carried out on a Tsvet-301 liquid chromatograph with a dielectric constant detector and a stainless-steel column (67 m × 4 mm I.D.). Silica gel C-3[1] with a specific surface area of 250 m^2/g was used as the adsorbent support. A layer of β,β'-oxydipropionitrile was deposited from solution on to this silica gel. After this modification, the new specific surface area was determined. *n*-Hexane was used as the eluent and analyses were carried out at room temperature.

RESULTS AND DISCUSSION

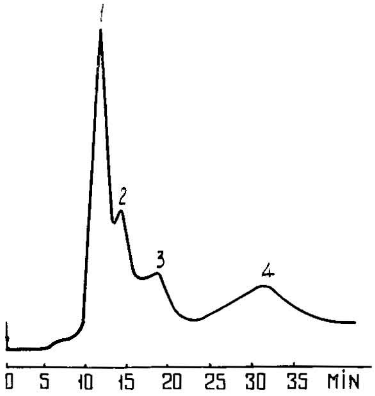
Our investigations showed that the best separation of the oxidation products is achieved on a column with 5% of β,β'-oxydipropionitrile deposited on silica gel C-3. This amount of liquid phase corresponds to a surface coverage of approximately 0.2 mg/m^2, which is much less than the dense monolayer capacity of silica gel surface. It has been shown[2,3] that such an amount is adsorbed fairly well. When the amount of β,β'-oxydipropionitrile on silica gel is increased to 10, 20 and 30% (that is, to 1.2 mg/m^2), the efficiency of column also increases but the selectivity of separation decreases. With 5, 20 and 30% of β,β'-oxydipropionitrile, the specific surface area of the adsorbent decreases to 205, 185 and 175 m^2/g, respectively.

The chromatographic analysis of the reaction mixture after the oxidation of phenol IV in pure oxygen and in the presence of potassium hexacyanoferrate(III) revealed that the mixture consists of four components. The main products were identified and are shown in the chromatogram in Fig. 1. The products of the thermal decomposition of the purified peroxide VIII under vacuum at 140° were also studied. The main product appears to be 2,6-di-*tert.*-butylbenzoquinone-1,4.

Fig. 2 shows a chromatogram of the reaction mixture after the oxidation of phenol I in pure oxygen and in the presence of potassium hexacyanoferrate(III). The main products were identified by using an internal standard. It is difficult to extract peroxides from phenol oxidation reaction mixtures by using conventional methods because of their thermal instability. Therefore, in the present study preparative liquid chromatography was used. The preparatively extracted substance (first peak in Fig. 2) is the product of re-grouping of



IX



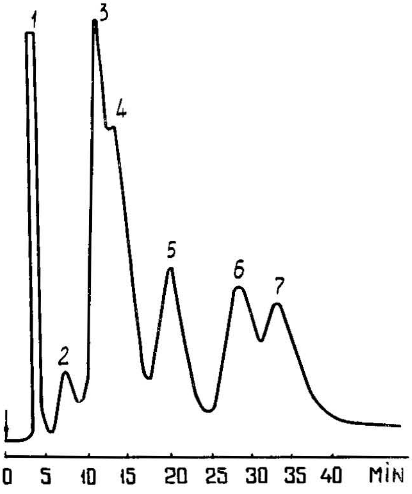
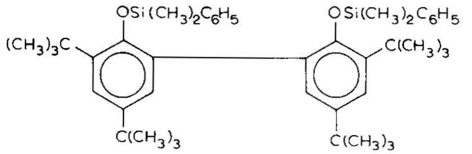


Fig. 1. Chromatogram of the reaction mixture after the oxidation of phenol IV in pure oxygen and in the presence of potassium hexacyanoferrate. Chromatograph: Tsvet-301 with a dielectric constant detector. Adsorbent: β,β'-oxydipropionitrile (5%) deposited on silica gel C-3 (particle size 30–50 μm). Eluent: *n*-hexane. Column: 67 cm × 0.4 cm I.D. Flow-rate: 1.2 cm^3/min; ambient temperature. Peaks: 1 = peroxide VIII; 2 and 3 = unidentified; 4 = 2,6-di-*tert.*-butylbenzoquinone-1,4.

Fig. 2. Chromatogram of the reaction mixture after the oxidation of phenol I in pure oxygen and in the presence of potassium hexacyanoferrate. Separation conditions as in Fig. 1 but with flow-rate 2.07 cm^3/min. Peaks: 1 = IX; 2, 3, 5, 6 = unidentified; 4 = peroxide V; 7 = 2-trimethylsilyl-6-*tert.*-butylbenzoquinone-1,4.

and the fourth peak on this chromatogram corresponds to peroxide V. After recrystallization of the prepared peroxide from methanol, the pure peroxide V was extracted, the presence and purity of which were confirmed chromatographically by using an internal standard and by IR spectroscopy.

Examination of the thermal decomposition products of peroxide V showed that the main product appears to be 2-trimethylsilyl-6-*tert.*-butylbenzoqinone-1,4.
the presence of potassium hexacyanoferrate, as in the oxidation of the above phenols, the following compounds were identified (under the same conditions): peroxide VI, 2-dimethylphenylsilyl-6-*tert.*-butylbenzoqinone-1,4 and the product of re-grouping of



X

Fig. 3 shows a chromatogram of the products of oxidation of phenol III. The

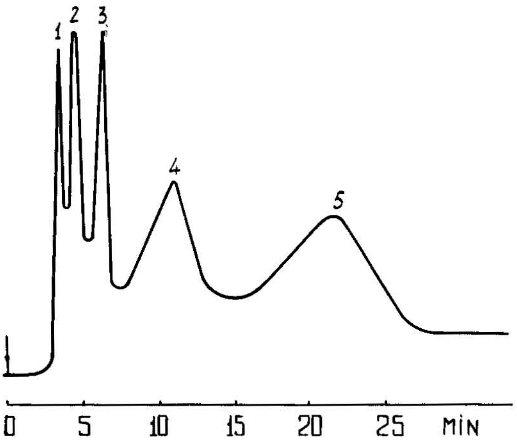
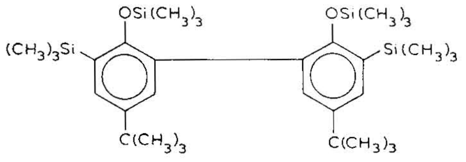


Fig. 3. Chromatogram of the reaction mixture after the oxidation of phenol III in pure oxygen and in the presence of potassium hexacyanoferrate(III). Separation conditions as in Fig. 2. Peaks: 1 = XI; 2, 3 = unidentified; 4 = peroxide VII; 5 = 2-trimethylsilyl-6-*tert.*-butylbenzoquinone-1,4.

main products of this oxidation are peroxide VII, 2-trimethylsilyl-6-*tert.*-butylbenzoquinone-1,4 and the products of re-grouping of



XI

The chromatograms of the phenol oxidation products show that the smallest retention times are those of products of re-grouping while quinones have the greatest retention times; peroxides have intermediate values. This order can be attributed to the fact that specific intermolecular interactions with the adsorbent weaken with a transition from substituted quinones (whose active groups are not all screened with *tert.*-butyl radicals or other substituents) to peroxides (the active groups of which are all screened by the corresponding substitutes).

REFERENCES

1 A. V. Kiselev, Yu. S. Nikitin, G. E. Zajtseva and Ya. I. Yashin, *Neftekhimia*, 8 (1968) 484.
2 N. Hadden, F. Baumann, F. Macdonald, M. Munk, R. Stevenson, D. Gere and F. Zamakoni *Basic Liquid Chromatography*, Varian Aerograph, Palo Alto, 1971.
3 J. J. Kirkland (Editor), *Modern Practice of Liquid Chromatography*, Wiley-Interscience, New York, 1971, p. 161.

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CHROM. 8847

Short Communication

Separation and identification of unsaturated steroids by combined gas chromatography–mass spectrometry with Silanox-type glass open tubular columns

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(Received October 27th, 1975)

A combined gas chromatograph–mass spectrometer equipped with packed columns is a uniquely powerful tool for the simultaneous separation and identification of the components of complex organic mixtures. After some earlier difficulties, a number of investigators have reported procedures for the preparation of reproducible, thermostable glass open tubular chromatographic columns[1–4]. Injectors which conform to the critical requirements of these columns have been developed[5,6] and the impact of these has led to a burgeoning of applications of these columns to biochemical problems. Several successful techniques for the connection of open tubular columns to mass spectrometers have demonstrated a significantly enhanced performance of the combined instrument in the analysis of complex mixtures[7,8]. Advances in analytical techniques based on glass open tubular columns have been reported in a recent symposium[9]. We report the application of Silanox-type glass open tubular columns to the gas chromatographic–mass spectrometric (GC–MS) analysis of two mixtures of steroids differing in respect of stereochemistry and/or unsaturation within the steroid nucleus.

EXPERIMENTAL

Open tubular glass spirals were drawn from 6-mm light-walled Pyrex glass tubing in a device constructed according to the design of Desty *et al.*[10] to an internal diameter of 0.5 ± 0.01 mm. The column blanks were inactivated by a gas phase silanization method[11] and were coated with 6–10-μm Silanox® (Cabot Corp., Billericia, Mass., U.S.A.), a finely divided silylated fumed silicon dioxide, and OV-1 methyl silicone stationary phase (Applied Science Labs., State College, Pa., U.S.A.) by the method of German and Horning[2].

These columns were installed in an LKB 9000 gas chromatograph–mass spectrometer (LKB, Stockholm-Bromma, Sweden) with a "falling needle" type dry injector device[6]. The discrepancy between column effluent flow-rate (4–6 ml/min) and the flow requirement for optimum performance of the two-stage stainless steel jet-orifice separator (30 ml/min) was overcome by the addition of gas to the effluent at the column outlet. This was accomplished without significant reduction in chromatographic efficiency by means of a "make-up" device which added the extra gas to the column effluent in a laminar fashion[12]. The carrier gas (helium) flow-rate through the column was maintained at 5 ml/min, and the supplementary flow of helium at approximately 25 ml/min. MS conditions were: separator and source temperature, 270°; filament emission current, 70 μA; electron energy, 70 eV.

Packed-column GC was carried out on a Perkin-Elmer Model 881 gas chromatograph equipped with a flame ionization detector. A 9-ft. glass spiral column of 3.5 mm I.D. was packed with 1% OV-1 coated on 100–120 mesh Gas-Chrom Q (Applied Science Labs.) and the carrier gas (nitrogen) flow-rate was 40 ml/min.

Reference materials were obtained from the following sources: Δ^{5}-androsten-3β-ol and 5α-androstan-3β-ol, Medical Research Council Steroid Reference Collection (Westfield College, London, Great Britain); $\Delta^{5,16}$-androstadien-3β-ol, Δ^{16},5α-androsten-3α-ol, Δ^{16},5α-androsten-3β-ol and Δ^{16},5β-androsten-3α-ol, Dr. G. F. Woods, Organon Research Labs. (Newhouse, Great Britain); 5α-androstan-3α-ol, Ikapharm (Ramat-Gan, Israel); Δ^{2}, Δ^{3} and $\Delta^{8(9)}$-5α-cholestenes and Δ^{4}- and Δ^{5}-cholestene: Dr. P. Bladon (University of Strathclyde, Glasgow, Great Britain).

Trimethylsilyl ethers were prepared in neat bis(trimethylsilyl)acetamide at 65° for 30 min, and excess reagent was removed under a stream of dry nitrogen. Solutions for analysis were in nanograde hexane at a concentration of 1 μg/μl.

DISCUSSION

Fig. 1A illustrates the separation of a six-component mixture consisting of Δ^{16},5α-androsten-3α-ol (a), Δ^{16},5β-androsten-3α-ol (b), $\Delta^{5,16}$-androstadien-3β-ol (c), Δ^{16},5α-androsten-3β-ol (d), Δ^{5}-androsten-3β-ol (e) and 5α-androstan-3β-ol (f), as their trimethylsilyl ethers. As expected, the 3α/3β hydroxy epimers (a, d) and the 5α/5β

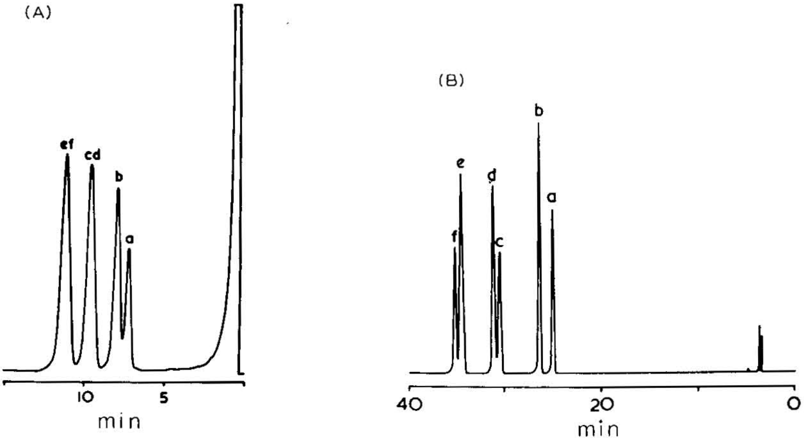
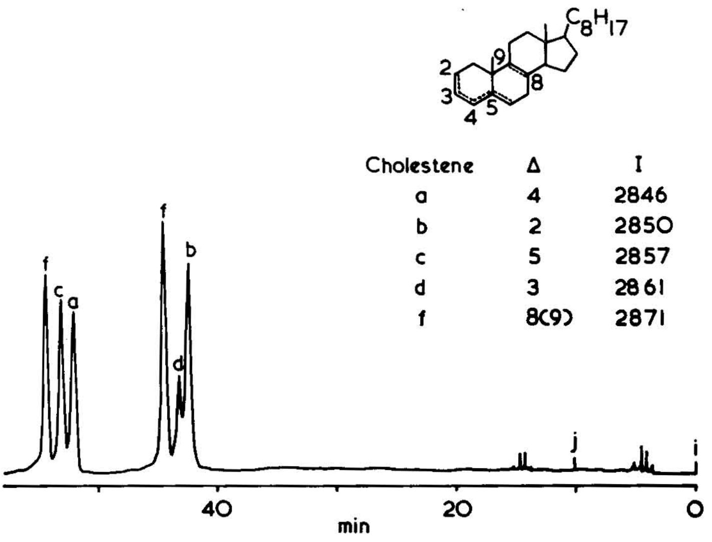


Fig. 1. Packed column chromatogram with a 9-ft. column packed with 1% OV-1 (A), and total ionization current chromatogram with a 50-m glass open tubular column coated with OV-1 over Silanox (B) of a synthetic mixture consisting of the trimethylsilyl ethers of the following compounds: a = Δ^{16},5α-androsten-3α-ol (I = 2223); b = Δ^{16},5β-androsten-3α-ol (I = 2277); c = $\Delta^{5,16}$-androstadien-3β-ol (I = 2290); d = Δ^{16},5α-androsten-3β-ol (I = 2296); e = Δ^{5}-androsten-3β-ol (I = 2330); f = 5α-androstan-3β-ol (I = 2335). I = Kováts retention indices for the C_{19} alcohol trimethylsilyl ethers on the open tubular column at 230°. Conditions: (A), carrier gas (nitrogen) flow-rate, 40 ml/min; column temperature, 170°; flame ionization detection; (B), carrier gas (helium) flow-rate, 5 ml/min; column temperature, 230°.

epimers (a, b) were well separated by packed-column chromatography. However, the 3β-hydroxy-Δ^{16} and ring D saturated $\Delta^5/5\alpha$ pairs (c, d and e, f respectively) were eluted as single unresolved peaks. Fig. 1B illustrates the marked improvement in chromatographic resolution afforded by a 50-m glass open tubular column. Allsix components were well resolved, and mass spectra obtained at the apex of each component corresponded closely to those recorded for the individual derivatives.

This model separation has relevance for the analytical characterisation of the Δ^{16}-steroids that occur as porcine pheromones[13,14].

Fig. 2 represents the separation of two mixtures of closely related cholestenes. The dry injection system, whereby samples are introduced without solvent, obviates the need for isolation of the spectrometer ion source during analysis. The undisturbed baseline also permits the use of intercalated injections (as in Fig. 2) affording efficient use of instrument time. An additional convenience is the option of loading the injector with a number of separate solutions, removing the solvent and injecting the combined sample.



Cholestene	Δ	I
a	4	2846
b	2	2850
c	5	2857
d	3	2861
f	8(9)	2871

Fig. 2. Total ionization chromatogram with 50-m glass open tubular column coated with OV-1 over Silanox of two successive injections (at i and j). a = Cholest-4-ene; b = 5α-cholest-2-ene; c = cholest-5-ene; d = 5α-cholest-3-ene; f = 5α-cholest-8(9)-ene. Carrier gas (helium) flow-rate, 5 ml/min; column temperature, 240°.

The mixtures in Fig. 2 represent difficult chromatographic separations which with the use of conventional packed columns would result in largely unresolved peaks. The facility demonstrated here is being applied in the analysis of mixtures of natural steroids.

ACKNOWLEDGEMENTβ

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REFERENCES

1 G. A. F. M. Rutten and J. A. Luyten, *J. Chromatogr.*, 74 (1972) 177.
2 A. L. German and E. C. Horning, *J. Chromatogr. Sci.*, 11 (1973) 76.
3 J. A. Luyten and G. A. F. M. Rutten, *J. Chromatogr.*, 91 (1974) 393.
4 P. van Hout, J. Szafranek, C. D. Pfaffenberger and E. C. Horning, *J. Chromatogr.*, 99 (1974) 103.
5 A. L. German and E. C. Horning, *Anal. Lett.*, 5 (1972) 619.
6 P. M. J. van den Berg and T. P. H. Cox, *Chromatographia*, 5 (1972) 301.
7 J. G. Leferink and P. A. Leclercq, *J. Chromatogr.*, 91 (1974) 385.
8 B. F. Maume and J. A. Luyten, *J. Chromatogr. Sci.*, 11 (1973) 607.
9 R. E. Kaiser (Editor), *Proceedings of the first international symposium on glass capillary chromatography including glass micro-packed columns, Hindelang/Allgäu, May 1975*, Institut für Chromatographie, Bad Dürkheim, 1975.
10 D. H. Desty, J. N. Haresnape and B. H. F. Whyman, *Anal. Chem.*, 32 (1960) 302.
11 M. Novotný, L. Blomberg and K. D. Bartle, *J. Chromatogr. Sci.*, 8 (1970) 390.
12 A. Linnarson, *LKB Application Note*, LKB-Produkter, Stockholm-Bromma, May 10, 1967.
13 D. B. Gower, *J. Steroid Biochem.*, 3 (1972) 45.
14 D. C. Bicknell and D. B. Gower, *J. Chromatogr.*, 110 (1975) 210.

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CHROM. 8639

Note

Hydrophobic interaction in sugar solutions

Results from gel interaction study

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(First received June 9th, 1975; revised manuscript received August 6th, 1975)

Sugars can prevent the thermal denaturation of proteins in solution[1–4] as well as reduce the extent of their denaturation by urea[5]. The possible mechanism by which sugars induce stability to denaturation in protein molecules is not known.

We have recently observed[6] that the solubility of N-acetyl ethyl esters of aromatic amino acids, which have been considered as models[7] for the relevant portion present in a protein molecule, is lower in glucose and sucrose solutions than in water. This decreased solubility has been termed the "sugaring out" effect by analogy with the "salting out" phenomenon which describes the decrease in solubility of non-electrolytes in salt solutions. The increased activity coefficients of the esters in sugar solutions and the positive free energy of transfer of the molecules from water to these solutions have been interpreted as arising from the increased hydrophobic interaction which is probably responsible for the increased stability of the proteins in sugar solutions.

In a recent communication[8], the elution of the N-acetyl amino acid ethyl esters from aqueous Sephadex LH-20 (hydroxypropyl derivative of Sephadex G-25) was found to depend on the hydrophobic interaction between the gel and the ester molecules. The conclusion was obtained from an observed increased retention of the esters in the gel at higher temperatures. The increased retention of the esters in the presence of electrolytes indicated an increased hydrophobic interaction between the esters and the gel; this supported Kauzmann's suggestion that the hydrophobic interaction increases in salt solution[9]. The present communication reports a study of the interaction between the ester molecules and the Sephadex LH-20 gel in sucrose, glucose and fructose solutions.

Sucrose and fructose, like glucose[10], are not retained in the gel. The void volume, V_0, of the gel is not effected in sugar solutions. Swelling of the gel is reduced in sugar solutions compared to its swelling in water (Fig. 1). Of the three sugars, sucrose reduces the swelling most, the effects of glucose and fructose being about equal.

The retention of the esters in sugar solutions increases, as can be seen from the upper part of Fig. 2, where the elution volume of N-acetyl-L-tyrosine ethyl ester (ATYE) in 1.5 M sucrose is 50 ml greater than its elution volume in water. To compare the retention at different sugar concentrations, an affinity number[11] $A = (V_e - V_t)/g$ is used where V_e is the elution volume of the solute, V_t is the bed volume of the

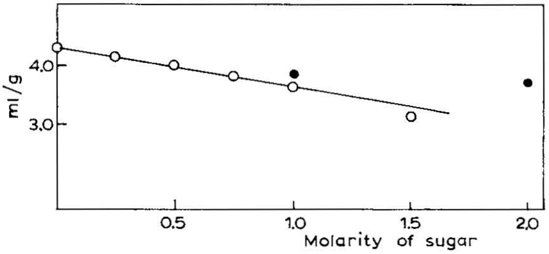

Fig. 1. The swelling of Sephadex LH-20 *versus* the concentration of sugar in the swelling solvents; ○, sucrose; ●, glucose and fructose. pH 6.6 (distilled water); temperature, 25°.

gel, and g is the dry weight of the gel in grams (Fig. 2). The affinity of the esters for the gel (i) increases with sugar concentration, the increase being more rapid at higher concentrations of sugar, and (ii) increases in sucrose solution as compared with those containing glucose and fructose, the affinity in these latter two solutions being nearly equal. The retention pattern is similar to observations made in salt solutions[8]. The retention of the esters in the gel takes place by hydrophobic interaction. The increased retention in sugar solutions probably indicates stronger hydrophobic interaction

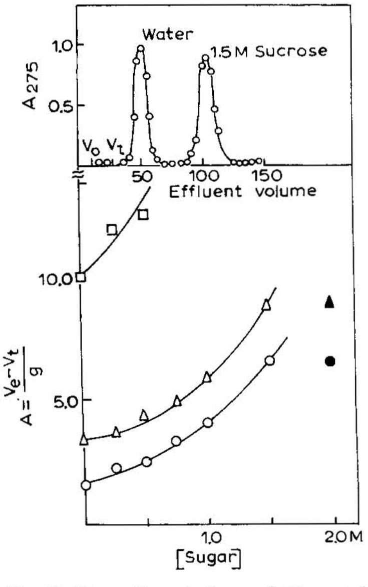

Fig. 2. Top: the elution of N-acetyl-L-tyrosine ethyl ester (ATYE) in water (50 ml) and 1.5 *M* sucrose solution (102 ml). V_0 = 11 ml; V_t = 28 ml. 3-ml fractions were collected except near the peak, where the fractions were 2.5 ml. Column dimensions 1.8 × 11 cm. V_0 was determined by Blue Dextran 2000, V_t by weighing the amount of water equal to the volume of the gel level in the column. Flow-rate, 25 ml/h; pH 6.6 (distilled water); temperature, 25°. Bottom: the affinity number, A, for N-acetyl-L-phenylalanine ethyl ester (APE), ATYE and N-acetyl-L-tryptophan ethyl ester (ATRE) chromatographed in Sephadex LH-20 *versus* the concentration of sugar in the elution; g = weight of dry gel. Open symbols, sucrose; closed symbols, glucose and fructose. Concentrations of the esters in the fractions were determined by measuring absorbance at 257, 275, and 278 nm for APE, ATYE and ATRE respectively. pH 6.6 (distilled water); temperature, 25°. □, ATRE; △, ▲, ATYE; ○, ●, APE.

between ester molecules and the gel. This indicates that hydrophobicity increases in sugar solutions, with the hydroxypropyl groups of the gel probably being the sites where the molecules interact. However, the possibility also exists that the solubilities of the esters in the mobile and stationary phases are different in the absence and presence of sugars, and this may explain the present results*.

