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THEORY OF CONSECUTIVE PULSE SAMPLE FEED ELUTION CHROMATOGRAPHY

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(First received December 23rd, 1974; revised manuscript received February 17th, 1975)

SUMMARY

Moments analysis was used to characterize the elution chromatographic peak from a column, in which the longitudinal dispersion in the mobile phase, the radial dispersion inside the porous spherical bed packing and the sorption on the internal surface of the spherical bed packing are simultaneously taking place. A sample feed and an elution step are fed into the column as a consecutive pulse alternately, so that a continuous operation of chromatography can be performed.

INTRODUCTION

The present paper describes a theory of consecutive pulse sample feed elution chromatography which simultaneously takes into account the longitudinal dispersion in the mobile phase, the radial dispersion inside the porous spherical bed packing, and the sorption on the internal surface of the spherical bed packing. The theory is a modification of Kučera's[1] linear non-equilibrium elution chromatography by changing a customarily used injection feed represented by a δ-function to a square pulse train sequence, so that a sample feed and an elution step can be alternated consecutively for an automated continuous operation of chromatography.

FORMULATION OF PROBLEMS

We consider an infinitely long column with a section uniformly filled with the porous spherical bed packing material. The void volume fraction in the column is designated as ε_m and the porosity of spherical radius R packing material is ε_s. The velocity profile of the mobile phase was assumed to be a plug flow with the average carrier velocity U. The dispersion in the mobile phase was assumed to be in a longitudinal direction only and its dispersion coefficient, D_m, was also assumed to be a constant. Then the solute transfer into the stationary phase through the interphase layer by a mass transfer process with a constant mass transfer coefficient k_M. The solute dispersed further in the stationary phase into the interior of each spherical packing with a constant dispersion coefficient D_s. Finally, on those porous spherical

packings adsorption and desorption were taking place for the solute component. Then the elution will proceed. The equations describing the above chromatographic process are characterized by the following mass balance equations

$$\frac{\partial C_m}{\partial t} + U\frac{\partial C_m}{\partial z} - D_m\frac{\partial^2 C_m}{\partial z^2} + k_M(KC_m - C_s\,|_{r=R}) = 0 \tag{1}$$

for the mobile phase, and

$$\frac{\partial C_s}{\partial t} - D_s\left(\frac{\partial^2 C_s}{\partial r^2} + \frac{2}{r}\frac{\partial C_s}{\partial r}\right) = -\frac{\partial n}{\partial t} \tag{2}$$

for the stationary phase. Assuming a finite rate of adsorption on the internal porous surface of spherical bed packing with a linear isotherm, one has

$$\frac{\partial n}{\partial t} = k_A C_s - k_D n \tag{3}$$

for adsorption kinetics. C denotes the solute concentration, and subscripts m and s denote mobile and stationary phases, respectively. n is the solute concentration adsorbed on the porous surface. K is the partition coefficient of the solute, and k_A and k_D are the rate constants for adsorption and desorption, respectively.

The initial and boundary conditions for the pulse sample feed train are

$$C_m(z,t) = 0 \qquad \text{for } t = 0 \tag{4}$$

$$C_s(r,z,t) = 0 \qquad \text{for } t = 0 \tag{5}$$

$$n(r,z,t) = 0 \qquad \text{for } t = 0 \tag{6}$$

$$C_m(z,t) = C_0[u(t) - u(t - t_0)] \qquad \text{for } z = 0 \tag{7}$$

$$C_m(z,t) = 0 \qquad \text{for } z = \infty \tag{8}$$

$$\varepsilon_m k_M(KC_m - C_s\,|_{r=R}) = -\frac{3(1-\varepsilon_m)\,\varepsilon_s}{R}\cdot D_s\left.\frac{\partial C_s}{\partial r}\right|_{r=R} \qquad \text{for } r = R \tag{9}$$

$$\left.\frac{\partial C_s}{\partial r}\right|_{r=0} = 0 \qquad \text{for } r = 0 \tag{10}$$

where $u(t)$ is a unit pulse function, t_0 is the time required for one sample leading into the packed section of the column and the expression $3(1 - \varepsilon_m)\cdot\varepsilon_s/R$ gives the surface area of a spherical porous particle per unit volume of the column.

THE LAPLACE TRANSFORMS AND MOMENTS ANALYSIS

Ideally, one would like to obtain an exact solution of $C_m(z,t)$. The set of differential equations subjecting to those initial and boundary conditions is difficult to solve analytically. However, the chromatographic peak can be completely charac-

terized by the statistical moments[2–7]. The moments of $C_m(z,t)$ can be found by using either the Laplace or the Fourier transformation. In the Laplace domain the equations describing the system presented above are given as

$$s\bar{C}_m + U\frac{\mathrm{d}\bar{C}_m}{\mathrm{d}z} - D_m\frac{\mathrm{d}^2\bar{C}_m}{\mathrm{d}z^2} + k_M(K\bar{C}_m - \bar{C}_s|_{r=R}) = 0 \tag{11}$$

$$s\bar{C}_s - D_s\left(\frac{\mathrm{d}^2\bar{C}_s}{\mathrm{d}r^2} + \frac{2}{r}\frac{\mathrm{d}\bar{C}_s}{\mathrm{d}r}\right) = -s\bar{n} \tag{12}$$

$$s\bar{n} = k_A\bar{C}_s - k_D\bar{n} \tag{13}$$

The initial conditions, eqns. 4–6, were used in obtaining those transformations. The boundary conditions in the Laplace domain are

$$\bar{C}_m(0,s) = C_0\left(\frac{1}{s} - \frac{\mathrm{e}^{-t_0 s}}{s}\right) \tag{14}$$

$$\bar{C}_m(\infty,s) = 0 \tag{15}$$

$$\varepsilon_m k_M(K\bar{C}_m - \bar{C}_s|_{r=R}) = -\frac{3(1-\varepsilon_m)\varepsilon_s}{R}\cdot D_s\frac{\mathrm{d}\bar{C}_s}{\mathrm{d}r}\bigg|_{r=R} \tag{16}$$

$$\frac{\mathrm{d}\bar{C}_s}{\mathrm{d}r}\bigg|_{r=0} = 0 \tag{17}$$

Elimination of $\bar{n}$ from eqns. 12 and 13 gives

$$\frac{\mathrm{d}^2\bar{C}_s}{\mathrm{d}r^2} + \frac{2}{r}\frac{\mathrm{d}\bar{C}_s}{\mathrm{d}r} - a\bar{C}_s = 0 \tag{18}$$

in which

$$a = \frac{1}{D_s}\left[\frac{s^2 + (k_A + k_D)s}{s + k_D}\right] \tag{19}$$

The solution of eqn. 18 together with the boundary conditions eqns. 16 and 17 is obtained as

$$\bar{C}_s = A_1\cdot\bar{C}_m[a^{\frac{1}{2}}r]^{-\frac{1}{2}}\cdot I_{\frac{1}{2}}(a^{\frac{1}{2}}r) \tag{20}$$

where

$$A_1 = \frac{k_M K\varepsilon_m R^2[a^{\frac{1}{2}}R]^{\frac{1}{2}}}{3(1-\varepsilon_m)\varepsilon_s D_s a^{\frac{1}{2}}RI_{3/2}(a^{\frac{1}{2}}R) + k_M\varepsilon_m R^2 I_{\frac{1}{2}}(a^{\frac{1}{2}}R)} \tag{21}$$

Substituting eqn. 20 into eqn. 11, one obtains

$$\frac{\mathrm{d}^2\bar{C}_m}{\mathrm{d}z^2} - \frac{U}{D_m}\frac{\mathrm{d}\bar{C}_m}{\mathrm{d}z} - \frac{A_2}{D_m}\bar{C}_m = 0 \tag{22}$$

in which

$$A_2 = s + \frac{3k_M K(1 - \varepsilon_m)\varepsilon_s D_s \alpha^{\frac{1}{2}} R\, I_{3/2}(\alpha^{\frac{1}{2}} R)}{3(1 - \varepsilon_m)\varepsilon_s\, \alpha^{\frac{1}{2}} R I_{3/2}(\alpha^{\frac{1}{2}} R) + k_M \varepsilon_m R^2 I_{\frac{1}{2}}(\alpha^{\frac{1}{2}} R)} \tag{23}$$

The solution for $\bar{C}_m$ with the boundary conditions, eqns. 14 and 15, is

$$\bar{C}_m(z,s) = C_0 \frac{1 - e^{-t_0 s}}{s} \exp\left\{\frac{U}{2D_m} - \left[\frac{U}{2D_m} + \frac{A_2}{D_m}\right]^{\frac{1}{2}}\right\} \cdot z \tag{24}$$

Since we are interested in $\bar{C}_m(z,s)$ at a particular point L (at the exit of the column) we can replace z in eqn. 24 by L. It is difficult, if at all possible, to invert the equation back to the time domain. However, the expression similar to Kučera's[1] expansion

$$C_m(t) = C_m(z,t)\mid_{z=L} = \sum_{n=0}^{\infty} a_n H_n\left(\frac{t - \bar{\mu}_1}{\sqrt{2\mu_2}}\right) \exp\left[-\frac{(t - \bar{\mu}_1)^2}{2\mu_2}\right] \tag{25}$$

can be found by using Hermite polynomials[7]

$$H_n(x) = \sum_{j=0}^{N} \frac{(-1)^j n!}{j!(n-2j)!} \cdot (2x)^{n-2j} \tag{26}$$

where $N = n/2$ for even n; $N = (n-1)/2$ for odd n. The expansion coefficients a_n are given by the equation

$$a_n = \frac{1}{2^n n! \sqrt{\pi}} \int_0^{\infty} C_m(t) H_n\left(\frac{t - \bar{\mu}_1}{\sqrt{2\mu_2}}\right) \frac{dt}{\sqrt{2\mu_2}} \tag{27}$$

based on the orthogonality of Hermite polynomials. In eqns. 25 and 27, μ_K and $\bar{\mu}_K$ are the Kth moment of function $C_m(t)$ defined by the equation

$$\bar{\mu}_K = m_K / m_0 \tag{28}$$

where

$$m_K = \int_0^{\infty} t^K C_m(t) dt \tag{29}$$

For convenience, all moments higher than the first around the center of gravity of $C_m(t)$ are generally used. The Kth central moment is defined by

$$\mu_K = \frac{1}{m_0} \int_0^{\infty} (t - \bar{\mu}_1)^K C_m(t) dt = \sum_{i=0}^{\nu} \binom{\nu}{i} (-\bar{\mu}_1)^{\nu - i} \cdot \bar{\mu}_i; \quad K > 1. \tag{30}$$

By use of the property of the Laplace transform

$$\int_0^{\infty} t^K C_m(t) dt = (-1)^K \lim_{s \to 0} \frac{\partial^K \bar{C}_m(s)}{\partial s^K} \tag{31}$$

the moments of $C_m(t)$ are obtained. The expansion coefficients a_n expressed in terms of central moments μ_K are

$$a_0 = \frac{m_0}{\sqrt{2\pi\mu_2}} \tag{32}$$

$$a_1 = a_2 = 0 \tag{33}$$

$$a_n = \frac{m_0}{\sqrt{2\pi\mu_2}} \sum_{j=0}^{N} \frac{(-1)^j \mu_{n-2j}\mu_2{}^j}{j!(n-2j)!\, 2^j (2\mu_2)^{\frac{n}{2}}} \tag{34}$$

The resulting moments up to the third central moments are presented by

$$\mu_0 = \bar{\mu}_0 = 1 \tag{35}$$

$$\mu_1 = 0; \quad \bar{\mu}_1 = \frac{t_0}{2} + \frac{L}{U}(1 + \varphi) \tag{36}$$

where

$$\varphi = \frac{K(1 - \varepsilon_m)\varepsilon_s(k_A + k_D)}{\varepsilon_m k_D} \tag{37}$$

$$\mu_2 = \frac{t_0}{12} + \frac{2D_m L}{U^3}(1 + \varphi)^2 + \frac{L}{U}\left[\frac{13\varphi^2}{10k_M K} + \frac{13R^2\varepsilon_m\varphi^2}{160K\varepsilon_s D_s(1-\varepsilon_m)} + \frac{2k_A\varphi}{k_D(k_A + k_D)}\right] \tag{38}$$

$$\mu_3 = \frac{12D_m L}{U^5}(1+\varphi)^3 + \left(\frac{2L^2}{U^2} + \frac{4D_m L}{U^3}\right)\left(1+\varphi + \frac{2D_m L}{U^3}\right)\left[\frac{13\varphi^2}{10k_M K} + \frac{13\varepsilon_m R^2\varphi^2}{160K\varepsilon_s(1-\varepsilon_m)D_s} + \frac{2k_A\varphi}{k_D(k_A+k_D)}\right] + \frac{L}{U}\left[\frac{4137\varphi^3}{640k_M^2K^2} + \frac{4R^2\varepsilon_m\varphi^3}{5k_M K^2\varepsilon_s D_s(1-\varepsilon_m)} + \frac{16\varepsilon_m^2R^4\varphi^3}{315\varepsilon_s^2(1-\varepsilon_m)^2K^2D_s^2} + \frac{117k_A\varphi^2}{15k_M k_D(k_A+k_D)K} + \frac{39R^2\varphi k_A}{80D_s k_D^2} + \frac{16k_A\varphi}{(k_A+k_D)}\right] \tag{39}$$

DISCUSSION

The first moment as indicated by Grushka *et al.*[8] is the retention time t_R of the solute, *i.e.*, the time when the maximum concentration is registered at the point of detection. In this case, the retention time is given by

$$t_R = \bar{\mu}_1 = \frac{t_0}{2} + \frac{L}{U}(1+\varphi) \tag{40}$$

It is interesting to note that t_R is unaffected by the dispersions in the longitudinal direction and in the porous spherical bed packing and that the parameter φ in eqn.

37 is a modification of Kučera's parameter[1] $K(1 - \varepsilon_m)/\varepsilon_m$ by multiplying with a factor of $\varepsilon_s(k_A + k_D)/k_D$. The peak does depend on the bed structure and adsorption and desorption coefficients. Probably, the sequence of pulse sample feed and elution trains eliminates the effect of dispersions, and the kinetic coefficients and the bed structure are becoming more important factors in the process. The result is similar to the one obtained by Grushka[9], but without assuming the term $2D_m/U^2$ being negligible. The second moment is the peak variance which leads to a direct derivation of the plate height, H,

$$H = \frac{L\mu_2}{\bar{\mu}_1^2} = \frac{2D_m}{U} + \frac{U}{(1+\varphi)^2}\left[\frac{13\varphi^2}{10k_M K\varepsilon_s} + \frac{13\varepsilon_m R^2\varphi^2}{160KD_s\varepsilon_s} + \frac{2k_A\varphi}{k_D(k_A+k_D)}\right] \quad (41)$$

In obtaining eqn. 41, the loading time t_0 was assumed to be very small compared with the other terms of eqns. 36 and 38. The theoretical plate height depends on all factors characterizing the column, its filling and transport of the given compound through the column. It increases with the increasing coefficient of the longitudinal dispersion, the partition coefficient, and the size of bed packing particles R. It decreases with the increasing of the dispersion coefficient in the porous bed packing, the mass transfer coefficient from the mobile phase to the stationary phase and the porosity of the bed packing.

With the information of t_0 and t_R, one should be able to program the sequence for sample feed–elution schedule for automated continuous operation of the chromatography.

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COMPORTEMENT DES ÉTHERS

III. CORRÉLATIONS DE DONNÉES CHROMATOGRAPHIQUES DES ÉTHERS AVEC CELLES D'AUTRES POPULATIONS CHIMIQUES

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SUMMARY

Behaviour of ethers. III. Relationships between the retention data of ethers and those of other chemical populations

The comparative chromatographic behaviour of various chemical populations can be examined by the following simple linear relationship.

$$\log t'_{R\,(RZ)_{\varphi_2}} = a \cdot \log t'_{R\,(RZ')_{\varphi_1}} + b$$

By this relationship retention parameters can be fairly accurately determined.

INTRODUCTION

Parmi les premières études de relations prévisionnelles des temps de rétention, il faut relever les travaux de Rohrschneider[1] qui conduisent à une relation à cinq paramètres et qui ont fait depuis l'objet d'un réexamen approfondi[2,3]. De son côté, McReynolds[4] a proposé une équation à sept paramètres. Tout récemment, Weiner et Howery[5] ont mis au point une équation à huit paramètres basée sur l'analyse factorielle et permettant la prévision d'indices de rétention.

Une autre méthode d'approche est constituée par l'établissement de relations "comportement–structure"[6–8]. Nos propes études[9,10] sur les éthers rentrent dans ce cadre et font apparaître l'existence d'une relation simple entre les temps de rétention des alkyloxyalcanes et ceux des allyloxyalcanes. De même, nous avons pu établir des relations linéaires régissant le comportement des alkyloxyalcanes vis-à-vis de phases stationnaires diverses.

Dans le présent mémoire nous allons montrer que l'approche précédente peut être généralisée et qu'il est possible d'une part de déduire des grandeurs de rétention d'une famille de composés chimiques RZ à partir de celles d'une autre RZ', en

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utilisant pour toutes les mesures une même phase stationnaire φ_1 et d'autre part de prévoir, à partir du comportement d'une famille RZ vis-à-vis d'une phase stationnaire φ_1, celui d'une autre famille RZ′ vis-à-vis d'une phase stationnaire φ_2.

MÉTHODE ET RÉSULTATS

La méthode d'analyse de données que nous préconisons est basée sur la comparaison d'un ensemble de temps de rétention à l'aide de la relation générale

$$\log t'_{R\,(\mathrm{RZ})_{\varphi_2}} = a \cdot \log t'_{R\,(\mathrm{RZ}')_{\varphi_1}} + b \qquad (1)$$

dans laquelle Z et Z′ représentent les fonctions chimiques des deux populations confrontées, R le groupe variable différenciant entre eux les membres d'une famille donnée, φ_1 et φ_2 les phases stationnaires.

Cette approche englobe deux comparaisons: (i) Celle du comportement d'une famille de substances, mises successivement en présence de deux phases stationnaires φ_1 et φ_2. Dans ce cas $Z = Z'$ et la relation 1 se simplifie en

$$\log t'_{R\,(\mathrm{RZ})_{\varphi_2}} = a_1 \cdot \log t'_{R\,(\mathrm{RZ})_{\varphi_1}} + b_1 \qquad (2)$$

Cette relation nous a permis de montrer[9] que la pente a_1 traduit la polarité relative des phases stationnaires. (ii) Celle du comportement de deux familles de composés mis en présence d'une même phase stationnaire. Dans ce cas $\varphi_1 = \varphi_2$ et la relation 1 se transforme en

$$\log t'_{R\,(\mathrm{RZ})_{\varphi_1}} = a_2 \cdot \log t'_{R\,(\mathrm{RZ}')_{\varphi_1}} + b_2 \qquad (3)$$

Si cette dernière est satisfaite, cela signifie que la contribution du groupe variable R à la valeur log t'_R est semblable pour les deux séries de substances RZ et RZ′.

En utilisant comme données de référence celles relatives aux éthoxyalcanes[9], l'analogie de comportement se traduit par

$$\log t'_{R\,(\mathrm{RZ})_{\varphi_1}} = a_2 \cdot \log t'_{R\,(\mathrm{ROEt})_{\varphi_1}} + b_2$$

Pour étudier cette analogie, nous avions à notre disposition des grandeurs de rétention, déterminées pour six phases stationnaires de polarité croissante, et concernant d'une part les éthoxyalcanes[9] que nous avons choisis comme termes de référence et pour lesquels nous avons effectué les mesures de rétention à 120°, d'autre part les résultats relatifs à une série de phénoxyalcanes[11], d'acétates[12], d'aldéhydes[12], de méthylcétones[12] et d'alcools[12].

Les résultats obtenus sont rassemblés dans le Tableau I. Nous observons dans tous les cas que les facteurs statistiques de fiabilité (r = coefficient de corrélation et ψ = test d'Exner[13]) sont d'autant meilleurs que la phase stationnaire utilisée est moins polaire. Dans certains cas ($r \leqslant 0.990$ et $\psi \geqslant 0.16$), la qualité de l'information n'est pas suffisante pour aboutir à une corrélation réelle, mais seulement pour affirmer une tendance à la corrélation (cas des phases stationnaires les plus polaires).

TABLEAU I

COMPARAISON DU COMPORTEMENT DES ÉTHOXYALCANES À CELUI D'AUTRES FAMILLES DE SUBSTANCES

Famille	*Phase*	a_2	b_2	r	ψ
Φ–OR	Apiezon L	0.826	−0.010	0.994	0.11
	SE-30	0.765	0.012	0.996	0.09
R = Me, Et, *n*Pr,	Ucon polar	0.734	−0.001	0.990	0.15
iPr, *n*Bu,	Carbowax 20M	0.712	−0.040	0.979	0.23
iBu, *s*Bu,	XF-1150	0.657	−0.035	0.967	0.28
*n*Pent, iPent	DEGS	0.593	−0.014	0.976	0.24
MeCOOR	Apiezon L	0.998	0.025	0.995	0.10
	SE-30	0.934	0.039	0.994	0.12
R = Me, Et, *n*Pr,	Ucon polar	0.966	0.046	0.990	0.16
iPr, *n*Bu, iBu,	Carbowax 20M	0.943	−0.024	0.978	0.23
*s*Bu, *t*Bu,	XF-1150	0.998	0.062	0.985	0.19
*n*Pent, iPent	DEGS	0 827	0.060	0 938	0 39
RCHO	Apiezon L	0 950	−0 148	0 996	0 10
	SE-30	0.882	−0.121	0.996	0.09
R = Et, *n*Pr, iPr,	Ucon polar	0.929	−0.094	0.993	0.12
*n*Bu, iBu, *s*Bu,	Carbowax 20M	0.919	−0.132	0.979	0.22
*t*Bu, *n*Pent,	XF-1150	0.942	−0.036	0.996	0.10
*n*Hex	DEGS	0.896	0.016	0.981	0.21
MeCOR	Apiezon L	0.891	−0.142	0.999	0.05
	SE-30	0.850	−0.127	0.999	0.03
R = Et, *n*Pr, iPr,	Ucon polar	0.890	−0.104	0.999	0.03
*n*Bu, *s*Bu, iBu,	Carbowax 20M	0.877	−0.124	0.998	0.06
*t*Bu, *n*Pent,	XF-1150	0.872	−0.035	0.998	0.05
*n*Hex, *n*Hept	DEGS	0.836	0.063	0.995	0.11
ROH	Apiezon L	0.991	0.069	0.992	0.13
	SE-30	0.955	0.009	0.990	0.14
R = Me, Et, *n*Pr, iPr,	Ucon polar	1.018	−0.023	0.976	0.23
*n*Bu, iBu, *s*Bu,	Carbowax 20M	0.986	−0.092	0.961	0.29
*t*Bu, *n*Pent,	XF-1150	0.971	0.021	0.975	0.23
iPent, *s*Pent,	DEGS	0.874	−0.004	0.961	0.29
*t*Pent, Me-2 Bu,					
[*s*.iPent, néoPent,					
*n*Hex, *n*Hept, *n*Oct					

Le coefficient a_2 dépend à la fois de la nature de la phase stationnaire, de la fonction chimique, de l'environnement fixe de cette dernière (Tableau II) et de la température (Tableau III). En d'autres termes a_2 est un coefficient de nature composite, lié aux phénomènes d'interaction soluté–solvant. Ces considérations restent valables en ce qui concerne le terme b_2.

L'étude des résultats obtenus par application de la relation 3 indique qu'il est difficile de réaliser une incrémentation simple conduisant à la détermination, pour une même phase stationnaire, des temps de rétention des composés d'une population donnée à partir de ceux d'une famille de référence. Ceci reste vrai pour l'équation 1. Celle-ci présente cependant des avantages dans le domaine pratique où il n'est pas toujours possible de sélectionner une phase stationnaire qui aurait le meilleur pouvoir séparateur possible vis-à-vis de toutes les fonctions chimiques. L'intérêt de la rela-

TABLEAU II

COMPARAISON DU COMPORTEMENT DES ESTERS[14] À CELUI DES ÉTHOXYALCANES

Phase stationnaire, SE-30; température de mesure, 150°.
R = Me, Et, *n*Pr, iPr, *n*Bu, iBu, *n*Pent, iPent.

Esters	a_2	b_2	r	ψ
HCOOR	0.827	0.053	0.994	0.12
MeCOOR	0.769	0.013	0.998	0.07
EtCOOR	0.767	0.010	0.996	0.10
*n*PrCOOR	0.704	0.021	0.997	0.09
iPrCOOR	0.710	0.017	0.997	0.08
*n*BuCOOR	0.693	0.015	0.996	0.10
iBuCOOR	0.702	0.037	0.996	0.11
*n*AmCOOR	0.683	0.014	0.997	0.09
iAmCOOR	0.691	0.019	0.997	0.09

TABLEAU III

COMPARAISON DU COMPORTEMENT DE DIVERSES FAMILLES CHIMIQUES À CELUI DES ÉTHOXYALCANES —INFLUENCE DE LA TEMPÉRATURE

Phase stationnaire, Apiezon L. Séries identiques à celles du Tableau I. (Bibl. 12).

Famille	a_2		b_2		r		ψ	
	120°	*160°*	*120°*	*160°*	*120°*	*160°*	*120°*	*160°*
ROH	0.991	0.851	0.069	0.079	0.992	0.991	0.13	0.14
MeCOOR	0.998	0.801	0.025	0.030	0.995	0.997	0.10	0.08
RCHO	0.950	0.601	−0.148	−0.015	0.996	0.981	0.10	0.22
MeCOR	0.891	0.749	−0.142	−0.102	0.999	0.996	0.05	0.09

tion 1 est illustré par les données du Tableau IV. Celui-ci fait cependant apparaître l'existence d'interactions spécifiques[3] dans le cas des amines et des alcools, interactions qui diminuent la précision de la corrélation.

Par ailleurs, nous avons pu vérifier qu'il est possible d'établir des relations de type 1 en se basant sur les grandeurs de rétention relatives non réduites. Par exemple, on peut relier les alcènes-1 (squalane, 27°)[19] aux méthylcétones (SE-30, 210°)[20] par la relation

$$\log t_{R(\text{R-CH=CH}_2)} = 0.298 \log t_{R(\text{MeCOR})} - 0.309$$

où

$$r = 0.997 \text{ et } \psi = 0.09$$

CONCLUSION

Le moyen d'approche que nous proposons dans le présent mémoire permet, même à partir de données expérimentales d'auteurs différents, d'établir des relations linéaires entre les temps de rétention de deux ensembles de substances R–Z et R–Z'. Ces temps de rétention peuvent être déterminés aussi bien dans des conditions isothermes que par programmation linéaire[21]. Ce type de relation ne saurait préjuger du modèle d'interaction soluté–solvant.

TABLEAU IV

COMPORTEMENT COMPARÉ DES ÉTHOXYALCANES (φ_1 = APIEZON L) ET D'AUTRES POPULATIONS CHIMIQUES (φ_2 = DIVERS) -VÉRIFICATION DE LA RELATION 1

R–Z/Phase φ_2	*Bibl.*	*a*	*b*	*r*	ψ	*p*
R–CH=CH$_2$ Squalane	15	1.675	0.138	0.993	0.13	10
(E) R–CH=CH–Me Squalane	15	1.711	0.073	0.993	0.13	7
R–I Tricrésylphosphate	16	0.889	0.057	0.996	0.09	12
R–OH Célite/PEG 1500	17	1.014	−0.121	0.976	0.24	12
R–NH$_2$ Carbowax 20M Célite/KOH	18	1.124	−0.039	0.989	0.16	12
R–CHO Hallcomid M 18 ol	12	0.945	−0.181	0.995	0.11	9
R–CO–Me Hallcomid M18 ol	12	0.890	−0.168	0.999	0.03	11
R–COOMe SE-30	14	0.719	0.055	0.995	0.11	8
Me–COOR Hallcomid M18 ol	12	0.954	0.027	0.989	0.16	15
Me–CH=C (R)–COOMe OV-210	22	0.559	0.019	0.995	0.10	10
R–C(Me)=CH–COOMe XF-1150	22	0.539	−0.018	0.998	0.07	10
p-R–Φ–OH Tri-(2,4-xylényl) phosphate	23	0.831	0.230	0.994	0.12	7
o-R–Φ–OH Tri-(2,4-xylényl) phosphate	23	0.724	0.110	0.991	0.15	7

Il ressort de notre étude que les groupes alkyles R ont un comportement analogue, quelle que soit la fonction chimique de la population examinée. De ce fait, la part que prend le groupement R dans l'interaction de premier ordre dipôle–dipôle devrait pouvoir être logiquement examinée à l'aide de paramètres polaires et stériques, tels que ceux de Taft.

RÉSUMÉ

Le comportement chromatographique comparé de diverses populations chimiques peut être abordé grâce à des relations simples du type

$$\log t'_{R\,(RZ)_{\varphi_2}} = a \cdot \log t'_{R\,(RZ')_{\varphi_1}} + b$$

Cette relation permet une précision raisonnable des grandeurs de rétention.

NOTE AJOUTÉE À LA LECTURE DES ÉPREUVES

À la fin de la grève des services postaux français nous avons eu connaissance d'un article par Ladon[24]. L'analyse que Ladon développe dans ce mémoire est basée

sur une relation parallèle à la nôtre par sa forme, mais appliquée à des recherches divergentes des nôtres.

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COMPORTEMENT DES ÉTHERS

IV. ÉTABLISSEMENT DE RELATIONS LINÉAIRES EXTRATHERMODYNAMIQUES ENTRE PARAMÈTRES STRUCTURAUX ET GRANDEURS DE RÉTENTION

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SUMMARY

Behaviour of ethers. IV. Determination of linear extrathermodynamic relationships between structural parameters and retention data

The linear extrathermodynamic relationship $\log t'_R = \varrho^* \sigma^* + \delta E_s + h(n-3)$ between polar and steric factors of a group of compounds and their relative retention data has been established for a series of esters, ketones, aldehydes, alcohols and ethers. It seems to be applicable to all groups of chemical compounds. In all the cases examined the polar factor ϱ^* prevails.

MÉTHODE ET RÉSULTATS

L'existence[1–3] d'une relation linéaire de type

$$\log t'_{R\,(\text{R-Z})_{\varphi_2}} = a \cdot \log t'_{R\,(\text{ROEt})_{\varphi_1}} + b$$

entre les logarithmes des temps de rétention réduits relatifs des éthoxyalcanes et ceux de substances RZ implique, pour chacune de telles relations, une contribution analogue des groupements alkyles R aux grandeurs de rétention. Pour représenter la variation du terme $\log t'_R$, il y a lieu par conséquent de confronter cette grandeur avec des paramètres structuraux propres à chacun des substituants R. Compte-tenu du fait que les interactions entre soluté et phase stationnaire peuvent être considérées comme des phénomènes polaires entre autres de type dipôle–dipôle[4] nous avons tenté d'établir une relation

$$\log t'_{R\,(\text{R-Z})_{\varphi_1}} = \text{f}(\sigma^*)$$

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entre le comportement des substances et le paramètre d'action que constituent les constantes d'effet polaire σ^*, affectées aux groupes R. Nous avons vérifié que cette relation était applicable à diverses familles chimiques et obtenu des graphes dont les deux exemples suivants (Fig. 1, relatif aux amines[5], Fig. 2, relatif aux alcools[6]), traduisent l'allure générale.

Dans tous les cas l'ensemble des points se répartit le long de trois droites à peu près parallèles. Les intervalles qui séparent la première droite du point singulier que constitue le groupe méthyle ou qui séparent deux droites voisines sont tous du même ordre de grandeur.

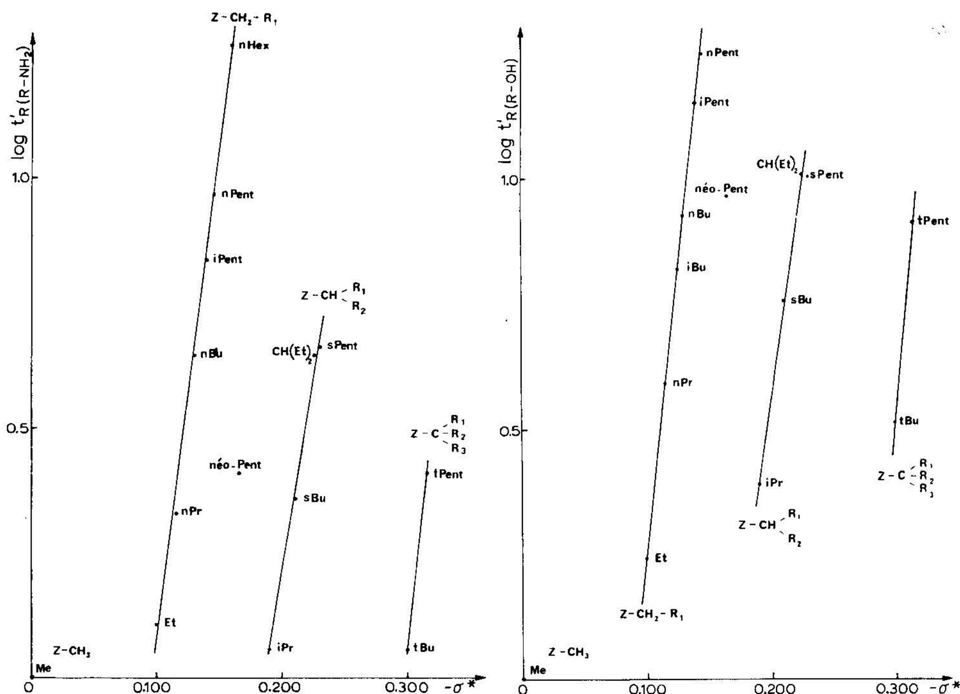

Fig. 1. Variation des grandeurs de rétention en fonction des constantes d'effet polaire. Cas des amines[5].

Fig. 2. Variation des grandeurs de rétention en fonction des constantes d'effet polaire. Cas des alcools[6].

On observe que les groupes alkyles rassemblés le long d'une droite se différencient de ceux de la droite voisine par le degré de substitution de l'atome de jonction avec la fonction chimique Z. Cela signifie qu'il existe un effet stérique qui se manifeste essentiellement par le nombre de ramifications au niveau de cet atome de jonction.

Si le parallélisme des droites montre que la sensibilité de l'effet polaire est la même, quelle que soit la structure du groupe R, la régularité des intervalles entre ces droites suggère de son côté que le remplacement successif des hydrogènes du carbone adjacent à Z par des groupes alkyles quelconques entraîne des variations quantifiées. C'est la raison pour laquelle, nous basant sur une représentation de Hancock *et al.*[8,9] nous avons remplacé le terme E_s de l'expression classique de Taft par une grandeur corrigée, tenant compte de notre observation

$$E_{s(\text{corrigé})} = E_s + k(n_H - 3)$$

où n_H représente le nombre d'atomes d'hydrogène portés par le carbone de jonction.

La relation entre le comportement et les paramètres d'action devient alors

$$\log t'_{R\,(R-Z)_{\varphi_1}} = \varrho^*\sigma^* + \delta E_s + h(n_H - 3)$$

où $h = \delta k$.

Les coefficients ϱ^*, δ et h sont indépendants de la structure du groupement R et caractérisent la sensibilité du phénomène chromatographique aux effets polaire et stériques. Dans le Tableau I, nous avons regroupé les valeurs de ces coefficients pour huit familles de substances chimiques et des phases stationnaires variées.

TABLEAU I

VARIATION DES COEFFICIENTS ϱ^*, δ, k ET h AVEC LA NATURE DE LA FAMILLE R–Z

r = Coefficient de corrélation; ψ = test d'Exner; p = nombre de points.

R–Z/Phases	*Bibl.*	ϱ^*	δ	k	h	r	ψ	p
R–CH=CH$_2$ Squalane	10	−33.07	0.026	106.9	2.78	0.999	0.03	9
(E)R–CH=CH–Me Squalane	10	−32.52	0.047	58.94	2.77	0.999	0.02	6
R–I Tricrésylphosphate	11	−18.58	0.008	193.7	1.55	0.999	0.05	8
R–OH Célite/PEG 1500	6	−21.53	0.113	17.61	1.99	0.995	0.12	9
R–NH$_2$ Carbowax 20M Célite/KOH	5	−21.59	0.039	50.26	1.96	0.992	0.16	9
R–CO–Me Hallcomid M18ol	7	−19.99	0.114	14.04	1.60	0.997	0.11	7
R–COOMe SE-30	12	−14.39	0.064	18.44	1.18	0.997	0.09	7
MeCOOR Hallcomid M18ol	7	−20.02	0.074	23.11	1.71	0.997	0.09	9

Les valeurs élevées de ϱ^*, comparées à celles de δ, montrent que la sensibilité à l'effet polaire est le facteur dominant dans le phénomène chromatographique. Le facteur stérique n'intervient de façon importante qu'au niveau du carbone de jonction avec la fonction chimique Z sans que, toutefois, le produit δE_s soit négligeable. Les valeurs négatives de ϱ^* traduisent l'effet donneur d'électrons des groupements alkyles R.

Pour vérifier dans quelle mesure la sensibilité à l'effet polaire du phénomène chromatographique était liée à l'interaction soluté–solvant, nous avons regroupé dans le Tableau II et pour six phases stationnaires les valeurs de ϱ^* relatives à sept populations.

Les données de base pour l'établissement de ce tableau proviennent en partie des travaux de McReynolds[7] et en partie des recherches de notre laboratoire[13–15]. Il apparaît nettement que pour une famille de substances déterminée ϱ^* varie avec la nature de la phase stationnaire employée et croît avec la polarité relative de celle-ci. Par ailleurs, l'exemple des éthers montre que si pour une fonction chimique donnée

TABLEAU II

VARIATION DES COEFFICIENTS ϱ^*, δ ET h AVEC LA NATURE DE LA PHASE STATIONNAIRE

Pour l'établissement de chacune des corrélations, nous avons utilisé un minimum de huit substances.

Phase	*Familles R–Z*		ϱ^*	δ	h	r	ψ
Apiezon L	MeCOOR	(7)	−20.09	0.060	1.710	0.998	0.07
	MeCOR	(7)	−20.09	0.089	1.615	0.998	0.08
	RCHO	(7)	−21.56	0.060	1.730	0.998	0.08
	ROH	(7)	−21.70	0.017	1.873	0.997	0.09
	EtOR	(13)	−21.24	0.148	1.829	0.996	0.11
	$CH_2=CH-CH_2OR$	(14)	−18.71	0.139	1.591	0.996	0.11
	ΦOR	(15)	−16.89	0.085	1.467	0.998	0.08
SE-30	MeCOOR		−17.54	0.048	1.485	0.998	0.08
	MeCOR		−17.61	0.081	1.420	0.998	0.08
	RCHO		−18.43	0.052	1.482	0.997	0.10
	ROH		−18.95	0.026	1.653	0.997	0.10
	EtOR		−19.33	0.120	1.665	0.995	0.12
	$CH_2=CH-CH_2OR$		−16.91	0.086	1.446	0.996	0.09
	ΦOR		−14.30	0.052	1.227	0.997	0.09
Ucon polar	MeCOOR		−18.12	0.080	1.560	0.997	0.09
	MeCOR		−17.97	0.117	1.459	0.997	0.09
	RCHO		−19.67	0.080	1.595	0.998	0.08
	ROH		−20.03	0.052	1.809	0.997	0.09
	EtOR		−19.21	0.130	1.673	0.996	0.12
	$CH_2=CH-CH_2OR$		−16.19	0.115	1.376	0.996	0.11
	ΦOR		−13.91	0.100	1.221	0.997	0.09
Carbowax 20M	MeCOOR		−15.38	0.103	1.356	0.997	0.09
	MeCOR		−15.25	0.145	1.257	0.997	0.09
	RCHO		−16.85	0.105	1.380	0.999	0.05
	ROH		−16.83	0.074	1.562	0.997	0.08
	EtOR		−17.10	0.166	1.476	0.995	0.12
	$CH_2=CH-CH_2OR$		−14.71	0.150	1.260	0.996	0.11
	ΦOR		−11.72	0.117	1.051	0.995	0.12
XF-1150	MeCOOR		−14.57	0.059	1.282	0.999	0.06
	MeCOR		−14.22	0.114	1.172	0.997	0.09
	RCHO		−15.55	0.077	1.293	0.999	0.05
	ROH		−15.61	0.065	1.426	0.997	0.09
	EtOR		−15.80	0.187	1.392	0.992	0.11
	$CH_2=CH-CH_2OR$		−13.02	0.121	1.120	0.996	0.11
	ΦOR		−10.00	0.122	0.917	0.991	0.17
DEGS	MeCOOR		−12.02	0.088	1.084	0.996	0.10
	MeCOR		−12.23	0.094	1.045	0.962	0.35
	RCHO		−13.68	0.094	1.155	0.998	0.07
	ROH		−12.95	0.075	1.223	0.990	0.17
	EtOR		−14.81	0.208	1.311	0.993	0.15
	$CH_2=CH-CH_2OR$		−11.86	0.152	1.012	0.995	0.12
	ΦOR		− 8.94	0.135	0.823	0.976	0.27

on change l'environnement en remplaçant des groupes alkyles saturés par des groupes plus riches en électrons (liaisons éthyléniques ou cycle benzénique), le facteur ϱ^* est affecté et varie dans le même sens que précédemment; cela signifie qu'il y a renforce-

ment de l'interaction soluté–solvant. Il ressort également de l'étude du Tableau II que les acétates et les méthylcétones d'une part, les aldéhydes, les alcools et les éthers aliphatiques d'autre part ont des comportements analogues.

CONCLUSION

En conclusion, il nous a été possible d'établir une relation linéaire extrathermodynamique entre le comportement chromatographique de diverses populations chimiques et les paramètres d'effets stériques et polaire. La sensibilité à ce dernier constitue le facteur dominant dans le phénomène chromatographique. L'expression que nous avons établie permet une prévision raisonnable des logarithmes des temps de rétention réduits relatifs.

RÉSUMÉ

La relation linéaire extrathermodynamique $\log t'_R = \varrho^*\sigma^* + \delta E_s + h(n - 3)$ entre le comportement d'une population chimique et les paramètres d'effets stériques et polaire a été établie à partir d'une série d'esters, de cétones, d'aldéhydes, d'alcools et d'éthers. Elle permet une prévision raisonnable des grandeurs de rétention. Il apparaît que dans le phénomène chromatographique le facteur polaire intervient d'une manière prépondérante.

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COMPORTEMENT DES ÉTHERS

V. RELATIONS DE TOPOLOGIE–INFORMATION ENTRE LES GRANDEURS DE RÉTENTION ET LA STRUCTURE DES MÉTHOXY- ET ÉTHOXY-ALCANES

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SUMMARY

Behaviour of ethers. V. Topology–information relationships between the retention parameters and the structure of methoxy- and ethoxyalkanes

The logarithms of the relative retention times of methoxy- and ethoxyalkanes have been correlated with the structures of these substances, using the DARC topological system. The correlation shows the contribution of each carbon atom of the alkyl groups to the value of log t'_R.

INTRODUCTION

Dans le phénomène de rétention chromatographique l'interaction entre le soluté et le solvant est liée entre autres aux effets polaire et stériques des différents groupements alkyles R du soluté. Dans notre précédent mémoire, nous avons montré qu'il était possible d'évaluer l'intervention de ces effets[1].

Si les relations linéaires extrathermodynamiques que nous avons ainsi établies traduisent l'action des groupements alkyles sous l'angle de leur structure globale, nous voudrions montrer ici qu'il est possible, grâce à la méthode de topologie–information DARC[2,3], d'expliciter les contributions respectives de chacun des atomes de carbone constitutifs (sites) d'un groupement R au phénomène chromatographique.

En effet, sans préjuger du mécanisme intime de l'interaction, la méthode DARC permet de faire apparaître la participation individuelle des différents sites d'un groupement alkyle.

Dans le cadre de cette méthode topologique, les molécules sont appréhendées par leur topomodèle qui rassemble, à côté du foyer FO, caractérisant le site privilégié commun à tous les individus de la population examinée, les parties extérieures à ce site qui constituent l'environnement *E* et qui se développent le long de "directions de

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TABLEAU I

CONTRIBUTIONS DES SITES TOPOLOGIQUES DES ÉTHOXYALCANES

r = Coefficient de corrélation; ψ = test d'Exner; ( ) = variance × 10^2.

Phase stationnaire	*Paramètres*												r	ψ
	I_{A_1}	I_{A_2}	I_{A_3}	$I_{B_{11}}$	$I_{B_{12}}$	$I_{B_{13}}$	$I_{C_{111}}$	$I_{C_{112}}$	I_D	I_E	I_F	I_G		
Apiezon L	0.183	0.188	0.223	0.326	0.200	0.065	0.317	0.217	0.347	0.328	0.334	0.326	0.999	0.05
	(0.122)	(0.037)	(0.064)	(0.049)	(0.046)	(0.116)	(0.046)	(0.116)	(0.116)	(0.158)	(0.158)	(0.158)		
SE-30	0.123	0.210	0.218	0.316	0.214	0.078	0.316	0.221	0.331	0.313	0.280	0.276	0.999	0.05
	(0.095)	(0.029)	(0.050)	(0.038)	(0.036)	(0.090)	(0.036)	(0.090)	(0.090)	(0.124)	(0.124)	(0.124)		
Ucon polar	0.107	0.161	0.199	0.303	0.161	−0.003	0.302	0.208	0.329	0.291	0.275	0.296	0.999	0.04
	(0.077)	(0.023)	(0.041)	(0.031)	(0.029)	(0.074)	(0.029)	(0.074)	(0.074)	(0.101)	(0.101)	(0.101)		
Carbowax 20M	0.105	0.119	0.159	0.256	0.200	−0.050	0.263	0.156	0.279	0.266	0.258	0.273	0.999	0.07
	(0.123)	(0.037)	(0.065)	(0.049)	(0.046)	(0.117)	(0.046)	(0.117)	(0.117)	(0.159)	(0.159)	(0.159)		
XF-1150	0.021	0.114	0.091	0.260	0.098	−0.076	0.229	0.167	0.276	0.215	0.240	0.288	0.997	0.13
	(0.402)	(0.124)	(0.214)	(0.162)	(0.153)	(0.384)	(0.153)	(0.384)	(0.384)	(0.521)	(0.521)	(0.521)		
DEGS	−0.019	0.049	0.115	0.223	0.040	−0.166	0.223	0.234	0.254	0.234	0.225	0.224	0.998	0.098
	(0.216)	(0.666)	(0.115)	(0.872)	(0.821)	(0.206)	(0.821)	(0.206)	(0.206)	(0.280)	(0.280)	(0.280)		

développement” à partir d’origines dont le nombre est lié au choix et à la structure du foyer.

Pour atteindre une description correcte de l’environnement E il faut que celui-ci soit ordonné. Nous désignerons par empreinte de la population l’environnement ordonné qui rassemble les sites occupés au moins une fois dans les environnements constitutifs de la population[3]. À chaque environnement E est associé un vecteur “topologie” $\overrightarrow{T(E)}$ de composantes $t_1, t_2, \ldots t_n$, tel que $t_n = 1$ si le $n$ième site de l’empreinte est occupé dans l’environnement du composé considéré et $t_n = 0$ dans le cas contraire.

De leur côté, les propriétés physico-chimiques ou chimiques sont représentées par l’information $I(E)$ dont les composantes $I(m)$ sont déterminées à partir des m composés constituant la population clé et permettant de chiffrer la contribution de chacun des sites topologiques.

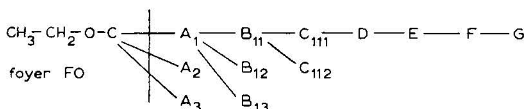
A partir des grandeurs $T(E)$ et $I(m)$ on aboutit à une “relation de topologie–information”

$$I(E) = < T(E) \mid I(m) >$$

dans laquelle l’information $I(E)$ est reliée à la contribution de l’environnement du foyer.

CONTRIBUTION DES SITES

Dans un premier temps, nous avons opéré sur une série de 18 éthoxyalcanes dont l’empreinte est la suivante



Les valeurs de rétention de ces différentes substances ont été publiées par ailleurs[4]. Les contributions des différents sites topologiques à la valeur du terme log t'_R, déterminées pour six phases stationnaires qui couvrent l’ensemble du domaine des polarités, sont rassemblées dans le Tableau I. A côté de chaque contribution nous avons fait figurer entre parenthèses la valeur de la variance attachée à ce terme.

TABLEAU II

SYSTÈME SIMPLIFIÉ DES CONTRIBUTIONS DES SITES TOPOLOGIQUES DES ÉTHOXY-ALCANES

Phase stationnaire	*Paramètres*							r	ψ
	I_{A_1}	I_{A_2}	I_{A_3}	$I_{B_{12}}$	$I_{B_{13}}$	$I_{C_{112}}$	I_p		
Apiezon L	0.179	0.187	0.226	0.196	0.069	0.204	0.330	0.999	0.05
SE-30	0.156	0.192	0.209	0.202	0.068	0.211	0.304	0.998	0.06
Ucon polar	0.114	0.156	0.197	0.158	−0.004	0.197	0.300	0.999	0.05
Carbowax 20M	0.100	0.117	0.161	0.114	−0.049	0.148	0.266	0.998	0.06
XF-1150	0.023	0.116	0.093	0.102	−0.068	0.158	0.247	0.994	0.13
DEGS	−0.023	0.045	0.118	0.033	−0.163	0.220	0.231	0.997	0.09

TABLEAU III

CONTRIBUTION DES SITES TOPOLOGIQUES DES MÉTHOXYALCANES

Nous avons introduit le site A_1 dans le foyer de telle sorte que le premier terme de la série soit identique pour les deux populations.