The reduction in the swelling of the gel in sugar solution probably arises as follows. The ether oxygen in the Sephadex LH-20 is capable of hydrogen bonding with water molecules to form an oxonium structure which results in the swelling of the gel[12]. Sugar molecules, which are capable of forming hydrogen bonds even more strongly than water[13], compete favourably for water molecules with the ether oxygens to result in gel shrinkage. Alternatively, by analogy with the shrinkage of the gel at high temperatures due to increased hydrophobic interaction[12], the hydrophobic interaction of hydroxypropyl side chains in sugar solution may increase resulting in the observed gel shrinkage.

The present result indicates that addition of sugars to water increases hydrophobic forces between additional groups and molecules present in the system.

NOTE BY THE EDITOR

We doubt whether a general "sugaring out" effect exists. Experiments in quite another field, namely the paper chromatography of $HAuCl_4$ in aqueous 0.1 *N* HCl–glucose solutions, were entirely negative.

REFERENCES

1 A. Beilinson, *Biochem. Z.*, 213 (1929) 399.
2 C. D. Ball, D. T. Hardt and W. J. Duddles, *J. Biol. Chem.*, 151 (1943) 163.
3 C. R. Hardt, I. F. Huddelson and C. D. Ball, *J. Biol. Chem.*, 163 (1946) 211.
4 M. Kerkes, *Biochim. (Rumania)*, 6 (1963) 245.
5 R. B. Simpson and W. Kauzmann, *J. Amer. Chem. Soc.*, 75 (1953) 5193.
6 T. S. Lakshmi and P. K. Nandi, *J. Phys. Chem.*, in press.
7 P. K. Nandi, *J. Phys. Chem.*, 78 (1974) 1197.
8 V. Prakash and P. K. Nandi, *J. Chromatogr.*, 106 (1975) 23.
9 W. Kauzmann, *Adv. Protein Chem.*, 14 (1959) 1.
10 H. Determann and I. Walter, *Nature (London)*, 219 (1968) 604.
11 H. Determann and K. Lampert, *J. Chromatogr.*, 69 (1972) 123.
12 K. Lampert and H. Determann, *J. Chromatogr.*, 56 (1971) 140.
13 J. B. Taylor and J. S. Rowlinson, *Trans. Faraday Soc.*, 51 (1955) 1183.

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* We are thankful to the reviewer of the paper for pointing out this possibility.

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CHROM. 8721

Note

A simple method for rapid alkali flame ionisation detector optimisation

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(Received August 18th, 1975)

A major disadvantage of alkali flame ionisation detectors (AFIDs) is that sensitivity is critically dependent upon a number of parameters, in particular, the alkali salt–flame separation distance[1]. The practical operation of an AFID requires in many cases, that the salt–flame separation is optimised daily; usually by a tedious, time-consuming sequence of alternate sample injections and adjustment of a detector parameter. This note reports a simple method for rapid AFID optimisation (AFIDO).

The principle of the method is to introduce a slowly varying amount of sample material to an AFID and to adjust continuously a detector parameter, such as: alkali salt position[1], polarising voltage[2] or flame gas flow-rates[3], until a maximum response is obtained. For convenience, sample material is introduced using a hypodermic syringe needle, inserted into the injection port in the usual manner.

EXPERIMENTAL

A Pye Unicam GCV chromatograph, fitted with a GCV nitrogen AFID and a 1.5-m 10% E30 glass column were set up according to the manufacturer's instructions. The nitrogen detector was of the three-electrode (RbCl) type. The flame was set with the following gas flow ratios: hydrogen–nitrogen–air (30:30:300). The AFIDO apparatus was constructed by filling a 7-cm, 100-μl syringe needle with 2,6-dinitrotoluene.

Chromatographic conditions were as follows: detector temperature, 200°; column temperature, 170°; injector temperature, 100°.

The AFIDO was initiated by inserting the filled syringe needle to a depth of 5 cm into the injection port of the chromatograph. This allowed 2,6-dinitrotoluene to "bleed" slowly onto the column. Penetration of the column packing material by the needle, was found not to affect the optimisation.

After a period of 10 min, during which time the flow of 2,6-dinitrotoluene to the nitrogen detector was established, the position of the alkali salt was carefully adjusted up and down, by means of an external screw, until a maximum response was obtained. The time taken for this stage of the optimisation was about 20 sec. The AFIDO needle was then removed, leaving the nitrogen detector in a fully optimised condition, as shown by analysing a mixture of pyridine, *p*-nitrotoluene and *n*-hexylamine. Each compound gave the maximum response.

CONCLUSIONS

The AFIDO is a simple, rapid method of general applicability, for obtaining the most favourable response from an AFID. By a suitable choice of sample material, this method of optimisation may be extended to other chromatographic detector systems, such as the flame ionisation detector and the flame photometric detector, which may require optimisation at regular intervals to ensure maximum sensitivity.

ACKNOWLEDGEMENT

The author wishes to thank the Management of Pye Unicam Ltd., for permission to publish this paper.

REFERENCES

1 W. Ebig, *Chromatographia*, 1 (1968) 382.
2 W. A. Aue, C. W. Gehrke, R. C. Tindle, D. L. Stalling and C. D. Ruyle, *J. Gas Chromatogr.*, 5 (1967) 381.
3 D. J. David, *Gas Chromatographic Detectors*, Wiley, New York, 1974, p. 132.

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CHROM. 8718

Note

Preparation of octadecyl Porasil for reversed-phase liquid chromatography

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(Received September 2nd, 1975)

In an earlier communication, we described the use of reversed-phase liquid chromatography in the separation of various indole alkaloids[1]. Owing to the expense of commercially available bonded phase packings, we sought a reproducible method for the preparation of the octadecyl phase (C_{18}-Porasil) used in this work. Several methods for the preparation of bonded phases have been published previously, including a helpful recent general paper by Majors and Hopper[2], and the subject has recently been reviewed[3], but comparison with currently available packing materials is not available for most of these. This note reports a simple method for the preparation of a bonded octadecyl phase packing which approximates closely in its chromatographic properties a widely available commercial packing.

EXPERIMENTAL

Chemicals

Octadecyltrichlorosilane and trimethylchlorosilane were obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and were used as supplied. Porasil B® and Bondapak C_{18}/Porasil B® were purchased from Waters Assoc. (Milford, Mass., U.S.A.). Toluene was reagent grade and was dried over calcium hydride and distilled before use.

Octadecyl Porasil B

Porasil B (300 g) was heated for 12 h at 100° in a mixture of 500 ml concentrated nitric acid and 500 ml concentrated sulfuric acid. After cooling and decantation of excess acid, the residue was washed with distilled water by decantation until neutral, and then dried at 110° for 12 h. The dry Porasil B was suspended in 800 ml dry toluene and 90 ml octadecyltrichlorosilane, and the mixture heated under reflux for 14 h. The excess toluene and chlorosilane were decanted off, the residue washed with dry toluene to remove any remaining chlorosilane, and the bonded chlorosilane was then hydrolyzed by stirring the Porasil with 50% aqueous acetonitrile for 2 h at room temperature. The aqueous mixture was then decanted and the residue washed thoroughly with water and dried. Final protection of free hydroxyl groups was achieved by reaction of the dry powder with 10% trimethylchlorosilane in dry toluene (500 ml) for 2 h under reflux, followed by final washes with toluene and methanol and drying at 110° for 3 h.

RESULTS

The octadecyl Porasil B obtained by the above procedure was compared with the commercially available Bondapak C_{18}/Porasil B by elemental analysis and by chromatographic properties. The results of elemental analysis are shown in Table I, and clearly the extent of coverage of the bonded phase is very similar for the commercial material and for the homemade material.

TABLE I

ELEMENTAL ANALYSIS

Packing	*Carbon (%)*	*Hydrogen (%)*
Bondapak C_{18}/Porasil B	11.07	1.95
Octadecyl Porasil B	11.21	1.98

The homemade material was also compared with the commercial material as a chromatographic packing. Columns (2 ft. × 1/8 in.) of each material were packed by the tap-fill method, and evaluated using a test mixture of benzene, naphthalene and biphenyl. The chromatogram obtained with the homemade material using a solvent of methanol–water (75:25) was essentially identical to that obtained with the commercial material using an 80:20 mixture of the two solvents. The homemade material thus can serve as an acceptable substitute for the commercial packing. In addition, the bonding technique described here should be applicable in the preparation of bonded phases of microparticulate materials such as the Partisils®, Spherisorb®, and μBondapak C_{18}®, where the octadecyl bonded phase packings are not yet commercially available.

ACKNOWLEDGEMENT

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REFERENCES

1 D. G. I. Kingston and B. T. Li, *J. Chromatogr.*, 104 (1975) 431.
2 R. E. Majors and M. J. Hopper, *J. Chromatogr. Sci.*, 12 (1974) 767.
3 A. Pryde, *J. Chromatogr. Sci.*, 12 (1974) 486.

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CHROM. 8730

Note

New improved method for separation of fecal bile acids by thin-layer chromatography

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Many solvent systems have been reported for free and methylated fecal bile acid separation by thin-layer chromatography (TLC)[1–4]. None as yet have provided an adequate procedure for removing the fatty acids present in the fecal extract before separating the bile acids. Fatty acids interfere with gas–liquid chromatographic (GLC) analyses of bile acids and can also interfere with spectrophotometric analysis.

To overcome these problems, new and existing TLC methods of both free and methylated bile acids have been modified in our laboratory. Separation of free bile acids was required for a total enzymatic determination using techniques employed by Weber *et al.*[4] and Engert and Turner[5]. The fatty acids were removed by a modification of the TLC solvent system used by Weber *et al.*[4] and the bile acids subsequently separated into three bands according to the amount of hydroxyl functions present using a modification of the TLC solvent system described by Sundaram *et al.*[1].

The solvent system used for removing fatty acids from free bile acid extracts was found to work equally well for removing fatty acids from methylated bile acid extracts. A second solvent system was utilized to complete the separation of the bile acids after the method described by Reddy *et al.*[6].

MATERIALS AND METHODS

The TLC plates used were pre-coated silica gel GF, 250 μm thick, 20 × 20 cm, obtained from Analtech (Newark, Del. U.S.A.; Cat. No. 2011). A standard rectangular tank was used with a heavy glass cover. Cholic acid, deoxycholic acid, lithocholic acid, palimitic acid and their methyl esters were used as standards. All standards were obtained from Applied Science Labs. (State College, Pa., U.S.A.). To allow for solvent saturation of the developing chamber, Whatman No. 1 qualitative filter paper was used to line the walls of the tank.

The free bile acids were extracted from the feces after the methods of Reddy *et al.*[6] and methylated overnight at ambient temperature with a freshly prepared ethereal–alcoholic solution of diazomethane.

A 5-cm vertical portion of the plate was separated by scoring the media for the standard spots so as to insure that no mixing of the standards with the sample could occur. Another portion was scored 2 cm from the upper border of the plate and the solvent front allowed to proceed up to this point. Samples were streaked 2 cm from the lower border of the plate and placed directly into the developing chamber which had previously been allowed to become saturated with solvent vapor for approximately one hour. The first development for both free and methylated bile acids was carried out in a modification of the system reported by Weber *et al.*[4] and consisted of hexane–chloroform–diethyl ether–*n*-butanol–acetic acid (40:10:10:3:0.5). The plates were removed, dried, and viewed under a short wave ultraviolet lamp to visualize the fatty acid band. A line was scored just below the fatty acids and the plates developed in their respective second baths up to this point. The second development for the free bile acids was carried out in a modification of a solvent system reported by Sundaram *et al.*[1] and consisted of isooctane–ethyl acetate–*n*-butanol–*n*-propanol–acetic acid (20:10:3:3:3). For methylated bile acids, the second system consisted of isooctane–isopropanol–acetic acid (120:40:1). When separating the free bile acids, tailing was often encountered with deoxycholic acid. This was alleviated by increasing the volume of acetic acid from 15 to 20 parts and excluding *n*-propanol. After the second development the plates were dried and sprayed with 0.1 % 8-hydroxy-1,3,6-pyrene-trisulfonic acid trisodium salt in water or methanol when the plates were to be eluted and subsequently analyzed. For photographic purposes, the plates were sprayed with 0.1 % 1,2-dichlorofluorescein in ethanol. Both dyes were obtained from Eastman (Rochester, N.Y., U.S.A.; Cat. Nos. 373 and P728, respectively).

RESULTS AND DISCUSSION

The resolution of the described solvent systems is shown in Fig. 1. Previous methods using a chloroform–benzene (1:1) solvent system for removing the fatty acids in methylated samples required two and sometimes three successive developments to attain the separation that our new system affords us after only one development. The fatty acids in all cases moved a minimum of 10 cm from the origin and usually 12–13 cm.

The actual separation of both free and methylated bile acids was accomplished by the second solvent system. Free and methylated bile acids were separated into three bands corresponding to the number of hydroxyl functions in the molecule. As was mentioned, occasional tailing of deoxycholic acid occurred in both the standard spots and samples causing overlap of deoxycholic and cholic acid. To eliminate this tailing, the solvent system used by Sundaram *et al.*[1] was modified by increasing the volume of acetic acid and excluding *n*-propanol. This variation allowed the deoxycholic acid to migrate up the plate with no significant tailing as shown in Fig. 2.

Removal of fatty acids from samples which are to be analyzed later by GLC is essential as they would interfere with bile acid analysis. Separation of bile acids according to those modified methods and separate elution of each band allowed us to use TLC and GLC procedures to identify the bile acids. The system used for free bile acids was designed to obtain adequately pure bile acids for a total enzymatic determination using a combination of methods used by Weber *et al.*[4] and Engert and Turner[5]. One can assay each band separately or altogether, the decision being left up to the individual investigator. We felt that these new systems allow for a rapid and precise method for separating free and methylated bile acids from fecal extracts in adequately pure form to be taken right to a GLC or enzymatic analysis.

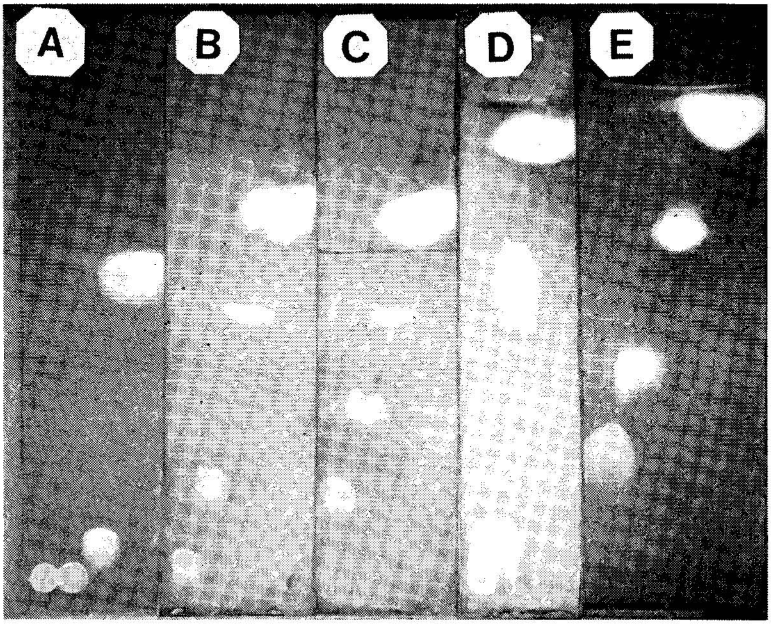

Fig. 1. Separation in one development of (A) methylated bile acids with chloroform–benzene (1:1); (B) methylated bile acids with hexane–diethyl ether–chloroform–*n*-butanol–acetic acid (40:10:10:3:0.5); (C) methylated bile acids with isooctane–isopropanol–acetic acid (120:40:1); (D) free bile acids, solvent system as in (B); (E) free bile acids with isooctane–ethyl acetate–*n*-butanol–*n*-propanol–acetic acid (20:10:3:3:3).

Fig. 2. Separation of free and methylated bile acids with (A) Isooctane–ethyl acetate–*n*-butanol–acetic acid (20:10:3:3) (tailing of deoxycholic acid spot) and (B) Isooctane–ethyl acetate–*n*-butanol–acetic acid (20:10:3:4) (tailing of deoxycholic acid reduced).

ACKNOWLEDGEMENTS

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REFERENCES

1 G. S. Sundaram, H. Singh and H. S. Sodhi, *Clin. Chim. Acta*, 34 (1971) 425.
2 A. Bruusgaard, *Clin. Chim. Acta*, 28 (1970) 495.
3 A. F. Hofmann, *J. Lipid Res.*, 3 (1962) 127.
4 A. M. Weber, L. Chartrand, G. Doyon, S. Gordon and C. C. Roy, *Clin. Chim. Acta*, 39 (1972) 524.
5 R. Engert and M. D. Turner, *Anal. Biochem.*, 51 (1973) 399.
6 B. S. Reddy, J. Weisburger and E. L. Wynder, *J. Nutrition*, 105 (1975) 878.

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CHROM. 8710

Note

Gas chromatographic method for the quantitative determination of lidocaine and its metabolite monoethylglycinexylidide in plasma

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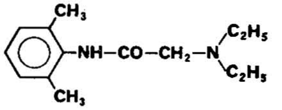
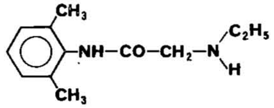
(Received August 27th, 1975)

Lidocaine has been used extensively for treatment of cardiac arrhythmias accompanying acute myocardial infarction. Therapeutic[1–3] and toxic[3] plasma levels of lidocaine have been reported. Monoethylglycinexylidide (MEGX), a dealkylated metabolite formed after the administration of lidocaine, has been reported to be approximately 80% as potent an anti-arrhythmic agent as the parent drug while the didesethylated metabolite glycinexylidide (GX) had only about 10% the potency[4]. Further, MEGX has been suggested as a contributing factor in the occurrence of adverse reactions that sometimes arise after the administration of lidocaine[5]. Therefore, the monitoring of plasma levels of lidocaine and MEGX in postmyocardial infarction patients receiving lidocaine may be of benefit in the control of antiarrhythmic therapy.



LIDOCAINE



MEGX

A number of gas chromatographic (GC) methods have been reported for the determination of lidocaine alone[6–10], and of lidocaine and its dealkylated metabolites[5,11–14] in biological material. The latter procedures, however, appear to suffer from some disadvantages. Di Fazio and Brown[11] described a 10% UCW 98 on Chromosorb W system but the lidocaine, MEGX and GX peaks were not completely resolved and no mention was made of the accuracy of the method. Keenaghan and Boyes[5] reported the quantitative chromatography, without the use of an internal standard, of heptafluorobutyryl derivatives of lidocaine and its metabolites. Mass fragmentography was the detection method used for the GC estimation of lidocaine, MEGX and GX as described by Strong and Atkinson[12] and Strong *et al.*[13]. The stationary phase employed was 3% SE-30–OV-17 (6:1) coated on a Chromosorb support.

However, the peak shapes of the primary and secondary amine metabolites were asymmetrical and the calibration curve for MEGX was non-linear below about 0.5 μg/ml. Adjepon-Yamoah and Prescott[14] used a single-column temperature-programming technique to determine lidocaine and its metabolites after they had been converted to their acetyl derivatives. This paper describes a quantitative method for the isothermal GC of underivatised lidocaine and MEGX following extraction from plasma samples.

MATERIALS AND METHODS

Materials

Lidocaine hydrochloride, MEGX hydrochloride and GX were supplied by Astra Chemicals (Sydney, Australia). Benzhexol hydrochloride (Cyanamid Australia, Melbourne, Australia) was used as an internal standard. All reagents and solvents were analytical reagent grade except diethyl ether, which was anaesthetic grade (BP). The diethyl ether was distilled immediately before use. Ucon 75-H-90,000 (Union Carbide Australia, Sydney, Australia) was the stationary phase employed.

Gas chromatography

A Hewlett-Packard Model 5710 A gas chromatograph equipped with a flame ionization detector was used. The column was glass (2.5 ft. × $\frac{1}{4}$ in. O.D.) packed with 2% Ucon 75-H-90,000–2% KOH on 80–100 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). A pan coating method[15] using methanol as the solvent was the procedure used to coat the KOH and Ucon onto the solid support. The packed column was conditioned for 3 h at 195° (no nitrogen flow) followed by 18 h at 185° (50 ml/min nitrogen flow). The operating conditions were: injection port temperature, 250°; column temperature, 175°; detector temperature, 250°; carrier gas (nitrogen) flow-rate, 50 ml/min; hydrogen flow-rate, 60 ml/min; air flow-rate, 240 ml/min.

Extraction procedure and sample preparation

Plasma (1 ml) and aqueous internal standard solution (5.0 μg/0.1 ml) were placed in a 15-ml glass-stoppered centrifuge tube. After addition of 0.5 ml of 10 *M* NaOH and 5 ml diethyl ether the tube was mixed for 2 min (vortex mixer) and centrifuged for 5 min at 1500 *g*. The tube was cooled in a dry ice–acetone bath for 1 min and the ether layer decanted into a second 15-ml glass-stoppered centrifuge tube containing 1 ml of a 1 *M* HCl solution. The ether and HCl mixture was mixed (1 min) and centrifuged as described above and the ether phase discarded. Following the addition of 0.5 ml 10 *M* NaOH the aqueous phase was extracted with 5 ml diethyl ether by mixing for 2 min. After centrifugation, the separated ether phase was transferred to a 15-ml tube with a 100-μl elongated bubble at the base, a silica boiling chip was added and the ether was evaporated by immersion of the tube in a waterbath at 40°. When no liquid ether was left the tube was stoppered and plunged into ice to condense the remaining ether vapour and wash down the sides of the tube. This evaporation and condensation procedure was repeated until approximately 10 μl of ether solution remained and 5–10 μl were injected into the gas chromatograph.

Calibration curves

Following chromatography, the heights of the peak corresponding to lidocaine, MEGX and benzhexol were measured. Calibration curves of peak height ratio against amount of lidocaine hydrochloride or MEGX hydrochloride were established simultaneously by assaying drug-free plasma to which known amounts of the compounds had been added.

RESULTS

Performance of analytical procedure

Symmetrical chromatographic peaks were obtained for all compounds (Fig. 1) and the retention times of lidocaine, MEGX and benzhexol were 4.8, 7.6 and 10.0 min, respectively. The peak shape of GX was also good and with a retention time of 11.9 min it was resolved from the other three peaks. However, due to its greater polarity the primary amine metabolite could not be quantitatively extracted with diethyl ether.

Calibration curves for lidocaine hydrochloride and MEGX hydrochloride in plasma were constructed over the range 0.05–25 μg and 0.05–5 μg, respectively, and are shown in Figs. 2 and 3. In each case the plot was linear and passed through the ori-

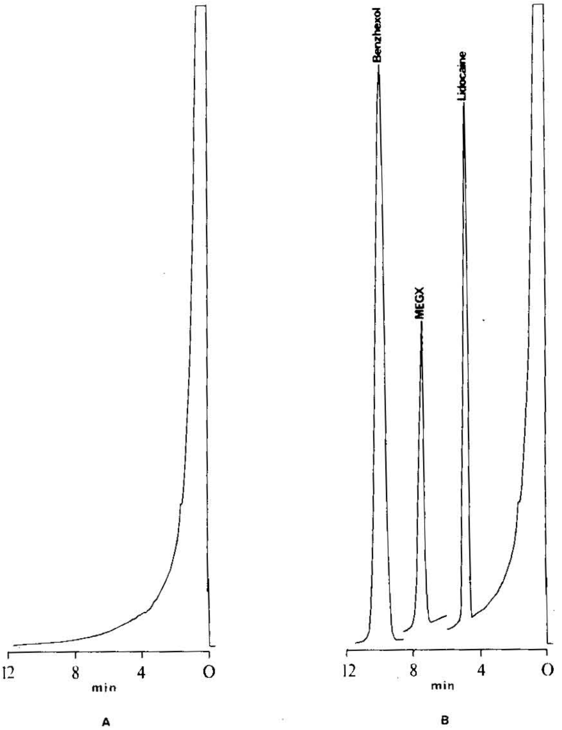


Fig. 1. Gas chromatograms of (A) blank plasma extract and (B) extract of plasma taken from a patient receiving lidocaine by intravenous infusion. The attenuation setting was × 160 for lidocaine and benzhexol and × 80 for MEGX.

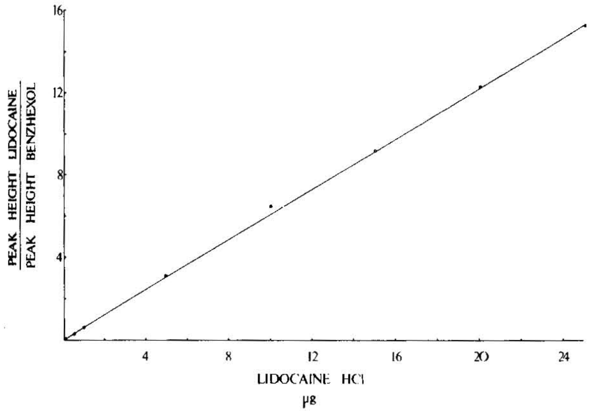


Fig. 2. Calibration curve for lidocaine hydrochloride in human plasma.

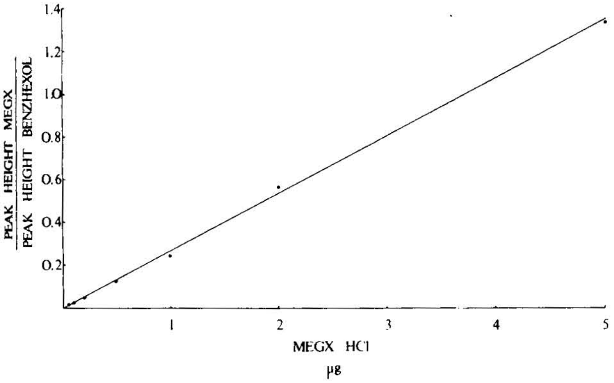


Fig. 3. Calibration curve for MEGX hydrochloride in human plasma.

gin. The reproducibility of replicate analyses of plasma samples containing different concentrations of the two compounds is recorded in Table I.

Application of the GC assay to biological samples

The assay procedure outlined has been used in a clinical setting to study the

TABLE I

REPRODUCIBILITY OF REPLICATE SIMULTANEOUS ANALYSES (MEANS FROM FIVE SAMPLES AT EACH CONCENTRATION) OF LIDOCAINE HYDROCHLORIDE AND MEGX HYDROCHLORIDE ADDED TO HUMAN PLASMA

Compound	*Plasma concentration (μg/ml)*	*Standard deviation (%)*
Lidocaine hydrochloride	0.25	3.09
	1.5	0.82
MEGX hydrochloride	0.1	5.21
	1.0	7.03

plasma levels of lidocaine and MEGX in patients who were being treated for cardiac arrhythmias with intravenous lidocaine infusions. Venous blood samples were collected in tubes containing 100 units of ammonium heparin together with blood separation granules. Plasma was separated by centrifugation as soon after sampling as possible and was stored at −20° until assayed. In some cases blood samples were collected from the patients during the lidocaine infusion. More commonly, however, blood samples were collected just prior to cessation of the infusion and for a number of hours subsequent to this. The plasma levels of lidocaine and MEGX in one patient in the latter category are illustrated in Fig. 4. No interfering chromatographic peaks were noted in plasma samples obtained before commencement of lidocaine therapy.

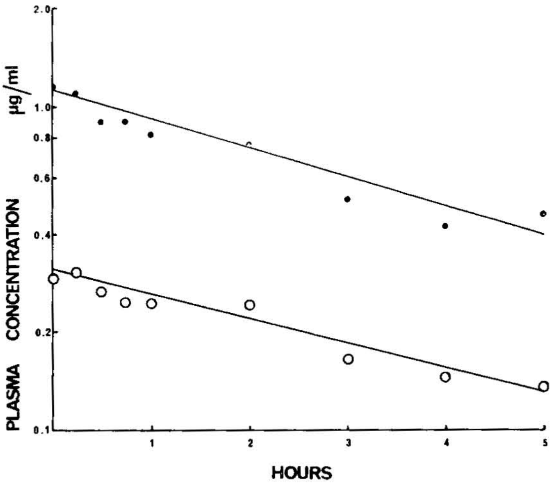


Fig. 4. Plasma levels of lidocaine hydrochloride (●) and MEGX hydrochloride (○) for 5 h after the cessation of the lidocaine infusion.

DISCUSSION

The method is sensitive and accurate for the determination of lidocaine and MEGX in plasma, and has been found to be satisfactory for clinical application. At this stage the technique has not been used to quantitate GX in plasma since, as stated earlier, this metabolite has much less pharmacological activity. However, if estimation of GX was required the use of a more polar organic solvent for extraction should be the only modification necessary. The use of the KOH-treated solid support in the GC system minimised adsorption of the amines and obviated the need for derivatisation procedures to ensure symmetrical peak shapes. The method has also been used to quantitate lidocaine and MEGX in urine.

ACKNOWLEDGEMENTS

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REFERENCES

1 R. Gianelly, J. O. van der Groeben, A. P. Spivak and D. C. Harrison, *New Engl. J. Med.*, 277 (1967) 1215.
2 D. E. Jewitt, Y. Kishon and M. Thomas, *Lancet*, i (1968) 266.
3 D. C. Harrison and E. L. Alderman, in D. B. Scott and D. G. Julian (Editors), *Lidocaine in the Treatment of Ventricular Arrhythmias*, E. & S. Livingstone, Edinburgh, London, 1971, Ch. 18, p. 186.
4 R. G. Burney, C. A. Di Fazio, M. J. Peach, K. A. Petrie and M. J. Silvester, *Amer. Heart J.*, 88 (1974) 765.
5 J. B. Keenaghan and R. N. Boyes, *J. Pharmacol. Exp. Ther.*, 180 (1972) 454.
6 G. Svinhufvud, B. Ortengren and S. E. Jacobsson, *Scand. J. Clin. Lab. Invest.*, 17 (1965) 162.
7 J. B. Keenaghan, *Anesthesiology*, 29 (1968) 110.
8 F. Reynolds and A. H. Beckett, *J. Pharm. Pharmacol.*, 20 (1968) 704.
9 M. Rowland, P. D. Thomson, A. Guichard and K. L. Melmon, *Ann. N.Y. Acad. Sci.*, 179 (1971) 383.
10 N. Benowitz and M. Rowland, *Anesthesiology*, 39 (1973) 639.
11 C. A. Di Fazio and R. E. Brown, *Anesthesiology*, 34 (1971) 86.
12 J. M. Strong and A. J. Atkinson, *Anal. Chem.*, 44 (1972) 2287.
13 J. M. Strong, M. Parker and A. J. Atkinson, *Clin. Pharmacol. Ther.*, 14 (1973) 67.
14 K. K. Adjepon-Yamoah and L. F. Prescott, *J. Pharm. Pharmacol.*, 26 (1974) 889.
15 H. M. McNair and E. J. Bonelli, *Basic Gas Chromatography*, Varian-Aerograph, Walnut Creek, Calif., 1968, p. 64.

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Note

Gas chromatographic separation of allylbarbital and butabarbital

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Allylbarbital (5-allyl-5-isobutylbarbituric acid) and butabarbital (5-ethyl-5-*sec.*-butylbarbituric acid) are among the commonly abused barbiturates. The gas–liquid chromatographic (GLC) columns used in many toxicology laboratories do not differentiate between these two barbiturates either as free drugs or as their methylated derivatives[1–5]. We have investigated a number of columns to obtain their best separation.

EQUIPMENT

A Varian gas chromatograph equipped with two columns, dual differential electrometer and two flame ionization detectors was used in this study. The columns used were 6 ft. × 2 mm I.D. glass, packed with the various materials described.

RESULTS AND DISCUSSION

The results of our study are summarized in Table I. Data show that a number of columns commonly in use for free and methylated barbiturates[1–5] cannot differentiate between allylbarbital and butabarbital. Analysis procedures relying on these columns cannot possibly be accurate for the two drugs.

Of the columns tested for free acid barbiturates, only 4% OV-210 could separate free allylbarbital from butabarbital. Fig. 1 shows that this column also gives clear separation of other commonly used barbiturates such as amobarbital, pentobarbital, phenobarbital, secobarbital and ibomal, which was used as an internal standard.

Four columns were found that could clearly separate methylated derivatives of allylbarbital and butabarbital: 3% OV-17, 3% OV-25, 4% OV-210, and 3% Poly-

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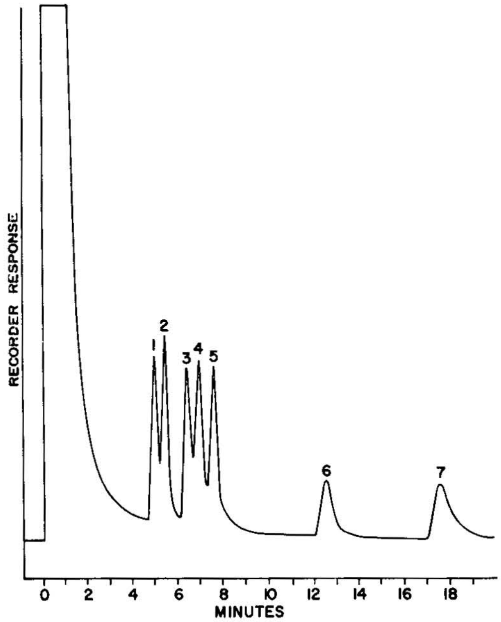
TABLE I

SEPARATION OF ALLYLBARBITAL AND BUTABARBITAL

Column	*Free acids**	*Methylated derivatives***
3% SE-30 on Chromosorb W, 80–100 mesh	no	no
3.8% UC W-98 on Chromosorb W, 80–100 mesh	no	no
10% UC W-98 on Chromosorb Q, 80–100 mesh	no	no
10% DC-200 on Chromosorb Q, 80–100 mesh	no	no
5% OV-1 on Chromosorb W, 80–100 mesh	no	no
3% SE-30–3% trimesic acid on Chromosorb W, 80–100 mesh	no	no
3% OV-17 on Chromosorb W, 80–100 mesh	no	yes
3% OV-25 on Chromosorb W, 80–100 mesh	no	yes
4% OV-210 on Chromosorb W, 80–100 mesh	yes	yes
3% Poly-A-103 on Chromosorb Q, 100–120 mesh	no	yes
5% QF-1 on Chromosorb W, 80–100 mesh	no	no
3% XE-60 on Chromosorb W, 80–100 mesh	no	no
5% Hi-Eff 8B on Chromosorb Q, 80–100 mesh	no	no
0.5% Versamide on Chromosorb W, 80–100 mesh	no	no
3% Poly-S-179 on Chromosorb Q, 100–120 mesh	no	no

* Column temperatures ranging from 165–210°.

** Column temperatures ranging from 140–190°.



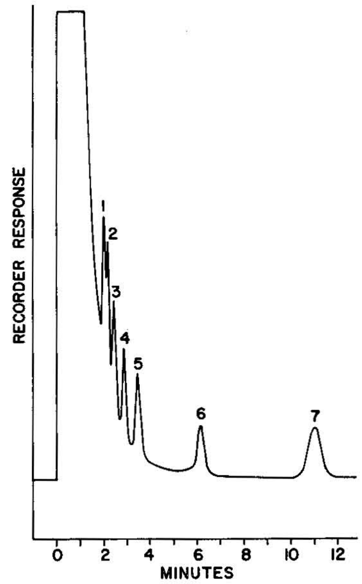


Fig. 1. Separation of barbiturates on 4% OV-210. Column, glass, 6 ft. × 2 mm I.D., column temperature, 180°; injector temperature, 220°; detector temperature, 230°; range, 10^{-11}; attenuation, ×32; injection, 1 μl containing 1 μg/ml each of (1) allylbarbital, (2) butabarbital, (3) amobarbital, (4) pentobarbital, (5) secobarbital, (6) ibomal (I.S.), and (7) phenobarbital.

Fig. 2. Separation of methylated barbiturates on 3% OV-17. Column, glass, 6 ft. × 2 mm I.D.; column temperature, 175°; injector temperature, 250°; detector temperature, 260°; range, 10^{-11}; attenuation, ×32; injection, 1 μl containing 1 μg/ml each of (1) allylbarbital, (2) butabarbital, (3) amobarbital, (4) pentobarbital, (5) secobarbital, (6) ibomal (I.S.), and (7) phenobarbital.

TABLE II

RELATIVE RETENTION TIMES OF METHYLATED DERIVATIVES OF BARBITURATES

Column temperature, 185°; injector temperature, 225°; detector temperature, 250°.

Barbiturate	*3% OV-17*	*3% OV-25*	*3% Poly-A-103*	*4% OV-210*
Allylbarbital	0.35	0.32	0.30	0.42
Butabarbital	0.38	0.35	0.36	0.48
Amobarbital	0.42	0.38	0.37	0.56
Pentobarbital	0.50	0.44	0.45	0.61
Secobarbital	0.58	0.52	0.51	0.63
Ibomal	1.00	1.00	1.00	1.00
Phenobarbital	1.73	1.84	1.79	1.67
Retention time of ibomal, min	6.0	6.3	6.4	5.8

A-103. The relative retention times of common barbiturates on these columns are shown in Table II. The table illustrates that although 4% OV-210 and 3% Poly-A-103 give the greatest separation of allylbarbital and butabarbital, they do not give good separation of some of the other common barbiturates. Poly-A-103 does not clearly separate butabarbital from amobarbital and OV-210 does not separate pentobarbital from secobarbital. The best separation of all the common barbiturates is accomplished by OV-17, as shown in Fig. 2.

In conclusion, 4% OV-210 is found to separate most free barbiturates, including allylbarbital and butabarbital, whereas 3% OV-17 and 3% OV-25 are most suitable to distinguish their methylated derivatives.

REFERENCES

1 I. Sunshine, R. Maes and B. Finkle, *Clin. Toxicol.*, 1 (1968) 281.
2 E. Watson and S. M. Kalman, *Clin. Chim. Acta*, 38 (1972) 33.
3 E. A. Fiereck and N. W. Tietz, *Clin. Chem.*, 17 (1971) 1024.
4 T. Inaba and W. Kalow, *J. Chromatogr.*, 69 (1972) 377.
5 L. R. Goldbaum, P. Santinga and A. M. Dominguez, *Clin. Toxicol.*, 5 (1972) 369.

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Note

Gas chromatography and high-pressure liquid chromatography of commercial hydroxyoxime copper extractants

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Derivatives of hydroxyoximes have been developed specifically for solvent extraction of copper in hydrometallurgical processes in the past few years and their use is increasing steadily. Recently Ashbrook[1] described the composition of the LIX® reagents produced by General Mills (Tucson, Ariz., U.S.A.). These included LIX 64, LIX 64N, LIX 65N, LIX 70, LIX 71 and LIX 73. Separations were performed by column and thin-layer chromatography (TLC) and the structures of the components were determined. The parent compounds are (a) 5,8-diethyl-7-hydroxy-6-dodecanone oxime[2,3], (b) 5-dodecyl-2-hydroxybenzophenone oxime, (c) 2-hydroxy-5-nonylbenzophenone oxime, and (d) 3-chloro-5-nonyl-2-hydroxybenzophenone oxime. The LIX products are composed of the preceding compounds as follows: LIX 63 is (a), LIX 64 is a mixture of (a) and (b), LIX 65N is (c), LIX 64N is a mixture of (a) and (c), LIX 70 of (a) and (d), LIX 71 of (c) and (d), and LIX 73 of (a), (c) and (d). Ashbrook showed by TLC that the aliphatic hydroxyoxime is a minor component when used in mixtures and that the *anti* and *syn* forms of the aromatic hydroxyoximes can be separated.

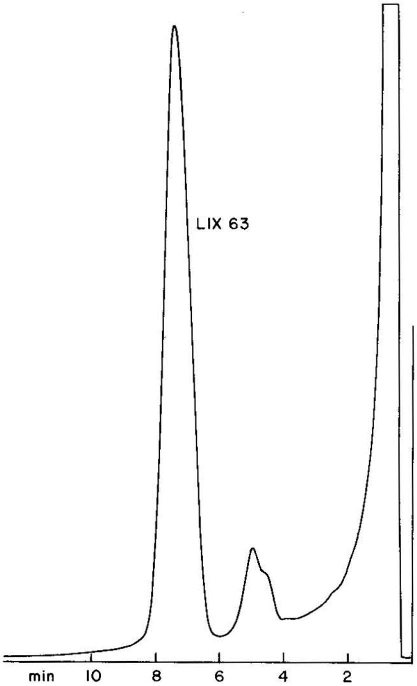
In addition to the LIX reagents there are now available Shell 529® (Shell, Amsterdam, The Netherlands), Acorga P-17® and Acorga P-50® (Acorga, Hamilton, Bermuda). The active ingredient in Shell 529 is 2-hydroxy-5-nonylacetophenone oxime[4] while Acorga P-17 and Acorga P-50 contain 2-hydroxy-5-nonylphenylbenzylketone oxime and 2-hydroxy-5-nonylbenzaldehyde oxime, respectively[5].

This note communicates the applicability of gas chromatography (GC) and high-pressure liquid chromatography (HPLC) to investigations involving commercial hydroxyoxime copper extractants.

EXPERIMENTAL

LIX 63, 64N, 65N, 70, 71, and 73, and Shell 529 and Acorga P-17 were commercial samples.

For GC, *ca.* 25 mg of material was treated with 1 ml of trimethylsilylimidazole reagent Tri-Sil Z (Pierce, Rockford, Ill., U.S.A.) for 10 min and 1-μl samples were injected into a Varian Aerograph Model 1200 gas chromatograph equipped with a flame ionization detector. The column was 5 ft. × 1/8 in. O.D. stainless steel packed with 5% SE-30 on Chromosorb W AW DMCS, 60–80 mesh. The carrier gas (nitrogen) flow-rate was 50 ml/min. The aliphatic hydroxyoxime LIX 63 was chromatographed



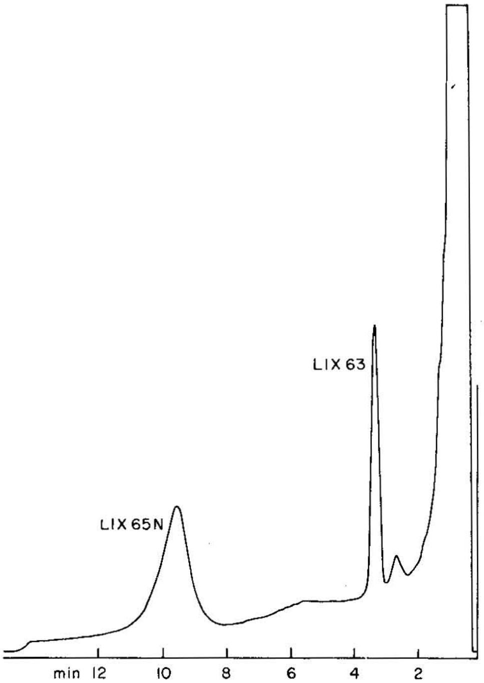
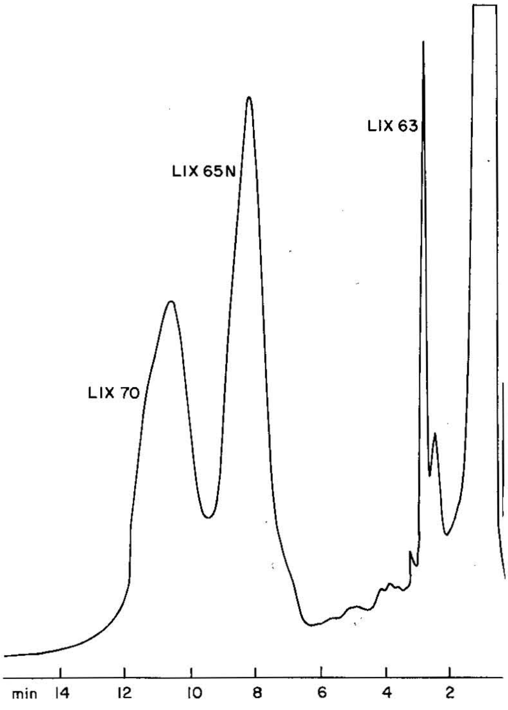


Fig. 1. GC of LIX 63.

Fig. 2. GC of LIX 64N.



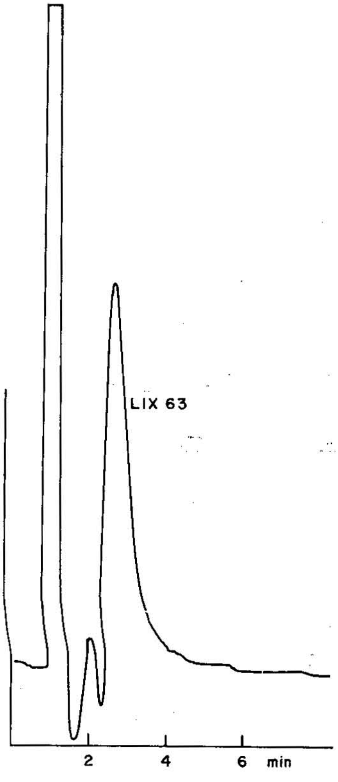


Fig. 3. GC of LIX 73.

Fig. 4. HPLC of LIX 63 with refractive index detector.

at 170° while mixtures of aliphatic and aromatic LIX reagents were separated by linear temperature programming at 4°/min from 170° to 225°. Temperatures for Shell 529 and Acorga P-17 were 200° and 225°, respectively.