Phase stationnaire	*Paramètres*											r	ψ
	I_{A_2}	I_{A_3}	$I_{B_{11}}$	$I_{B_{12}}$	$I_{B_{13}}$	$I_{C_{111}}$	$I_{C_{112}}$	I_D	I_E	I_F	I_G		
Apiezon L	0.232	0.280	0.329	0.214	0.067	0.332	0.230	0.357	0.308	0.325	0.323	0.999	0.06
	(0.035)	(0.060)	(0.045)	(0.043)	(0.108)	(0.430)	(0.108)	(0.108)	(0.147)	(0.147)	(0.147)		
SE-30	0.190	0.238	0.327	0.187	0.062	0.279	0.208	0.312	0.314	0.307	0.289	0.998	0.10
	(0.093)	(0.161)	(0.122)	(0.115)	(0.288)	(0.115)	(0.288)	(0.288)	(0.392)	(0.392)	(0.392)		
Ucon polar	0.199	0.255	0.287	0.179	0.024	0.311	0.205	0.334	0.291	0.294	0.298	0.998	0.10
	(0.071)	(0.123)	(0.093)	(0.088)	(0.221)	(0.088)	(0.221)	(0.221)	(0.301)	(0.301)	(0.301)		
Carbowax 20M	0.158	0.249	0.263	0.132	−0.029	0.282	0.189	0.282	0.271	0.261	0.256	0.999	0.08
	(0.050)	(0.086)	(0.065)	(0.062)	(0.155)	(0.062)	(0.155)	(0.155)	(0.211)	(0.211)	(0.211)		
XF-1150	0.142	0.176	0.254	0.092	−0.061	0.228	0.138	0.257	0.230	0.248	0.260	0.998	0.10
	(0.072)	(0.126)	(0.095)	(0.090)	(0.255)	(0.089)	(0.225)	(0.225)	(0.306)	(0.306)	(0.306)		
DEGS	0.084	0.201	0.211	0.049	−0.139	0.226	0.109	0.245	0.231	0.217	0.239	0.996	0.16
	(0.162)	(0.280)	(0.213)	(0.200)	(0.503)	(0.200)	(0.503)	(0.503)	(0.683)	(0.683)	(0.683)		

TABLEAU IV

SYSTÈME SIMPLIFIÉ DES CONTRIBUTIONS DES SITES TOPOLOGIQUES DES MÉTHOXYALCANES

Nous avons introduit le site A_1 dans le foyer de telle sorte que le premier terme de la série soit identique pour les deux populations.

Phase stationnaire	*Paramètres*						r	ψ
	I_{A_2}	I_{A_3}	$I_{B_{12}}$	$I_{B_{13}}$	$I_{C_{113}}$	I_P		
Apiezon L	0.228	0.279	0.211	0.064	0.226	0.330	0.998	0.06
SE-30	0.192	0.241	0.194	0.072	0.198	0.305	0.996	0.10
Ucon polar	0.193	0.255	0.168	0.019	0.197	0.304	0.997	0.08
Carbowax 20M	0.153	0.246	0.127	−0.034	0.189	0.271	0.997	0.08
XF-1150	0.143	0.178	0.095	−0.054	0.131	0.244	0.996	0.10
DEGS	0.081	0.203	0.041	−0.139	0.097	0.228	0.991	0.15

La relation "rétention–structure" établie à partir de 18 éthers fait intervenir 12 paramètres. L'examen des vecteurs information met en évidence la nécessité de tenir compte de termes d'interaction. En effet, la valeur de la contribution d'un maillon méthylène de la chaîne linéaire n'est pas rigoureusement constante, comme le montrent les valeurs de I (A_1, B_{11}, C_{111}, D, E, F, G). Néanmoins, dans une première approximation nous pouvons admettre que les n sites P (B_{11}, C_{111}, D, E, F et G) ont une contribution analogue.

Cette simplification qui permet de limiter à sept le nombre de paramètres de l'environnement est compatible avec la loi d'additivité de James–Martin[5,6], ce qui revient à utiliser la relation $\log t'_R = a \cdot n_c + b$ pour les éthers linéaires (n_c étant le nombre d'atomes de carbone de cette chaîne).

Cette simplification trouve sa justification dans la validité des corrélations ainsi redéterminées (Tableau II).

Les mêmes arguments peuvent être repris pour une série de 17 méthoxyalcanes (Tableau III). Il apparaît à nouveau qu'il est possible de procéder à la réduction du nombre de paramètres (Tableau IV).

DISCUSSION DES RÉSULTATS

Pour les deux séries, les valeurs statistiques des régressions obtenues ne sont pas affectées d'une manière sensible par la réduction du nombre de paramètres et nous voyons que la relation de topologie information conserve sa validité.

La proférence[3] des relations ainsi établies permet une estimation raisonnable des temps de rétention de 102 éthoxyalcanes et de 99 méthoxyalcanes, dans un intervalle de confiance de 95%. Notons cependant que dans le cas où les incréments n'englobent pas les interactions entre le foyer et le groupe alkyle R, les prévisions, même si elles sont basées sur des déterminations statistiques excellentes, peuvent perdre une partie de leur signification. Dans notre approche, où le temps de rétention est considéré comme une résultante entre les contributions du foyer et du groupement R, les écarts observés expriment les distorsions de nature géométrique ou électronique.

La variation du temps de rétention réduit relatif avec les changements de structure des éthers est due entre autres à l'action des forces de dispersion et aux interactions dipôle–dipôle qui dépendent à la fois de l'induction de la phase stationnaire et de l'environnement E du foyer de la molécule.

Pour les deux séries de substances et l'ensemble des phases stationnaires sélectionnées, la relation de topologie information s'écrit

$$\log t'_{R_{\varphi_1}} = \sum_{i}^{3} A_i I_{A_i} + \sum_{i=2}^{3} B_{1i} I_{B_{1i}} + C_{112} I_{C_{112}} + nPI_P$$

Dans le cas des méthoxyalcanes le premier terme de l'expression est sommé sur deux sites, alors qu'il l'est sur trois dans le cas des éthoxyalcanes.

Dans notre approximation on constate qu'entre les deux séries d'éthers étudiés les vecteurs information I_P des n sites P sont très voisins pour chacune des phases stationnaires. De plus, la comparaison entre les vecteurs I_P et le coefficient a de la relation de James–Martin montre une concordance des résultats (Tableau V). Le faible écart entre a et I_P indique que la contribution d'un site méthylène n'est que faiblement perturbée lorsqu'on substitue ce site.

TABLEAU V

COMPARAISON DU COEFFICIENT a DE LA RELATION DE JAMES–MARTIN AVEC LE VECTEUR INFORMATION I_P

Phase stationnaire	*Série*			
	MeOR		*EtOR*	
	a	I_P	a	I_P
Apiezon L	0.327	0.330	0.317	0.330
SE-30	0.300	0.305	0.291	0.304
Ucon polar	0.302	0.304	0.285	0.300
Carbowax 20M	0.268	0.271	0.252	0.266
XF-1150	0.242	0.244	0.227	0.247
DEGS	0.223	0.228	0.210	0.231

CONCLUSION

Le système DARC permet d'exprimer convenablement la variation des logarithmes des temps de rétention réduits relatifs des éthers méthyliques et éthyliques. Une relation de topologie information qui fait intervenir les contributions de chacun des sites carbonés des molécules examinées a été déterminée à partir de 35 éthers; elle peut s'appliquer à 201 composés. Compte-tenu de la règle de James–Martin, cette relation peut se simplifier, tout en conservant une précision suffisante à la prévision du log t'_R. Dans cette approximation la grandeur du vecteur contribution I_P d'un chaînon méthylène est très voisine de la valeur du coefficient a de l'expression $\log t'_R = a\, n_c + b$. À la différence de cette dernière, la relation de topologie information reste valable pour des molécules ramifiées.

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Nous tenons à remercier Monsieur J. Chrétien et l'équipe de recherche d'informatique chimique du Professeur J. E. Dubois pour l'aide qu'ils nous ont apportée dans la résolution de certains calculs pour lesquels la configuration de notre ordinateur était insuffisante.

RÉSUMÉ

La méthode de topologie–information permet d'exprimer la variation de log t'_R des alcoxyalcanes en fonction de leur structure. La relation de topologie information dégagée s'applique à 201 composés. Elle a été établie grâce à la détermination des contributions des sites d'une population de 35 éthers.

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PROGRAMMED MULTIPLE DEVELOPMENT

LATERAL SPOT RECONCENTRATION

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SUMMARY

Normal programmed multiple development (PMD) causes longitudinal spot reconcentration, but does not counter lateral diffusion. If the periodic evaporation of solvent characteristic of PMD is caused to occur preferentially along the center line of the developing chromatogram, solvent from each side flows with a component toward that first-drying center line. This flow produces lateral spot reconcentration. Normal PMD complemented by lateral spot reconcentration is called centered PMD. An easily reproducible and usable mask produces centered PMD, which is demonstrated on ordinary precoated plates. Grooves in the thin-layer bed can also produce centered PMD. A centered-PMD spot produced on an ordinary pre-coated plate has a minimized, round area 1–3 mm in diameter. With a given plate, this area reflects the whole of a given molecular population. Such a spot does not spread with longer developments, but merely separates further from its neighbors.

INTRODUCTION

Programmed multiple development (PMD)[1-9] is a form of thin layer chromatography (TLC)[10,11]. In PMD, the TLC plate remains at all times in contact with the solvent. Therefore throughout each PMD the solvent moves by capillary action toward the solvent front. The location of the solvent front is governed by the rate of solvent evaporation.

The rate of solvent evaporation is varied automatically, in accordance with the given PMD program chosen by the operator. In consequence, the solvent front moves (characteristically for PMD) farther up the plate with each solvent advance, but returns to the point of spot deposition with each solvent removal.

Each time the solvent front moves up or down across a spot, the spot becomes reconcentrated. During solvent advance, molecules behind the front move toward molecules not yet reached. During solvent removal, molecules behind the front move toward molecules that have been deposited by the receding solvent front. Thus, twice per PMD cycle, each spot is reconcentrated longitudinally.

Heretofore, no method has been reported for countering the lateral diffusion

of spot molecules, except channeled plates. Channels mechanically limit the lateral diffusion of the spot molecules. However, channels do not cause lateral spot reconcentration, in which the spot molecules actually move laterally toward the centers of their respective spots.

This paper reports a convenient PMD method that, while preserving normal PMD longitudinal spot reconcentration, at the same time achieves lateral spot reconcentration on conventional, precoated, unchanneled TLC plates.

EXPERIMENTAL

Conventional TLC equipment

Pre-coated silica gel G TLC plates, calibrated micropipettes, and dye mixtures were purchased from Camag, New Berlin, Wisc., U.S.A. For use, the plates were cut to the sizes desired.

PMD equipment

The Model 2000 Programmer, the Model 222 Developer, PTFE spacers, and facing plates, all from the Regis Chemical Company, Morton Grove, Ill., U.S.A., were used.

Masks

The masks were hand-made, cut by a razor from brown kraft paper and household aluminum foil.

Each mask was made as wide as the plate with which it was to be used, but 20 mm less in length. Thus, in use, a mask does not dip into the solvent.

Each mask contained three or more slots, mutually aligned, and vertical in use. Each slot was 3 mm wide and extended to about 5 mm from the top and the bottom of the mask. The slot centers were spaced regularly, 15 mm or more apart (see Fig. 1).

Test solutions

The dye mixture to be used was prepared by mixing approximately equal volumes of Camag dye mixtures I, II, and IIN. From this full-strength composite, a one-tenth-strength solution was prepared by dilution with reagent-grade benzene.

Spotting

Each spot was made by applying 1 μl of the chosen solution to a point 25 mm from the edge of the plate.

If a mask was to be used, it was placed under the plate during spotting. The spots were then placed over the mask slots, which could be easily seen through the plate.

Mask use

In use, a mask is held between the TLC plate and the radiator, its reflective side toward the radiator.

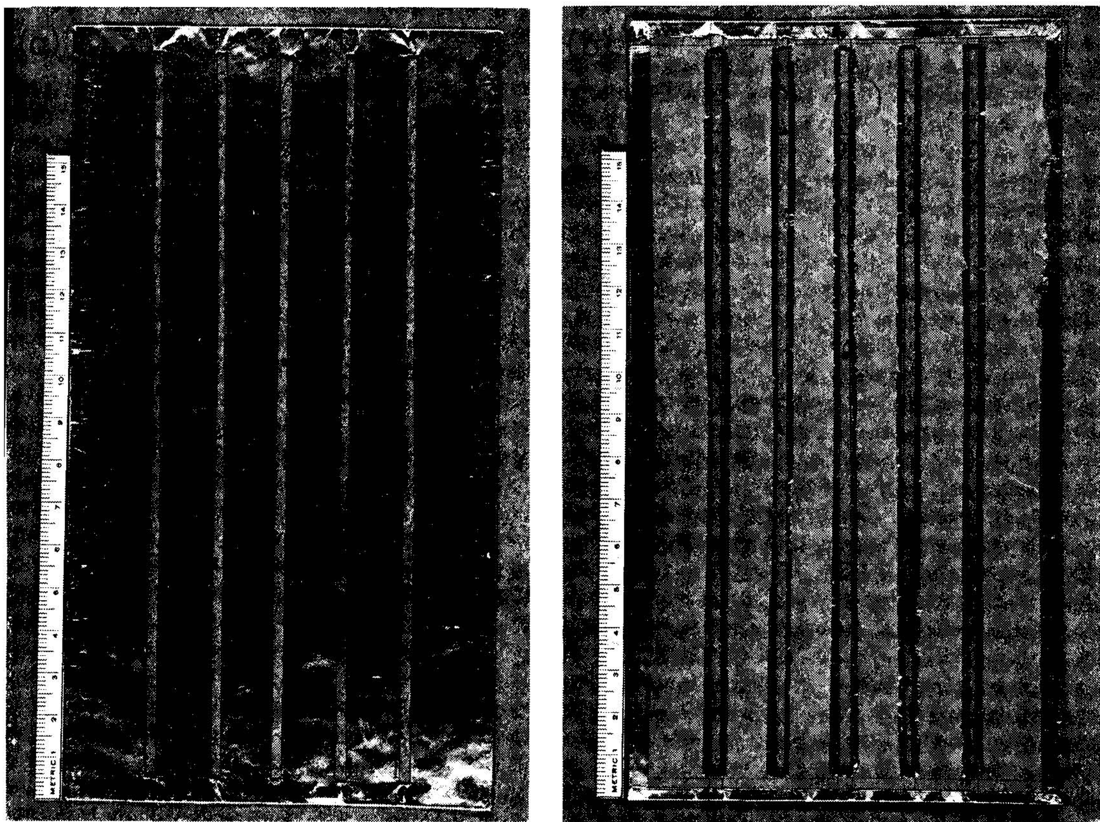

Fig. 1. Usable mask, made from kraft paper covered by aluminum foil. (a) Reflective aluminum surface faces radiators in use; (b) back of mask faces TLC plate in use.

TLC plate preparation and use

The TLC plates were prepared and developed in the fashion normal for PMD. A 1-mm wide strip of bed is removed 10 mm from each vertical edge of the plate. In use, the TLC plate is separated from a matching glass facing plate by a PTFE spacer; spring clamps hold the assembly, including a mask if one is used (Fig. 2).

Also, a vapor-trapping 15-mm wide aluminum foil strip around the outside of the assembly is advisable with either conventional or PMD developments if the plate measures 200 mm in the vertical direction. Otherwise, the solvent loss as vapor through the sides of the chamber can approach or equal the solvent flow upward when the solvent front is far up the plate; the solvent front will in consequence either advance abnormally slowly or stop completely. (The outside strip stops short of dipping into the solvent.)

Developments

The development conditions for the plates are given in Table I.

A rough guide for correlation of the developed spots is given in Table II.

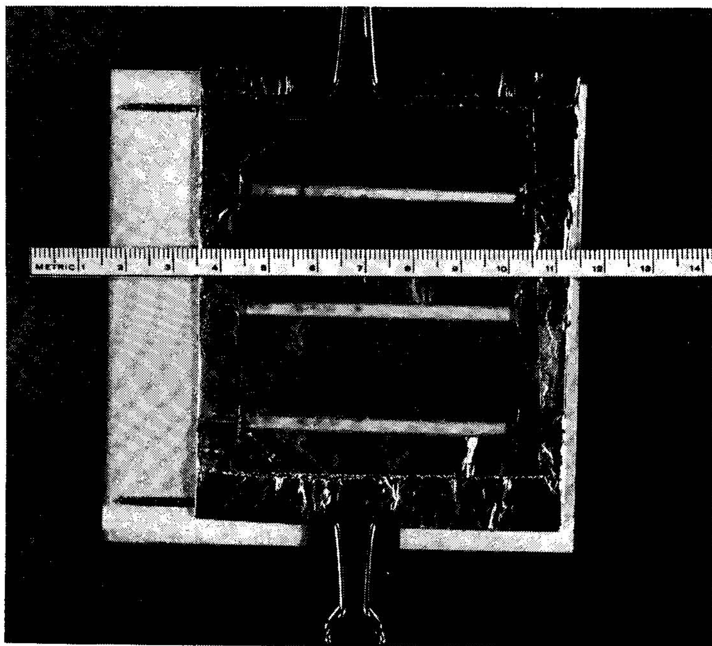

Fig. 2. The mask in use. The mask is then merely included as part of the plate assembly. The reflective foil is placed away from the plate, toward the radiator. The spots are placed under the slots in the mask.

TABLE I

DEVELOPMENT CONDITIONS FOR THE PLATES

Figure	*Method*	*Cycles*	*Unit times (sec)*		*Overall time (h)*
			Solvent advance	*Solvent removal*	
3	Conv.*				1.3
4	PMD**	9***	100	100§	1.5
5	PMD**	32***	100	20§§	17.6

* Developed by benzene; solvent front marked.

** Developed by chloroform–carbon tetrachloride (1:2).

*** Cycles in Mode 1. In Mode 1, the solvent advance time T in a given cycle is $T = n\,t$, for a given cycle of number n and unit advance time t. Thus for the unit advance time of 100 sec, the successive advance times are 100, 200, 300, ··· (100 n) sec.

§ Fixed time. Fixed solvent removal times are invariant.

§§ Scheduled time. Scheduled solvent removal times follow the solvent advance Mode law***.

RESULTS AND DISCUSSION

We shall call that PMD that includes lateral as well as longitudinal spot reconcentration, centered PMD.

The results of applying centered PMD are illustrated in Figs. 4 and 5. It can

TABLE II

CORRELATION OF THE DEVELOPED SPOTS

*Spot**	*Position (mm)*		
	Fig. 3	*Fig. 4*	*Fig. 5*
4, 5	97	45	98 (spot 4), 91 (spot 5)
6	80	42	85
7, 8	70	38	77 (spot 7), 73 (spot 8)
9	60	35	62
10	50	32	51
11	37	28	43

* For further correlation and origin of spot numbers, see Figs. 6 and 7 in ref. 12.

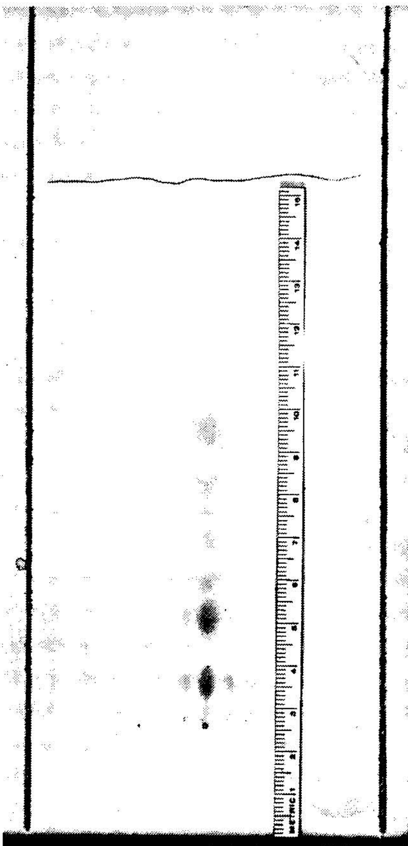

Fig. 3. Three conventionally developed, 80-min chromatograms. The center chromatogram was made from the full-strength dye solution; the outer two chromatograms were made from the same dye mixture at one-tenth strength.

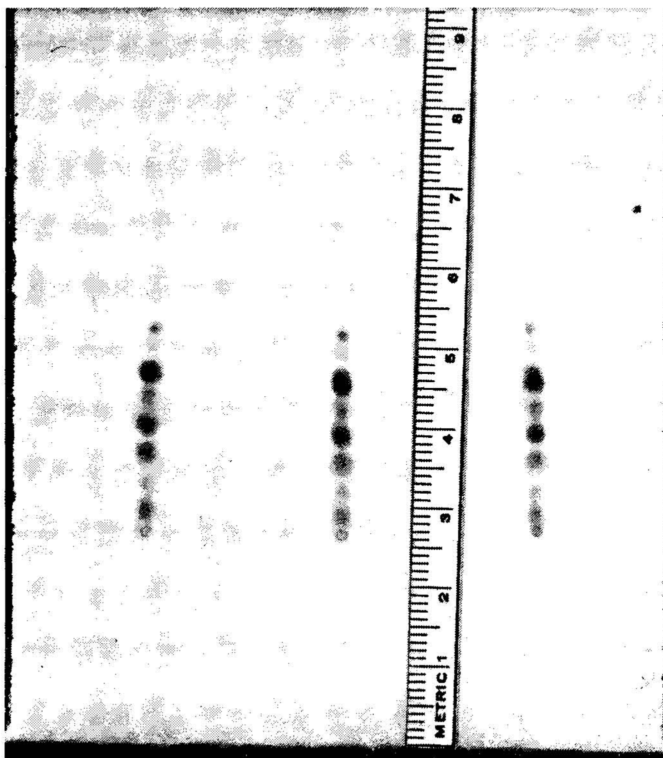

Fig. 4. Centered-PMD chromatograms, 90 min, each corresponding to each of the two outer chromatograms of Fig. 3. The spot diameter roughly indicates the relative concentration of a dye component, as can be seen. The minimized spot diameter, shown by spots at approximately 50 and 52 mm, is 1 to 2 mm. Chromatograms are all made from one-tenth-strength dye solution.

be seen that with centered PMD, spot area greater than a certain minimum reflects only one variable: spot loading.

Mechanism

The mechanism that leads to lateral spot reconcentration is easily explained. The thin-layer bed on each side of the developing chromatogram is shielded from direct infrared radiation. Therefore, when the radiator comes on to effect solvent removal, the solvent under each slot is directly heated by the radiation. This solvent begins to evaporate considerably faster than the cooler, shaded solvent to each side.

The solvent front under each slot soon develops a characteristic dip. This dip shows the desired, preferential evaporation and ultimate depletion of solvent from the line of the chromatogram.

The dips in the solvent front deepen as the radiant heating continues. Solvent, however, always flows normally with respect to the local solvent front. The direction of solvent flow into either side of one of these characteristic dips therefore has a lateral, chromatogram-centered component. Moving with such solvent flow, spot molecules move toward their respective centers. All the spots along the chromatogram are laterally reconcentrated as the cusped solvent front recedes to the origin.

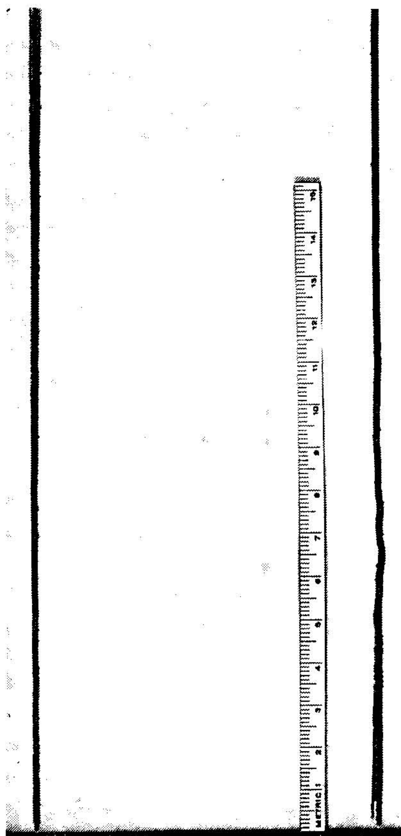

Fig. 5. Centered-PMD chromatograms, 17 h, corresponding to the chromatograms of Fig. 4 and also to the outer two chromatograms of Fig. 3. The oblong, Fig. 4 spot at 45–47 mm has become the Fig. 5 spots at 93 and 98 mm. Spot diameters are not functions of R_F or development time, but primarily of molecular population. Chromatograms are all made from one-tenth-strength dye solution.

After solvent removal, the radiator is usually turned off for solvent advance. It need not be. The chromatogram shown in Fig. 4, for instance, was made with the radiator kept on at lowest power (1.25% of maximum) during solvent advance. However, the advancing solvent front was not cusped. The effect of such radiant heating during solvent advance is essentially the same with centered as with normal PMD: the chromatogram is compressed longitudinally. Thus, lateral spot reconcentration seems to be primarily a result of interaction of the receding solvent front with the spot.

Solvent removal has been shown to be more effective than solvent advance in longitudinal spot reconcentration involving heating[9]. Also, the slower the solvent removal, the more effective the resultant longitudinal spot reconcentration[1,9]. Therefore both longitudinal and lateral spot reconcentration are more effective if solvent removal is carried out with scheduled rather than fixed time.

Techniques

The slots in the mask apparently need not be narrower than those used here. Slot widths from 1 mm to 10 mm were tried, though not tested rigorously. The 3-mm

slots are reasonably easy to make, reproducible from slot to slot and level to level, and wider than the spots they produce. The lateral width of the spots from 3-mm wide slots depends primarily on loading, as shown particularly in Fig. 5.

Spots that are reconcentrated both longitudinally and laterally are usually round. With normal PMD, the top-to-bottom spot width approaches a minimum of perhaps 25 particle diameters with zero loading. With plates such as these, that is about 0.8 mm. Thus the round spots generated here similarly show diameters that approach an 0.8 mm minimum.

The spacing of the slots was not investigated. A spacing closer than the 15 mm minimum used here may be quite adequate. However, enough slots should be used to cover the width of the plate, so as to have a reasonably uniform (though cusped) receding solvent front.

For those developments in which the solvent does not advance more than 30 or 40 mm past the spot origin, the narrow rectangular slots of the masks used in this work are adequate. For longer solvent advances, slots shaped narrow at the top and wide at the bottom would be better. With these, solvent front recession would start desirably slowly but eventually, and necessarily for PMD, continue to the origin.