For HPLC a Waters Assoc. Model ALC 202 instrument with ultraviolet (254 nm) and refractive index detectors was used. The packing was Durapak Carbowax 400 on Porasil C in a 2 ft. × 1/8 in. O.D. column. For LIX 63 the solvent was 2,2,4-trimethylpentane–chloroform (11:3) at a flow-rate of 2.0 ml/min while for the other extractants the solvent was pentane–chloroform (10:3) at 2.0 ml/min. Generally 5-μl samples were injected using 10% solutions of LIX 63 and 1% solutions of the other extractants.

RESULTS AND DISCUSSION

Some typical GC separations are shown in Figs. 1, 2 and 3 for LIX 63, LIX 64N and LIX 73, respectively. The procedure is particularly effective for LIX 63 which is a minor but important component of LIX 64N, LIX 70 and LIX 73, as reported by Ashbrook. The GC procedure readily separates the aliphatic oxime (a) and two aromatic oximes (c) and (d) which are in currently marketed LIX reagents. The hydroxyoxime in Shell 529 had a retention time of 4 min at 200°, while Acorga P-17

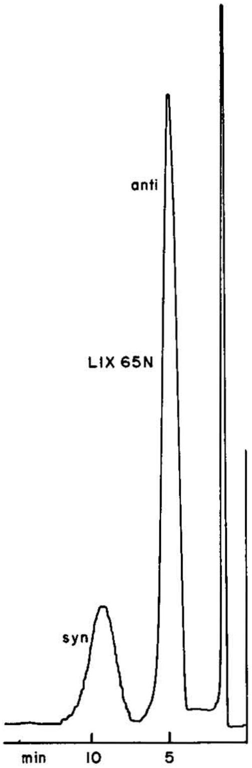


Fig. 5. HPLC of LIX 65N.

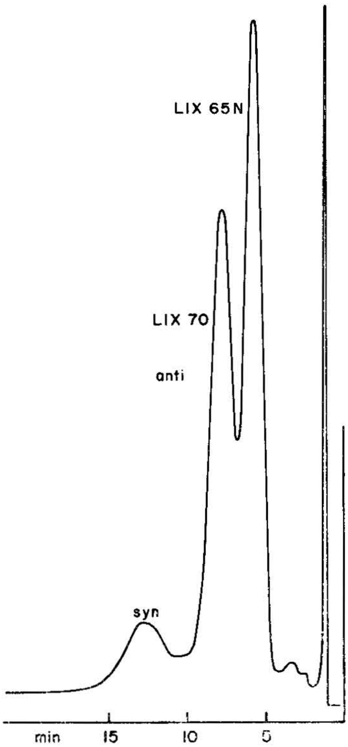


Fig. 6. HPLC of LIX 71.

appeared at 6 min at 225°. The volatility of the Shell 529 extractant is between that of LIX 63 and LIX 65N in accord with its mixed aromatic–aliphatic structure. Under the conditions used, Acorga P-17 is not separated from LIX 65N. The *anti* and *syn* isomers of the hydroxyoximes also are not separated by the GC procedure.

HPLC separations are shown in Fig. 4, 5 and 6 for LIX 63, LIX 65N and LIX 71, respectively. For LIX 63, the refractive index detector was used while the aromatic hydroxyoximes were detected by ultraviolet (UV). The *anti* and *syn* isomers of the aromatic hydroxyoximes are well separated (Fig. 5) and the mixed nature of LIX 71 is clearly indicated (Fig. 6). The major (*anti*) isomers of the hydroxyoximes in Shell 529 and Ocorga P-17 have retention times of 5 min and 10 min, respectively. The ability of HPLC to separate and analyse *anti* and *syn* isomers easily and rapidly is useful in investigations since the isomers differ in their chelating properties. HPLC has the advantage of separating extractants directly without prior conversion to derivatives. The separation of isomeric oximes by HPLC may be added to the previously reported techniques of column adsorption chromatography[6] and TLC[7].

ACKNOWLEDGEMENT

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REFERENCES

1 A. W. Ashbrook, *J. Chromatogr.*, 105 (1975) 141.
2 R. R. Swanson, *U.S. Pat.*, 3,224,837, 1965.
3 D. S. Flett, *Proc. Int. Solvent Extr. Conf., The Hague, 1971*, Soc. Chem. Ind., London, 1971, p. 204.
4 A. J. van der Zeeuw, *Hydrometallurgy Symposium, Manchester, 1975, Institution of Chemical Engineers Symposium Series (London)*, No. 42 (1975) 16.1.
5 R. Price and J. A. Tumilty, *Hydrometallurgy Symposium, Manchester, 1975, Institution of Chemical Engineers Symposium Series (London)*, No. 42 (1975) 18.1.
6 L. Zechmeister, W. H. McNeely and C. Solyom, *J. Amer. Chem. Soc.*, 64 (1942) 1923.
7 M. Hranisavljević-Jakovljević, I. Pejović-Tadić and A. Stojiljković, *J. Chromatogr.*, 12 (1963) 70.

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Note

High-pressure liquid chromatography of naturally occurring xanthones

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Xanthones occupy an important position in the chemistry of natural compounds. Their structure is related to that of flavonoids and their chromatographic behaviour is also similar. Whereas flavonoids are frequently encountered in nature, xanthones have been found in only a few families. They always occur in Guttiferae and Gentianaceae and can be considered characteristic of these plants. In the last few years, a large number of new naturally occurring xanthones have been identified in the genera Canscora[1], Swertia[2] and Gentiana[3–5] (Gentianaceae). The growing interest in these compounds is easily explained by their pharmacological activity[6] (anti-psychotic action, monoamine oxidase inhibition, anti-tubercular activity) as well as their importance in chemotaxonomy.

Xanthones are usually separated by thin-layer chromatography (TLC)[7,8], classical column chromatography[3] or by gas–liquid chromatography as the trimethylsilyl ethers[9]. The work reported here deals with the separation of naturally occurring xanthones isolated from the genus Gentiana and of their methyl ethers by high-pressure liquid chromatography (HPLC). Emphasis is given to the separation of xanthones which are poorly separated by the other chromatographic methods.

EXPERIMENTAL

Materials

All the naturally occurring xanthone samples were available from our previous work on extracts from Gentiana. The completely methylated xanthones have been obtained by treatment of the natural products by an excess of diazomethane. One exception was the 1,3,6,7-tetramethoxy xanthone which has been synthesized in our laboratory. The samples have been dissolved in chloroform to a concentration of 1 μg/μl. Since these compounds absorb well in the ultraviolet (UV), injections of microgram quantities gave good chromatographic results.

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* To whom requests for reprints should be addressed.

High-pressure liquid chromatography

The separations were made on a Varian Model 8500 instrument with gradient capability. The columns used were stainless-steel, 25 cm × 2.2 mm I.D., slurry-packed with microporous chemically bonded silica gel (Micropak CN or Micropak NH_2 from Varian with 10 μm average particle size). Detection was made by UV absorption at 254 nm with a Variscan multiple wavelength detector. Solutions (1–10 μl) were injected with an SGE 10-μl syringe and the Varian stop-flow injector.

RESULTS AND DISCUSSION

We have begun our work by investigating the separation of tetramethoxy xanthones because they are not separated well by TLC on either silica gel or polyamide.

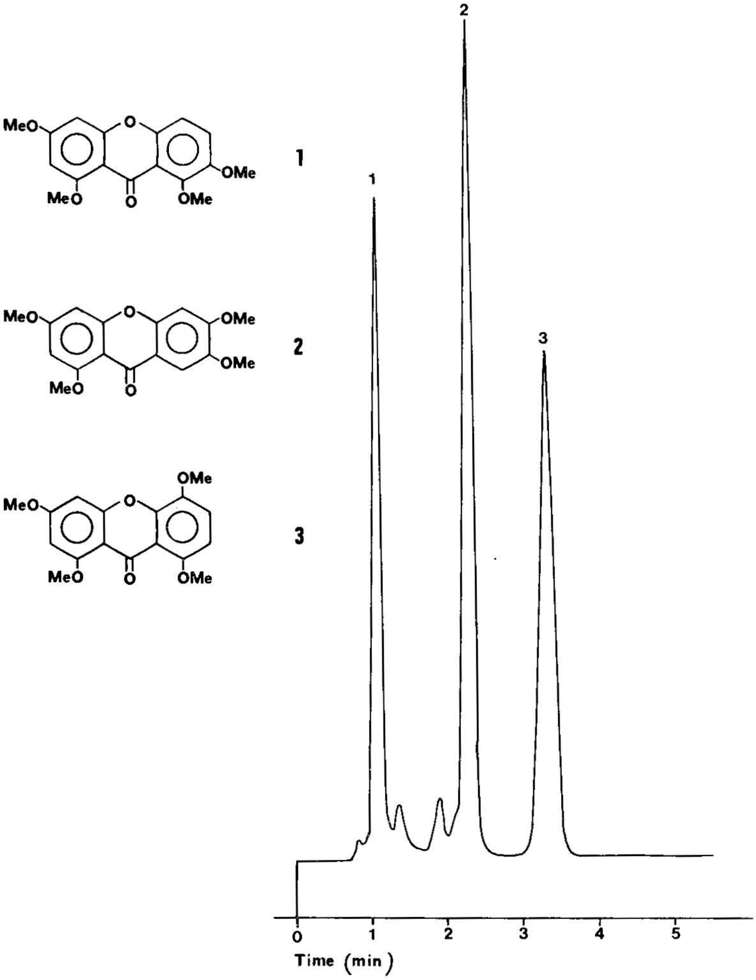


Fig. 1. Tetramethoxy xanthone isomers. Column, 25 cm × 2.2 mm Micropak CN; solvent, *n*-hexane–chloroform (13:7); flow-rate, 60 ml/h; pressure, 70 atm; sample, 5 μl (approx. 1 μg) of each compound; detector, 254 nm. Me = Methyl.

These TLC techniques have been well developed in our laboratory for xanthones and reported elsewhere[3–5].

We initially tried both 5- and 10-μm porous silica gel columns. Even with a variety of solvents and gradient systems no separation of isomers was possible. A Varian Micropak CN column gave the excellent separation of three tetramethoxy xanthone isomers shown in Fig. 1. Both the speed and high resolution confirm the advantages of HPLC for difficult isomer separations such as shown here. Fig. 2 shows the separation of a more complex mixture. This sample contains three tetramethoxy xanthone isomers as well as a trimethoxy xanthone and a tetramethoxy flavone. This mixture is typical of plant extracts which have been methylated.

Methylation of xanthones is a useful technique for structural determinations[8]. HPLC using chemically bonded phases offers a rapid separation of these compounds.

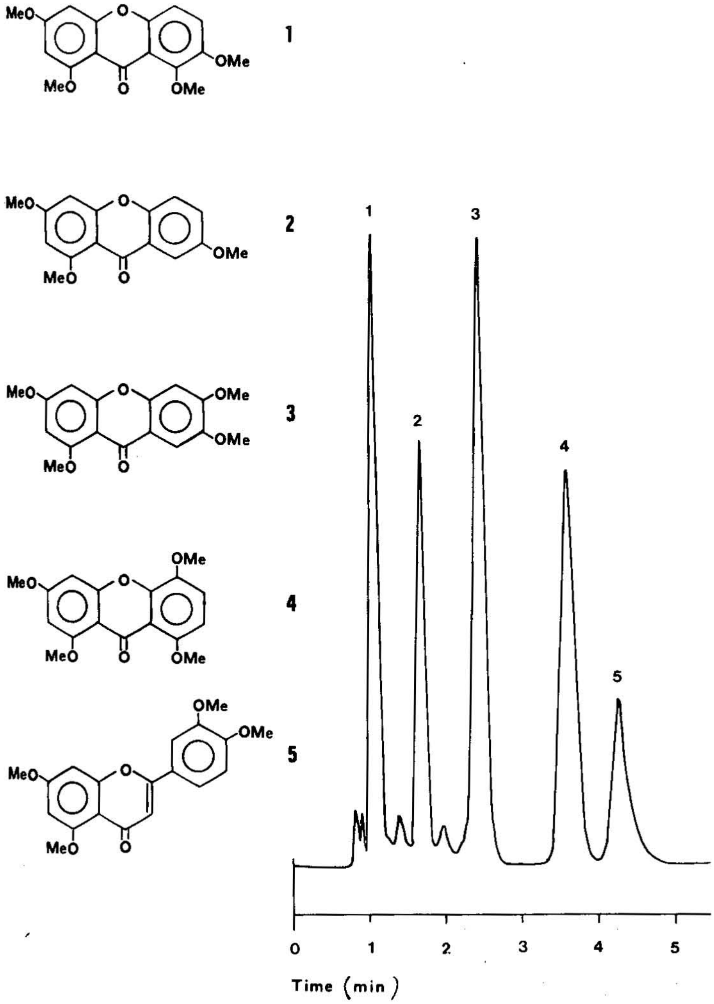
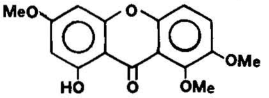


Fig. 2. Tri- and tetramethoxy xanthones and a tetramethoxy flavone. Same conditions as Fig. 1.



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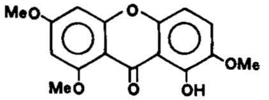

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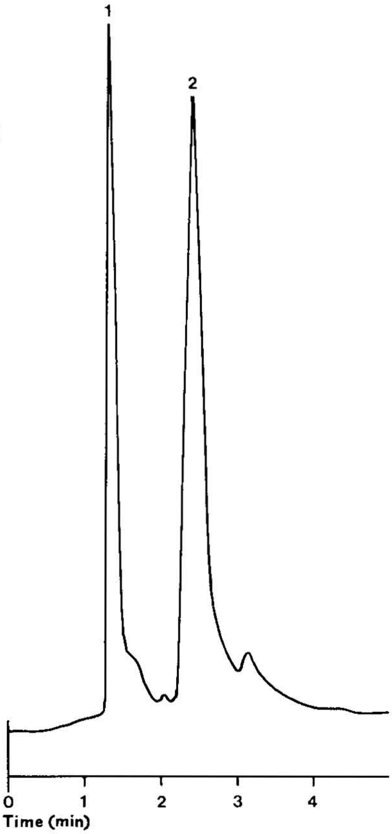

Fig. 3. Monohydroxy xanthone isomers. Conditions are the same as in Fig. 1 except the solvent which is *n*-hexane–chloroform (2:3).

It is necessary however to separate xanthones containing free hydroxyl groups as well. To date we have been only moderately successful in the separation of the more polar xanthones. Fig. 3 shows the separation of two monohydroxy isomers. Note that a more polar solvent is required. Fig. 4 shows the results obtained with dihydroxy xanthones. On the Micropak CN column the third peak tails badly, conditions of solvent polarity and flow-rate are critical. Also shown in Fig. 4 is the same sample on a Micropak NH_2 column. The active functional group is a weaker $-NH_2$ group and we would expect less retention of the polar samples on this column. This conclusion is verified by the result shown in both Figs. 4 and 5. Fig. 5 shows the separation of a tri- and tetrahydroxy xanthone. These are the most polar xanthones separated to date, but in future work we will use reverse-phase columns in an attempt to increase the range of xanthones handled rapidly by HPLC. Attempts to separate positional isomers of either tri- or tetrahydroxy xanthones under conditions similar to Fig. 5 were unsuccessful.

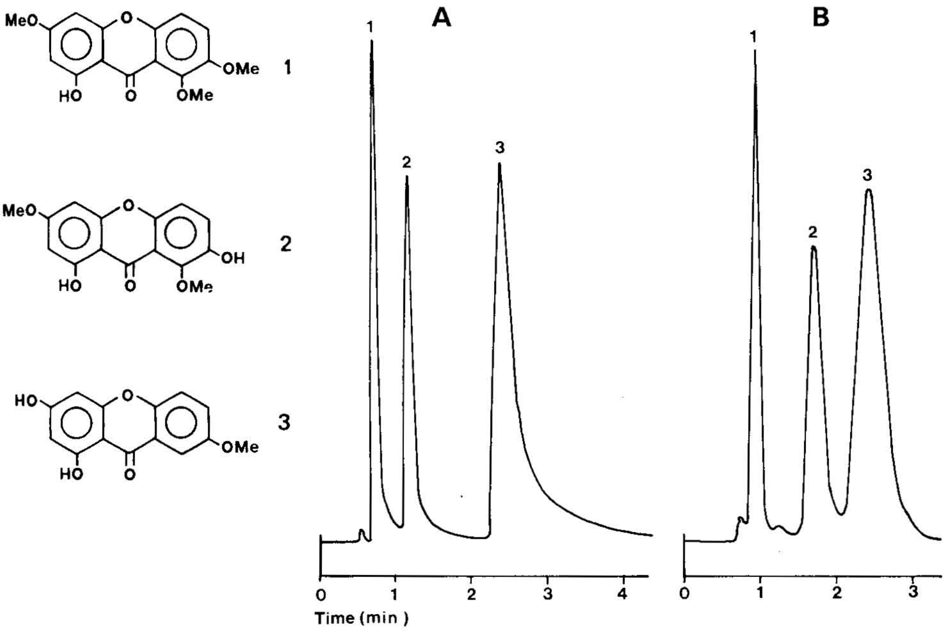

Fig. 4. Dimethoxy xanthones on Micropak CN and Micropak NH_2 columns. A: Column, 25 cm × 2.2 mm Micropak CN; solvent, cyclohexane–chloroform (1:4); flow-rate, 80 ml/h; pressure, 70 atm. B: Column, 25 cm × 2.2 mm Micropak NH_2; solvent, isooctane–chloroform (3:17); flow-rate, 70 ml/h; pressure, 60 atm.

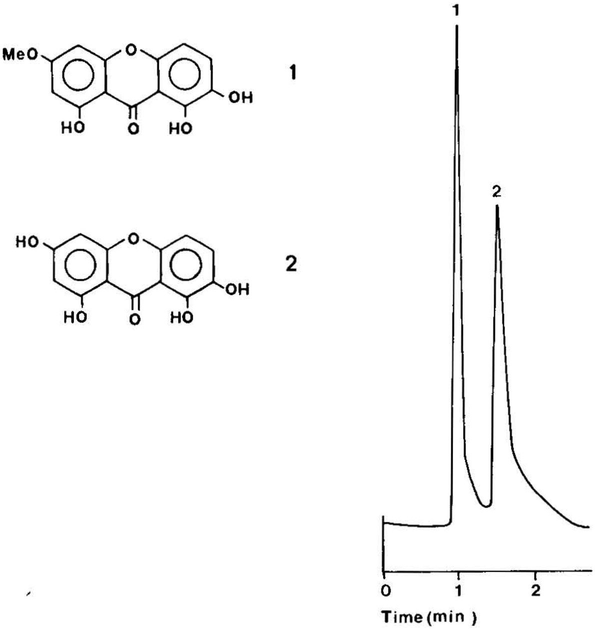

Fig. 5. Tri- and tetrahydroxy xanthones. Column, Micropak NH_2; solvent, dioxane–dichloromethane (1:9); flow-rate, 70 ml/h; pressure, 60 atm.

CONCLUSION

HPLC is used to separate naturally occurring xanthones isolated from the genus Gentiana and their methyl ethers. Positional isomers, as well as mono-, di-, tri- and tetrahydroxy xanthones, are resolved on two chemically bonded phases: Micropak NH_2 and Micropak CN.

HPLC offers the separation of xanthone isomers not possible by TLC or column chromatography. Results in our laboratory with microporous silica gel have shown essentially no selectivity for positional isomers of xanthones. More polar xanthones, with three and four free hydroxyl groups, require more polar solvents and the less polar Micropak NH_2 column. Reversed-phase columns may prove even more useful.

ACKNOWLEDGEMENTS

We are happy to acknowledge the loan of the liquid chromatograph from Varian AG, Zug, Switzerland. One of the authors, H.M.M., happily acknowledges financial support from the "Convention intercantonale romande pour l'enseignement du 3e cycle en chimie", and the hospitality and encouragement of Professor Jacot-Guillarmod, Director of the Institute of Chemistry, University of Neuchâtel, where this research was conducted.

REFERENCES

1 S. Ghosal, R. K. Chaudhuri and A. Nath, *J. Pharm. Sci.* 62 (1973) 137.
2 S. Ghosal, P. V. Sharma and R. K. Chaudhuri, *Phytochemistry*, 14 (1975) 1393.
3 K. Hostettmann, R. Tabacchi and A. Jacot-Guillarmod, *Helv. Chim. Acta*, 57 (1974) 294.
4 K. Hostettmann and A. Jacot-Guillarmod, *Helv. Chim. Acta*, 57 (1974) 1155.
5 M. Kaldas, K. Hostettmann and A. Jacot-Guillarmod, *Helv. Chim. Acta*, 57 (1974) 2557.
6 S. Ghosal and R. K. Chaudhuri, *J. Pharm. Sci.*, 64 (1975) 888.
7 P. Arends, *J. Chromatogr.*, 47 (1969) 550.
8 N. A. M. Saleh, *J. Chromatogr.*, 92 (1974) 467.
9 A. Jefferson, C. I. Stacey and F. Scheinmann, *J. Chromatogr.*, 57 (1971) 247.

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Note

Chromatography of saturated steroid hydrocarbons (steranes) on alumina

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(Received August 11th, 1975)

The isolation of pure components from the complex mixtures of steranes obtained from petroleum and rock extracts is a formidable problem. The differences in their physical properties are small, and chemical derivatives cannot be made. Clathrate formation with thiourea and preparative gas–liquid chromatography (GLC) are widely used, but even this powerful combination fails to separate some of the mixtures encountered. We have found adsorption chromatography on active alumina to be a promising additional technique (*cf.* ref. 1) and report here its application to a range of isomeric and homologous steranes, including epimeric pairs of 24-alkyl-5α-cholestanes, which are inseparable by other methods.

EXPERIMENTAL

The apparatus consisted of two jacketed columns in series, each 1.4 m × 5 mm I.D., connected via a flow refractometer (Waters Assoc., Model R403) to a fraction collector, and filled under pentane with aluminium oxide (Woelm, activity I, 60 g). The columns and refractometer were thermostatted at 15° or 18°. Steranes (1–10 mg) were applied in a few drops of pentane and were eluted with pentane under *ca.* 0.5 atm pressure (flow-rate 30 ml/h). (Hexane has also been used, with similar results.) 2,6,10,14-Tetramethylpentadecane (pristane) was sometimes added to the steranes as a marker. The columns could be used for many consecutive runs without cross-contamination or appreciable loss of resolution.

RESULTS AND DISCUSSION

Results for a series of synthetic reference compounds are presented in Table I and Fig. 1 (A–C). All the compounds produced positive peaks (refractive index of eluates higher than that of pentane). The response to unit amounts of the different compounds appeared to vary somewhat, though this has not been quantified. Analysis of the collected fractions confirmed the separations indicated by the refractometer traces. The observed relative retention volumes of steranes are structure dependent, flatness of the carbon skeleton and length of the side chain being the major factors. Comparison of these values with relative retention volumes on a common GLC stationary phase such as SE-30 (Table I) shows that some steranes which are not

TABLE I

RELATIVE RETENTION VOLUMES OF STERANES AND TRITERPANES ON GAS–LIQUID AND ON LIQUID–SOLID CHROMATOGRAPHY COLUMNS

Compound	*Formula*	*Relative retention volume*	
		*GLC**	*Adsorption***
5α-Androstane	$C_{19}H_{32}$	0.14	0.55–0.57
5β-Androstane	$C_{19}H_{32}$	0.13	
5β-Cholestane	$C_{27}H_{48}$	0.92	
5α,14β-Cholestane	$C_{27}H_{48}$	0.87	
5α,17β(H)-Cholestane	$C_{27}H_{48}$	0.73	
(20*S*)-5α,17β(H)-Cholestane	$C_{27}H_{48}$	0.80	
(24*R*)-24-Methyl-5β-cholestane	$C_{28}H_{50}$	1.16	
(24*S*)-24-Methyl-5β-cholestane	$C_{28}H_{50}$	1.16	
Dammarane***	$C_{30}H_{54}$	1.49 1.55	0.63
5α-Pregnane	$C_{21}H_{36}$	0.23	0.68–0.69
20-Methyl-5α-pregnane	$C_{22}H_{38}$	0.30	
5α,8α,14β-Cholestane	$C_{27}H_{48}$	0.98	
24,24-Dimethyl-5α-cholane	$C_{26}H_{46}$	0.75	0.72–0.76
(20*S*)-5α-Cholestane	$C_{27}H_{48}$	0.91	
Lupane	$C_{30}H_{52}$	1.83	
23-Methyl-5α-cholane	$C_{25}H_{44}$	0.57	0.86–0.89
24,24-Diethyl-5α-cholane	$C_{28}H_{50}$	1.29	
(24*R*)-24-Methyl-5α-cholestane	$C_{28}H_{50}$	1.29	
(24*R*)-24-Ethyl-5α-cholestane	$C_{29}H_{52}$	1.64	
(24*S*)-24-Ethyl-5α-cholestane	$C_{29}H_{52}$	1.64	
5α-Cholestane	$C_{27}H_{48}$	1.00	1.00
4α-Methyl-5α-cholestane	$C_{28}H_{50}$	1.17	1.03–1.07
4β-Methyl-5α-cholestane	$C_{28}H_{50}$	1.27	
(24*S*)-24-Methyl-5α-cholestane	$C_{28}H_{50}$	1.29	

* 1.8 m × 3.2 mm, 5% SE-30 on Chromosorb W, 250°.

** 2.8 m × 5 mm, aluminium oxide, pentane eluent, conditions described in text. Retention volumes measured from time of application of sample.

*** 1:1 Mixture of (20*R*)- and (20*S*)-epimers.

separated by preparative GLC are separated on alumina, and *vice versa*. Chromatography on alumina may also prove useful for isolating triterpanes, two of which are included in Table I.

While it should be possible to improve the separations, using longer columns or microparticulate alumina, even the simple, low-resolution apparatus described here is of practical value. Thus, (20*S*)-5α-cholestane has been isolated[2] from a preparative GLC fraction derived from a crude oil and containing at least four other, as yet unidentified, steranes which have smaller retention volumes on alumina (Fig. 1D).

Of special significance is the ability of the alumina column to separate (24*R*)-24-methyl-5α-cholestane (5α-campestane) and (24*S*)-24-methyl-5α-cholestane (5α-ergostane), illustrated in Fig. 1E by the partial resolution of a pure 24-methyl-5α-

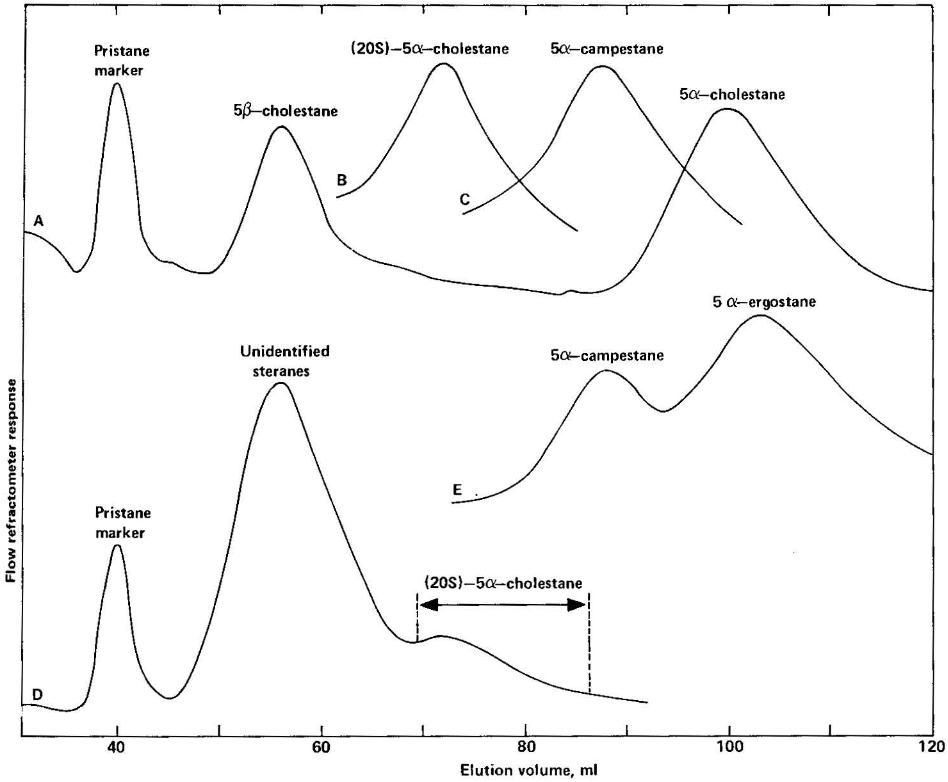

Fig. 1. Chromatography of steranes on alumina. Apparatus and conditions are described in text. A, B, and C, some reference steranes. D, sterane mixture obtained from Rozel Point, Utah, crude oil; the fractions which were combined to yield (20*S*)-5α-cholestane are indicated. E, the 24-methyl-5α-cholestane fraction obtained from Green River shale, Colorado.

cholestane fraction obtained from an oil shale into the two isomers. By re-chromatography of the middle fractions, the two components were obtained nearly free from each other, the relative quantities agreeing with the ratio 1:3 estimated by 220 MHz proton magnetic resonance analysis of the original mixture[2]. The corresponding 5β-steranes are not separated. A mixture of (24*R*)- and (24*S*)-24-ethyl-5α-cholestane (5α-stigmastane and 5α-poriferastane, respectively) gave a single peak on the refractometer trace, but proton magnetic resonance analysis revealed enrichment of the earlier fractions in the (*R*)-epimer, and of the later fractions in the (*S*)-epimer, indicating that some resolution had occurred. No chromatographic separations of (24*R*)- and (24*S*)-24-alkylcholestanes, nor of epimer pairs of the parent sterols, have previously been reported. The configuration at C-24 is important in studies of plant sterols[3,4]. Reduction of 24-methylsterols to hydrocarbons which can be separated and quantified provides an accurate method of determining the 24*R*:24*S* ratio in such sterols, which could be extended to the 24-ethyl compounds if alumina columns of higher resolution are used.

ACKNOWLEDGEMENTS

I thank Dr. L. J. Mulheirn for providing the synthetic steranes used in this study[4,5] and Mrs P. R. Jennings and Miss S. Drewitt for technical assistance.

REFERENCES

1 T. C. Hoering, *Carnegie Inst. Washington, Geophys. Lab., Yearbook*, (1969) 303.
2 L. J. Mulheirn and G. Ryback, *Nature (London)*, 256 (1975) 301.
3 L. J. Goad, J. R. Lenton, F. F. Knapp and T. W. Goodwin, *Lipids*, 9 (1974) 582.
4 L. J. Mulheirn, *Tetrahedron Lett.*, (1973) 3175.
5 L. J. Mulheirn and G. Ryback, *J. Chem. Soc. Chem. Commun.*, (1974) 886.

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Note

New spray for the detection of sulfonyl chlorides

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(First received May 21st, 1975; revised manuscript received July 21st, 1975)

In pursuing the chlorination of sulfur compounds as our primary research interest, we have encountered a variety of sulfur compounds which could not be visualized on chromatographic plates. In the past, we have reported[1,2] the development of sprays which facilitate the detection of thiols, sulfides, sulfoxides (particularly α-polychlorosulfoxides) and sulfinate esters. Both hexanitratoammonium cerate(IV)[1] and chromium trioxide[2] sprays are oxidizing sprays and are therefore unable to detect sulfones or sulfonyl chlorides.

Recently we have developed a method for the preparation of α,α-dichloro-α-sulfonyl sulfonyl chlorides. The desire to characterize these compounds on thin-layer chromatography (TLC) plates along with our longer-standing desire to detect simpler sulfonyl chlorides, which we have been isolating for some time[3,4], has led to a search for such a spray.

Potassium iodide has been employed for the detection of compounds containing the sulfinyl group, *i.e.* thiosulfinates and sulfinic acids[5,6]. The sensitivity of the spray may be enhanced by spraying the plate with starch solution after spraying with iodide, since the iodine–starch complex gives a stronger color than simple molecular iodine[6].

EXPERIMENTAL

Chemicals

Compounds 1–3 and 13 (see Table I) were prepared by the method we have developed recently[7]; compound 4 was prepared by the method of Opitz[8]; compound 5 was prepared as we have previously outlined[4]; compounds 7, 8 and 11 were prepared by the exhaustive chlorination of the corresponding symmetric sulfides in aqueous acetic acid utilizing the conditions we have developed for the conversion of α-chlorosulfides to sulfonyl chlorides[4]; compound 12 was prepared as we have described elsewhere[9].

Compounds 6, 9 and 10 were purchased from Baker Chemicals (Phillipsburg, N.J., U.S.A.). Silica gel GF_{254} was purchased from Merck (Darmstadt, G.F.R.).

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TABLE I

RESULTS FOR SULFONYL CHLORIDES, α-DICHLORO-β-DISULFONES AND SULFENYL CHLORIDES

All compounds gave amber spots on a colorless background at room temperature after development with chloroform. I = Immediate appearance; TF = appearance after 3–5 min.

No.	*Compound*	*Appearance*	*R_F value*	*Amount applied (μg/spot)*
	Sulfonyl chlorides			
1	$CH_3–SO_2–CCl_2–SO_2–Cl$	I	0.48	115
2	$C_6H_5–SO_2–CCl_2–SO_2–Cl$	I	0.63	129
3	$ClCH_2–SO_2–CCl_2–SO_2–Cl$	I	0.69	120
4	$CH_3–SO_2–CH_2–SO_2–Cl$	I	0.04	490
5	Chloromethane	TF	0.91	253
6	Methane	TF	0.53	563
7	Ethane	TF	0.73	437
8	Propane	TF	0.83	500
9	Benzene	TF	0.75	246
10	*p*-Toluene	TF	0.86	258
11	Benzyl	TF	0.57	590
	α-Dichloro-β-disulfones			
12	$CH_3–SO_2–CCl_2–SO_2–CH_2–C_6H_5$	TF	0.40	635
	Sulfenyl chlorides			
13	$CH_3–SO_2–CCl_2–S–Cl$	I	0.47	238

General procedure

The plates were made with silica gel GF_{254} (750 μm thick) using a Desaga TLC applicator. Plates were air dried 24 h before use. The spray reagent was prepared by dissolving sodium iodide (10 g) in acetone (100 ml). Plates were spotted with the appropriate amount of compound (see Table I), developed 15 cm, air dried and sprayed with sodium iodide solution. Amber spots appeared on a colorless background within 5 min of spraying.

RESULTS AND DISCUSSION

For some time, we have been isolating alkanesulfonyl and arylsulfonyl chlorides from reaction mixtures utilizing column and/or preparative TLC without loss of material due to hydrolysis*. However, when the α-sulfonyl sulfonyl chloride (compound 3) was developed on preparative TLC plates and isolated it was found to be 21% sulfonyl chloride and 79% $ClCH_2–SO_2–CCl_2H$**. The presence of the trichlorosulfone indicates that the α,α-dichloro-α-sulfonyl sulfonyl chloride is very sensitive to traces of moisture and hydrolyzes to furnish the sulfone by a new dechlorosulfonylation reaction which we have recently reported in detail[7]. Thus the sensitivity of the spray reagent for compounds 1–3 is some five times greater than is indicated by the amount of material which must be applied to observe a spot after development.

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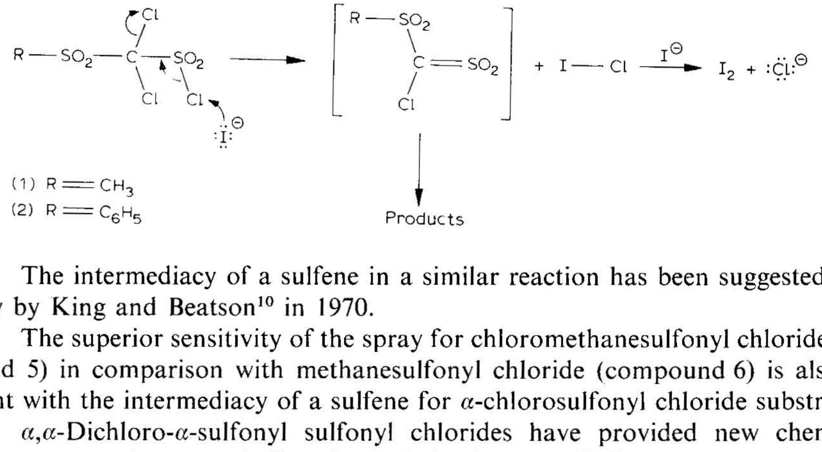
* For example, methanesulfonyl chloride has been quantitatively separated from benzyl chloride by column chromatography[7].

** Sodium iodide spray does not detect simple α-polychlorosulfones.

Table I depicts the results obtained on the sulfur compounds examined, including the α,α-dichloro-α-sulfonyl sulfonyl chlorides (compounds 1, 2 and 3) which were of primary interest to us. The mode of action of the iodide spray clearly involves reduction of the sulfur compounds. Plates spotted more heavily with compounds 1, 2 and 3 gave black-purple spots and iodine vapour could be observed rising from the surface of the plate.

Two possible pathways for the reduction suggested themselves. Firstly, attack by iodide ion on the chlorine atom attached to sulfur would result in the formation of iodine and the sodium salt of the sulfinic acid. In order to find support for this possibility, we examined a variety of sulfonyl chlorides (compounds 4–11) and found that the results were positive but the sensitivity of the spray was impaired. The second possibility involves attack by iodide ion on one of the chlorine atoms attached to carbon, which would furnish iodine and the α-chloro-α-sulfonyl sulfonyl chloride. We chose to investigate this possibility by examining a compound which contained carbon bound chlorine in a very similar electronic environment, namely the α,α-dichloro-β-disulfone (compound 12). Once again the result was positive although the sensitivity of the spray was impaired.

The results on compound 4 demonstrate that the α-chlorine atoms of compounds 1, 2 and 3 play an important role in the interaction between the spray and these compounds. In order to rationalize the greater sensitivity of the spray and faster appearance of the spots when compounds 1, 2 and 3 are visualized, we are proposing the following mechanism involving both types of chlorine atom:



The intermediacy of a sulfene in a similar reaction has been suggested previously by King and Beatson[10] in 1970.

The superior sensitivity of the spray for chloromethanesulfonyl chloride (compound 5) in comparison with methanesulfonyl chloride (compound 6) is also consistent with the intermediacy of a sulfene for α-chlorosulfonyl chloride substrates.

α,α-Dichloro-α-sulfonyl sulfonyl chlorides have provided new chemistry[7], new spectrometric properties[11] and new behaviour on TLC.

CONCLUSIONS

The development of sodium iodide–acetone solution as a spray reagent for the detection of sulfonyl chlorides has been reported. It reduces the sulfonyl chlorides forming iodine which is visible as an amber spot on the plate. A novel mechanism involving sulfene formation is proposed in order to explain the enhanced sensitivity of the spray for α,α-dichloro-α-sulfonyl sulfonyl chlorides, a new and interesting subclass of sulfur compounds.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the kindness of Dr. T. T. Tidwell during the course of this work as well as the technical assistance of Mr. Richard Langler, Sr. Mr. M. M. Bharadwaj provided details for the preparation of compound 3. We thank the University of Toronto for financial support.

REFERENCES

1 J. S. Grossert and R. F. Langler, *J. Chromatogr.*, 97 (1974) 83.
2 R. F. Langler, *J. Chromatogr.*, 104 (1975) 228.
3 J. S. Grossert and R. F. Langler, *Chem. Commun.*, (1973) 49.
4 J. S. Grossert, W. R. Hardstaff and R. F. Langler, *Chem. Commun.*, (1973) 50.
5 C. de Marco, *Nature (London)*, 198 (1963) 683.
6 A. Senning (Editor), *Sulfur in Organic and Inorganic Chemistry*, Vol. 2, Marcel Dekker, New York, 1972, p. 223.
7 W. R. Hardstaff, R. F. Langler, J. Leahy and M. J. Newman, *Can. J. Chem.*, in press.
8 G. Opitz, *Angew. Chem. Int. Ed. (Eng.)*, 6 (1967) 107.
9 W. R. Hardstaff and R. F. Langler, *Org. Mass Spectrom.*, 10 (1975) 215.
10 J. King and R. Beatson, *Chem. Commun.*, (1970) 663.
11 W. R. Hardstaff, R. F. Langler and M. J. Newman, *Org. Mass Spectrom.*, 9 (1974) 1156.

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Note

Separation of 4-N,N-dimethylaminoazobenzene-4′-sulfonyl amino acids on polyamide sheets

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(Received July 21st, 1975)

4-N,N-Dimethylaminoazobenzene-4′-sulfonyl chloride (DABS-Cl) is a sensitive chromophoric azo compound synthesized from the reaction of methyl orange and PCl_5[1]. The sulfonyl group of DABS-Cl readily reacts with primary and secondary amino groups, thiols, imidazoles, phenols and aliphatic hydroxyl groups. The effectiveness of DABS-Cl in the qualitative and quantitative identification of amino acids (AAs) and in the determination of the N-terminal amino acids of peptides and proteins using silica gel plates, and the investigation of the physical properties of seventeen DABS-AAs have been reported[1]. The intense chromophoric DABS-AAs permit the detection of amino acids as colored spots in the range of 10^{-10} to 10^{-11} moles visible directly on the thin-layer plate. But due to the diffusion problem on silica gel plates which decreases the sensitivity and causes unsatisfactory separation of certain DABS-AAs, such as Leu, Ile and Val, Met and Phe, Ala and Pro, we endeavoured to find a better separation of DABS-AAs and in this report investigated polyamide sheets for the purpose.

EXPERIMENTAL

Cheng Chin polyamide layer sheets were purchased from Pierce and were cut into 5 × 5-cm squares prior to use. All solvents used were commercial analytical grade without further purification.

50 nmoles of each amino acid dissolved in 50 μl of 0.2 *M* $NaHCO_3$ was allowed to mix with 50 nmoles of DABS-Cl in 50 μl acetone and reacted at 70° for 5–10 min. About 10–15 picomoles of each DABS-AA was successively applied to the origin of the plate. In order to obtain a better separation, the diameter of the original spot should be confined to 1.0–1.3 mm by using a mild hair dryer.

Two-dimensional development in a covered jar was performed by ascending solvent flow[2,3]; solvent 1, water–2-chloroethanol–formic acid (100:60:3.5, v/v/v) for the first dimension development and solvent 2, benzene–acetic acid (6:1, v/v) for the second dimension.

RESULTS AND DISCUSSION

Fig. 1 shows the original developed plate after being exposed to HCl vapor.

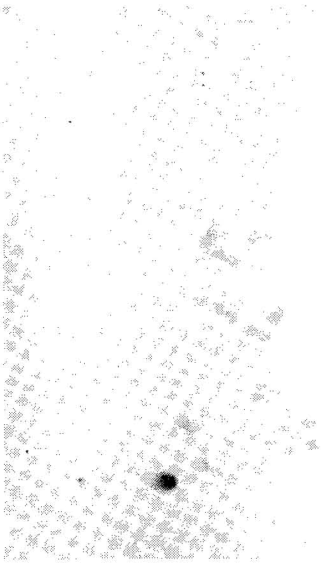


Fig. 1. Photograph of a two-dimensional development. 10–15 picomoles of each DABS-AA was applied. The spots appeared on the sheet as red colored after exposure to HCl vapor.

It shows that the separation between many DABS-AAs was quite satisfactory. Fig. 2 is a schematic representation of 30 different DABS-AAs on a two-dimensional plate. However, only 23 individual spots appeared on the plate, the other 7 DABS-AAs were incompletely separated and give four spots, *i.e.*, $CysO_3H$ and DABSOH; Hyp,

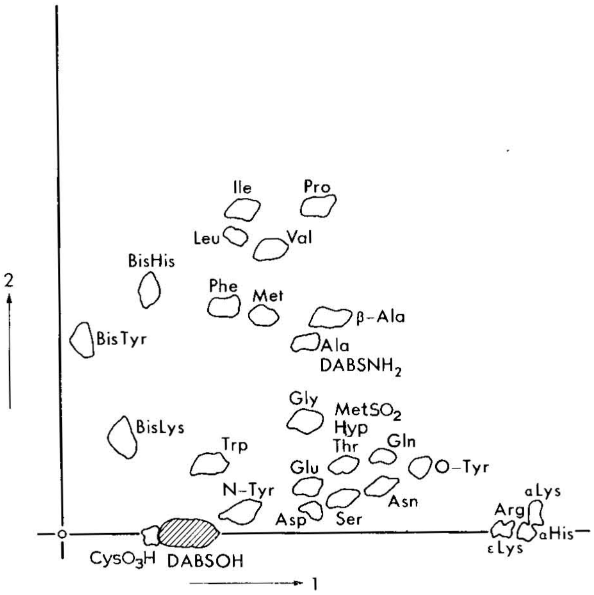
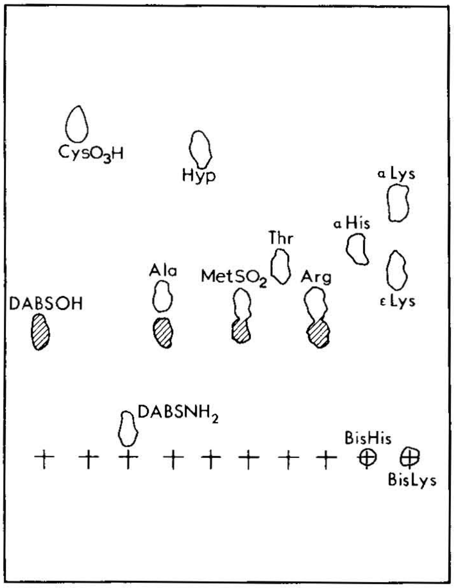


Fig. 2. A schematic representation of the two-dimensional chromatography of 30 DABS-AAs. Details are given in the text.



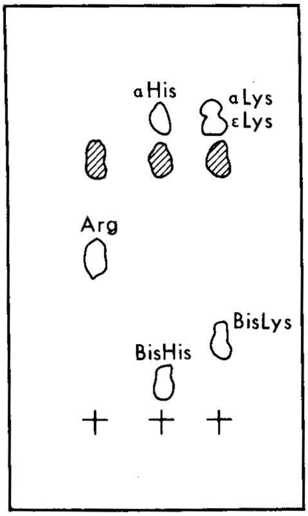


Fig. 3. One-dimensional separation of four overlaps: DABSOH and $CysO_3H$; $DABSNH_2$ and Ala; $MetSO_2$, Hyp and Thr; α-monoHis, α-monoLys, ε-monoLys and Arg by solvent 4. Details: see text.

Fig. 4. One-dimensional chromatography using solvent 3 (see text) for the identification of Arg from mono-substituted Lys and His.