Spot area and molecular population

The centered-PMD chromatograms of Figs. 4 (90 min) and 5 (17 h) were made with one-tenth-strength dye solution, as were the outer two conventional chromatograms (80 min) in Fig. 3. Diffusion, uncountered either longitudinally or laterally in the two outer conventional chromatograms, quickly renders them essentially unusable. Even with normal PMD, uncountered lateral diffusion would seriously diminish the sensitivity of higher-R_F spots if the development were continued for a number of hours, say, over 10.

Under centered PMD, molecules can no longer leave their respective spots. Therefore developing spots, once isolated, thereafter remain constant in area. The molecular populations of fully separated spots change only by adventitious, well-known, but always unwelcome mechanisms such as photodecomposition. One must accept this possibility and be ready to take counter-measures such as, with photodecomposition, protecting the developing plate from possibly harmful ultraviolet or even visible light.

Centered PMD makes possible the use of conventional unchanneled plates for separations that require very high numbers of multiple developments, without any loss of sensitivity through diffusion. Fig. 5 suggests the potential of this approach, which has not previously been available. Therefore a further demonstrative study of such an extended separation has been prepared[12].

Centered PMD further improves the sensitivity enhancement of normal PMD[3]. This improvement will be more closely defined in a forthcoming study.

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CHROM. 8302

THE DETECTION OF ORGANIC SOLVENT PRESERVATIVES IN WOOD BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A simple and rapid method for the detection of common organic solvent wood preservative materials is described. The method uses thin-layer chromatography after leaching of the preservative from sections of timber with chloroform. R_F values and the sensitivity of the method are discussed and a routine procedure for identification of the organic and organometallic preservatives is proposed. Methods of achieving greater sensitivity for particular applications are also described.

INTRODUCTION

A number of thin-layer chromatographic (TLC) methods have been developed in recent years for the detection of various fungicides and insecticides in a range of matrices. Methods have been suggested for the determination of chlorinated pesticides[1–4], organophosphorus and carbamate insecticides[5,6] and organomercury fungicides[7].

Challen and Kučera[8] devised a method which was applicable to PCP, OPP, PCN, TBTO and γ-BHC*, but the limits of detection which they found (50 μg) were too high for detection of preservative in small sections of wood. They used a four hour Soxhlet extraction to dissolve the preservative, a lengthy procedure which is unsuitable for routine work. They also encountered considerable problems associated with extractives in the wood and inconsistent R_F values were obtained. Specific TLC methods for PCP, MCN and γ-BHC have been published[9].

The present paper describes a routine TLC method for the determination of the fungicides and insecticides commonly used in wood preservative formulations. Despite the plethora of commercial preservatives which are available today, the basic components of the organic and organo-metallic preservatives are relatively few. The

* Abbreviations used: BHC = benzenehexachloride; CuN = copper naphthenate; LPCP = pentachlorophenyl laurate; MCN = monochloronaphthalene; OPP = *o*-phenylphenol; PCN = polychloronaphthalene; PCP = pentachlorophenol; TBTO = tributyl tin oxide; ZnN = zinc naphthenate.

method was therefore oriented towards detection of the following ten preservative materials: PCP, OPP, LPCP, MCN, PCN, γ-BHC, dieldrin, ZnN, CuN and TBTO.

These preservatives are usually applied as solutions in hydrocarbon solvents by brushing, spraying or immersion, and in some cases by special plant (*e.g.*, double vacuum process). They are often present in small amounts located at or close to the surface of the timber to which they are applied, and methods of detection need to be sensitive and applicable to small samples. It must also be remembered that some of the chemicals used as wood preservatives are mixtures rather than pure compounds. In some cases this may lead to several spots for one ingredient, and to streaking, and it is important to compare the chromatograms with those for known materials.

Despite an exhaustive search it was found impossible to separate all ten preservatives using one solvent/plate system, and a two-plate/two-solvent system was therefore employed.

APPARATUS AND REAGENTS

Apparatus

Chromatographic tanks: Shandon Chromatanks 25 cm × 10 cm × 20 cm lined with Whatman No. 1 chromatography paper. TLC plates: Merck (Darmstadt, G.F.R.) pre-coated chromatography plates, cellulose F (0.1 mm) and silica gel 60 (0.25 mm) without fluorescent indicator. Two plate sizes (20 × 20 cm and 5 × 20 cm) were used. Disposable micro pipettes: Drummond Microcaps (1 μl and 5 μl). Spray units: Pulvérisateur Armand Vaast from Etab^{TS} Vaast (Paris, France). Ultraviolet (UV) lamp: Hanovia.

Reagents

Silver nitrate solution: 0.25 g of silver nitrate in 100 ml of 66% acetone. Catechol violet solution: 0.05 g catechol violet in 100 ml ethanol. Chrome azurol solution: 0.5 g of chrome azurol S and 5.0 g of anhydrous sodium acetate in 100 ml ethanol. Dithizone solution: 0.01 g of dithizone in 100 ml of chloroform. Brentamine solution: 0.5 g of brentamine fast red G in 100 ml of 50% acetone. Sodium acetate–carbonate solution: 12.5 g of anhydrous sodium acetate and 5 g anhydrous sodium carbonate in 100 ml water. Standard solutions: 0.5 g preservative in 100 ml chloroform (ten separate solutions, store away from direct sunlight). Developing solvent 1: cyclohexane–acetone–liquid paraffin 15:3:2. Developing solvent 2: ethyl acetate–glacial acetic acid (2:1).

NORMAL DETECTION PROCEDURE

Extraction

A chloroform extract of the wood was prepared by removal of the surface millimetre of the sample with a chisel, rasp or microtome and saturation of the shavings with analytical-reagent grade chloroform in a 50 ml beaker covered with a watch glass: the mixture was heated on a hot plate at 50° for ten minutes, care being taken that the wood remained saturated with chloroform but the volume of the extract being kept to a minimum to achieve maximum sensitivity. The extract was then allowed to cool to room temperature and divided into two portions, before application to

TABLE I

R_F VALUES

Compound	Plate	Developing solvent	R_F values	
			Unwashed plate	Washed plate
OPP	Silica gel	1	0.21	0.37
PCP	Silica gel	1	0.25	0.32
γ-BHC	Silica gel	1	0.50	0.69
Dieldrin	Silica gel	1	0.61	0.76
MCN	Silica gel	1	0.68	0.85
PCN	Silica gel	1	0.78	0.94
LPCP	Silica gel	1	0.87	0.96
TBTO	Cellulose	2	0.98	1.00
CuN	Cellulose	2	0.89	0.80
ZnN	Cellulose	2	0.75	0.30

the TLC plates. If the presence of the anti-stain agent sodium pentachlorophenate is being investigated then the extractions must be carried out using methanol.

Organic preservatives

PCP, OPP, LPCP, PCN, MCN, γ-BHC and dieldrin were separated on silica gel plates using developing solvent 1. 1–5 μl of the chloroform extract was spotted on to the start line of the chromatographic plate (2 cm from the bottom edge of the plate). 1-μl volumes of suitable standards were also spotted at 1-cm intervals along the start line. After drying in an air stream for 10 sec the plates were developed using developing solvent 1 in a Shandon Chromatank. The solvent front was allowed to travel 14–16 cm for maximum separation (approx. 1½ h). The plate was allowed to air-dry for 5 min and exposed to unfiltered UV light for 30 min. Then the plate was sprayed uniformly with silver nitrate solution and irradiated for a further period of 30 min using filtered UV light. The preservatives were then visible as dark brown spots on a light brown background. The R_F values obtained using this system are shown in Table I.

It was necessary to carry out a specific test for OPP as this compound is not made visible using the above spray reagent. If OPP was present, a purple-brown spot was observed after the first exposure period. Although this provides an identification of the compound by comparison with a standard, a confirmatory test should be applied. By spraying the immediate area of the spot with brentamine solution, a bright yellow spot is obtained which on further spraying with sodium acetate–carbonate solution after drying of the plate produces a purple spot. The use of this spray is worthwhile to avoid masking of the spot by wood extractives which travel between 1/5 and 1/4 of the distance moved by the solvent up the plate. The detection limits using this system for organic preservatives are shown in Table II. The sensitivities obtained thus are adequate for most routine determinations of preservatives in wood, but a more sensitive technique is described later.

Organometallic preservatives

TBTO, ZnN and CuN were separated on cellulose plates using developing

TABLE II

DETECTION LIMITS

Compound	*Detection limit (μg), unwashed plates*	*Detection limit (μg), washed plates*
OPP	0.70	0.40
PCP	0.06	0.025
γ-BHC	1.0	0.40
Dieldrin	0.45	0.20
MCN	0.2	0.2
PCN	0.85	0.10
LPCP	0.30	0.30
TBTO	0.04	0.015
CuN	0.07	0.03
ZnN	0.05	0.01

solvent 2. 1–5 μl of the chloroform extract was spotted on to the start line of the chromatographic plate. 1-μl volumes of suitable standards were also spotted at 1-cm intervals along the start line. After drying in an air stream the plate was developed in a Shandon Chromatank. The solvent front was allowed to travel 14–16 cm (1 h). The plate was allowed to air-dry for 5 min and exposed to unfiltered UV light for 30 min. The plate was divided into three approximately equal parts by two pencil lines parallel to the base line. The top third was sprayed with catechol violet solution. If tin was present it was visualised as a sky blue spot on a light yellow background. The central portion of the plate was sprayed either with chrome azurol S solution or with catechol violet solution to visualise the copper. Catechol violet was the most sensitive of the two reagents, but chrome azurol S solution should be used if a high concentration of tin is present in the sample. The lower portion of the plate was sprayed with dithizone solution to visualise the zinc, as a pink streak on a blue background. The R_F values obtained using this system are shown in Table I. Both copper and zinc produce a streak rather than a discrete spot presumably due to the diversity of naphthenic compounds present in commercial preparations. R_F values for the leading edge are given as an indication of position on the plate, rather than a definitive value for these two preservatives, as the R_F tends to change by up to 20% depending on the concentration and composition of the naphthenate mixtures. The detection limits obtained for the organometallic preservatives were generally better than for the organic preservatives and are shown in Table II.

Spray reagents for copper and zinc can be applied directly to treated wood but this method does not distinguish between salt and organic solvent type preservatives.

HIGHER-SENSITIVITY TECHNIQUE

The method as described so far is adequate for most routine procedures. However, in some cases greater sensitivity may be required and this can be achieved by use of a more elaborate procedure. The main factors which are significant for the improvement of sensitivity are:

(a) Use of pre-washed plates: by allowing a solution of 80% acetone to traverse the full length of the plate in a Chromatank a considerable amount of the background

colouration of the plate can be removed. The plate should be allowed to air-dry before use after washing. Removal of the impurities from the plate causes a considerable change in R_F values and the R_F values found using a washed plate are shown in Table I.

(b) Longer irradiation time: for the organic preservatives the lengthening of the second period of exposure to UV light from 30 to 60 min improves sensitivity.

(c) Use of back-light viewer: in some cases, particularly on washed plates the use of a back-light viewer was found to improve sensitivity slightly.

(d) Use of Stahl (TAS) oven[10]: for small samples (10–15 mg) where extraction would be difficult, the use of a Stahl (TAS) oven is recommended. All the volatile preservatives can be transferred directly from sample to the plate by this procedure (but not CuN or ZnN) and direct spotting onto the plate is an obvious concentration factor. The method avoids contamination by most wood extractives but separate tests are necessary if the presence of copper or zinc naphthenates is suspected.

INTERFERENCES

Wherever possible samples should be taken from sapwood areas. The sapwood usually contains more preservative than the heartwood and causes little problem with extractives. However, identification is still adequate when heartwood samples are used. Care must be taken when paint or other finishes have been applied to the timber since these often contain both copper and zinc, but again identification is not seriously affected. Commercial preparations of PCP often contain lower chlorophenols as impurities; these will resolve themselves on the plate as a series of decreasing shadow spots directly ahead of the pentachlorophenol spot.

SCOPE OF THE METHOD

The method has been applied over a period of several years in this laboratory to a variety of timber samples. It has been found to be rapid, efficient and reproducible. The technique has been applied to the detection of anti-staining agents at very low level, and to establishing of the composition of preservative formulations.

ACKNOWLEDGEMENT

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CHROM. 8256

ÉLECTROPHORÈSE PRÉPARATIVE DES GLYCOSAMINOGLYCANES-PEPTIDES

APPLICATION AU FRACTIONNEMENT DES GLYCOSAMINOGLYCANES-PEPTIDES DE LA PAROI ARTÉRIELLE

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SUMMARY

Preparative electrophoresis of peptidic glycosaminoglycans. Application to the fractionation of peptidic glycosaminoglycans of arterial wall

A new procedure for the fractionation of glycosaminoglycans by electrophoresis on Pevikon has been described. Mixtures of glycosaminoglycans were fractionated by preparative electrophoresis on Pevikon in pyridine formate or glycine–HCl buffers. By this procedure, 200 mg of a mixture of hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate isolated from arterial wall could be successfully separated without loss of material. The purified fractions were analysed by enzymatic and chemical procedures. The molar ratios of uronic acid to hexosamine and of sulphate to hexosamine and the amino acid content of each glycosaminoglycan have been determined. The peptidic content (less than 1%) is represented by five amino acids, *viz.* serine, glycine, alanine, aspartic acid, and glutamic acid.

INTRODUCTION

De nombreuses méthodes analytiques permettent de caractériser les différents glycosaminoglycanes mais la préparation de ces substances à l'état pur se révèle souvent laborieuse. Des méthodes préparatives ont été préalablement envisagées par fractionnement sur colonne de cellulose[1], ECTEOLA-cellulose[2,3,21] et DEAE-cellulose[4,5]. Ces méthodes ont été décrites avec succès pour l'isolement des glycosaminoglycanes de cartilage, tissu ne renfermant que deux types différents de glycosaminoglycanes. Ces méthodes sont difficiles à utiliser dans le cas de tissus se distinguant par une grande variété dans leur composition en glycosaminoglycanes. L'abondance des fractions obtenues ne permet pas d'obtenir avec un bon rendement des glycosaminoglycanes à l'état pur.

Pour ces raisons, nous nous sommes proposés de décrire une méthode de fractionnement électrophorétique permettant d'obtenir avec un rendement satisfaisant des glycosaminoglycanes à partir de mélanges complexes tels que la paroi artérielle en renferme. En outre, dans toutes les méthodes de fractionnement décrites, les différents glycosaminoglycanes présents dans les fractions étaient identifiés par des méthodes souvent peu spécifiques et les déterminations des rapports hexosamines/acides uroniques ne pouvaient constituer des critères de pureté satisfaisants. Dans le présent travail, au cours du fractionnement nous avons eu recours à des tests enzymatiques spécifiques pour l'identification de nos produits.

MATÉRIEL ET MÉTHODES

Préparation des glycosaminoglycanes-peptides

Les artères aortes de porc sont prélevées, lavées dans une solution de NaCl à 0.9%. Les différentes tuniques (média-intima, adventice) sont séparées. Les tissus finement coupés sont délipidés à +4° par lavage dans trois bains d'acétone. Les glycosaminoglycanes-peptides sont obtenus après digestion protéolytique de la poudre acétonique par la pronase: 1 g pour 100 g de tissu sec en 0.1 *M* Tris–0.004 *M* $CaCl_2$ (pH 8.5) à 56°.

La digestion s'effectue pendant 96 h; l'enzyme est renouvelé toutes les 24 h. Ce temps d'hydrolyse a été retenu après une étude cinétique systèmatique de la dégradation des protéoglycanes par la pronase.

Après inactivation de l'enzyme par la chaleur, le milieu de protéolyse est centrifugé puis dialysé contre de l'eau courante et de l'eau distillée. Les glycosaminoglycanes-peptides sont ensuite précipités par le chlorure de cétyl-pyridinium à la concentration finale de 1% en 0.03 *M* NaCl. Le précipité est solubilisé en 1.25 *M* NaCl–0.3% CPC. Les glycosaminoglycanes-peptides sont alors précipités par trois volumes d'éthanol pendant une nuit à +4° en présence d'acétate de sodium à 5%.

Séparation des glycosaminoglycanes-peptides

Dans un but préparatif, les glycosaminoglycanes-peptides sont fractionnés par électrophorèse sur un copolymère de chlorure de polyvinyle et d'acétate de polyvinyle (Pévikon).

Préparation du support électrophorétique

Le Pévikon est mis en suspension dans de l'eau distillée préalablement chauffée à 50°. Après décantation, le surnageant est prélevé par aspiration. L'opération est renouvelée plusieurs fois de manière à assurer une granulation homogène. Après la dernière décantation, le Pévikon étalé sur plusieurs épaisseurs de papier filtre est séché à l'étuve à 37°. Le Pévikon séché est mis en suspension dans le tampon quelques heures avant l'utilisation. Après décantation, l'excès de tampon est prélevé par aspiration. Le Pévikon est alors étalé sur la plaque réfrigérée d'un appareil d'électrophorèse à haut voltage de type Pherograph.

Le support électrophorétique ainsi constitué mesure 350 mm de long sur 150 mm de large et 15 mm d'épaisseur. Une gouttière de 50 mm de long sur 10 mm de large et 10 mm d'épaisseur est creusée à 100 mm de l'extrémité cathodique de la plaque. Dans cette gouttière est déposée la solution de glycosaminoglycanes à

séparer préalablement reprise par du Pévikon sec. Latéralement et dans le prolongement de cette gouttière sont également creusées deux gouttières annexes de 10 mm de long sur 10 mm de large et 10 mm d'épaisseur destinées à recevoir les glycosaminoglycanes marqueurs préparés selon le protocole décrit ci-dessous.

Préparation des marqueurs colorés

La migration électrophorétique est conduite en présence de marqueurs colorés. Dans le cas présent ce seront des glycosaminoglycanes d'aorte préparés selon la méthode de Dudman et Bishop[6]. À cette fin 10–20 mg de glycosaminoglycanes totaux isolés de paroi artérielle sont colorés par le rouge procion (I.C.I., Macclesfield, Grande Bretagne) soumis à une gel filtration sur Sephadex G-25 pour éliminer l'excès de colorant et les sels, comme cela a été décrit par ailleurs[7]. Les glycosaminoglycanes ainsi colorés sont concentrés. Leur mobilité électrophorétique reste inchangée après coloration.

Modalités de l'électrophorèse

L'électrophorèse est effectuée à 0° sous une différence de potentiel de 600 V pendant 5 h dans les systèmes tampons suivants: glycocolle–HCl, 0.05 *M*, pH 2.0; acétate de baryum, 0.015 *M*, pH 7.0; formate de pyridine, 0.05 *M*, pH 2.3. Le dépôt de glycosaminoglycanes totaux est compris entre 50 et 100 mg. La présence de marqueurs colorés déposés latéralement permet de suivre aisément la migration.

Après migration, la plaque de Pévikon est découpée par zones de 1 cm de largeur. Les glycosaminoglycanes sont élués par de l'eau distillée. Le Pévikon est alors éliminé par filtration sur verre frité. Les solutions aqueuses de glycosaminoglycanes sont alors évaporées à sec et pour chacune d'entre elles des contrôles analytiques sont effectués.

Identification des glycosaminoglycanes

L'identification des glycosaminoglycanes et le contrôle de leur pureté s'effectuent par des analyses électrophorétiques associés à des tests enzymatiques par des mucopolysaccharidases specifiques —hayluronidase et chondroïtinase— selon la technique précédemment décrite[8,9].

Hydrolyse par l'hyaluronidase

Elle s'effectue dans les conditions expérimentales décrites par Thunell[10]. L'incubation a lieu en présence d'hyaluronidase (Sigma, St. Louis, Mo., États Unis; Type I) dans 80 μl d'une solution tampon de MacIlvaine à pH 7 contenant entre 20 et 50 μg de glycosaminoglycanes à identifier. L'hydrolyse est effectuée à 37° pendant 2 h; un tube témoin ne contenant pas l'enzyme est incubé dans les mêmes conditions.

Hydrolyse par les chondroïtinases

La digestion par ces enzymes est réalisée dans les conditions expérimentales décrites par Yamagata *et al.*[11]. Le milieu d'incubation est constitué par 10 μl d'une solution de tampon Tris enrichi à pH 8.0 à laquelle sont ajoutés 25–60 μg de glycosaminoglycanes, et 0.1 unité de chondroïtinase. L'incubation, dont le volume final ne dépasse pas 100 μl, est réalisée à 37° pendant 30 min. Un tube témoin sans enzyme est incubé dans les mêmes conditions.

Analyse des constituants glucidiques et peptidiques

Constituants glucidiques. Les concentrations en hexosamines, acides uroniques et sulfates sont déterminées pour chacun des glycosaminoglycanes-peptides. Les acides uroniques sont dosés par la méthode au carbazol de Dische[12]. Les hexosamines sont dosées après une hydrolyse réalisée en 4 *N* HCl, 4 h à 100° selon la méthode modifiée d'Elson et Morgan[13]. Les sulfates sont dosés après une hydrolyse réalisée en 4 *N* HCl, 4 h à 100° selon la méthode de Terho et Hartiala[14].

Constituants peptidiques. Les amino acides sont dosés après une hydrolyse acide en 6 *N* HCl, 100° durant 24 h, sur un analyseur Beckman Unichrom selon la technique de Devenyi[15].

Détermination des masses moléculaires

Les masses moléculaires des différents glycosaminoglycanes ont été déterminées selon la méthode de Mathews et Decker[16]. L'électrophorèse a été réalisée dans un gel de polyacrylamide à 5% en tampon 0.05 *M* phosphate à pH 8.0 pendant 4 h, 5 mA par gel. Des glycosaminoglycanes-peptides de masses moléculaires connues, généreusement donnés par Mathews et Decker, sont pris comme référence.

Après électrophorèse, les gels sont colorés par une solution aqueuse au bleu d'Alcian 0.5%(w/v) contenant 3% (v/v) d'acide acétique pendant 1 h. La décoloration s'effectue pendant 15 h dans une solution d'acide acétique à 7% (v/v).

RÉSULTATS

Profil d'élution des glycosaminoglycanes d'aorte sur Pévikon

La séparation des glycosaminoglycanes, effectuée par électrophorèse préparative sur Pévikon dans les différents systèmes tampons, donne par ordre de mobilité croissante: l'acide hyaluronique, l'héparane sulfate, le dermatane sulfate puis les chondroïtines sulfates A et C, dont les mobilités relatives par rapport au chondroïtine sulfate sont respectivement 0.19, 0.53 et 0.75.

La Fig. 1 représente le profil d'élution des différents glycosaminoglycanes-peptides de la média de paroi artérielle de porc. Une seule électrophorèse préparative réalisée en tampon glycocolle–HCl nous permet de séparer les trois principaux glycosaminoglycanes de la média.

Le dermatane sulfate étant présent à l'état de traces dans la média aortique de porc, il est préférable de s'adresser à l'adventice pour préparer ce glycosaminoglycane avec de bons rendements. La Fig. 2 montre l'obtention du dermatane sulfate à partir d'un mélange de glycosaminoglycanes d'adventice.

Contrôle de la pureté des fractions

Les différentes fractions isolées après électrophorèse préparative ont été systématiquement soumises à des contrôles enzymatiques et à des analyses chimiques. Pour contrôler leur pureté, ces fractions ont été mises en présence des diverses mucopolysaccharidases: chondroïtinase AC, chondroïtinase ABC et hyaluronidase. Des électrophorèses analytiques sur cellogel ont été pratiquées avant et après l'action des enzymes sur ces fractions. La Fig. 3 montre les résultats de cette étude.

Des fractions pures ne renfermant qu'un seul glycosaminoglycane sont obtenues avec des rendements satisfaisants. Le Tableau I montre qu'après une électro-

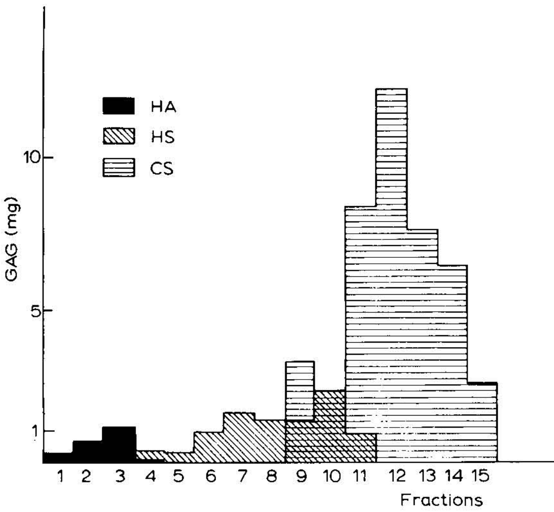

Fig. 1. Profil d'élution des glycosaminoglycanes (GAG) de média d'aorte de porc sur Pévikon. HA = Acide hyaluronique; HS = héparane sulfate; CS = chondroïtine sulfate.

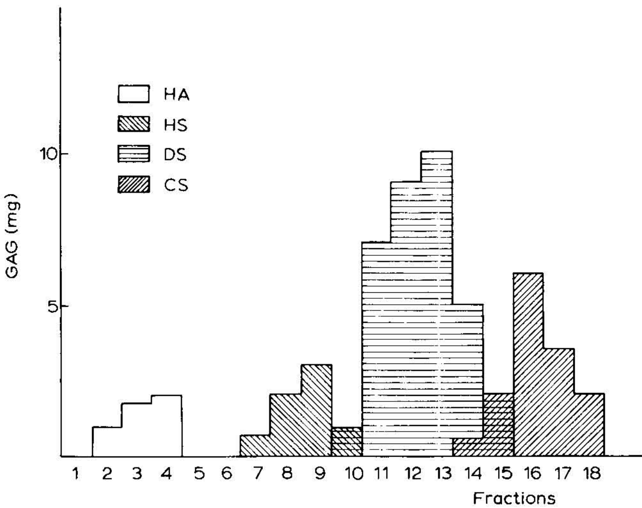

Fig. 2. Profil d'élution des glycosaminoglycanes (GAG) d'adventice d'aorte de porc sur Pévikon. HA = Acide hyaluronique; HS = héparane sulfate; DS = dermatane sulfate; CS = chondroïtine sulfate.

phorèse préparative les glycosaminoglycanes-peptides sont récupérés avec un rendement qui peut atteindre 90%. Plus de 60% de chacun des glycosaminoglycanes est obtenu à l'état pur.