$MetSO_2$ and Thr; Ala and $DABSNH_2$; α-monoHis, α-monoLys, ε-monoLys and Arg. Solvent 4, water–pyridine–ammonia (28%)–formic acid (100:20:10:2), was found to be extremely satisfactory for resolving these four overlaps and solvent 3, water–ammonia (28%)–ethanol (9:1:10), for identification of arginine (Figs. 3 and 4). The identification of the mono-substituted bifunctional amino acids tyrosine and lysine was carried out by dabsylation and hydrolysis of carbobenzoxy, benzyl and acetyl protected amino acids.

α-Monohistidine was confirmed by hydrolyzing BisHis in 6 *N* HCl for 2 h. The diffusion problem on polyamide has been proved to be much less than that on silica gel and hence increases the sensitivity of detection on polyamide plate. The diameters of the separated spots were limited to approximately 2 mm when the diameter of the original spot was confined to 1.0–1.3 mm. In addition, the whiter background of polyamide sheet has facilitated the observation of the separated spots. Under these circumstances, we have been able to detect the spots of DABS-AAs up to picomole level without any need for an ultraviolet lamp or chemical indicators. Furthermore, this sensitive colorimetric method was expected to offer a much more convenient procedure in quantitative analysis and column chromatographic separation of DABS derivatives.

REFERENCES

1 J. K. Lin and J. Y. Chang, *Anal. Chem.*, 47 (1975) 1634.
2 B. S. Hartley, *Biochem. J.*, 119 (1970) 805.
3 K. R. Woods and K. T. Wang, *Biochim. Biophys. Acta*, 113 (1967) 369.

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Note

Thin-layer chromatography of apomorphine and its analogs

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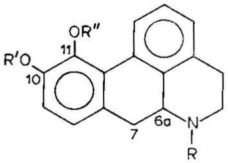
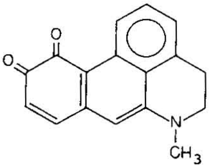
As part of systematic studies concerning the mammalian metabolism of aporphine alkaloids[1–3], recent investigations in these laboratories have focused on the metabolic fate of apomorphine analogs which are potential anti-parkinsonian prodrugs[4–7].



(I) Apomorphine: $R = CH_3$; $R' = R'' = H$.
(II) Apocodeine: $R = R' = CH_3$; $R'' = H$.
(III) Isoapocodeine: $R = R'' = CH_3$; $R' = H$.
(IV) Apomorphine dimethyl ether: $R = R' = R'' = CH_3$.
(V) 10,11-Dihydroxy-6-*n*-propylnoraporphine: $R = (CH_2)_2CH_3$; $R' = R'' = H$.

(VI) 10-Hydroxyaporphine: $R = CH_3$; $R' = H$.
(VII) 10-Methoxyaporphine: $R = R' = CH_3$.
(VIII) 10-Hydroxy-6-*n*-propylnoraporphine: $R = (CH_2)_2CH_3$; $R' = H$.



(IX) Apomorphine orthoquinone.

The instability of compound I due to air oxidation of the catechol moiety[8], and the sensitivity toward oxidation of the C_{6a} and C_7 positions of aporphines[9], are well known. Accordingly, preliminary investigation indicated that significant decomposition of these materials occurs during typical microsomal metabolism studies, *e.g.*, during both microsomal incubation and drug metabolite isolation procedures. Because of this labile nature, methods were developed to prevent air oxidation of these materials during each of the steps encountered in metabolic studies. Decomposition in aqueous media or under microsomal incubation conditions can be effectively prevented by use of antioxidants (sodium bisulfite or dithiothreitol) while decomposition during isolation procedures can be prevented by rigorous nitrogenation of the organic solvents used to extract and concentrate samples prior to analysis. Employing these techniques, thin-layer chromatography (TLC) of I–VIII yields single, characteristic spots for each of the compounds. TLC solvent systems have been developed to separate the metabolic substrates II–VIII from I and/or other probable metabolites. These systems and structural characterization of the major decomposition product of I are described in this report.

MATERIALS AND METHODS

Materials

Apomorphine hydrochloride hemihydrate was purchased from Penick. Apocodeine (II) was prepared as described previously[3]. Isoapocodeine (III) and 10,11-dimethoxyaporphine (IV) were synthesized according to the procedure of Cannon *et al.*[2]. Compounds V and VIII were obtained through the scheme of Neumeyer *et al.*[10] while VI and VII were made using procedures described by Borgman[11]. Compound IX was prepared according to the method of Linde and Ragab[8]. When the melting point of IX was determined in the usual way, it appeared to decompose without melting, consistent with the reports of previous workers. However, IX rapidly melted when placed in a pre-heated oil bath (200°). All solvents and reagents were reagent grade. Sodium bisulfite SO_2 lot analysis was 59.9%.

Standard solutions

1–5-mg quantities of compounds I–VIII as their hydrohalide salts were dissolved in 1 ml of rigorously nitrogenated water. The aqueous media were neutralized with 1 ml 1.0 *M* Tris–HCl buffer (pH 7.2) and extracted with 2 ml nitrogenated ethyl acetate. Ethyl acetate solutions were used immediately to spot thin-layer plates.

Thin-layer chromatography

Silica gel GF_{254} plates, 250 μm thick (Analtech), were scored into 1-cm channels and spotted with 1–5 μl of standard (ethyl acetate) or 0.25–0.50 ml of sample [concentrated microsomal extracts; chloroform– methanol (1:1)] solutions. Spotted plates were developed 10 cm in the following solvent systems: A, benzene–methanol (4:1); B, acetone–methanol (1:1); C, benzene–ethyl acetate–diethylamine (6:3:1); D, chloroform–acetone (8:2). Visualization was done by quenching of plate fluorescence while irradiated with ultraviolet (UV) light (254 nm) and by use of diazotized sulfanilic acid (DSA) and 2,6-dichloroquinone-4-chloroimide (DCQ) spray reagents. DSA spray reagent was generated immediately prior to use by combining equal volumes of 0.5% aqueous sulfanilic acid hydrochloride (5 g sulfanilic acid and 53 ml conc. HCl in 1 l of water) and 0.5% aqueous sodium nitrite stock solutions. DCQ spray reagent contained 2% DCQ in methanol. After spraying plates with DCQ, a 15-min interval was allowed for color production at room temperature. The plates were then warmed over a hot plate and colors again noted.

Simulated microsomal metabolism studies

2-μmole quantities of I–VIII in 0.5 ml of 0.01 *N* HCl, were added to simulated microsomal metabolic incubation media containing 300 μmoles Tris–HCl buffer (pH 7.4) and 0.5 g liver tissue (Sprague Dawley rat liver 10,000 *g* supernatant[6,7]) in 3 ml water. Media also contained 0.05% sodium bisulfite or 2 μmole dithiothreitol. Mixtures were incubated under air at 37° for 30 min in a Dubnoff metabolic shaker. Protein was removed from these media by acidification with 0.5 ml of 1 *N* HCl followed by either filtration or centrifugation (2000 *g* for 5 min) and retention of the supernatant. Deproteinated aqueous media were neutralized[5] by adding 2 ml of 1.0 *M* Tris–HCl buffer (pH 7.2) and extracted five times with 6-ml portions of rigorously nitrogenated ethyl acetate. Extracts were maintained under nitrogen and after combination, evaporated to dryness under vacuum. The resulting residues were taken up in 1 ml nitrogenated chloroform–methanol (1:1) and these solutions concentrated (to appropriate volumes for TLC application) by gentle warming under rigorous nitrogenation.

Nuclear magnetic resonance (NMR) studies

NMR spectra were obtained in deuterochloroform (Silanor-C, Merck) as saturated solutions with TMS as an internal standard. Proton spectra were recorded with a Varian A-60 NMR spectrometer and ^{13}C spectra were recorded with a Brucker WH-90 FT spectrometer operated at a frequency of 22.615 MHz.

RESULTS AND DISCUSSION

Fig. 1a illustrates a thin-layer chromatogram of I obtained from a simulated microsomal metabolism study where no precautions were taken to prevent air oxidation. The marked decomposition is apparent. The chromatogram in Fig. 1b is obtained from simulated metabolic studies in which rigorously nitrogenated organic solvents, used to extract and concentrate samples prior to their TLC analysis, were employed. The same chromatogram is obtained from aqueous solutions of I which undergo green discoloration as decomposition progresses. The spot pattern is characteristic for I in a variety of solvent systems and may be referred to as the "apomorphine fingerprint". The material with an value of R_F 0.45 is visible under ordinary light as a dark-green spot and is more intense for aqueous solutions of I which have undergone extensive decomposition. The material with R_F 0.35 is initially visible only under UV irradiation. However, after exposure to the air, it gradually turns green and can then be seen in ordinary light. These data suggested that the spot with R_F 0.35 is I and that the spot with R_F 0.45 results from air oxidation of I. Two-dimensional TLC confirmed that the spot with R_F 0.45 could be obtained from the material with R_F 0.35 if 15-min exposure to air were allowed between developments. It was also suspected that the material with R_F 0.45 possessed structure IX.

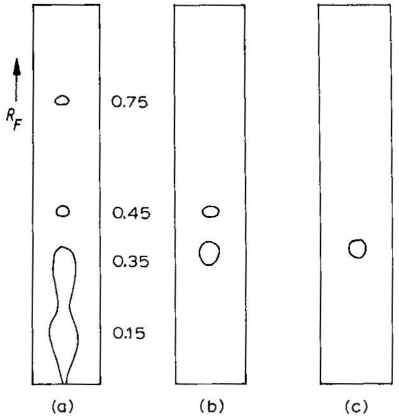


Fig. 1. Thin-layer chromatograms of apomorphine obtained after simulated microsomal metabolism studies. Silica gel G_{254} TLC plates were developed in benzene–methanol (4:1). a, Without antioxidants or nitrogenation; b, with nitrogenation of organic solvents; c, with antioxidants present during microsomal incubation and nitrogenation of organic solvents.

The preparation of IX from I has been reported using either mercuric chloride[8] or potassium dichromate[12]. Repetition of the former procedure produced a dark-green crystalline compound similar to that described by Linde and Ragab[8]. This material possessed R_F values identical to the suspected spot in the apomorphine fingerprint in a variety of developing systems. However, the mass spectrum of this material showed both an expected[8] peak at 263 *m/e* (for $C_{17}H_{13}NO_2$) and an additional unreported peak at 265 *m/e*. The relative abundance of these peaks was found to vary from sample to sample. Although M + 2 peaks are not unusual in the mass spectra of quinones[13,14], it was felt that this descrepancy required further study. Furthermore, the reported[8] lack of a melting point below 350° seemed unusual for a structure such as IX. Since the infrared spectrum and a negative ferric chloride test indicated that phenolic hydroxyl groups were not present, it was suspected that a compound having two additional mass units might then be structure IX where the C_{6a}–C_7 double bond is saturated. NMR studies were conducted to test this possibility. Both proton and ^{13}C spectra were obtained. The proton spectrum was identical to that reported by Linde and Ragab[8] and was appropriate for structure IX. The ^{13}C spectrum is illustrated in Fig. 2, and is also appropriate for structure IX. In this spectrum, the resonances at 183.2 and 176.5 δ may be assigned to C_{11} and C_{10} since these chemical shifts are predicted[15] for α,β-unsaturated ketones and occur at too a low field for aromatic carbons. Other characteristic features are the resonances at 50.4, 28.9 and 40.2 δ which are in the regions expected[16] for C_5, C_4 and N–CH_3 respectively. These studies suggest, then, that the additional peak at 265 *m/e* is an M + 2 artifact which occurs for the

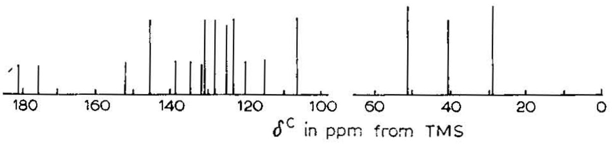


Fig. 2. ^{13}C NMR spectrum of apomorphine orthoquinone obtained in C^2HCl_3.

same material obtained by Linde and Ragab[8] and that the material with an R_F value of 0.45 is indeed IX.

Fig. 1c represents the thin-layer chromatogram of I obtained from simulated microsomal metabolism studies in which antioxidants (either 0.05% sodium bisulfite or equimolar dithiothreitol) were employed during microsomal incubation and rigorous nitrogenation of all organic solvents was employed during subsequent extraction prior to TLC analysis. It is apparent that when metabolic studies are performed in this fashion, even the characteristic spot resulting from air oxidation of I to IX can be eliminated and a single-spot chromatogram of apomorphine can be obtained. Furthermore, there is no evidence of decomposition occurring during the TLC development *per se* as evidenced by single spots on plates developed under conditions described under *Thin-layer chromatography*. To minimize decomposition prior to TLC, plates are rapidly spotted (using solutions prepared with nitrogenated solvents) and immediately developed. Again, two dimensional TLC with 15-min air exposure between developments confirmed that the characteristic apomorphine fingerprint (Fig. 1b) could be obtained from the chromatogram in Fig. 1c.

Since I is one of the more labile compounds in the series I–VIII, the procedures used to prevent its decomposition provide a means for the utilization of TLC methods in metabolism studies of II–VIII as well.

The four solvent systems A–D were designed to accomplish specific goals based upon anticipated results of metabolic studies of I–VIII. Systems A, C, and D separate II, III, IV, VI, and VII from their potential metabolite I. System D separated VIII from V (a potential hydroxylated metabolite of VIII) while systems A, C, and D separated IV from I–III (possible O-demethylated metabolites of IV) and VII from VI (possible O-demethylated metabolite of VII). Based on a previous report[1] it is anticipated that system B will be useful in distinguishing I–VIII from their N-dealkylated metabolites. R_F values for I–VIII appear in Table I. In most cases, these values were maintained when anticipated metabolic mixtures of I–VIII were spotted, *e.g.* a mixture of IV with II and III. However, slightly lower R_F values were obtained for VII and for II when these materials were co-chromatographed with their O-dealkylated analogs (VI and I, respectively). This lowering of the R_F value is thought to result from an associative interaction between the two molecular species involved.

TABLE I

TLC OF APOMORPHINE AND ANALOGS

For composition of solvent systems A–D, see text. R_F values are averages of 4–7 determinations.

Structure	*R_F × 100*			
	Solvent A	*Solvent B*	*Solvent C*	*Solvent D*
I	35	65	10	10
II	55	60	60	20
III	25	45	40	10
IV	60	65	65	25
V	50	60	40	20
VI	50	60	35	15
VII	60	60	65	25
VIII	50	60	40	30

Ambiguity resulting from this phenomena is not anticipated during metabolic studies since the use of spray reagents dramatically distinguishes between these compounds.

Although visualization by UV irradiation was effective, the spray reagents DSA and DCQ were also found to be useful in characterizing and distinguishing certain of the compounds I–VIII. The behaviors of I–VIII toward DSA and DCQ are summarized in Table II. Interestingly, the reactivity of the phenolic systems present in certain of these aporphines is such that the spray reagents effectively couple without requiring activation by subsequent spray with a basic reagent[17].

TABLE II

BEHAVIOR OF APOMORPHINE AND ANALOGS TOWARD DSA AND DCQ SPRAY REAGENTS

Structure	*Color*		
	DSA	*DCQ**	*DCQ, heat***
I	gray	gray	darker
II	orange	blue	darker
III	pink	none	brown
IV	pink	none	brown
V	brown	gray	darker
VI	brown	none	brown
VII	pink	none	brown
VIII	brown	none	brown

* When DCQ is used after development in solvent system C, entire plates darken and no useful information is obtained.

** After spraying with DCQ, plates were gently warmed over a hot-plate.

ACKNOWLEDGEMENTS

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REFERENCES

1 R. V. Smith and S. P. Sood, *J. Pharm. Sci.*, 60 (1971) 1654.
2 J. G. Cannon, R. V. Smith, A. Modiri, S. P. Sood, R. J. Borgman, M. A. Aleem and J. P. Long, *J. Med. Chem.*, 15 (1972) 273.
3 R. V. Smith and A. W. Stocklinski, *J. Chromatogr.*, 77 (1973) 419.
4 R. V. Smith, M. R. Cook and A. W. Stocklinski, *J. Chromatogr.*, 87 (1973) 294.
5 R. V. Smith and A. W. Stocklinski, *Anal. Chem.*, 47 (1975) 1321.
6 R. V. Smith and P. W. Erhardt, *Anal. Chem.*, in press.
7 R. V. Smith, P. W. Erhardt and S. W. Leslie, *Res. Commun. Chem. Path. Pharmacol.*, 12 (1975) 181.
8 H. H. A. Linde and M. S. Ragab, *Helv. Chim. Acta*, 51 (1968) 683.
9 M. P. Cava, A. Venkateswarlu, M. Srinivasan and D. L. Edie, *Tetrahedron*, 28 (1972) 4299.

10 J. L. Neumeyer, F. E. Granchelli, K. Fuxe, U. Ungerstedt and H. Corrodi, *J. Med. Chem.*, 17 (1974) 1090.
11 R. J. Borgman, *J. Heterocycl. Chem.*, 12 (1975) 599.
12 K. Rehse, *Arch. Pharm. (Weinheim)*, 302 (1969) 487.
13 F. Bohlmann and K. M. Kleine, *Chem. Ber.*, 99 (1966) 885.
14 R. F. Muraca, J. S. Whittick, G. D. Daves, Jr., P. Friis and K. Folkers, *J. Amer. Chem. Soc.*, 89 (1967) 1505.
15 G. A. Gray, *Anal. Chem.*, 46 (1975) 546A.
16 E. Wenkert, J. S. Bindra, C. Chang and D. W. Cochran, *Acc. Chem. Res.*, 7 (1974) 46.
17 S. Siggia, *Instrumental Methods of Organic Functional Group Analysis*, Wiley-Interscience, New York, 1972, pp. 21–23.

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Note

The separation of glyoxalase I and glyoxalase II by paper electrophoresis

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The glyoxalase enzyme system, consisting of the two enzymes glyoxalase I (S-lactoylglutathione methylglyoxal lyase; Gl. I) (E.C. 4.4.1.5) and glyoxalase II (S-2-hydroxyacylglutathione hydrolase; Gl. II) (E.C. 3.1.2.6) catalyses the following reactions[1]:

$$\text{methylglyoxal} + \text{glutathione} \xrightarrow[\text{Gl.I}]{} \text{S-lactoylglutathione} \xrightarrow[\text{Gl. II}]{} \text{D-lactate} + \text{glutathione}$$

Investigations on the physico-chemical properties of the glyoxalases have been reported[2–13], but no data on the electrophoretic properties of these enzymes have been published.

This paper reports the results of investigations on the feasibility of separating the enzymes of this system by electrophoresis, and on the factors that determine the electrophoretic behaviour of the glyoxalases, particularly Gl. I.

MATERIALS AND METHODS

Yeast Gl. I and glutathione (GSH) were purchased from Boehringer (Mannheim, G.F.R.) and crystalline albumin from BDH (Poole, Great Britain). Methylglyoxal was obtained by distillation of glyceraldehyde (Reanal, Budapest, Hungary)[14] and S-lactoyl-GSH was prepared enzymatically[15]. All other reagents were of analytical purity.

Ox liver homogenates (1:6 in 0.15 *N* potassium chloride solution) were prepared by using a Potter–Elvehjem homogenizer and centrifuged in a refrigerated centrifuge at 20,000 *g* for 30 min. The supernatant solutions were used for electrophoretic studies.

Purified Gl. I and Gl. II were obtained by the molecular filtration method as described by Jerzykowski *et al.*[13]. The fractions with maximum activity were used for electrophoretic studies (both before and after precipitation with ammonium sulphate as described in the text).

Electrophoresis was conducted with a standard apparatus for serum paper electrophoresis, using Whatman No. 1 paper, at 220 V for 5.5 hours with an ionic strength of 0.1. Paper strips 3 mm wide were eluted in 2 ml of phosphate buffer (1/15 *M*, pH 6.8) by shaking them in the solution for 1 h.

Gl. I and Gl. II in the eluates from the paper strips and in the glyoxalase preparations (column fractions) were determined by the method of Racker as described by Jerzykowski *et al.*[12,13]. Units of enzyme activity are expressed as μmoles of lactoyl-GSH produced (Gl. I) or decomposed (Gl. II) in 1 min (initial velocity) at room temperature under the experimental conditions used.

Protein concentrations were determined by the method of Warburg and Christian[16].

RESULTS AND DISCUSSION

Liver glyoxalases

Most of the experiments were carried out with the supernatant solutions from ox liver homogenates, which exhibited considerable activity of both glyoxalases. Several preliminary tests on the influence of pH on glyoxalase mobility were conducted at pH 5–10 and with veronal, Tris–hydrochloric acid and phosphate buffers. The electrophoresis time (5.5 h), voltage (220 V) and ionic strength (0.1) were constant. The purpose of these tests was to determine as far as possible the optimal conditions for the separation of Gl. I and Gl. II in supernatant solutions from ox liver homogenate. These conditions were found to be the use of veronal buffer at pH 8–9.

The relative electrophoretic mobility of both glyoxalases in veronal buffer (pH 8.6) in comparison with human serum (Fig. 1a) is shown in Fig. 1d. In this instance, Gl. I migrates to the anode, exhibiting a mobility similar to that of the albumin fraction in the electrophoresis of blood serum, while Gl. II apparently remains in the initial position. Fig. 2 shows the distribution of enzyme activity after electrophoresis at pH 8.0. At room temperature, about 50% of the initial activity of Gl. I and about 50% of the activity of Gl. II can be obtained from electropherogram analysis, and the enzyme separation is complete. This suggests the feasibility of using this buffer and pH to achieve enzyme separations by continuous curtain electrophoresis. Further investigations were conducted in the selected range of pH.

Red cell glyoxalases

Both Gl. I and Gl. II are present in blood red corpuscles. Electrophoretic testing of the supernatant solution from haemolized human red corpuscles (after prior centrifugation and repeated washing with 0.9% sodium chloride solution) showed a glyoxalase activity distribution similar to that found for glyoxalases from ox liver homogenate.

Purified yeast glyoxalase I

Several experiments were performed with yeast Gl. I obtained from Boehringer. It can be seen from Fig. 1b that yeast Gl. I moves toward the cathode. This migration is only apparent, as actually the dextran determined line of zero flow[17] is in the position marked by the arrow (Fig. 1a), which provides evidence that under these conditions Gl. I does not migrate owing to the presence of a charge.

Purified liver glyoxalase I

A behaviour similar to that described above was found for Gl. I purified by a molecular filtration method and then precipitated with ammonium sulphate at a

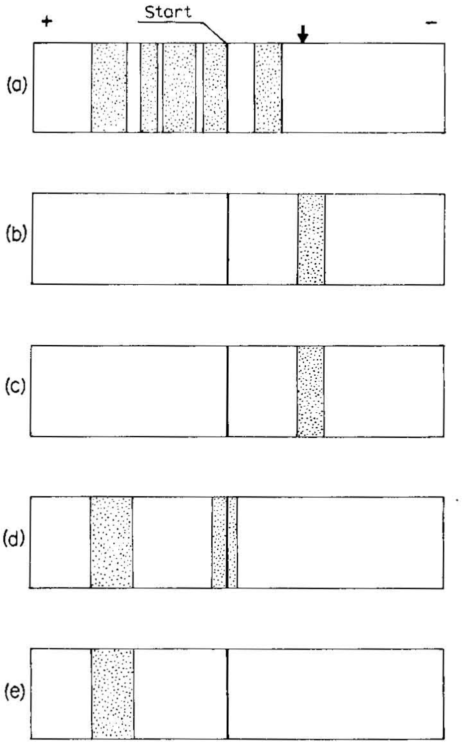

Fig. 1. Low-voltage electropherograms of glyoxalases (veronal buffer, pH 8.6, ionic strength 0.1, 220 V, time 5.5 h, paper electrophoresis). (a) human serum; (b) purified yeast Gl. I; (c) Gl. I precipitated with ammonium sulphate; (d) Gl. I and Gl. II from liver homogenate supernatant; (e) Gl. I purified by molecular filtration, after the addition of ox albumin.

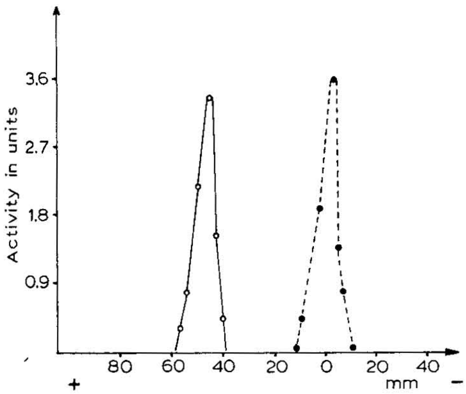

Fig. 2. Distribution of Gl. I (○—○) and Gl. II (●---●) activity after paper electrophoresis (veronal buffer, pH 8.0, ionic strength 0.1, 220 V, time 5.5 h).

degree of saturation >0.7 (Fig. 1c). This glyoxalase preparation exhibits a specific activity five times greater than that of Gl. I from the supernatant solution. However, if this preparation is diluted with water, or if the degree of saturation with ammonium sulphate is less than *ca.* 0.7, the Gl. I moves to the anode, in the same manner as Gl. I from homogenate. The mobility is the same when the dilution is made with 8 *M* urea solution, or Mg^{2+} ions (magnesium sulphate, 10^{-3} *M* solution). However, a lack of mobility, or more accurately an apparent mobility towards the cathode, is found after adding EDTA (10^{-3} *M* solution).

An exceptional behaviour is shown by Gl. I prepared by molecular filtration and not precipitated with ammonium sulphate. This preparation is probably completely deactivated by electrophoresis, as no enzyme activity can be detected over the whole electropherogram, despite the high activity of the initial preparation. It should be noted that GSH ($5 \cdot 10^{-2}$ *M*), which is a substrate for Gl. I, shows no protective effect on the behaviour of this preparation of Gl. I. However, this preparation maintains its activity when ox serum albumin is added to it. In this instance, the enzyme moves towards the anode and can be detected in the same position in which it is found after direct electrophoresis of ox liver preparation (Fig. 1e). Hence, it was reasonable to assume that the enzyme migrated in association with the added protein. In order to test this assumption, a detailed comparative analysis was made of the molecular weights of Gl. I and mixtures of Gl. I and albumin (by the method described by Jerzykowski *et al.*[13]). These experiments provided no evidence that albumin combined with Gl. I. However, it should be stressed that the conditions under which the molecules migrate during electrophoresis do not correspond precisely to the conditions that exist during the determination of molecular weights. This aspect appears to require further elucidation.

CONCLUSIONS

Electrophoretic tests on glyoxalase I preparations have been described. The electrophoretic mobility shown by this enzyme depends on the method of preparation. It was demonstrated that under the electrophoretic conditions determined (paper electrophoresis, veronal buffer, ionic strength 0.1, pH 8.0), Gl. I and Gl. II can be completely separated. Studies were conducted using glyoxalases from ox liver, human red corpuscles and also yeast Gl. I.

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REFERENCES

1 E. J. Racker, *J. Biol. Chem.*, 190 (1951) 685.

2 K. Ekwall and B. Mannervik, *Arch. Biochem. Biophys.*, 137 (1970) 128.

3 L. Uotila and M. Koivusalo, *Abstracts of Communications presented at the 8th Meeting of FEBS, Amsterdam, 1972*, p. 438.

4 B. Górna, *Abstracts of Communications presented at the 8th Meeting of FEBS, Amsterdam, 1972*, p. 394.

5 D. L. Vander Jagt, Liang-Po B. Han and Ch. H. Lehman, *Biochemistry*, 11, No. 20 (1972) 3735.
6 R. Vince and W. B. Wadd, *Biochem. Biophys. Res. Commun.*, 35 (1969) 593.
7 R. A. Strzinek, V. E. Scholes and S. J. Norton, *Cancer Res.*, 12 (1972) 2359.
8 K. Ekwall, L. Lindström and B. Mannervik, *Abstracts of Communications presented at the 8th Meeting of FEBS, Amsterdam, 1972*, p. 168.
9 K. A. Davis and G. R. Williams, *Biochim. Biophys. Acta*, 113 (1966) 393.
10 K. A. Davis and G. R. Williams, *Can. J. Biochem.*, 47 (1969) 553.
11 T. Bartfai, K. Ekwall and B. Mannervik, *Biochemistry*, 12, No. 3 (1973) 387.
12 T. Jerzykowski, R. Winter and W. Matuszewski, *Biochem. J.*, 135 (1973) 713.
13 T. Jerzykowski, W. Matuszewski and R. Winter, *FEBS Lett.*, 1 (1968) 159.
14 I. W. Patterson and A. Lazarow, *Methods Biochem. Anal.*, 2 (1955) 270.
15 T. Wieland, B. Sandman and G. Pfleiderer, *Biochem. Z.*, 328 (1956) 239.
16 O. Warburg and W. Christian, *Biochem. Z.*, 310 (1942) 384.
17 M. Macheboeuf and P. Rebeyrotte, *Bull. Soc. Chim. Biol.*, 35 (1953) 334; cited by W. Ostrowski, *Elektroforeza w Badaniach Biochemicznych i Klinicznych*, PWN, Warsaw, 1970.

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CHROM. 8650

Note

Chromogenic response of aqueous cobalt thiocyanate to lipophilic drugs

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(First received March 13th, 1975; revised manuscript received July 22nd, 1975)

Two percent aqueous cobalt thiocyanate (CTC) has found widespread application in the analysis of cocaine[1]. The test relies on a change in the color of the reagent from pink to blue in the presence of drug. Its relatively poor specificity for cocaine was brought to our attention by local drug control agencies and led to the development of a superior test not based on CTC[2].

It was of interest to determine if CTC might make useful distinctions among drug types as a spray reagent in drug screening procedures based on thin-layer chromatography (TLC). Two percent CTC in acetone has been reported to distinguish certain alkaloids on the basis of the extent and rate of color development[3]. The advantages of acetone as a solvent were not described nor was a positive test response associated with any particular drug property.

Our earlier efforts in this direction[4] pointed to a unique quality of CTC in responding to the more highly substituted lipophilic homologs of certain drugs. Heroin responds readily to the aqueous reagent, codeine less so, and morphine not at all; dimethyltryptamine responds but not tryptamine, methamphetamine responds weakly but more than amphetamine. We suggested that a ligand-sensitive thiocyanate–isothiocyanate linkage isomerism may be implicated in explaining these results based on analogy with the steric and polar effects operating in related transition metal thiocyanate complexes[5].

It is apparent that CTC is responsive to the lipophilic character of drugs, a property the importance of which in drug transport and action is increasingly evident[6]. We report here our further experience with the nature and scope of this reagent in drug testing.

EXPERIMENTAL

Chromatography

The results reported here were obtained using precoated silica gel F-254 TLC plates (Merck, Darmstadt, G.F.R.). While the choice of adsorbent had little if any effect on the CTC–drug response, background interference was significant with Florisil which develops an immediate blue color indistinguishable from the test color. Adsorbents based on silica gel and, to a lesser extent, alumina will also develop a blue background color as the plate dries. This generally necessitates that test results be interpreted within 5–10 min after spraying. However, background color may be repeatedly and selectively removed by exposure to a water-saturated atmosphere. Test color or persistence did not change when commercial grades of acidic, basic, and neutral alumina were used as TLC adsorbents.

Test drugs were spotted in graded concentrations on the TLC plates and developed for a solvent front distance of 10 cm with 0.5% ammoniacal methanol. The CTC reagent was insensitive to the acidity, basicity, or any other property of the developing solvent as long as the usual amount of forced air or oven drying preceded the application of the reagent.

Test reagent

A less than saturating application of 2% aqueous cobaltous thiocyanate (K & K Labs., Plainview, N.Y., U.S.A.) was applied to the dried plate with a conventional spray applicator. Since water will ultimately wash out a positive test result, solutions more dilute than 2% were self-defeating and more concentrated solutions served no useful purpose. No serious effort was made in this study to modify the reagent in order to optimize or alter the test result.

That water is an important part of the test reagent is brought out by the use of 2% CTC in acetone. This reagent has been reported useful in making distinctions between certain alkaloids in TLC applications[3]. In our hands, test responses were significantly weaker and less persistent. The blue test and background colors are completely bleached out after standing for several days whereas the colors deriving from the aqeuous reagent are stable indefinitely. No advantages to the use of the acetone reagent were observed.

Test drugs

Test drugs were obtained either in unadulterated form from commercial sources or in prescribed dosage form from the Pharmacy of the Marcy Psychiatric Center (Marcy, N.Y., U.S.A.) Cannabis extract, methaqualone, and diacetylmorphine were obtained from confiscated material which had been submitted to our laboratory for analysis. Dimethoxyphenethylamine (DMPEA) heptanoate was synthesized by unambiguous means from commercial DMPEA and was isolated in crystalline form. Arctigenin was isolated by column chromatography of the chloroform extract of Arctium lappa seeds collected locally.

RESULTS AND DISCUSSION

The drugs tested in this study were arbitrarily divided into hydrogen donor drugs (Table I) and hydrogen acceptor drugs (Table II) by familiar criteria[7]. Drugs with mixed donor–acceptor properties were included in Table I as hydrogen donors. Available measured or calculated octanol–water partition coefficients[7] are listed in Tables I and II as drug parameters which correlate reasonably well with CTC–drug response. The average partition coefficient of those drugs responding to CTC at the level of 20 μg or less CTC (+ + and + in Tables I and II) in this study was 3.05 ± 0.30 (standard error of the mean). The average partition coefficient of those drugs that did not respond to CTC, or whose response required more than 20 μg, was 1.21 ± 0.20. This difference was significant at the $p < 0.001$ level by t-test, two-tailed.

As lipophilic or hydrophobic character further increases, CTC response di-

TABLE I

RESPONSE OF HYDROGEN ACCEPTOR* DRUGS TO COBALT THIOCYANATE

Response: ++, detectable after TLC development at the level of 10 μg or less; +, 10–20 μg; –, more than 20 μg or undetectable.

Drug	*Response to cobalt thiocyanate*	*Octanol–water partition coefficient**
Amitriptyline	++	
Amphetamine	–	1.63
Benzphetamine	++	2.47
Benztropine	+	
Caffeine	–	–0.07
Chlorpheniramine	+	
Chlorpromazine	+	5.16
Cocaine	+	2.73
Cyproheptadine	++	
Dextromethorphan	+	2.91
Diacetylmorphine	++	
Diazepam	++	2.82
Dihydrocodeinone	–	
Dimethoxyphenethylamine	–	
Dimethyltryptamine	++	
Diphenhydramine	++	3.30
Doxepin	++	
Ethopropazine	+	
Ethyl aminobenzoate	–	2.41
Imipramine	++	4.62
Laudanosine	+	
Lysergide	–	
Meperidine	+	
Mescaline	–	
Methadone	++	
Methamphetamine	–	2.12
Methapyrilene	+	
Methaqualone	–	
Methenamine	–	
Methylphenidate		
Nicotine	–	1.17
Nortriptyline	+	
Papaverine	++	
Phenmetrazine	–	1.73
Phenyltoloxamine	+	
Procaine	++	1.87
Promethazine	+	2.25
Propoxyphene	++	4.18
Protriptyline	++	
Strychnine	+	1.93
Tetracaine	++	3.73
Thiothixene	+	
Tripelennamine	++	
Tryptamine	–	

* As described in ref. 7.

TABLE II

RESPONSE OF HYDROGEN DONOR* DRUGS TO COBALT THIOCYANATE

Drug	*Response to cobalt thiocyanate***	*Octanol–water partition coefficient**
Acetaminophen	—	
Acetylsalicylic acid	—	1.23
Arctigenin	++	
Atropine	—	1.79
Bufotenine	—	
Cannabis hexane extract	—	
Chlordiazepoxide	—	2.44
Codeine	—	1.01
Colchicine	++	
Cyclandelate	—	
Dimethoxyphenethylamine heptanoate	++	
Ephedrine	—	0.93
Glutethimide	—	1.90
Haloperidol	+	
Hydromorphone	—	
Hydroxyzine	+	
Isocarboxazid	—	1.49
Lidocaine	+	
Meprobamate	—	0.70
Morphine	—	0.76
Nylidrin	+	
Oxazepam	—	
Pentazocine	+	
Phenobarbital	—	
Physostigmine	—	0.17
Procyclidine	++	
Quinine	+	1.73
Salsoline	—	
Scopolamine	—	
Sulfisoxazole	—	1.15
Theobromine	—	−0.78
Trihexyphenidyl	++	

* As described in ref. 7.
** As defined in Table I.

minishes to a weak, delayed response, such as we have observed in the case of fatty esters such as glyceryl tristearate, and finally to no response in the case of aliphatic or aromatic hydrocarbons. Nitrogen content is not essential since water-miscible solvents such as acetone and methanol give a strong blue test color as does the weakly acidic arctigenin (Fig. 1). Thus, a positive test result entails a rather narrow compromise between hydrophillic and lipophilic character in the substance tested.

It is apparent from this study that CTC–drug response can often be promoted by the synthesis of a suitable derivative. This is dramatically illustrated by the acetylation of morphine and by the N-methylation of tryptamine.

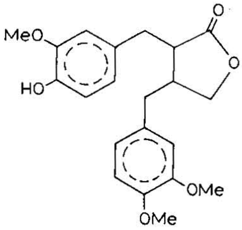

Fig. 1. Arctigenin.

ACKNOWLEDGEMENT

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REFERENCES

1 Anonymous, *Drug Enforcement*, 1 (1974) 26.
2 F. W. Grant, W. C. Martin and R. W. Quackenbush, *Bull. Narcotics*, 27 (1975) 33.
3 G. Zweig and J. Sherma, *Handbook of Chromatography*, Vol. 2, Chemical Rubber Company Press, Cleveland, Ohio, 1972, p. 112.
4 F. W. Grant and R. W. Quackenbush, *Abstracts of the Sixth Northeast Regional Meeting of the American Chemical Society, Burlington, Vt., August 19, 1974.*
5 W. Beck and W. P. Fehlhammer, *MTP Int. Rev. Sci., Inorg. Chem., Ser. 1*, Vol. 2, Butterworths, London, 1972, pp. 267–279.
6 R. N. Smith, C. Hansch and M. M. Ames, *J. Pharm. Sci.*, 64 (1975) 599.
7 A. Leo, C. Hansch and D. Elkins, *Chem. Rev.*, 71 (1971) 525.

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CHROM. 8640

Note

Thin-layer and short-column chromatography of epimeric alcohols of some quinolizidine derivatives

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(Received July 9th, 1975)

During our study of the stereochemical course of the reduction of some quinolizidin-2-one derivatives, it was found that the oxo group of 13-oxolupanine (I) could be stereoselectively transformed, depending on the medium used in catalytic hydrogenation [in the presence of platinum (IV) oxide], into either an equatorial or an axial hydroxy group[1]. Alternatively, the oxo group can be reduced in one stage to a methylene-group[1]. It was also found that, depending on the molar ratio of hydrochloric acid to I, catalytic hydrogenation results in a mixture of epimeric alcohols or, in another experiment, in a mixture of epimeric alcohols and the hydrogenolysis product[2]. The latter achievement was possible owing to an elaborate and efficient method of separation by chromatography. The use of thin-layer chromatography (TLC) permitted formation of the epimeric alcohols to be controlled, and their separation was achieved on a preparative scale by "short-column" chromatography[3].

In this paper, we report the application of TLC and "short-column" chromatography for the separation of two pairs of epimeric alcohols of the same quinolizidine derivatives, *viz.*, 13-hydroxylupanine (II) and *epi*-13-hydroxylupanine (III), and 13-hydroxy-α-isolupanine (IV) and *epi*-13-hydroxy-α-isolupanine (V).

EXPERIMENTAL

TLC was carried out on glass plates (5 × 20 cm) coated with a 0.25-mm thick layer of silica gel HF_{254} (Merck, Darmstadt, G.F.R.). The plates were air dried. After development, the compounds were located with iodine vapour. Several developing systems were used, of which three are considered here:

(A) Chloroform–ethanol (3:2)
(B) Benzene–methanol (4:1)
(C) Ethyl acetate–chloroform–benzene (2:2:1).

RESULTS AND DISCUSSION

II and III differ from IV and V only in the configuration of the C–D fragment of the molecule; II and III consist of two quinolizidine systems with *trans–cis* ring-fusion, whereas IV and V have two *trans*-quinolizidine systems.

The three solvent systems A, B and C were used with success to resolve the two pairs of epimeric alcohols. The equatorial epimers always had R_F values higher than those of the axial epimers. The epimeric alcohols from the *trans–trans* ring-fused quinolizidine were the most rapidly moving compounds in all of the solvent systems tested. The best resolution of both pairs of epimeric alcohols was achieved in system A.

PREPARATIVE-SCALE RESOLUTION OF EPIMERS II AND III

For this purpose, "short-column" chromatography was used[3], with a column of silica gel HF_{254} and system A as mobile phase. The separation was achieved on a 25 × 2 cm column; a cylindrical vessel with a sintered disc was used as column support, and the column was topped by a head connected with an aquarium-aeration pump.

A Whatman 41 filter-paper was laid over the sintered disc, and 7 g of the silica gel was shaken briskly for 1 min in a stoppered flask with 15 ml of solvent A; the moderately thin slurry was then carefully transferred to the column with a large pipette, which reached well down into the vessel. The column was left vertical and undisturbed until the gel had bedded down, which took about 1 h; it was ready for use by the time the surface of the descending liquid had reached the top of the gel. The height of the gel layer after bedding down was 4 cm.

An open-texture filter-paper (Whatman 41) was then placed over the surface, and the supernatant solvent was allowed to drain into the gel, then an even layer (1.5 mm thick) of washed sand was added to facilitate uniform distribution of the material to be chromatographed. This material (100 mg of the mixture of alcohols II and III) was dissolved in 1 ml of chloroform, and the solution was added centrally, by pipette, to the surface of the sand. The column was then very carefully filled up with solvent, and the pump was started. Twenty-five fractions, each of about 3 ml, were collected, and the flow-rate was 0.8 ml/min. The separation was quantitative, 75 mg of III and 25 mg of II being obtained.

Separation of the epimers by "short-column" chromatography was much more efficient than that achieved on conventional columns, and was attained in only one twentieth of the time needed with a conventional column. Also, the volume of solvent required was only 2% of that necessary with a larger column. This method has also been successfully applied to the resolution of other epimeric alcohols. Results for compounds II, III, IV and V are shown in Table I.

TABLE I

SEPARATION OF EPIMERIC ALCOHOLS OF SOME QUINOLIZIDINE DERIVATIVES

For compositions of the mobile phases A, B and C see text.

Compound	R_F		
	A	*B*	*C*
II	0.92	0.93	0.87
III	0.76	0.82	0.83
IV	0.83	0.83	0.79
V	0.54	0.75	0.66

REFERENCES

1 W. Wysocka and M. Wiewiórowski, *Bull. Acad. Pol., Sci., Ser. Sci. Chim.*, 10 (1974) 831.
2 W. Wysocka, *Bull. Acad. Pol. Sci., Ser. Sci. Chim.*, in press.
3 B. J. Hunt and W. Rigby, *Chem. Ind. (London)*, (1967) 1868.

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CHROM. 8704

Note

Buffer system for ion-exchange chromatography of amino acid mixtures containing methionine sulphone

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(Received August 15th, 1975)

In a study of sulphur nutrition of ryegrass at this laboratory, it was necessary to determine (a) free amino acids, extracted from the leaves by 80% aqueous ethanol, and (b) the amino acid composition of the leaf protein (in the residue after ethanol extraction), especially the proportions of cystine and methionine. To minimize the losses of the sulphur amino acids during hydrolysis of the leaf protein, the vacuum-dried samples were treated with performic acid to convert cystine and methionine residues to cysteic acid and methionine sulphone, respectively[1]. Analyses were by ion-exchange chromatography. However, when the buffer system developed for the analysis of the free amino acids[2] was used for the hydrolysates, methionine sulphone was incompletely separated from aspartic acid and neither could be determined accurately (Fig. 1A). It would have been possible to use the systems of Thomas[3] or Redman[4], but this would have required two sets of buffers, one for the free amino acids[2] and the other for the protein hydrolysates, which would be inconvenient and uneconomical. The effects of starting the chromatography with a buffer of lower pH than 2.875 were therefore studied.

EXPERIMENTAL AND RESULTS

A Technicon NC-1 single-column amino acid analyser equipped with a nine-chamber Autograd was used. The 140 × 0.6 cm I.D. column was packed to a height of 126 cm with "Chromobeads" Type A and the column temperature was 60°. As both Thomas[3] and Redman[4] used a starting buffer of pH 2.5, this was tried, but in the first chamber only, the other buffers remaining the same as in the earlier work[2]. The modified buffer gradient increased the time interval between the methionine sulphone and aspartic acid peaks from 6 to 21 min but the peaks were broadened and skewed. The threonine, serine and glutamic acid peaks were similarly distorted. Obviously the reduction of pH in the starting buffer was too great. An adequate separation of 11 min between methionine sulphone and aspartic acid was obtained when the pH in the first chamber was adjusted to 2.72, peak broadening being slight (Fig. 1B). Furthermore, an analysis of a standard mixture of amino acids found in ethanolic plant extracts, including phenylalanine, ethanolamine, γ-aminobutyric acid and ammonium, showed that the resolution of all peaks was still perfect. The modified buffer system

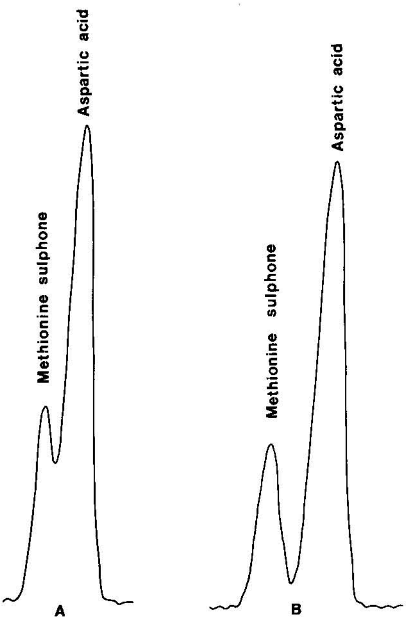

Fig. 1. Effect of pH of starting buffer on the separation of methionine sulphone and aspartic acid. (A) pH 2.875; (B) pH 2.72.

was therefore satisfactory for the determination of amino acids in both oxidised protein hydrolysates and non-protein ethanolic extracts.

It was not found necessary to equilibrate the resin with pH 2.72 buffer before loading the sample on the column and starting the analysis; pH 2.875 buffer could be used for this as previously. The pH 2.72 buffer was conveniently prepared by readjusting some of the pH 2.875 solution with 5 *N* hydrochloric acid.