Caractéristiques chimiques des glycosaminoglycanes

Pour chaque type de glycosaminoglycane répondant aux critères de pureté électrophorétique et enzymatique décrits précédemment, les principales caractéristiques chimiques de composition ont été précisées.

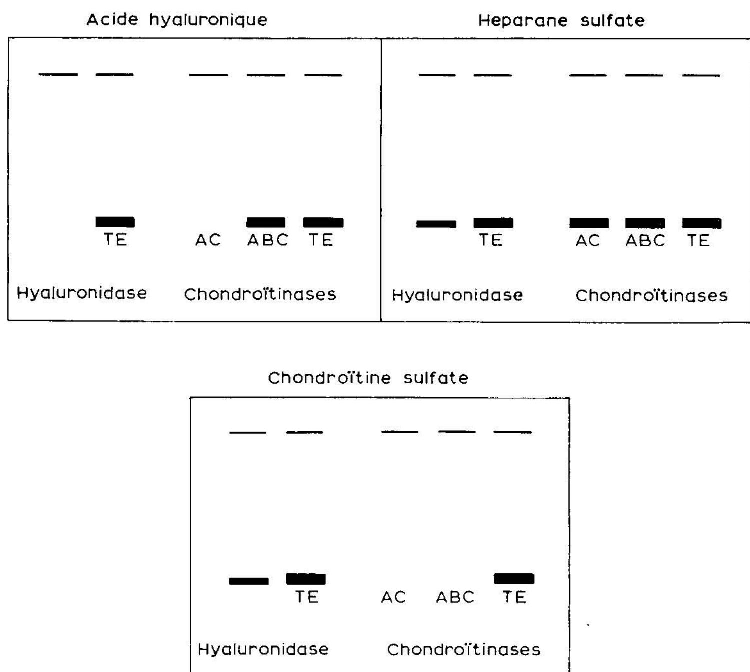

Fig. 3. Electrophorèses sur cellogel des glycosaminoglycanes d'aorte de porc séparés sur Pévikon après action des mucopolysaccharidases.

TABLEAU I

FRACTIONNEMENT ÉLECTROPHORÉTIQUE PRÉPARATIF DES GLYCOSAMINO-GLYCANES DE MÉDIA AORTIQUE DE PORC

(I) Proportions relatives des différents glycosaminoglycanes exprimées en mg pour 100 mg d'un mélange. (II) Récupération des glycosaminoglycanes après électrophorèse sur Pévikon; (a) en mg pour 100 mg du mélange, (b) rendement relatif (%) à chaque glycosaminoglycane. (III) Quantité de glycosaminoglycanes obtenus à l'état pur: (a) en mg pour 100 mg du mélange, (b) rendement relatif (%) à chaque glycosaminoglycane.

Glycosaminoglycane	*I*	*II*		*III*	
		a	*b*	*a*	*b*
Acide hyaluronique	5	4.6	92	4	87
Héparane sulfate	21	17	81	8	47
Chondroïtine sulfate	74	70	94.5	42	67

Composition en hexosamine, acide uronique et sulfate des chaines de glycane. La composition en acide uronique, hexosamine et sulfate figure sur le Tableau II. Les résultats indiquent que les rapports molaires acide uronique/hexosamine sont tous voisins de l'unité. Seul l'héparane sulfate présente un rapport plus élevé égal à 1.5 en accord avec des déterminations récentes[17]. Le rapport molaire sulfate/hexosamine est voisin de l'unité pour l'héparane sulfate et le chondroïtine sulfate. Il est légèrement plus faible pour le dermatane sulfate.

TABLEAU II

COMPOSITION EN HEXOSAMINE, ACIDE URONIQUE ET SULFATE DES DIFFÉRENTS GLYCOSAMINOGLYCANES ISOLÉS DE L'ARTÈRE AORTE DE PORC APRÈS SÉPARATION ÉLECTROPHORÉTIQUE SUR PÉVIKON

Glycosaminoglycane		*Hexosamine*	*Acide uronique**	*Sulfate**	*Hexosamine***	
Artère aorte	*Cartilage*				*Glc N*	*Gal N*
Acide hyaluronique		1	1.05	0	95.4	4.6
Héparane sulfate		1	1.50	0.93	97.4	2.6
Dermatane sulfate		1	1.07	0.70	2.0	98.0
Chondroïtine sulfate		1	0.87	0.98	2.0	98.0
	Chondroïtine sulfate	1	1.09	0.94	2.4	97.6

* Les résultats sont exprimés en mole pour une mole d'hexosamine.
** Proportions relatives des deux hexosamines après identification et dosage à l'auto analyseur Beckman. Les résultats sont exprimés en moles pour 100 moles du mélange des deux hexosamines.

L'hexosamine de l'acide hyaluronique et de l'héparane sulfate est représentée à plus de 98 % par la glucosamine. La galactosamine caractérise le chondroïtine sulfate et le dermatane sulfate.

Composition en acides aminés. La composition en acides aminés est indiquée sur le Tableau III. Elle reste très voisine quelque soit le type de glycosaminoglycane considéré. Elle est caractérisée par une forte proportion en glycocolle (un acide aminé sur 3 ou 4), en sérine (un acide aminé sur 5), en acide aspartique, acide glutamique et alanine (un acide aminé sur 10). Il n'y a pas d'acides aminés soufrés, et en général peu d'acides aminés basiques et aromatiques. Toutefois l'acide hyaluronique et l'héparane sulfate renferment une lysine pour dix acides aminés et le chondroïtine sulfate contient une phénylalanine pour six acides aminés.

Sur le Tableau III figure, à titre de comparaison, les compositions en acides aminés des chondroïtines sulfates de cartilage de porc et de rat préparés au laboratoire par la même méthode de fractionnement. La composition en acides aminés des glycosaminoglycanes varie peu selon la nature et l'origine tissulaire du glycosaminoglycane.

Peu de variations importantes sont notées en fonction de l'espèce, la composition en acides aminés des chondroïtines sulfates de cartilage de porc et de rat étant très voisines (voir Tableau III). Pour tous ces glycosaminoglycanes-peptides, l'acide aspartique, la sérine, l'acide glutamique, le glycocolle et l'alanine représentant près de 80 % des résidus d'acides aminés.

Néanmoins, le contenu peptidique de ces glycosaminoglycanes reste faible; pour 100 moles d'hexosamine, l'héparane sulfate et le dermatane sulfate renferment 5 moles d'acides aminés et le chondroïtine sulfate 2.

Ces résultats montrent la présence dans l'acide hyaluronique d'un résidu peptidique dont la composition est proche de celle des autres résidus peptidiques des différents glycosaminoglycanes-peptides. Ces résultats sont en accord avec la présence d'un hyaluronate protéine dans le tissu conjonctif[18].

Détermination des masses moléculaires

L'analyse des glycosaminoglycanes préalablement séparés par électrophorèse

TABLEAU III

COMPOSITION EN ACIDES AMINÉS DES DIFFÉRENTS GLYCOSAMINOGLYCANES ISOLÉS DE L'ARTÈRE AORTE DE PORC ET DES CHONDROÏTINES SULFATES DE CARTILAGE DE PORC ET DE RAT APRÉS SÉPARATION ÉLECTROPHORÉTIQUE SUR PÉVIKON

Les résultats sont exprimés en nombre de résidus pour 100 résidus.

Acide aminé	*Glycosaminoglycane d'artère aorte de porc*				*Chondroïtine sulfate de cartilage*	
	Acide hyaluronique	*Héparane sulfate*	*Dermatane sulfate*	*Chondroïtine sulfate*	*Porc*	*Rat*
Asp	11.0	10.9	6.1	8.5	5.1	6.9
Thr	6.4	6.1	3.1	5.6	2.8	4.5
Ser	12.9	17.3	15.0	20.5	43.0	40.2
Glu	10.3	13.2	10.0	10.1	9.5	8.2
Pro	5.3	—	5.5	—	6.0	6.1
Gly	23.4	26.4	28.3	30.0	18.4	18.0
Ala	9.6	10.5	11.5	8.6	4.0	4.6
Val	6.5	5.2	3.2	—	—	—
Leu	3.8	3.6	2.5	—	4.5	4.8
Phe	—	—	7.3	14.0	4.5	4.7
Lys	10.8	10.5	1.8	3.0	2.5	2.0

préparative a été complétée par la détermination de leurs masses moléculaires qui figurent sur le Tableau IV. On voit que la masse moléculaire du chondroïtine sulfate de média artérielle est deux fois supérieure à la masse moléculaire du chondroïtine sulfate de cartilage préparée par la même méthode. La masse moléculaire de l'acide hyaluronique, supérieure à 90,000, ne peut être déterminée avec précision.

TABLEAU IV

MASSES MOLÉCULAIRES DES GLYCOSAMINOGLYCANES ISOLÉS D'ARTÈRE AORTE DE PORC ET DE CARTILAGE DE PORC ET DE RAT

Glycosaminoglycane	*Masse moléculaire (daltons)*
Héparane sulfate média	38,000
Dermatane sulfate adventice	16,000
Chondroïtine sulfate média	25,000
Chondroïtine sulfate cartilage (porc)	13,000
Chondroïtine sulfate cartilage (rat)	11,000

DISCUSSION

La présence de mélanges de glycosaminoglycanes dans le tissu conjonctif rend difficile la séparation de ces macromolécules. Si ces difficultés ont été surmontées d'un point de vue analytique[8], peu de méthodes préparatives apparaissent satisfaisantes. C'est ainsi que les méthodes chromatographiques sur DEAE-cellulose[4,5] ou sur colonne d'ECTEOLA-cellulose[2,3] ne présentent une bonne résolution que pour des quantités de produit n'excédant pas 5 mg. La méthode chromatographique sur colonne de CPC-cellulose proposée par Thunell *et al.*[1] a été appliquée à la séparation d'un mélange ren-

fermant 100 mg de glycosaminoglycanes aortiques[19]. Dans ces conditions, il est impossible d'obtenir à l'état pur l'héparane sulfate malgré l'importance quantitative de ce composé au niveau de la paroi artérielle.

La méthode proposée par électrophorèse préparative sur Pévikon présente l'avantage d'obtenir une séparation de tous les constituants d'un mélange de 200 mg de glycosaminoglycanes de paroi artérielle. Elle présente en outre une résolution et un rendement satisfaisants. Elle offre les mêmes avantages lorsqu'elle est appliquée à la séparation d'un mélange de glycosaminoglycanes n'excédant pas 10 mg.

Une seule électrophorèse préparative nous permet d'obtenir à l'état pur les quatre types de glycosaminoglycanes présents au niveau de la paroi artérielle. L'application de cette méthode à des fractionnements de glycosaminoglycanes provenant d'autres formations conjonctives nécessite des vérifications préalables. En effet la densité de charges anioniques d'un glycosaminoglycane peut subir des variations selon l'origine tissulaire du composé. Cette méthode d'excécution aisée est rapide et reproductible. L'identification et le contrôle de la pureté de nos produits ont été facilités par l'emploi de l'analyse électrophorétique sur cellogel associé à des tests enzymatiques et à des déterminations chimiques.

L'étude des principales caractéristiques chimiques des glycosaminoglycanes ainsi isolés, en particulier les rapports molaires acide uronique/hexosamine et sulfate/hexosamine, est en accord avec les données structurales proposées par différents auteurs[20].

Le résidu peptidique de tous ces glycosaminoglycanes est principalement représenté par cinq acides aminés: acide aspartique, sérine, acide glutamique, glycocolle et alanine totalisant à eux seuls 80% des résidus d'acides aminés. Les proportions élevées de ces acides aminés retrouvées par ailleurs par d'autres auteurs[22–24] plaident en faveur d'une séquence, Glu-Gly-Ser-Gly[25,26], envisagée au voisinage de la liaison O-glycosidique.

Nos résultats mettent aussi en évidence la présence d'un hyaluronate peptide dont l'existence dans les tissus conjonctifs n'a été que récemment évoquée[18].

Bien que la détermination des masses moléculaires des glycosaminoglycanes ne puisse être déterminée avec précision, comme dans le cas des protéines, Mathews a montré que les méthodes habituellement utilisées dans l'étude des masses moléculaires des glycosaminoglycanes (gel filtration, électrophorèse en gel de polyacrylamide) donnent des résultats concordants. Ces méthodes ont été discutées dans un précédent travail dans le cas de diverses mucines[7].

Les résultats que nous nous avons obtenus sont en accord avec les estimations habituelles des masses moléculaires des glycosaminoglycanes. Il faut remarquer en outre, que la masse moléculaire de l'acide hyaluronique est, comme usuellement, beaucoup plus élevée que celle des autres glycosaminoglycanes.

Les données analytiques rassemblées dans ce travail sont en faveur de la pureté des produits obtenus et justifient l'utilisation de cette méthode électrophorétique dont les avantages ont été rappelés.

REMERCIEMENTS

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RÉSUMÉ

Une nouvelle méthode de fractionnement est proposée pour préparer les différents glycosaminoglycanes par électrophorèse sur Pévikon. Par cette méthode, 200 mg d'un mélange de glycosaminoglycanes contenant de l'acide hyaluronique, de l'héparane sulfate, du dermatane sulfate et du chondroïtine sulfate peuvent être séparés avec une résolution et un rendement satisfaisants. La pureté de chacune des fractions a été contrôlée par des procédés chimiques et enzymatiques. Les rapports molaires acide uronique/hexosamine, sulfate/hexosamine et le contenu en acides aminés ont été déterminés. Le résidu peptidique inférieur à 1 % est représenté par cinq acides aminés principaux: la sérine, le glycocolle, l'alanine, l'acide aspartique et l'acide glutamique.

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GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF DI-, KETO- AND HYDROXYCARBOXYLIC ACID BENZYL ESTERS

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SUMMARY

The benzyl esters of di-(C_2–C_6)- and hydroxycarboxylic acids were prepared by reaction with N,N′-dicyclohexyl-O-benzylisourea or with phenyldiazomethane. The hydroxycarboxylic acids lactic and 3-hydroxybutyric acid were also alkylated with N,N′-dicyclohexyl-O-benzylisourea to give the corresponding O-benzyl ethers. The 2-oxocarboxylic acids pyruvic, oxaloacetic and 2-oxoglutaric acid were converted by means of O-benzylhydroxylamine hydrochloride into the corresponding O-benzyl oximes prior to esterification with phenyldiazomethane. The carboxylic acid benzyl esters were separated by gas chromatography on an SE-30 column. Mass spectra of the compounds were recorded with a MAT 111 GC–MS system. The main fragments at *m/e* 91 and *m/e* 107, found in all mass spectra, were due to the benzyl alcohol moiety of the esters. The general fragmentation pattern was similar to that of fatty acid and aromatic acid benzyl esters. The fragmentation scheme for this class of substances is discussed.

INTRODUCTION

Gas chromatographic–mass spectrometric (GC–MS) analysis of fatty acid benzyl esters has been described by us previously[1,2]. The method was proved to be particularly suitable for the gas–liquid chromatographical (GLC) separation of short and medium chain fatty acids[1]. Thus, for short chain fatty acids, an analytical procedure was developed that was as useful as the methylation method applied in the GLC of long chain fatty acids.

Despite continuous investigations[3–5] on the GLC separation of di- and polyfunctional carboxylic acids, difficulties remained and no standard method has been developed hitherto. The two derivatives of carboxylic acids most frequently used for GLC were the methyl and trimethylsilyl esters. Difficulties arose essentially from the preparation of the esters, because of the formation of by-products. Diazomethylation, for example, resulted in numerous compounds that interfered in gas chromatograms[4]. In particular, the 2-oxocarboxylic acids reacted to several products owing to the enolization of the 2-oxo group.

In this work, benzylation by means of phenyldiazomethane and N,N′-dicyclo-

hexyl-O-benzylisourea was extended to di- and polyfunctional carboxylic acids. Analytical investigations of these acids are of particular interest with regard to their biological occurence in the citrate cycle and related metabolic reactions. Various mixtures of carboxylic acid benzyl esters were separated by gas chromatography and the efficiency of the method was demonstrated.

After the GLC separations, mass spectra were recorded. The methyl esters of these acids have been analyzed systematically by mass spectrometry[4], while the benzyl esters were still unexplored in this respect. As expected, the mass spectra of these compounds showed the characteristic fragmentation pattern of benzyl esters reported for fatty acid benzyl esters. The mass spectra, however, were more complicated because of the additional functional groups present in the carboxylic acids investigated.

MATERIALS

Lactic, DL-3-hydroxybutyric, malic, 2-oxoglutaric, oxaloacetic, malonic, succinic, glutaric, adipic and maleic acids were obtained from E. Merck (Darmstadt, G.F.R.) and pyruvic and fumaric acids from Fluka (Buchs, Switzerland). All solvents (reagent grade) and chemicals were purchased from E. Merck.

The gas–liquid chromatograph (Model 402, Hewlett-Packard, Palo Alto, Calif., U.S.A.) was equipped with a flame ionization detector and recorder (Hewlett-Packard, Model 7127 A). Glass columns (1.70 m × 3 mm I.D.) were packed with 3% SE-30 on Chromosorb Q, 100–120 mesh, obtained from Applied Science Labs. (State College, Pa., U.S.A.). For GC–MS measurements, a MAT 111 GC–MS system (Varian-MAT, Bremen, G.F.R.) was used. Mass spectra were recorded with an Oscilloport light-point recorder (Siemens, Erlangen, G.F.R.). GC separations were achieved on a glass column (9 m × 2 mm I.D.) packed with 3% OV-101 on Gas-Chrom Q, 100–120 mesh (Serva, Heidelberg, G.F.R.).

METHODS

Preparation of carboxylic acid benzyl esters

Carboxylic acid benzyl esters were synthesized by esterification of carboxylic acids with phenyldiazomethane (PDM) or N,N′-dicyclohexyl-O-benzylisourea (DBI)[1]. All esters were prepared in chloroform–methanol (2:1, v/v). Hydroxy- and 2-oxocarboxylic acids were treated with PDM at 0°.

Preparation of benzoximes of 2-oxocarboxylic acids

The benzoximes were prepared in a similar manner to the analytical procedure described by Chalmers and Watts[6]. A 3-mmole amount of 2-oxocarboxylic acid and a four-fold excess of O-benzylhydroxylamine hydrochloride were stirred overnight in 50 ml of anhydrous pyridine. The solvent was removed by evaporation under reduced pressure, and the residue was dissolved in 100 ml of 1 *N* hydrochloric acid. The clear solution was extracted five times with 60-ml portions of diethyl ether and the combined ether extracts were evaporated to dryness. The crude product was re-dissolved in 50 ml of diethyl ether and dried for 3 h over anhydrous sodium sulphate.

The solution was filtered, the solvent removed and the crystalline benzoximes were washed with small volumes of benzene and dried over anhydrous calcium chloride.

Gas chromatography of carboxylic acid benzyl esters

The carboxylic acid benzyl esters were separated on an SE-30 column. Identical flow-rates were used for all separations: helium at 60 ml/min, synthetic air at 350 ml/min and hydrogen at 38 ml/min. For temperature programming, the temperatures of the injection port and the flame ionization detector were adjusted so as to be 30° above the final temperature.

GC–MS of single carboxylic acid benzyl esters

Carboxylic acid benzyl esters (2 μl of a 1% *n*-pentane solution) were separated from the solvent with the MAT 111 GC–MS system and the mass spectra were recorded using the following conditions: ionizing voltage, 80 V; current, 270 μA; ion source temperature, 300°; inlet tube temperature, 220°. Depending on the volatility of the carboxylic acid benzyl esters, different column and injection port temperatures were used.

Evaluation of mass spectra

The intensities of fragments were measured relative to the peak with the greatest intensity (100%). All peaks in the range *m/e* 40–250 that were at least 1% of the intensity of the base peak were plotted.

RESULTS

Esterification with PDM

Saturated dicarboxylic acids, hydroxycarboxylic acids, maleic acid and the benzoximes of the 2-oxocarboxylic acids gave almost quantitatively the benzyl esters upon esterification with PDM. The reaction of hydroxycarboxylic acids without the formation of by-products, however, was successful only at 0°. Fumaric acid formed the corresponding 4,5-dicarbobenzoxy-3-phenylpyrazoline with PDM. It was impossible, by esterification with PDM, to convert 2-oxocarboxylic acids, such as pyruvic, oxaloacetic and 2-oxoglutaric acids, into their benzyl esters. With different concentrations of the reagents and temperatures, a number of unidentified compounds always appeared.

Esterification with DBI

Most of the carboxylic acids investigated could be esterified upon treatment with DBI as alkylating agent, especially by reactions in boiling benzene or dioxan. 2-Oxoglutaric acid, oxaloacetic acid and their 2-benzoximes gave no benzyl esters. Lactic acid and 3-hydroxybutyric acid were esterified and additionally alkylated at their hydroxy function, leading to benzoxy ethers.

Gas–liquid chromatography

Fig. 1 shows the temperature-programmed separation of the benzyl esters of seven dicarboxylic acids simultaneously esterified with PDM.

Benzylation with PDM gave, in addition to the reaction artefact A, no com-

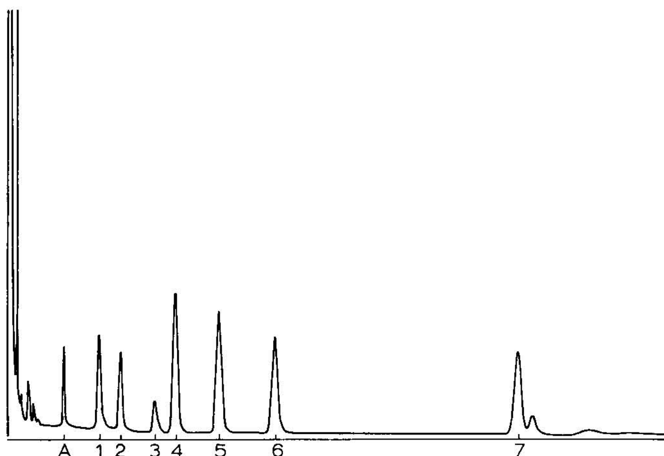


Fig. 1. Chromatogram of seven carboxylic acid benzyl esters on an SE-30 column programmed from 180° to 250° at 2°/min. A, Reaction artefact; 1, oxalic acid dibenzyl ester; 2, malonic acid dibenzyl ester; 3, maleic acid dibenzyl ester; 4, succinic acid dibenzyl ester; 5, glutaric acid dibenzyl ester; 6, adipic acid dibenzyl ester; 7, 4,5-dicarbobenzoxy-3-phenylpyrazoline.

pounds that showed interfering peaks in the range of the carboxylic acid benzyl esters. Low-boiling by-products appeared only between the solvent peak and the reaction artefact. No fumaric acid dibenzyl ester was formed, the pyrazoline peak being the last in the chromatogram. All peaks were well separated. Owing to the different volatilities of the compounds, it was not possible to run the gas chromatogram isotherm.

The separation of a mixture of benzyl esters of eleven carboxylic acids from

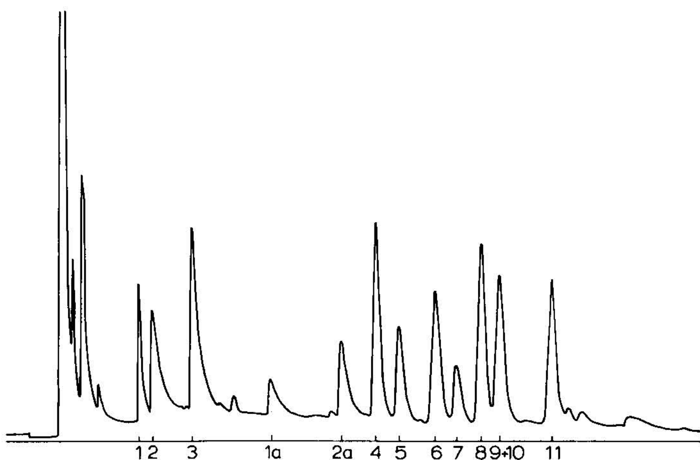


Fig. 2. Chromatogram of eleven carboxylic acid benzyl esters on an SE-30 column programmed from 95 to 185° at 5°/min, then from 185° to 220° at 2°/min. 1, Lactic acid benzyl ester; 2, pyruvic acid benzyl ester; 3, 3-hydroxybutyric acid benzyl ester; 4, oxalic acid dibenzyl ester; 5, malonic acid dibenzyl ester; 6, maleic acid dibenzyl ester; 7, succinic acid dibenzyl ester; 8, fumaric acid dibenzyl ester; 9, glutaric acid dibenzyl ester; 10, malic acid dibenzyl ester; 11, adipic acid dibenzyl ester; 1a, lactic acid benzyl ester O-benzyl ether; 2a, 3-hydroxybutyric acid benzyl ester O-benzyl ether.

lactic to adipic acid is shown in Fig. 2. All esters were prepared by the DBI method. Malic acid showed the same retention time as glutaric acid dibenzyl ester. The separation of these two esters could not be achieved, either by another temperature programme or by using EGSS-X as the stationary phase. The intensity of some interfering peaks in the chromatogram was very small compared with the intensity of the benzyl esters. Impurities of higher concentrations appeared only in the range between the solvent peak and the lactic acid benzyl ester peak. The benzyl esters and the benzyl ester O-benzyl ethers of the hydroxycarboxylic acids gave considerable tailing on the SE-30 column, the peaks of the other esters being satisfactorily separated. The three 2-oxocarboxylic acids (pyruvic, oxaloacetic and 2-oxoglutaric acids) were converted into the corresponding O-benzyloximes by means of O-benzylhydroxylamine, and separated by gas chromatography as their benzyl esters from a mixture of carboxylic acid benzyl esters (Fig. 3). Pyruvic acid benzoxime benzyl ester could not be separated from malonic acid benzyl ester.

Mass spectrometry

Fig. 4 shows the mass spectra of the benzyl esters of lactic, 3-hydroxybutyric, malic, oxalic, succinic, glutaric and adipic acids. Spectra could not be obtained for the benzyl esters of malonic, fumaric, maleic and the 2-oxocarboxylic acids. The mass spectrum of malic acid benzyl ester showed only a few characteristic peaks and could not be interpreted.

Lactic acid and 3-hydroxybutyric acid gave a molecular ion with 5% relative intensity. The other esters could be detected by their $M^+ - 91$ fragments, which

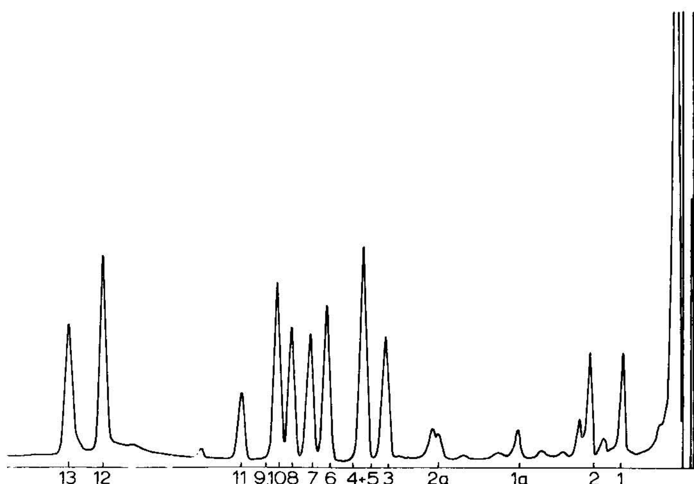
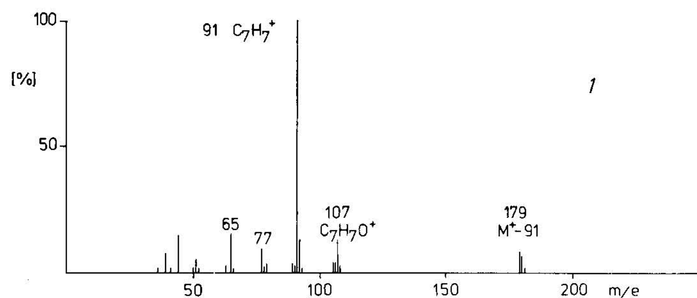
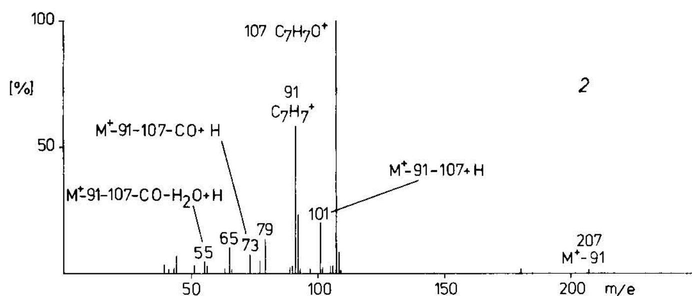
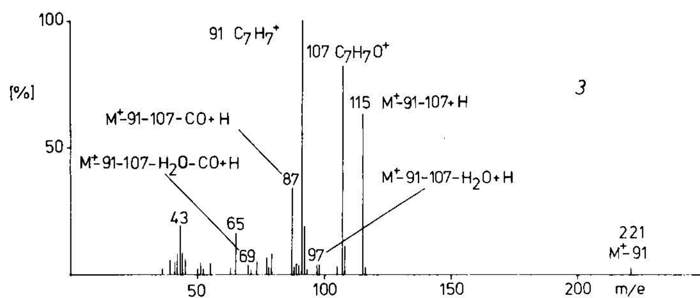


Fig. 3. Chromatogram of thirteen carboxylic acid benzyl esters on an SE-30 column programmed from 144° to 257° at 3°/min, then from 257° to 290° at 5°/min and isothermal at 290°. 1, Lactic acid benzyl ester; 2, 3-hydroxybutyric acid benzyl ester; 3, oxalic acid dibenzyl ester; 4, malonic acid dibenzyl ester; 5, pyruvic acid benzyl ester benzoxime; 6, maleic acid dibenzyl ester; 7, succinic acid dibenzyl ester; 8, fumaric acid dibenzyl ester; 9, glutaric acid dibenzyl ester; 10, malic acid dibenzyl ester; 11, adipic acid dibenzyl ester; 12, 2-oxoglutaric acid dibenzyl ester benzoxime; 13, oxaloacetic acid dibenzyl ester benzoxime; 1a, lactic acid benzyl ester O-benzyl ether; 2a, 3-hydroxybutyric acid benzyl ester O-benzyl ether.







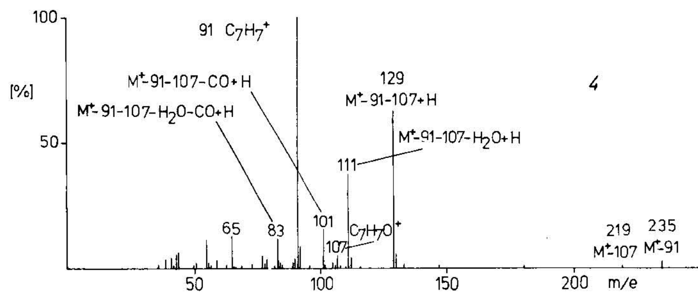
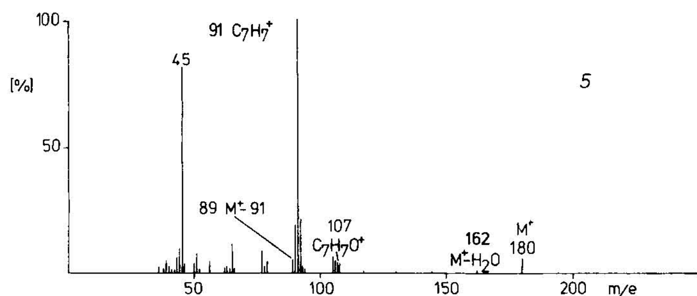
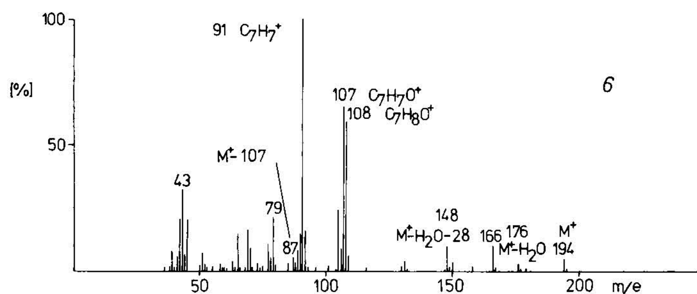


Fig. 4.





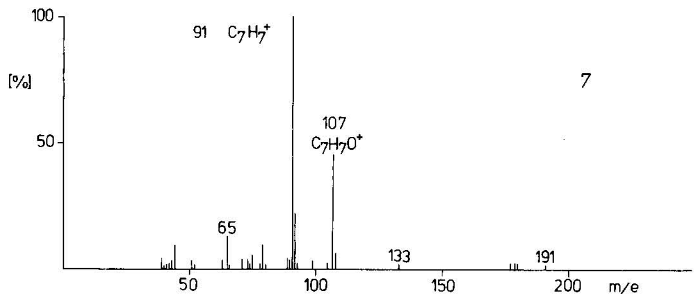


Fig. 4. Mass spectra of carboxylic acid benzyl esters. 1, Oxalic acid dibenzyl ester; 2, succinic acid dibenzyl ester; 3, glutaric acid dibenzyl ester; 4, adipic acid dibenzyl ester; 5, lactic acid benzyl ester; 6, 3-hydroxybutyric acid benzyl ester; 7, malic acid dibenzyl ester.

varied in intensity from 2 to 15%. Species of *m/e* 91 (tropylium) and *m/e* 107 (benzoxy radical cation) appeared in all mass spectra with high intensities. The species of *m/e* 107 was the base peak in the succinic acid benzyl ester spectrum, *m/e* 91 in all others. Tropylium and phenyl cations decomposed and gave the known aromatic fragments. $M^+ - 107$, produced by cleavage of the benzoxy radical from the molecular ion, appeared only in the spectrum of 3-hydroxybutyric acid and adipic acid benzyl ester. With hydroxycarboxylic acid benzyl esters, the cleavage of H_2O from M^+ to give $M^+ - 18$ was characteristic. For the decomposition of carboxylic acid benzyl esters, the following steps are suggested.

(1) Dicarboxylic acid benzyl esters:

(a) Cleavage of benzyl radicals from M^+ led to carboxyl cations ($M^+ - 91$).

(b) Cleavage of benzoxy radicals from the molecular ion produced acyl cations ($M^+ - 107$).

(c) Cleavage of benzyl radicals and benzoxy radicals with simultaneous capture of a hydrogen atom gave acyl carboxyl cations ($M^+ - 91 - 107 + H$).

(d) Cleavage of H_2O from acyl carboxyl cations produced acyl aldoketene cations ($M^+ - 91 - 107 - H_2O + H$).

(e) Cleavage of CO from acyl carboxyl cations produced alkylcarboxylic acid cations ($M^+ - 91 - 107 - CO + H$).

(f) Cleavage of CO from acyl aldoketene cations gave aldoketene cations ($M^+ - 91 - 107 - H_2O - CO + H$).

(g) Cleavage of H_2O from alkyl carboxylic acid cations led to the same aldoketene cations ($M^+ - 91 - 107 - CO - H_2O + H$).

(h) By cleavage of acyl radicals from M^+, the benzoxy radical cation at m/e 107 was formed.

(i) Cleavage of carboxylic radicals from M^+ produced a tropylium cation of m/e 91, which led to the fragments of m/e 65 and m/e 39.

(2) Hydroxycarboxylic acid benzyl esters:

The decomposition steps were the same as proposed in steps (a), (b), (h) and (i) for the dicarboxylic acid benzyl esters. However, some other fragments also appeared in these spectra, as follows.

(j) Cleavage of H_2O from the molecular ion produced a vinyl radical cation ($M^+ - H_2O$).

(k) Cleavage of hydroxyaldoketenes from M^+ and rearrangement of an H atom gave the benzyl alcohol radical cation of m/e 108.

(l) A fragment $M^+ - H_2O - 28$ was observed in the spectrum of 3-hydroxybutyric acid benzyl ester.

In no case could a cleavage of two tropylium cations or two benzoxy cations be observed.

DISCUSSION

The preparation of carboxylic acid benzyl esters by reaction of carboxylic acids with PDM or DBI as alkylating agents is a simple method which can be applied to many acids, and which converts the non-volatile carboxylic acids into derivatives suitable for gas chromatographic studies.

Esterification of saturated dicarboxylic acids, hydroxycarboxylic acids and maleic acid was achieved with both reagents without problems. Fumaric acid gave a pyrazoline derivative with PDM. An analogous reaction has been described by several workers in connection with the treatment of fumaric acid with diazomethane[3,7]. 2-Oxocarboxylic acids did not give benzyl esters in satisfactory yields with either PDM or with DBI. Pure pyruvic acid benzyl ester could be obtained in a yield of only 20% after successive column chromatography on silica gel. The high reactivity of the 2-oxo function was responsible for the formation of by-products, which differed between acids in both number and amount. Among others, glycidates and 2,2-

dibenzoxy derivatives were formed[4]. 2-Hydroxy and 3-hydroxy acids were alkylated by DBI not only at the carboxylic group but also at the hydroxy function. Vowinkel[8], who introduced this method, reported only alkylation of phenolic hydroxy functions, O-alkylation at unprotected alcoholic hydroxy functions not being observed.

The results for the 2-oxocarboxylic acids could be improved by converting the 2-oxo function with O-benzylhydroxylamine into a 2-benzoxime. Benzylation of the protected 2-oxoacid with PDM was then successful. The retention times of the 2-oxocarboxylic acid benzoxime benzyl esters did not interfere with the peaks of the other carboxylic acid benzyl esters in the gas chromatogram. Only pyruvic acid benzoxime benzyl ester and malonic acid dibenzyl ester exhibited the same retention time. The benzoximes of the 2-oxodicarboxylic acid dibenzyl esters were less volatile than the dibenzyl esters of the dicarboxylic acids and well separated from them.

The mass spectra, especially the dominating peaks of *m/e* 91, *m/e* 107 (108) and the $M^+ - 91$ fragment, were similiar to the spectra of benzyl esters described by Emery[9] and the spectra of fatty acid benzyl esters investigated by Hintze *et al.*[2]. In addition to the cleavage of a benzyl fragment or a benzoxy (benzyl alcohol) fragment, which was also found in the spectra of fatty acid benzyl esters, dicarboxylic acid benzyl esters split off a second fragment of *m/e* 91 or *m/e* 107 (108). If the first group to be split off were a benzyl group, then the second was a benzoxy group, and *vice versa*. Cleavage of two *m/e* 91 or two *m/e* 107 (108) fragments was not observed for the esters investigated. Following the cleavage of two aromatic fragments, the successive splitting of H_2O and CO or of CO and H_2O led to the same molecule, whereby the intermediates increased in intensity as the number of carbon atoms in the ester increased. A break within the hydrocarbon chain, which was observed in long chain fatty acid benzyl esters and methyl esters, was found only for adipic acid benzyl ester. Products of McLafferty rearrangements did not appear, not even with carboxylic acid benzyl esters that have a γ-hydrogen atom. Peaks of protonated acids were missing from the spectra. In the hydroxycarboxylic acid benzyl esters, the cleavage of H_2O from M^+ was remarkably strong. The $M^+ - H_2O$ peaks were more intense than in the corresponding methyl esters investigated by Ryhage and Stenhagen[10]. The resulting α,β-unsaturated benzyl esters were well stabilized by mesomeric structures. The hydroxycarboxylic acid benzyl ester O-benzyl ethers were also identified by mass spectrometry. In addition to the fragments of *m/e* 91 and *m/e* 107 (108), however, no similarity with the spectra of the other benzyl esters in the fragmentation pattern was found.

Owing to the lability of the compounds, no spectra could be obtained from malonic acid benzyl ester, unsaturated carboxylic acid benzyl esters and 2-oxocarboxylic acid benzyl esters. Malonic acid was decarboxylated easily under the influence of heat and metal catalysts. The other acid derivatives underwent side-reactions with their additional functional groups, a problem that was also observed in the alkylation with PDM and DBI. The conditions found in the GC–MS separator, the presence of metal catalysts and high temperatures made the decomposition of these benzyl esters unavoidable.

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GAS CHROMATOGRAPHIC BEHAVIOUR OF C_1–C_4 ALIPHATIC AMIDES AND THEIR N-METHYL AND N,N-DIMETHYL DERIVATIVES ON POROUS POLYMERS OF THE PORAPAK TYPE

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SUMMARY

Aliphatic amides can be analyzed, without any preliminary treatment, by gas chromatography on porous copolymers of the Porapak type. On all of the copolymers studied, amides gave symmetrical chromatographic peaks. The best separation was achieved on Porapak N; all unsubstituted, N-methyl and N,N-dimethylamides of formic, acetic, propionic and butyric acids were separated, except for the pair N-methyl- and N,N-dimethylformamide, which were separated on Porapak Q and T. The sorption of amides on Porapak P, Q, N, R and S and on Synachrom is due mainly to non-specific interactions. Only Porapak T displays appreciable polarity; the order of retention of amides suggests that Porapak T is both an electron donor and, to a lesser extent, an electron acceptor type of adsorbent. The differences in the retentions of amides on Porapak P and Q are in accordance with the difference in the specific surface areas of these sorbents, but the sorption enthalpies are almost identical. The solute–sorbent interaction is of the same nature in both instances, regardless of an order of magnitude difference in the specific surface areas. The excellent sorption properties of Porapaks permitted the reliable determination of the response factors of amides in their detection with flame ionization and thermal conductivity detectors.

INTRODUCTION

Amides are among the most polar organic compounds. Owing to the high propensity of the amide group to undergo hydrogen bonding, the molecules of amides are strongly associated with one another so that even amides of low molecular weight display very low volatilities. For instance, the simplest aliphatic amide, formamide, has been used as a gas chromatographic (GC) stationary phase[1].

In extremely dilute solutions of amides in non-polar solvents, the intermolecular association due to hydrogen bonding is almost zero and the volatilities of the dissolved amides are markedly increased (large positive deviation from Raoult's law). Hence, the GC of amides is feasible with the use of non-polar stationary phases, but the application of conventional GLC packings for this purpose is difficult owing

to the strong adsorption of amides on the surface of the support. The extent of peak tailing is usually intolerable even with the use of very inactive supports.

It follows that non-polar porous organic polymers can be very advantageous sorbents for the GC of free amides. Although the thermodynamic properties of a solute molecule adsorbed on a solid surface are different in many respects from those of the molecule dissolved in a liquid, the effect of the supression of the association of solute molecules applies in both instances.

Aliphatic amides have been analyzed by GC on a mixed stationary phase of orthophosphoric acid and poly(ethylene glycol succinate) deposited on Chromosorb W[2]. At the temperature employed (205°C), the amides were dehydrated to the corresponding nitriles on the stationary phase. A disadvantage of this method was that the conversion was incomplete. Metcalfe *et al.*[3] chromatographed aliphatic amides at 220°C on Apiezon L on Chromosorb W treated with sodium hydroxide. O'Donnell and Mann[4] separated lower aliphatic amides at 220°C on Dowfax 10 plus sodium hydroxide deposited on Chromosorb W. VandenHeuvel *et al.*[5] chromatographed amides after their conversion into more volatile fluoro derivatives.

Yasuda and Nakashima[6] used Porapak Q for the GC of aliphatic amides at 230°C; N-methyl derivatives of the amides were converted into nitriles on a pre-column with orthophosphoric acid prior to the GC analysis proper. Chromosorb 101 was employed by Nakagawa *et al.*[7] in the temperature-programmed GC of a mixture of C_2–C_4 fatty acids and their amides.

In this paper, we present the results of a systematic investigation of the chromatographic behaviour of C_1–C_4 aliphatic amides and their N-methyl and N,N-dimethyl derivatives on Porapak P ,Q, N, R, S and T and on Synachrom E-5 (ref. 8).

EXPERIMENTAL

Chemicals

Solutes. Formamide and N,N-dimethylformamide were commercial products (Lachema, Brno, Czechoslovakia), purified by distillation. The unsubstituted, N-methyl and N,N-dimethylamides of acetic, propionic and butyric acids were synthesized from the acids by esterification with ethanol and subsequent shaking of the esters with ammonia solution, methylamine and dimethylamine, respectively[9]. The isolation of pure amides from the reaction mixture was carried out by preparative GC on a Perkin-Elmer 900 (Norwalk, Conn., U.S.A.) gas chromatograph provided with an auxiliary preparative unit, employing a 6-m stainless-steel column, packed with Porapak Q, 80–100 mesh, maintained at 185°C. C_6–C_{10}, C_{14}, C_{16} and C_{18} *n*-alkanes (BDH, Poole, Great Britain) were used as reference solute compounds.

Chromatographic materials. The Porapaks employed were products of Waters Ass., Framingham, Mass., U.S.A., and Synachrom E-5 was obtained from the Research Institute of Plastics and Lacquers, Pardubice, Czechoslovakia. The batch numbers, particle sizes and specific surface areas of the polymers are given in Table I.

Instruments and procedures

Measurement of retention data. The measurement of the retention characterics of amides was carried out on a Chrom 2 gas chromatograph (Laboratorní Přístroje, Prague, Czechoslovakia) equipped with a flame ionization detector (FID),

TABLE I

SPECIFICATIONS OF THE SORBENTS INVESTIGATED

Sorbent	*Particle size (mesh)*	*Batch number*	*Specific surface area (m^2/g)*
Porapak P	80–100	596	68
Q	100–120	594	Not measured
	100–120	558	654
	80–100	638	Not measured
R	100–120	620	636
S	80–100	684	591
T	80–100	691	195
N	100–120	500	503
Synachrom E-5	60–70	--	290

using nitrogen as the carrier gas. The experiments were designed so as to provide the data necessary for calculating the specific retention volumes. The flow-rate of the carrier gas was measured with a differential manometer showing the pressure drop across a capillary, and the column inlet pressure was measured with a mercury manometer. Both devices were installed ahead of the sample inlet port in the carrier gas line. The flow-rate of the carrier gas was maintained within 0.7–1.0 ml/sec. U-shaped 60- or 30-cm (depending on the sorption capacity of the given material) brass columns of 7 mm I.D. were employed. All of the polymers investigated were purged with nitrogen for 20 h at 170°C prior to the measurements proper.

The amides were dissolved in acetone, and about 5-μl portions of the solutions were injected into the gas chromatograph. The charges contained about 1 μg of each amide, except for formamide, the amount of which had to be about ten times greater owing to the lower sensitivity of the FID towards this compound. All of the measurements were performed under isothermal conditions at several temperatures within the range 135–185°C. Methane was employed as the marker of the column hold-up time. Some examples of the separation of amides on Porapaks were provided on a Hewlett-Packard 402 gas chromatograph (*cf.*, measurement of FID response factors).

Measurement of response factors. The FID and thermal conductivity detector (TCD) response factors of amides were measured on a Hewlett-Packard 402 (Avondale, Pa., U.S.A.) and a Perkin-Elmer 900 gas chromatograph, respectively. In the work with the FID, the analyses were carried out on a 1 m $\times$ 6 mm I.D. glass column with Porapak P at 170°C. The operating conditions of the detector were as follows: carrier gas (nitrogen) flow-rate, 42–72 ml/min; hydrogen flow-rate, 109–125 ml/min; air flow-rate, 600 ml/min; detector temperature, 170°C; and sensitivity attenuation, 1/400–1/800. A disc integrator (Disk Instruments, Santa Ana, Calif., U.S.A.), built-in in the recorder, was employed for the evaluation of the peak areas. In the work with the katharometer, the samples were chromatographed on a 90 cm $\times$ 3.1 mm I.D. stainless-steel column with Porapak Q at 170°C, using hydrogen as the carrier gas. The operating conditions were as follows: carrier gas flow-rate, 19.2 ml/min; katharometer bridge current, 275 mA; detector temperature, 220°C; and full detector sensitivity. The chromatograms were recorded with a Honeywell-Brown (Philadelphia, Pa., U.S.A.) recorder at a chart speed of 0.2 in./min, and the

TABLE II

CONSTANTS a AND b OF THE EQUATION LOG $V_g = (a/T) - b$ FOR AMIDES ON PORAPAKS AND SYNACHROM

Solute*	n**	Sorbent													
		P		Q		N		R		S		T		SCH	
		a	b	a	b	a	b	a	b	a	b	a	b	a	b
1A	0	28.65	5.83	22.52	3.97	27.17	4.53	32.09	6.01	41.33	8.04	35.65	6.31	32.69	5.99
2A	1	31.61	6.33	29.13	5.10	29.65	4.81	43.21	8.25	39.21	7.26	43.23	7.80	42.94	7.90
3A	2	32.95	6.39	33.13	5.69	32.90	5.24	45.15	8.39	41.91	7.55	45.58	8.16	47.69	8.70
4A	3	36.05	6.83	36.51	6.10	37.90	6.01	49.67	9.07	45.84	8.09	48.77	8.65	47.41	8.34
1B	0	30.14	5.99	27.43	4.82	30.59	5.12	37.64	7.05	44.82	8.57	35.31	6.23	42.03	7.86
2B	1	33.33	6.57	30.85	5.31	32.41	5.31	44.15	8.30	41.51	7.58	39.08	6.94	49.62	9.33
3B	2	36.51	7.09	34.16	5.80	36.10	5.89	46.61	8.60	43.85	7.84	40.73	7.17	51.44	9.50
4B	3	37.79	7.13	38.39	6.44	42.41	6.97	53.70	9.88	47.06	8.25	43.61	7.60	51.25	9.15
1C	0	29.52	5.84	29.12	5.14	29.76	4.97	36.33	6.79	31.36	5.54	34.38	6.23	37.47	6.84
2C	1	32.79	6.35	34.37	5.98	36.48	6.17	47.64	9.00	42.15	7.65	37.11	6.62	40.56	7.19
3C	2	36.39	6.96	35.41	5.96	36.79	5.99	50.33	9.35	42.33	7.44	39.85	7.10	43.62	7.65
4C	3	39.83	7.50	38.67	6.39	41.28	6.69	55.33	10.18	48.58	8.52	42.73	7.56	46.81	8.09

* 1, 2, 3 and 4 represent formamide, acetamide, propionamide and butyramide, and A, B and C designate unsubstituted, N-methyl- and N,N-dimethyl-amides, respectively.