The new system is given in Table I. Buffer compositions are detailed in the earlier publication[2].

TABLE I

BUFFER GRADIENT

Autograd chamber No.	*Buffer pH*	*Amount of buffer (ml)*	*Methanol (ml)*
1	2.72	70	5
2	2.875	72	3
3	2.875	75	—
4	2.875	75	—
5	4.20	75	—
6	5.00	75	—
7	5.00	75	—
8	5.00	75	—
9	5.00	75	—

REFERENCES

1 D. S. Bidmead and F. J. Ley, *Biochim. Biophys. Acta*, 29 (1958) 562.
2 W. Lazarus, *J. Chromatogr.*, 87 (1973) 169.
3 D. C. Thomas, *Techniques in Amino Acid Analysis*, Technicon Monograph No. 1, Technicon International Division SA, Geneva, 1966, p. 116.
4 D. G. Redman, *J. Chromatogr.*, 46 (1970) 107.

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CHROM. 8649

Note

Determination of vanilmandelic acid and homovanillic acid in urine by high-speed liquid chromatography

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(Received July 21st, 1975)

Homovanillic acid (HVA) and vanilmandelic acid (VMA) are the main metabolic end-products of dopamine and norepinephrine or epinephrine, respectively. Levels of VMA and HVA in urine have often been of importance in the diagnosis of neuroblastoma and phaeochromocytoma, and in the course of treatment of these diseases and Parkinson's disease.

A number of methods for separating and identifying VMA and HVA in urine have been described such as paper partition chromatography[1–3], thin-layer chromatography[4,5] and electrophoresis[6,7], which are relatively time consuming and not quantitative. Gas chromatography[8,9] requires sample clean up from crude materials and conversion into volatile derivatives. Another method applicable for the analysis of body fluids is liquid chromatography. Mrochek *et al.*[10] determined many constituents of urine, including VMA and HVA, by high-resolution anion-exchange chromatography; twenty hours were required for a complete analysis. Änggård *et al.*[11] also separated methyl esters of VMA and HVA using a column of Sephadex LH-20. Lange *et al.*[12] separated a standard mixture of catecholamine metabolites by cation-exchange chromatography. Recently, Thomas *et al.*[13] separated VMA and 3-hydroxy-4-methoxymandelic acid (iso-VMA) by cation-exchange chromatography, but the method was not applied to biological materials.

In this paper we describe a rapid and simple quantitative method for the determination of VMA and HVA in urine by high-speed adsorption liquid chromatography.

EXPERIMENTAL

Materials

VMA and HVA were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). All of the other chemicals used were of reagent grade purity.

Instrument

A high-speed liquid chromatograph was assembled from a micro-pump of the

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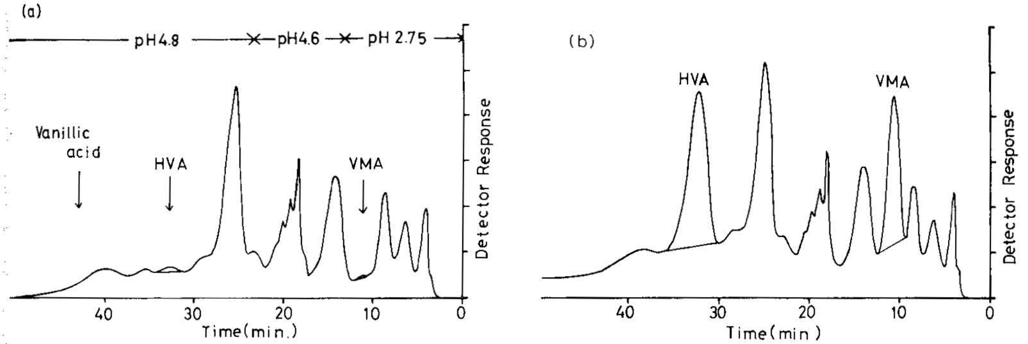


Fig. 1. Liquid chromatograms of 4-ml samples of (a) normal urine and (b) normal urine containing 0.43 mg VMA and 0.41 mg HVA.

double-plunger type (KWU-H, Kyowa Seimitsu, Tokyo, Japan), glass tubing (500 × 3 mm I.D.) packed with Hitachi Gel No. 3010 (spherical porous particles of styrene–divinylbenzene; particle diameter, *ca.* 25 μm), a UV monitor (LDC Model 1280; wavelength, 280 nm) and a recorder (Model EPR-2TC, Toa Electronics, Tokyo, Japan). The column temperature was maintained at 30°, and the flow-rate of the mobile phase was 1.3 ml/min. The meter ranges of the UV monitor and recorder were set at 0.02 absorbance units and 2.5 mV full scale, respectively.

Methods

VMA and HVA were extracted from urine according to the method of Dziedzic *et al.*[9]. 4 ml of urine in a 10-ml glass-stoppered centrifuge tube were acidified with 0.3 ml of 6 *N* hydrochloric acid, 1 g of sodium chloride was added and the resulting mixture was extracted three times with 5 ml of ethyl acetate by shaking for 2 min. The ethyl acetate in the collected upper phase was evaporated under reduced pressure and the residue was stored in a freezer until the chromatographic analysis. The residue was dissolved in 0.2 ml of methanol and 10 μl of the solution was injected on to the column. VMA and HVA were eluted with 0.05 *M* tartrate buffer–methanol (4:1). The pH of the buffer was varied as shown in Fig. 1.

RESULTS AND DISCUSSION

Under the present conditions, VMA and HVA were eluted at 10.5 and 31.1 min, respectively, and were well separated from the other constituents in normal urine. Calibration graphs of peak height against concentration of VMA or HVA in urine were linear as shown in Fig. 2. Additions of known amounts of VMA and HVA to samples of normal urine resulted in increased peak heights. The peak heights of VMA and HVA were directly proportional to the amounts added (Fig. 1). Vanillic acid, a metabolite of dietary vanillin, which resulted in too high a value for HVA in the colorimetric assay of Ruthven and Sandler[14], did not interfere with the present liquid chromatographic method. No attempt was made to measure iso-VMA and 3-hydroxy-4-methoxyphenylacetic acid (iso-HVA), because the excretion of these isomers in urine is said to be within a few percent of those of VMA and HVA[15,16].

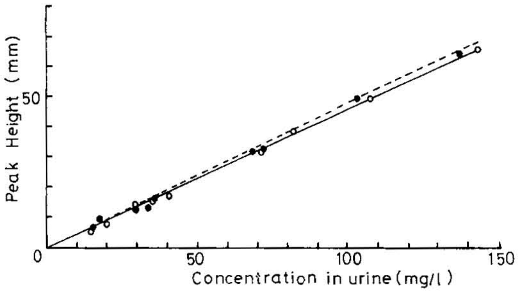
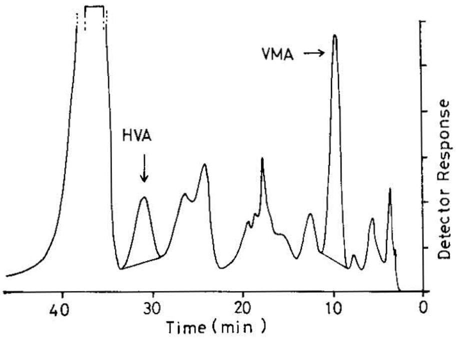


Fig. 2. Calibration graphs of peak height against concentration for VMA (○—○) and HVA (●---●) in urine.



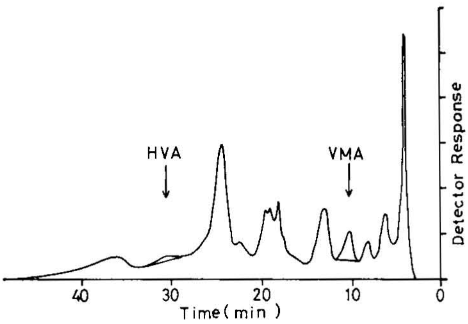


Fig. 3. Liquid chromatogram of urine from a patient with neuroblastoma.

Fig. 4. Liquid chromatogram of urine from a patient with phaeochromocytoma.

Our method was reproducible and the peak heights deviated by less than ± 5% when a solution containing 100 mg/l of the acids was repeatedly injected. The minimum detectable concentration for both acids was *ca.* 4 mg/l.

We applied this chromatographic technique to the urine of a patient with neuroblastoma. The results are shown in Fig. 3. The concentrations of VMA and HVA in urine were 125 mg/l (or 77.5 mg per day) and 43.5 mg/l (or 27 mg per day), respectively. The chromatogram of urine from a patient with phaeochromocytoma is shown in Fig. 4. The concentrations of VMA and HVA were 16 mg/l (or 15 mg per day) and 4 mg/l (or 3.7 mg per day), respectively.

The method seems to be promising for routine analysis of VMA and HVA in urine. All of the operations could be carried out within 2 h. After chromatographic separation, if necessary, the individual peaks may be analyzed by other methods such as gas chromatography or mass spectrometry.

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REFERENCES

1 M. D. Armstrong and A. McMillan, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 16 (1957) 146.
2 A. Vahidi, H. R. Roberts, J. S. Filippo, Jr. and D. V. S. Sankar, *Clin. Chem.*, 17 (1971) 903.
3 M. D. Armstrong, K. N. F. Shaw and P. E. Wall, *J. Biol. Chem.*, 218 (1956) 293.
4 J. S. Annino, M. Lipson and L. A. Williams, *Clin. Chem.*, 11 (1965) 905.
5 I. Sankoff and T. L. Sourkes, *Can. J. Biochem. Physiol.*, 41 (1963) 1381.
6 G. A. Hermann, *Amer. J. Clin. Pathol.*, 41 (1964) 373.
7 W. von Studnitz, *Klin. Wochenschr.*, 40 (1962) 163.
8 T. J. Sprinkle, A. H. Porter, M. Greer and C. M. Williams, *Clin. Chim. Acta*, 25 (1969) 409.
9 S. W. Dziedzic, L. M. Bertani, D. D. Clarke and S. E. Gitlow, *Anal. Biochem.*, 47 (1972) 592.
10 J. E. Mrochek, S. R. Dinsmore and D. W. Ohrt, *Clin. Chem.*, 19 (1973) 927.
11 E. Änggård, B. Sjöquist and R. Sjöström, *J. Chromatogr.*, 50 (1970) 251.
12 H.-W. Lange, H. F. K. Mannl and K. Hempel, *Anal. Biochem.*, 38 (1970) 98.
13 H. Thomas, H. Ponnath and D. Müller-Enoch, *J. Chromatogr.*, 103 (1975) 197.
14 C. R. J. Ruthven and M. Sandler, *Anal. Biochem.*, 8 (1964) 282.
15 P. Mathieu and L. Revol, *Bull. Soc. Chim. Biol.*, 51 (1969) 1347.
16 S. W. Dziedzic, L. B. Dziedzic and S. E. Gitlow, *J. Lab. Clin. Med.*, 82 (1973) 829.

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Note

Determination of 2-mercaptobenzothiazole in waste dump effluent by high-pressure liquid chromatography

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2-Mercaptobenzothiazole (MBT) has been known as a vulcanisation accelerator for rubber for many years[1]. It is also used as an intermediate for the preparation of *(inter alia)* other vulcanisation accelerators and has been demonstrated to have fungicidal properties[2].

The problem of the analysis of MBT came to our attention when samples of water which had been flowing from a refuse tip on which waste MBT was thought to have been disposed were submitted for analysis to this laboratory. A determination was required of the amount of MBT present in the samples.

The method of analysis for MBT which has been the subject of the greatest number of publications is the technique of amperometric titration, which uses metal salt solutions as titrants[3] or relies on oxidation by iodine[4] or iodine chloride[5]. Colorimetric methods have been reported in which bismuth nitrate[6] or selenious–sulphuric acid[7] are used as colour development reagents. MBT may also be determined by paper[8] and thin-layer[9,10] chromatography. Methods of analysis for the problem in hand which did not involve a separation step were deemed to be open to interference by other materials emanating from the tip, whilst thin-layer chromatographic techniques were insufficiently accurate for quantitation with the apparatus available. These problems were overcome by the development of a procedure involving the use of high-performance liquid chromatography, which resulted in a rapid and accurate method of analysis.

EXPERIMENTAL

Liquid chromatography was carried out using a chromatograph constructed in this laboratory. Mobile phase was supplied by an air-driven pressure intensification pump (Haskel, Burbank, Calif., U.S.A.) and the column was maintained at 30° by use of a water jacket and circulating thermostat (Haake, Type FE). The detector was a variable-wavelength UV monitor (Cecil Instruments, Cambridge, Great Britain) operated at a wavelength of 325 nm. Sample introduction was effected with a syringe using a stopped-flow injection system similar to that reported by Cassidy and Frei[11]. The column was constructed from stainless-steel tubing (15 cm × 4.6 mm I.D.) and was packed with Merckosorb SI 60 (5 μm) silica gel at 3,500 p.s.i. pressure from a slurry in 2,2,4-trimethylpentane. The packing material was retained in the column by stainless-steel wire mesh of nominal pore size 8 μm (Sankey Wire Weaving, Warrington, Great Britain) inserted into a drilled-out Swagelok coupling. A similar disc of wire mesh was pressed on the top of the column packing and was retained by a plug of silanised glass wool. Ethanol–2,2,4-trimethylpentane (1:9) was used as the mobile phase with a flow-rate of 1 ml/min.

All solvents used were of spectroscopic quality (Fisons, Loughborough, Great Britain), and the MBT was reagent grade (Hopkin and Williams, Chadwell Heath, Great Britain).

The aqueous sample (2 ml) was acidified with two drops of concentrated hydrochloric acid. This mixture was shaken with chloroform (2 ml) for 1 min using a flask shaker. Aliquots (2 μl) of the chloroform layer were used for the chromatographic analysis.

RESULTS AND DISCUSSION

Several column systems were investigated for the elution of MBT. These involved adsorption chromatography using silica gel and alumina, and reversed-phase chromatography using silica gel with bonded octadecyl groups. Of these columns, silica gel gave the best results. MBT was eluted from the column in 4 min with a capacity factor (k') of 1.4 and a height equivalent to a theoretical plate (HETP) of 20 μm.

A graph plotting peak height against sample size for this system (Fig. 1) showed a rectilinear relationship between the parameters up to at least 1.5 μg MBT injected: the minimum quantity detectable (defined as a peak with height equal to three times the noise level) was 0.6 ng. This compares favourably with the limit of 0.2 μg reported for the thin-layer chromatographic procedure[10].

Two procedures were investigated for the extraction of MBT from aqueous samples. The first involved acidification of the sample followed by several extractions with chloroform: this resulted in a quantitative recovery. Similar recoveries were

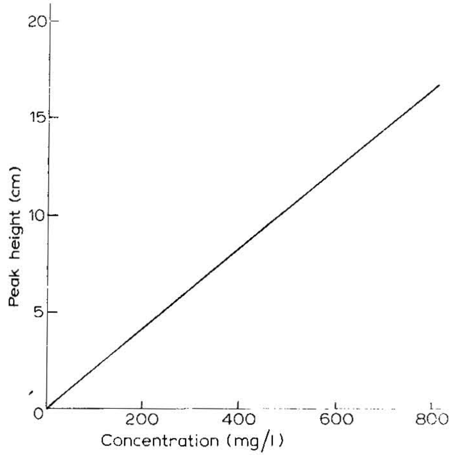
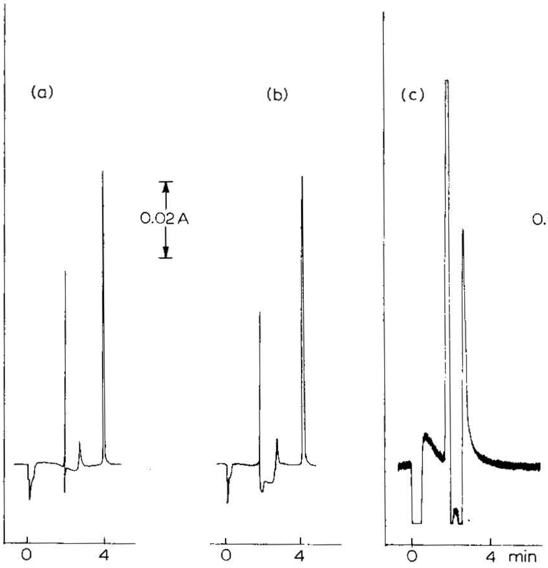


Fig. 1. Relation between peak height and concentration of MBT. For conditions, see text.

also obtained by the more convenient and rapid procedure of shaking equal volumes of acidified sample and chloroform for 1 min using a flask shaker. Effluents from two sites on the waste dump were analysed for MBT using the latter extraction procedure. No peak having the retention time of MBT was observed in either sample: the concentration of MBT in the samples was hence less than 0.3 mg/l.

Fig. 2 shows chromatograms obtained for a MBT standard and those obtained for the effluent and for the effluent spiked with MBT. MBT was detected in one of the samples, however, when the sensitivity was enhanced by a factor of 10 by the extraction of 100 ml of effluent with 10 ml of chloroform. This sample gave rise to a very small peak, with a height approximately double that of the baseline noise (see Fig. 3). Whilst under these conditions an accurate determination is not possible, calculation suggests that a concentration of the order of 0.03 mg/l of MBT was present in the sample.



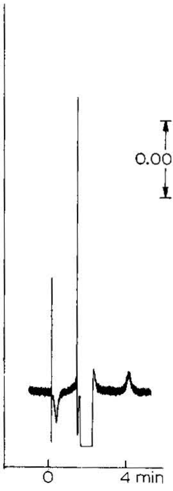


Fig. 2. Chromatography of MBT. (a) Standard, 80 mg/l. (b) Spiked effluent, 80 mg/l. (c) Unspiked effluent. For conditions, see text.

Fig. 3. Detection of MBT in effluent water. For conditions, see text.

A feature of the chromatograms is the lack of interfering peaks. Having regard to the nature of the samples, it was expected that many other components would be present in the samples. Fortunately, however, only a limited number of compounds have appreciable UV absorption at a wavelength of 325 nm, the absorption maximum of MBT: in consequence, other materials which may have been present were not detected.

ACKNOWLEDGEMENTS

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REFERENCES

1 L. B. Sebell and C. E. Boord, *Ind. Eng. Chem.*, 15 (1923) 1009.
2 D. Weight and D. A. L. Seiler, *Brit. Pat.*, 873,602 (1957).
3 N. Konopik, *Oester. Chem. Ztg.*, 55 (1954) 127.
4 W. Scheele and C. Ilsner-Gensch, *Kautsch. Gummi*, 8 (1955) WT55.
5 A. K. Zhdanov, V. A. Khadeev, A. I. Kubrakova and N. V. Bondarenko, *Uzb. Khim. Zh.*, (1961) 44.
6 A. Popov and V. Gydera, *C. R. Acad. Bulg. Sci.*, 12 (1959) 419.
7 V. E. Levine and M. Nachman, *J. Forensic Med.*, 10 (1963) 65.
8 M. Stepien and R. Gaczynski, *Chem. Anal. (Warsaw)*, 6 (1961) 1045.
9 L. Fishbein, J. Fawkes and P. Jones, *J. Chromatogr.*, 23 (1966) 476.
10 M. Ganeva, *Khig. Zdraveopaz.*, 14 (1971) 300.
11 R. M. Cassidy and R. W. Frei, *Anal. Chem.*, 44 (1972) 2250.

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Book Review

Manuel pratique de chromatographie en phase liquide, by R. Rosset, M. Caude and A. Jardy, Varian, Orsay, 1975, XV + 280 pp., price Ffr. 85.60.

La chromatographie liquide hautes performances (HPLC) est une discipline en pleine expansion dans le domaine de la chimie analytique; les articles et publications se rapportant à cette technique sont nombreux dans les revues scientifiques, mais le débutant risque d'être désorienté par leur abondance et leur diversité.

Il n'existait pas jusqu'ici d'ouvrage en langue française traitant de manière claire et précise de la HPLC.

C'est maintenant chose faite avec le *Manuel pratique de chromatographie en phase liquide* et le mérite de MM. Rosset, Caude et Jardy est grand, d'avoir écrit un livre pouvant servir de guide aussi bien pour l'analyste ayant une certaine expérience de la chromatographie liquide, que pour le nouveau venu dans cette discipline.

Ce manuel regroupe l'essentiel de ce que l'analyste doit connaître pour pratiquer efficacement la HPLC. En particulier, la très importante question du remplissage des colonnes avec des supports de fine granulométrie (5 et 10 μm) est décrite par les auteurs avec suffisamment de détails pour que les laboratoires pourvus d'un matériel adéquat, puissent envisager la fabrication de leurs propres colonnes.

Après un bref rappel des théories et des formulations qu'il est indispensable de connaître, les auteurs ont inclus des exercices montrant comment la théorie peut guider l'analyste dans la résolution d'un problème donné.

Les problèmes d'optimisation de temps d'analyse, de résolution, essentiels en HPLC, ont été traités avec beaucoup de soin et de clarté.

Enfin, hormis la chromatographie par perméation de gel, tous les autres modes de chromatographie sont traités complètement.

Nul doute que cet ouvrage puisse contribuer au développement de la chromatographie liquide en France.

Rouen (France) J. L. HORRÉARD

PUBLICATION SCHEDULE FOR 1976

Journal of Chromatography (incorporating *Chromatographic Reviews*)

MONTH	D 1975	J	F	M	A	M	J	J	A	S	O	N	D
JOURNAL	115/1 115/2	116/1 116/2	117/1 117/2	118/1 118/2	118/3 119	120/1 120/2	121/1 121/2	122 123/1	123/2	124/1 124/2	125/1 125/2	125/3 126	128/1 128/2
REVIEWS*					127/1				127/2			127/3	

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3 R. D. Marshall and A. Neuberger, in A. Gottschalk (Editor), *Glycoproteins*, Vol. 5, Part A, Elsevier, Amsterdam, 2nd ed., 1972, Ch. 3, p. 251.

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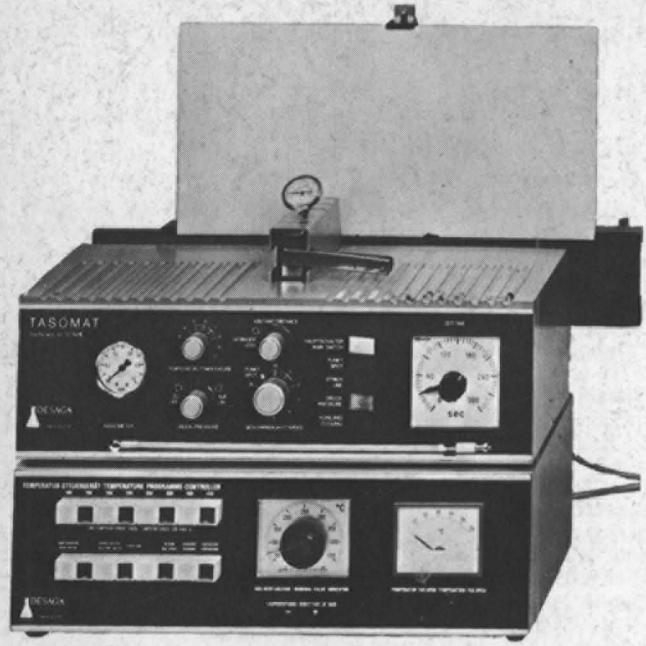

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