** In $C_nH_{2n+1}COR$, where R = $-NH_2$, $-NHCH_3$ or $-N(CH_3)_2$.

peak areas were measured with a planimeter. In both instances, defined volumes of solutions of known concentrations of the individual amides in acetone were introduced into the gas chromatograph, employing the on-column injection method. A Hamilton 701N syringe (Micromesure N.V., The Hague, The Netherlands) and a Zimmermann (Leipzig, G.D.R.) 40-μl syringe were used in the work with the FID and TCD, respectively. The concentrations of the amides in the model solutions were about 1% (w/v), except for formamide with the FID, where the concentration was about 10%. After each amide sample charge or after a series of three charges, defined amounts of C_8, C_9 and C_{10} *n*-alkanes (reference compounds) were injected and their chromatograms run under the same conditions.

RESULTS AND DISCUSSION

Retention characteristics

All of the retention data measured for the individual amides and reference hydrocarbons at different temperatures were expressed in the form of the absolute specific retention volumes[10], V_g, and these values were further processed by linear regression so as to obtain the constants a and b of the equation

$$\log V_g = (a/T) - b$$

where T is the absolute temperature of the system. The results are given in Table II.

The constant a is related to the standard molar enthalpy of sorption, ΔH^0, by the equation

$$a = \mathrm{d} \log V_g/\mathrm{d}(1/T) = -\Delta H^0/2.303\ R$$

where R is the gas constant. The values of ΔH^0 calculated as shown above for all of the amides on Porapak P and Q are listed in Table III. It is interesting that the ΔH^0 values on Porapak P and Q do not differ appreciably, although there is about an order of magnitude difference in the specific surface areas and the sorption capacities of these materials. Fig. 1 shows the temperature dependence of log V_g for acetamide and N,N-dimethylacetamide measured on Porapak P (Fig. 1a) and Porapak Q (Fig. 1b). The similarity of the ΔH^0 values and the difference in the sorption capacity are apparent from the slopes and positions of the lines for Porapak P and Q. Hence it follows that the solute–sorbent interactions are of the same nature with both types of Porapak, the difference in the sorption capacity being given merely by the difference in the entropy of sorption.

Table IV gives the Kováts retention indices, I_{170}, of the amides on the Porapaks at 170°C. The actual separation in the systems studied is demonstrated by the chromatograms in Fig. 2. It can be seen that the best separation is achieved on Porapak N. The pair N-methyl and N,N-dimethylformamide, which is unresolved on Porapak N, can be separated on Porapak Q and T. The sequence of the retention indices reflects the selectivity of the individual types of Porapak to the amide group. Thus, when considering propionamide and N,N-dimethylpropionamide, for instance, the orders of the selectivity towards the $-CONH_2$ and $-CON=$ groups are Q > S > R > P > N > SCH > T and S > R > Q > N > P > SCH > T, respectively. Porapak

TABLE III

STANDARD ENTHALPIES OF SORPTION ($\Delta H°$) OF AMIDES ON PORAPAK P AND Q

β is the 95% interval of confidence.

*Solute**	*Porapak P*		*Porapak Q*	
	$-\Delta H°$ *(kcal/mole)*	β *(kcal/mole)*	$-\Delta H°$ *(kcal/mole)*	β *(kcal/mole)*
1A	13.1	11.9–14.3	10.4	9.2–11.4
2A	14.5	13.3–15.7	13.3	12.3–14.4
3A	15.1	14.1–16.1	15.2	14.2–16.1
4A	16.5	15.2–17.8	16.7	15.3–18.1
1B	13.8	12.5–15.1	12.5	11.7–13.4
2B	15.2	14.5–16.0	14.1	13.2–15.0
3B	16.7	15.7–17.7	15.6	14.2–17.0
4B	17.3	16.3–18.3	17.6	15.6–19.5
1C	13.5	12.6–14.5	13.3	11.6–15.1
2C	15.0	13.6–16.4	15.7	14.3–17.2
3C	16.7	15.4–17.9	16.2	14.1–18.3
4C	18.2	16.9–19.5	18.0	15.4–20.0

* For solutes, see footnote to Table II.

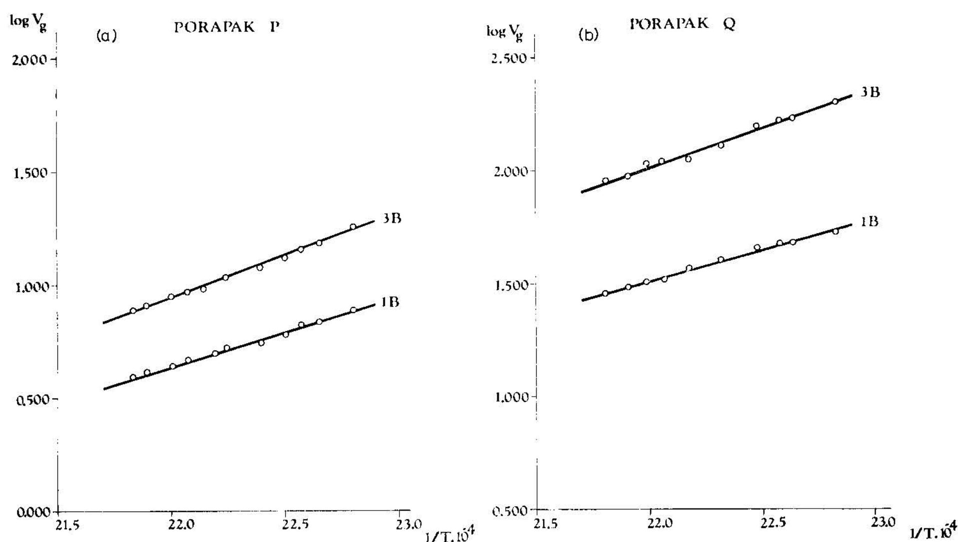

Fig. 1. Temperature dependence of log V_g for acetamide and N,N-dimethylacetamide measured on (a) Porapak P and (b) Porapak Q.

Q, R and S are virtually non-selective, a slight selectivity can be observed with Porapak P, N and with Synachrom (SCH), and the most pronounced selectivity is shown by Porapak T. The differences between the largest and the smallest values of the retention index of propionamide and N,N-dimethylpropionamide are 452 and 295, respectively. Provided that the least selective Porapaks are virtually non-selective, these two differences represent the selectivity of Porapak T towards the $-CONH_2$ and $-CON=$

TABLE IV

KOVÁTS RETENTION INDICES OF AMIDES ON PORAPAKS AND SYNACHROM AT 170 °C

*Solute**	I_{170}						
	P	*Q*	*N*	*R*	*S*	*T*	*SCH*
1A	758	654	756	687	666	1140	755
2A	816	744	837	760	755	1221	875
3A	898	834	922	848	841	1286	960
4A	988	936	1020	947	940	1371	1049
1B	818	718	806	750	740	1142	823
2B	865	796	871	808	809	1196	902
3B	936	872	942	884	884	1247	971
4B	1022	966	1044	977	976	1328	1065
1C	821	742	811	742	737	1064	824
2C	899	834	895	836	829	1149	920
3C	973	911	965	910	903	1198	992
4C	1052	998	1051	998	994	1271	1078

* For solutes, see footnote to Table II.

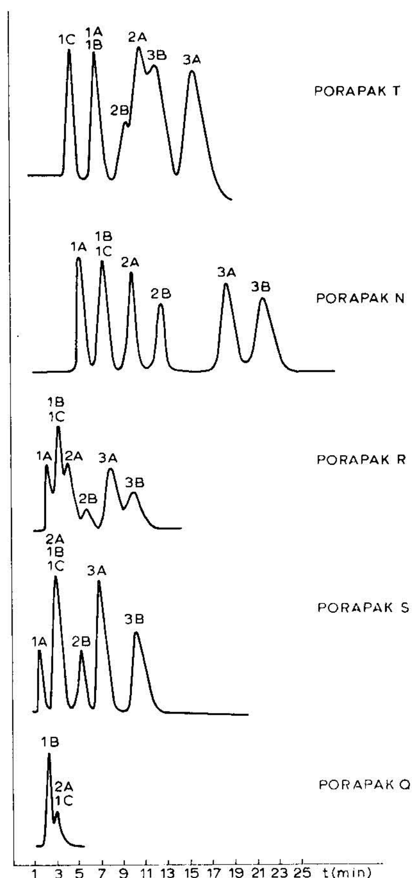

Fig. 2. Chromatograms of amides on Porapaks at 170 °C. For the designations of the peaks, see footnote to Table II.

groups, respectively. This indicates that Porapak T is both an electron donor and an electron acceptor type of adsorbent, but the electron donor character predominates. Note that while the retention of all of the amides on Porapak T increases with increasing length of the hydrocarbon chain attached to the CO group, the methylation of the NH_2 group results in a decrease in retention.

Response factors

Both the FID and TCD response factors of the amides (i) were expressed in the form of the relative molar response, $RMR_{i,r}$, employing n-decane and n-octane as reference compounds (r), respectively. The results were calculated by means of the equation

$$RMR_{i,r} = (A_i/N_i)/(A_r/N_r)$$

where A_i and A_r are the peak areas corresponding to N_i and N_r moles of the amide and the reference compound; N_i and N_r were introduced in separate charges into the gas chromatograph. The results, representing averages of three determinations, are given in Table V, where ΣC_{eff} is the number of effective carbon atoms[11]. The latter quantity was calculated from the equation[12]

$$RMR_{i,r} = (\Sigma C_{eff})_i/(\Sigma C_{eff})_r$$

where, in this work, $(\Sigma C_{eff})_r = 10$ (carbon number of decane).

With both the FID and TCD, the $RMR_{i,r}$ value is linearly proportional to the methylene carbon number within a homologous series of amides, as shown in Figs. 3

TABLE V

RELATIVE MOLAR RESPONSES ($RMR_{i,r}$) OF AMIDES IN THEIR DETECTION WITH THE FLAME IONIZATION DETECTOR AND KATHAROMETER

$(\Sigma C_{eff})_i$ is the sum of the effective carbon atoms of the solute compound (i) in its detection with the FID.

*Solute**	n**	*Mol. wt.*	*FID*		*TCD*
			$RMR_{i,r}$	$(\Sigma C_{eff})_i$	$RMR_{i,r}$
1A	0	45.04	0.0030	0.03	0.4585
2A	1	59.07	0.0798	0.80	0.5041
3A	2	73.09	0.1555	1.56	--
4A	3	87.12	0.2358	2.36	0.5906
1B	0	59.07	0.0765	0.76	0.4781
2B	1	73.09	0.1566	1.57	0.5259
3B	2	87.12	0.2360	2.36	0.5772
4B	3	101.15	0.3214	3.71	0.6212
1C	0	73.09	0.1568	1.57	0.5069
2C	1	87.12	0.2299	2.30	0.5694
3C	2	101.15	0.3252	3.25	0.5903
4C	3	115.18	0.4144	4.14	0.6556

* For solutes, see footnote to Table II.

** In $C_nH_{2n+1}COR$, where R = $-NH_2$, $-NHCH_3$ or $-N(CH_3)_2$.

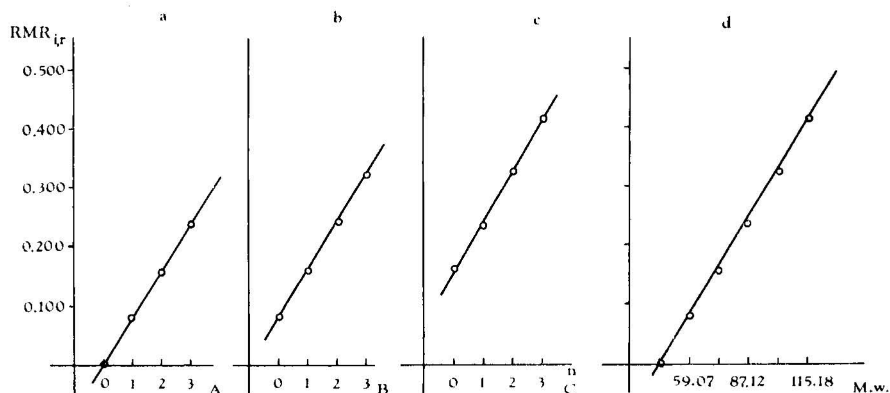

Fig. 3. Dependence of $RMR_{i,r}$ on the methylene carbon number for (a) unsubstituted, (b) N-methyl- and (c) N,N-dimethylamides and (d) on molecular weight for all of the amides in their detection with the FID.

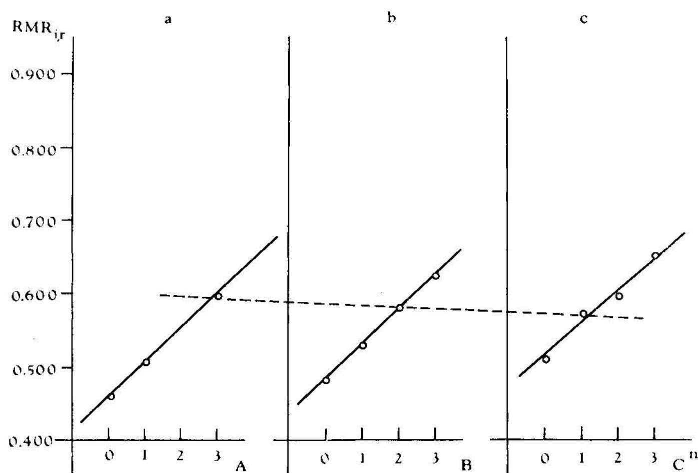

Fig. 4. Dependence of $RMR_{i,r}$ on the methylene carbon number for (a) unsubstituted, (b) N-methyl- and (c) N,N-dimethylamides in their detection with the TCD.

and 4. However, as concerns the dependence of the $RMR_{i,r}$ value on the structure of the amide molecule is concerned, the FID and TCD give different relationships. With the FID, the contribution of a CH_2 group to the $RMR_{i,r}$ value is the same, regardless of whether the group is part of the chain bound to the carbonyl group or of the substituted amide group. Thus, the $RMR_{i,r}$ values of all of the unsubstituted, N-methyl- and N,N-dimethylamides actually lie on a single line in the graph of $RMR_{i,r}$ *versus* methylene carbon number or molecular weight. Note that the line in Fig. 3d crosses the abscissa in the point corresponding to the molecular weight of formamide. The response to the $-CONH_2$, $-CONH-$ and $-CON=$ groups alone is almost zero. With the katharometer, the average contributions of a CH_2 group at the carbonyl and at the amide group to the $RMR_{i,r}$ value are about 0.044 and 0.027, respectively, under the given conditions.

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GAS–LIQUID CHROMATOGRAPHY ON FLUORINATED STATIONARY PHASES

I. HYDROCARBONS AND FLUOROCARBONS

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SUMMARY

Four fluorinated stationary phases have been examined for their retention behaviour towards hydrocarbons and their fluorinated derivatives. It is seen that the customary way of determining stationary phase polarities gives little information on the retention characteristics of highly fluorinated solutes. Of the phases examined, Krytox AC, a fluorinated polyether whose gas–liquid chromatographic properties have not previously been reported, demonstrated an exeptionally high affinity for fluorinated solutes enabling hydrocarbon–fluorocarbon separations to be carried out efficiently. Fluorolube oil proved an efficient phase for the separation of fluoro compounds of different degrees of fluorination, whilst Kel F oil proved to be the best stationary phase for the separation of compounds having an equal degree of fluorination and for which boiling point differences were the main criteria for separation.

INTRODUCTION

The gas–liquid chromatographic (GLC) behaviour of fluorinated compounds has received little attention as yet, there being but few published papers on the subject. Most of the work has been on the development of efficient stationary phases for fluorocarbon separations or on the separation and analysis of the low-boiling fluorine-containing compounds used in anaesthetics or aerosols. As early as 1955, Evans and Tatlow[1] separated 1H:3H-decafluorocyclohexane, the monohydroperfluorocyclohexenes and perfluorocyclohexadienes on a preparative column containing dinonylphthalate, the column was 16 ft. long for the preparative work but identification of peaks had been achieved on a 6-ft. column. These authors made the valuable suggestion that "other high-boiling liquids such as the chloro–fluoro oils and silicones..." would be useful stationary phases for fluorocarbon analysis. Hall *et al.*[2] used columns containing di-isodecyl phthalate or benzyl ether for the separation of fluothane (2-bromo-2-chloro-1,1,1-trifluoroethane) from diethyl ether but Reed[3] had previously investigated fluorocarbon separations on fluorine-containing stationary phases. He

used three fluorocarbons, perfluoropentane, -heptane and -nonane, studying their chromatographic behaviour on perfluorokerosine, on Kel F-90 grease which is a chlorofluorocarbon polymer, and on the ethyl ester of Kel F acid 8114. Reed stated that a fluorocarbon stationary phase gives better separations of fluorocarbon mixtures than does a hydrocarbon stationary phase; furthermore for some mixtures, chlorofluorocarbons (Kel F) have a better resolving power than the fluorocarbon stationary phases.

Reed's work led to Campbell and Gudzinowicz[4] comparing the stationary phase properties of Apiezon L, a methyl phenyl ether, di-isodecyl phthalate and a Kel F oil during an investigation into the pyrolysis of commercial PTFE. For the GLC of the mixture of fluoroalkanes and -alkenes obtained, the authors found that the three non-fluorinated stationary phases gave poor performance and suggested that Kel F oil was superior for the separation of high-boiling fluorocarbons.

Greene and Wachi[5] have studied the separation of low-molecular-weight fluorocarbons at 0° and recommend a (decafluoroheptyl) acrylic ester as the optimum stationary phase. Brown *et al.*[6] have studied several stationary phases and their behaviour towards fluorocarbons and fluoroalcohols. Considerable information on fluorocarbon retention on Apiezon L and on poly(ethylene glycol succinate) is given and, as the authors were concerned primarily with electron acceptor types, two useful electron acceptor phases (a fluoro ester and a fluoro phosphonitrilate) are described and their properties discussed.

In this work four fluorinated stationary phases have been examined against a squalane reference. Three of the phases, Kel F-90, Fluorolube 2000, and silicone QF-1, have been used as such for some time and information is available to a limited extent about their GLC properties. The fourth phase studied, Krytox 240 AC, is a fluorinated grease produced by DuPont as a high temperature lubricant and has not previously been used as a GLC stationary phase.

A range of aromatic fluorocarbons, fluorobenzenes and fluorotoluenes, have been studied chromatographically on the five stationary phases together with the parent hydrocarbons and the results are compared from the viewpoint of the Kováts retention indices obtained. A perfluoroparaffin and a perfluoroolefin, together with their parent hydrocarbons, have also been studied and the effects of introducing π-electron characteristics and chain branching into the solutes is seen on the various stationary phases and the solute–stationary phase interactions explained in terms of molecular structure. The concept of column efficiency is also examined in terms of the degree of fluorination of the solute and the results augment Reed's conclusions that fluorinated stationary phases are superior for fluorocarbon analysis but, as retention increments show, considerable versatility is demonstrated by the comparatively few fluorinated stationary phases studied here.

EXPERIMENTAL

All measurements were made on a Pye series 104 gas chromatograph with flame ionization detector. Columns were 1 m × 6 mm O.D. tubing packed with 20% w/w of stationary phase coated on acid-washed, DCMS-treated Celite, 80–100 mesh partical size. The carrier gas was nitrogen, the flow-rate 20 ml/min.

Phases

(1) Kel F-90 oil, a chlorofluorocarbon polymer (poly(chlorotrifluoroethylene)), obtained from Phase Separations, Queensferry, Great Britain.

(2) Fluorolube 2000 oil, chlorofluorocarbon, Phase Separations.

(3) Silicone QF-1, poly(methyl-3,3,3-trifluoropropyl siloxane), Phase Separations.

(4) Krytox 240 AC, a perfluoroalkyl polyether oil thickened with Vydax 1000 fluorocarbon telomer solids, obtained from DuPont Petroleum, Chem. Div., Wilmington, Del., U.S.A. As this material is completely insoluble in all common solvents, the column packing was prepared by making a slurry of the Celite with a solution of Krytox in perfluoro 1,3-dimethylcyclohexane and evaporating under vacuum in a rotary evaporator.

RESULTS AND DISCUSSION

Table I compares the efficiencies of the five columns studied in terms of HETP at 80° using benzene and perfluorobenzene. Using the hydrocarbon for evaluation shows that squalane is the most efficient and Krytox the least efficient column, with silicone QF-1 and Kel F-90 having similar and intermediate values. With perfluorobenzene however, the situation changes remarkably when squalane shows slightly lower efficiency and Krytox, now having almost twice the efficiency, being comparable to squalane. The silicone and Kel F oil columns are no longer equivalent, Kel F showing improved efficiency with the fluorocarbon whilst the silicone efficiency decreased. To complete the picture, the Fluorolube oil shows no change in efficiency from hydrocarbon to fluorocarbon. These results suggest which stationary phase would be best suited for certain applications. Thus, for the separation of hydrocarbon–fluorocarbon mixtures Krytox and Kel F should give high resolutions with preferential retention of fluorocarbon, the silicone will display preferential retention for the hydrocarbon whilst Fluorolube would separate in order of boiling points irrespective of the fluorine content of the compound.

TABLE I

A COMPARISON OF COLUMN EFFICIENCIES AS DETERMINED BY BENZENE AND PERFLUOROBENZENE AT 80°

Stationary phase	*HETP (cm)*	
	Benzene	*Perfluorobenzene*
Krytox	0.35	0.22
Silicone QF-1	0.33	0.41
Kel F-90	0.32	0.23
Fluorolube 2000	0.22	0.21
Squalane	0.16	0.19

Table II gives the McReynolds[7] constants for the stationary phases where X', Y', Z', U' and S' are column constants given by ΔI values for benzene, *n*-butanol, 2-pentanone, nitropropane and pyridine, respectively. All phases are of low polarity with Krytox the least polar and silicone QF-1 having the highest polarity. Krytox, with its low X' value, will separate hydrocarbons rapidly from molecules containing

TABLE II

McREYNOLDS CONSTANTS FOR THE STATIONARY PHASES

Stationary phase	*X′*	*Y′*	*Z′*	*U′*	*S′*
Krytox	4	148	86	147	93
Kel F-90	41	135	134	153	111
Fluorolube 2000	75	192	148	200	158
Silicone QF-1	130	225	336	432	281

TABLE III

RETENTION INDEX (*I*) VALUES OF HYDROCARBONS AND FLUOROCARBONS AT 100°

Compound	*Retention index*				
	Krytox	*Silicone QF-1*	*Kel F-90*	*Fluorolube 2000*	*Squalane*
Benzene	668	782	698	730	650
Fluorobenzene	716	817	725	752	643
1,2,4,5-Tetrafluorobenzene	815	859	767	744	604
Pentafluorobenzene	842	848	769	716	589
Perfluorobenzene	861	827	771	687	571
Toluene	767	889	818	839	756
o-Fluorotoluene	824	919	842	858	757
m-Fluorotoluene	829	939	848	864	756
p-Fluorotoluene	829	939	846	866	756
Cyclohexane	680	700	659	679	671
Methylcyclohexane	747	758	734	743	738
Perfluoro-1,3-dimethyl-cyclohexane	978	568	698	340	462
1-Octene	772	806	797	799	784
2-Methylpentane	591	618	604	570	573
2-Methylpent-2-ene	597	611	624	616	595
Perfluoro-2-methylpent-2-ene	704	454	498	225*	185*

* Approximate values.

a functional group and the high Y' value suggests that the phase may selectively retard alcohols. Silicone QF-1, with its high Z' value, selectively retards ketones hence its usefulness in steroid analysis.

Aromatics

Table III lists the Kováts retention indices of all hydrocarbons and fluorocarbons studied, whilst in Table IV an assessment of the degree of interaction between stationary phase and solute is made on the basis of solute retention increments on the fluorinated stationary phase as compared with squalane. The predictions made from Table I can be seen to be borne out, on Krytox benzene is rapidly eluted, the fluorinated benzenes having increasing retention indices as the percentage of fluorine present increases. This is not a regular increase but varies as expected, with substitution of the first fluorine into benzene giving rise to an increase of 48 units whereas introduction of the last fluorine (into pentafluorobenzene) gives a retention increase of only 19 units, Table III shows quite clearly that for the separation of unreacted benzene from

TABLE IV

SOLUTE–STATIONARY PHASE INTERACTIONS. RETENTION INCREMENTS, ΔI ($I_{\text{stationary phase}} - I_{\text{squalane}}$), FOR HYDROCARBONS AND FLUOROCARBONS AT 100°

Compound	ΔI			
	Krytox	*Silicone QF-1*	*Kel F-90*	*Fluorolube 2000*
Benzene	12	132	48	80
Fluorobenzene	73	174	82	109
1,2,4,5-Tetra-fluorobenzene	211	255	163	140
Pentafluorobenzene	253	259	180	127
Perfluorobenzene	290	256	200	116
Toluene	11	133	62	83
o-Fluorotoluene	67	162	85	101
m-Fluorotoluene	73	183	92	108
p-Fluorotoluene	73	183	90	110

its fluorination products, Krytox is the most efficient stationary phase. This effect is also seen when toluene and the three monofluoro derivatives are considered. Kel F oil is seen to have similar characteristics towards fluoroaromatics, however the effects are not as pronounced as with Krytox and a smoothing out is noted for this phase, the retention increment for substituting the first fluorine being 27 units and for the sixth fluorine a mere 2 units. This is seen clearly in Table IV where the solute–stationary phase interactions involving Kel F are relatively high for benzene but show a smaller increase to perfluorobenzene.

Fluorolube oil shows rather an unusual degree of interaction with the various fluorocarbons, the retention indices in Table III reflect both solute–stationary phase interaction and boiling point characteristics of the solutes so that monofluorobenzene has the highest retention increment. Consideration of Table IV however, where the boiling point characteristics have been eliminated, bears out the initial prediction that solute–stationary phase interactions on this phase will be of the same order of magnitude —in fact all the fluorobenzenes are included in the increment range 109–140.

Silicone QF-1 behaviour also follows that predicted from Tables I and II. The high retention of benzene relative to perfluorobenzene means that this would be the least effective stationary phase for such a separation. As this is the most polar phase, the highest retention indices are found on the silicone but a saturation, in terms of fluorine, is soon achieved by the solute molecule so that —as seen in Table IV— solute–stationary phase interactions involving tetra-, penta-, and perfluorobenzenes are very similar.

On considering the toluene and fluorotoluene data in the tables, it can be seen that no separation of parent hydrocarbon or three fluoro isomers is possible on squalane. As stated, the toluene could be separated from the fluorinated products on Krytox and silicone QF-1 would appear to be the most suitable phase for isolation of the *ortho* derivative. However, none of the phases examined could separate *meta* and *para* fluorotoluenes and the results indicate that a selective phase such as a Bentone or a metal stearate could be necessary to achieve this isomer separation.

Table V presents information on solute–stationary phase interactions on sub-

TABLE V

INFLUENCE OF SUBSTITUTING F FOR H IN THE SOLUTES. RETENTION INCREMENTS, ΔI ($I_{solute} - I_{parent\ hydrocarbon}$), FOR FLUOROCARBONS AT 100°

Compound	*ΔI*				
	Krytox	*Silicone QF-1*	*Kel F-90*	*Fluorolube 2000*	*Squalane*
Fluorobenzene	48	35	27	22	−7
1,2,4,5-Tetrafluorobenzene	147	77	69	14	−46
Pentafluorobenzene	174	66	71	−24	−61
Perfluorobenzene	193	45	73	−43	−79
o-Fluorotoluene	57	30	24	19	1
m-Fluorotoluene	62	50	30	25	0
p-Fluorotoluene	62	50	28	27	0

stituting one or more fluorine atoms for hydrogen in the solutes. This information is complementary to that in Table IV and it can be seen that whilst fluorine substitution makes a large positive contribution to interactions on Krytox, the effects after the fourth fluorine is introduced are negligible on Kel F, and are actually negative on Fluorolube and silicone QF-1. The largest negative contributions on fluorine substitution are, of course, on the hydrocarbon stationary phase squalane. Again, for the three monofluorotoluenes, Table V reveals that positional effects by the substituent are very small and, in the case of the *meta* and *para* isomers, are almost certainly too small to allow separation.

Table VI lists the number of theoretical plates required for unit resolution of three pairs of aromatic solutes using the Purnell[8] formula, Krytox is seen to be the best stationary phase for benzene–perfluorobenzene separation, Fluorolube however proving slightly more efficient than Krytox for the fluorobenzene–perfluorobenzene separation whilst Kel F is the optimum phase for a fluorobenzene–fluorotoluene separation.

TABLE VI

NUMBER OF THEORETICAL PLATES (n) REQUIRED FOR UNIT RESOLUTION OF TWO-COMPONENT MIXTURES AT 100°

Mixture	*n*			
	Krytox	*Silicone QF-1*	*Kel F-90*	*Fluorolube 2000*
Benzene–perfluorobenzene	108	426	199	250
Fluorobenzene–perfluorobenzene	153	>10,000	429	118
Fluorobenzene–*p*-fluorotoluene	244	94	40	53

Paraffins

The effect of chain branching is seen for 2-methylpentane in Table III where Krytox and Fluorolube behave in a manner similar to non-fluorinated stationary phases, showing a slight decrease in retention index when a branch point is incorporated into the paraffin. Kel F and silicone QF-1 behave uncharacteristically, a small

increase in retention being apparent. The changes in retention on cyclisation can be regarded as normal, Krytox giving a retention increment for cyclohexane over that for *n*-hexane of +80 units. In fact, the behaviour of Krytox for hydrocarbons generally is very close to the theoretical predictions obtained by the Kováts retention index system, thus the increment (ΔI) for introduction of $-CH_2-$ into benzene is +99 units, the increment for cyclisation of hexane is only slightly higher than the value obtained on squalane columns, and the increment for introduction of $-CH_2-$ into cyclohexane, +67 units, is also the value obtained using squalane. The effects of fluorinating 1,3-dimethylcyclohexane can be seen in Table III although the parent hydrocarbon was not available for study. By comparison of the fluorocarbon retention data on the fluorinated phases with that on squalane it is apparent that perfluoro paraffins show a high degree of interaction with Krytox, lower but significant degrees of interaction with Kel F and silicone QF-1 whilst perfluoro paraffins are rejected by Fluorolube oil in that their retention increments over squalane are negative.

Olefins

The availability of π-electrons to the fluorinated stationary phases upon introduction of a double bond into the solute is seen as enhanced retention. Krytox again demonstrates its indifference to the parent hydrocarbon, 2-methyl pent-2-ene, whereas the other three phases show enhanced retention. The effects of molecular structure on retention index are found not to be additive for this class of compound. From consideration of 1-octene and 2-methyl pentane results in Table III, increments for chain-branching and double bond introduction may be determined and theoretical retention indices for 2-methyl pent-2-ene calculated on the additive principle. The experimental values are considerably higher than the calculated values for three of the fluorinated phases and, even allowing for a large experimental error in the determination of such numerically low retention indices, this variation between experimental and calculated values is significant. Silicone QF-1 is the unusual phase in this respect, although the McReynolds constants in Table II show it to be the most polar phase studied and, in agreement with this, the retention index of 1-octene is higher than on the other phases, the value for 2-methyl pent-2-ene at 611 is lower than the values obtained on Kel F or Fluorolube. Furthermore, the calculated value of 624 is considerably higher than the experimental value obtained on this phase. The perfluoro analogue retention data shows close agreement with that predicted from the perfluoro paraffin data with the highest retention being on Krytox and retention indices decreasing in the order Kel F, silicone QF-1, Fluorolube, squalane. In perfluoro-olefins the factor contributing most to the retention is obviously the degree of fluorination and not the presence of the double bond as fluoro-olefins are much more similar to fluoro-paraffins than to fluoroaromatics.

CONCLUSIONS

Of the four low-polarity fluorinated stationary phases examined, the polarities increased in the order shown in Table II. However, a more important criterion for the selection of a stationary phase for the chromatography of fluorinated hydrocarbons would appear to be the extent of substitution of hydrogen by fluorine. Thus, for the three perfluorocarbons studied, Krytox, which has the lowest polarity, gives rise

to the highest retention indices —that is to say that it shows the highest degree of interaction with these solutes.

A mathematical treatment which is only an approximation nevertheless enables the performance of each stationary phase, towards substitution by fluorine into the solute, to be studied. If the retention index of each perfluorocarbon on squalane is subtracted from the retention index on each fluorinated stationary phase, the residual increment represents total solute–stationary phase interaction. This interaction, however, would seem to be dominated by the extent of fluorine substitution so, ignoring other contributions and making the broad assumption that all fluorine atoms in the molecule are equivalent (which they certainly are not), this increment may be divided by the number of fluorine atoms in the molecule to give an incremental effect on solute–stationary phase interaction of the substitution of a fluorine atom. The results of such a treatment are shown in Table VII.

TABLE VII

ΔI EFFECT, ON SOLUTE–STATIONARY PHASE INTERACTIONS, OF SUBSTITUTING ONE F FOR H IN THE SOLUTE (AVERAGED VALUES)

Solute	ΔI			
	Krytox	*Silicone QF-1*	*Kel F-90*	*Fluorolube 2000*
Aliphatic fluorocarbons (saturated)	32	6	15	−8
Olefinic fluorocarbons	44	21	24	2
Aromatic fluorocarbons	45	21	28	6

It can be seen that all values increase in the order aliphatic, olefinic, aromatic due to the ignored π-electron interaction. In the table, however, the similarities of the three classes of fluorocarbon on each stationary phase are apparent. Thus fluorine substitution has the largest effect on chromatographic retention on Krytox followed by Kel F, silicone QF-1 and Fluorolube in order of decreasing effect. There is such a wide spread between the large effects on Krytox and the almost negligible effects on Fluorolube that choice of one of these four stationary phases should allow most of the hydrocarbon–fluorocarbon–fluorinated hydrocarbon separations which may be necessary to be carried out efficiently.

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KINETICS OF HEROIN DEACETYLATION IN AQUEOUS ALKALINE SOLUTION AND IN HUMAN SERUM AND WHOLE BLOOD

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SUMMARY

A kinetic study of heroin hydrolysis in alkaline aqueous solution at room temperature was conducted by a gas chromatographic method to measure the consecutive reactions of diacetylmorphine to monoacetylmorphine and of monoacetylmorphine to morphine. A first-order reaction was observed in both instances, and the rate for the deacetylation of heroin was greater than that of monoacetylmorphine.

The rates of *in vitro* hydrolysis of diacetylmorphine in human whole blood and in serum were compared by the same method. Diacetylmorphine was hydrolyzed twice as rapidly in blood as in serum. While morphine was an end product of hydrolysis in the blood, it was absent in the serum.

INTRODUCTION

The hydrolysis of diacetylmorphine (heroin) to morphine occurs by a pathway of 6-monoacetylmorphine, as confirmed by manometric[1], colorimetric[2], and paper chromatographic[3] methods. However, the manometric and colorimetric procedures do not delineate the molecular entities produced in heroin degradation since the former measures the release of CO_2 during deacetylation and the latter quantitates the free phenolic group liberated from the cleavage of the acetyl groups from the heroin. The paper chromatographic approach does not provide a convenient measurement of the hydrolysis products.

Gas chromatographic (GC) techniques, on the other hand, are highly suited for kinetic studies, since consecutive reactions may be measured, the hydrolysis of intermediate products may be followed, and a precise quantitative analysis of reaction products is possible.

The lability of heroin in aqueous solution, particularly in concentrated solu-

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tions of hydroxyl and hydrogen ions, is of concern to the forensic chemist. Certain illicit drug preparations contain heroin in mixture with other drugs. Since the classical separation procedures described in The Bulletin on Narcotics[4] entail an alkaline treatment of samples prior to the extraction of basic drugs with organic solvents, it is possible that the heroin component of that sample would be subject to rapid degradation. The rate of that degradation would be of practical interest.

A comparative measurement of the rate of hydrolysis from diacetylmorphine to monoacetylmorphine and from monoacetylmorphine to morphine would be more meaningful than a graphic comparison by paper or thin-layer chromatography (TLC).

A kinetic study of the hydrolysis of heroin in an aqueous system can be used as a basis for further studies as a function of pH, of temperature, of prolonged storage, of relative amounts of humidity, and of enzyme catalysis in *in vitro* biological reactions. The present study was undertaken to elucidate the nature of heroin with respect to its structural stability in aqueous solution and in biological fluid as an initial step in establishing a method for its determination in blood.

Ellis[5] presented experimental evidence that the 6-acetyl and 3-acetyl groups of heroin were perhaps cleaved by separate enzymes in rabbit plasma.

Way *et al.*[6] noted the rapidity in which heroin was deacetylated in man and four other mammalian species when incubated in liver, kidney, blood, and brain; these investigators employed a countercurrent extraction system for separating 6-monoacetylmorphine from heroin. Nakamura and Ukita[7] demonstrated that heroin is deacetylated *in vivo* in dog within 10 min following an intravenous dose. Therefore, it seems futile to attempt detection of heroin in blood, and morphine would appear to be the metabolic product to be detected to imply the use of heroin. However, 6-monoacetylmorphine remained a possible entity for a systematic search in blood.

An *in vitro* study of heroin hydrolysis in human blood was undertaken to observe the rate of production and decay of heroin metabolites. A sample of fresh blood serum, obtained from a normal male subject who was not a drug user, and a sample of *post mortem* whole blood specimen freshly removed and heparinized were used. The specific objectives were to determine if complete enzymatic hydrolysis occurred in the serum or in the presence of blood cells, if postmortem blood was capable of degrading heroin and 6-monoacetylmorphine to morphine, if heroin was deacetylated by autohydrolysis in a buffer at physiological pH, and to study the rate of *in vitro* hydrolysis of heroin in blood.

EXPERIMENTAL

Reagents

Diacetylmorphine·HCl (heroin·HCl) was prepared according to a method described by Nakamura[8]. 6-Monoacetylmorphine was prepared according to the method of Wright[1]. It was found here that the crystallization of 6-monoacetylmorphine is hastened by dissolving heroin in a minimum amount of ethanol and then treating with an equivalent amount of hydroxylamine·HCl. Methadone·HCl USP was obtained from E. Lilly Labs. (Indianapolis, Ind., U.S.A.) and morphine sulfate USP from Mallinckrodt (St. Louis, Mo., U.S.A.).

Gas chromatography

A Microtek Model 220 dual-column gas chromatograph with a hydrogen flame ionization detector was used in this study. The 6 ft. × 4 mm I.D. column was treated with dimethyldichlorosilane. The liquid phase used was 3.8% silicone gum rubber UCW-98 (Hewlett-Packard), applied onto Chromosorb W HP, 80–100 mesh. The column was conditioned by a series of massive injections of free morphine (in methanol) to saturate its absorption sites. The temperature of the oven was maintained at 225°. The carrier gas (helium) flow-rate was 40 ml/min. The attenuation range was set at $10^2 \times 2$.

Mixed standard

Ten milligrams each of diacetylmorphine, 6-monoacetylmorphine, and morphine (each calculated as an anhydrous base) were dissolved in methanol containing 0.2 μg/ml methadone as internal standard. Two microliters were used for comparison purposes. A chromatogram of these standards is shown in Fig. 1.

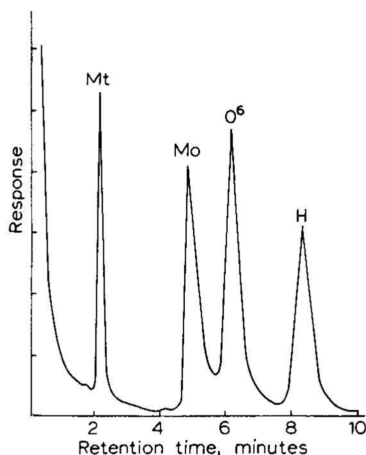


Fig. 1. A mixture of methadone (Mt), morphine (Mo), 6-monoacetylmorphine (O^6) and heroin (H) chromatographed under standard conditions. GC unit, Microtek 220 with flame ionization detector; column, 6 ft. × 4 mm glass, packed with 3.8% UCW-98 on Chromosorb W HP, 80–100 mesh. Injection temperature, 225°; detector temperature, 275°; carrier gas (helium) flow-rate, 40 ml/min; attenuation range, $10^2 \times 2$.

Aqueous solution

To 25 ml of water, 10 mg heroin hydrochloride and 25 ml of 1 *M* sodium carbonate were added. After various time intervals (1, 5, 10, 30, 60, 90, and 120 min), a 1-ml aliquot was removed and the pH was lowered to approximately 8.5 by adding 1 ml of saturated ammonium chloride to the aliquot. The mixture was quickly shaken with successive amounts of chloroform–isopropanol (8:2) in volumes of 25, 10, and 10 ml. The chloroform extracts were passed through filter paper to remove water and evaporated over a warm steam-bath under an air jet. Each extract was dissolved in 1 ml of methanol (GC-spectrograde, J. T. Baker, Phillipsburgh, N.J., U.S.A.) containing 0.1 mg methadone. One microliter was injected under standard conditions.

Hydrolysis in serum and whole blood, in vitro

To ten milliliters of fresh human serum and post-mortem whole blood, 1 ml of phosphate buffer, pH 7.4 and 20 and 30 mg, respectively, of diacetylmorphine hydrochloride were introduced. The mixture was incubated at 37.5° and a 1-ml aliquot was removed at intervals of 15, 30, 45, 90, and 120 min.

To each aliquot twelve drops of 40% dipotassium phosphate were added. The mixture was shaken with chloroform–isopropanol (9:1), first with 25 ml, then with 15 ml, and finally with 10 ml. Each succeeding extract was passed through a filter paper containing a pinch of powdered sodium sulfate and then evaporated to dryness on a warm steam-bath under an air jet. The residue was dissolved in 1 ml of methanol containing 0.1 mg of methadone. One microliter was injected under standard GC conditions.

Control

Ten milligrams of heroin hydrochloride were dissolved in 10 ml of 0.1 *M* phosphate buffer, pH 7.3. A 1-ml aliquot was treated with twelve drops of 40% K_2HPO_4 and then extracted and subjected to GC analysis in the same manner as that described for serum and blood hydrolysates.

Thin-layer chromatography

Each of the methanolic extracts from the above GC runs were evaporated to dryness and redissolved in 100 μl of methanol. The solution was quantitatively transferred to a TLC plate (Bakerflex silica gel 1B), each spot representing a particular hydrolysis time. The spots applied from the different hydrolysates were arranged on the plate according to a progressive sequence of time.

About 50 μg each of pure heroin, 6-monoacetylmorphine and morphine were applied as references on the same base line as the hydrolysates. These TLC plates were developed in a covered glass tank in an ascending mode in chloroform–methanol (8:2). The spots were revealed by spraying the plates with iodoplatinate spray reagent, as shown in Figs. 2 and 3. The TLC procedure was adapted from that used by Mary and Brochman-Hanssen[9].

RESULTS AND DISCUSSION

Gas chromatograms of a heroin hydrolysate (Fig. 4) of different time intervals from 1–120 min illustrate the deacetylation of heroin to O^6-monoacetylmorphine, as an intermediate product, and then to morphine, as the final product. The concentration values of reactants and products are shown in Table I. Fig. 5 shows the curves for the two-step series reactions for the degradation of heroin, resulting in a decrease in concentration, the appearance of 6-monoacetylmorphine, and its gradual degradation to morphine, and finally the increasing amount of the end product, morphine. Linear plots of reaction rates of heroin to monoacetylmorphine and of monoacetylmorphine to morphine, as a function of time, are shown in Fig. 6.

The plots of Fig. 5 make it quite evident that the acetyl group at the 3-carbon position, *i.e.*, on the phenolic hydroxyl group, is much more labile than that on the 6-carbon position of the alcoholic hydroxyl group. The reactants appear to be first-order, since a linear plot is obtained and the equation as shown below is obeyed, and

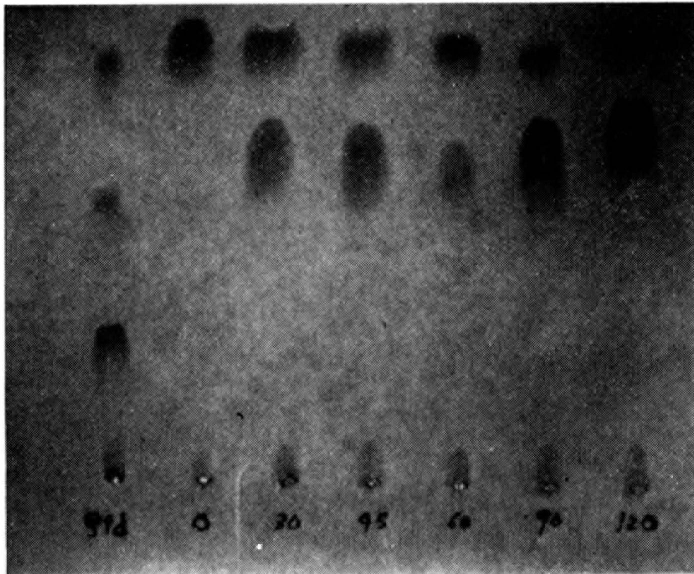


Fig. 2. Thin-layer chromatogram of series hydrolysis of heroin in serum, 0–120 min. From top to bottom: heroin, 6-monoacetylmorphine, and morphine. Std. = Reference standard.

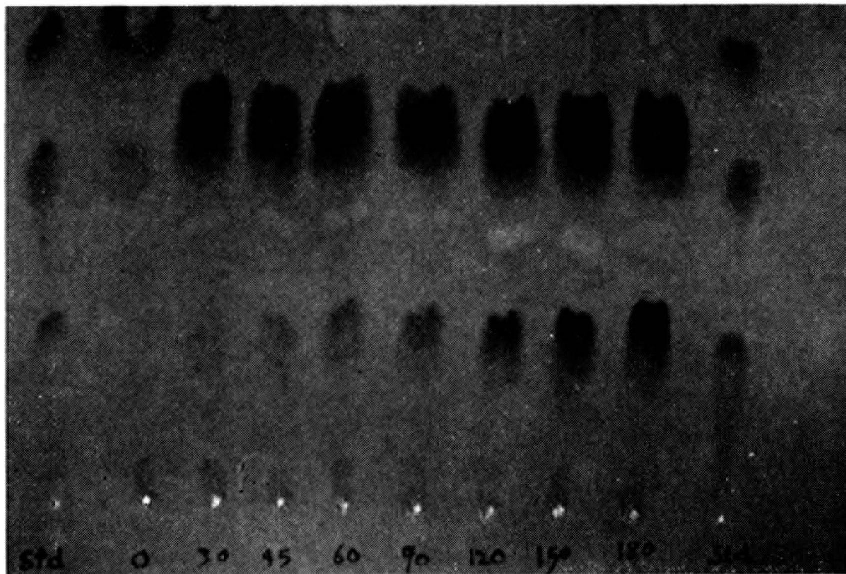


Fig. 3. Thin-layer chromatogram of series hydrolysis of heroin in whole blood, 0–180 min. From top to bottom: heroin, 6-monoacetylmorphine, and morphine. Std. = Reference standard.

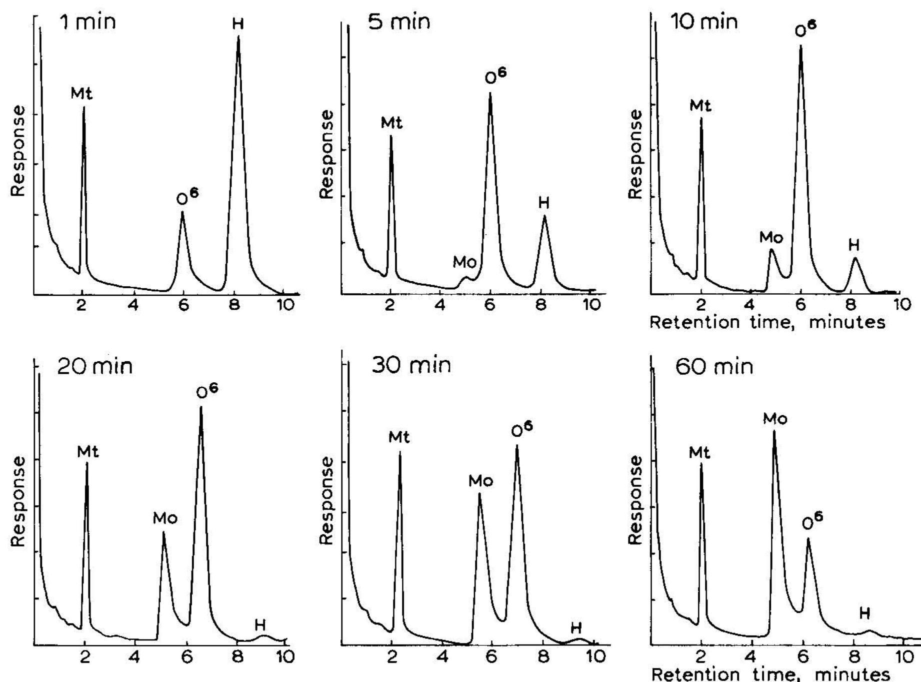


Fig. 4. GC profile of heroin and its hydrolysis products in 1 *M* Na_2CO_3 at different time intervals up to 60 min. The same analytical conditions, except for the length of hydrolysis, were observed in each case.

TABLE I

CONCENTRATION OF REACTANTS AND PRODUCTS IN HEROIN HYDROLYSIS MEASURED FROM GAS CHROMATOGRAMS

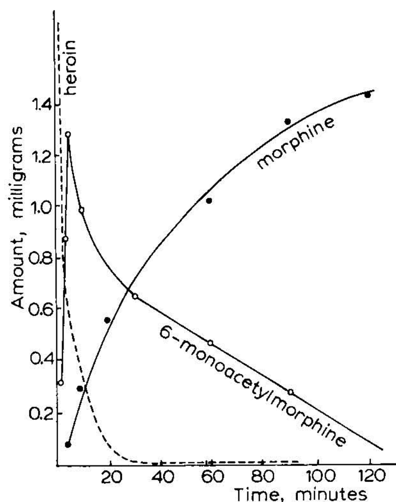
Mean values from two hydrolysis determinations are taken. Triangular method of area measurement made on GC peaks: $\frac{1}{2}$ (height × width) = area.

Time (min)	*Concentration (mg)*		
	Morphine	*6-Monoacetylmorphine*	*Heroin*
1	0	0.33	1.67
5	0.08	1.28	0.56
10	0.28	1.00	0.32
20	0.55	0.77	0.07
30	0.72	0.64	0.04
60	1.06	0.48	0.02
90	1.32	0.29	0.02
120	1.52	0.18	0

since heroin, as shown in Fig. 5, decreases in an exponential manner as morphine increases. Also the entire amount of the reactant is exhausted under standard analytical conditions in which an excess amount of alkali is present in the reaction mixture.

The rate study as shown in Fig. 6 was based on the following eqn.[9]:

$$\log \frac{a}{a - x} = \frac{kt}{2.303} \tag{1}$$



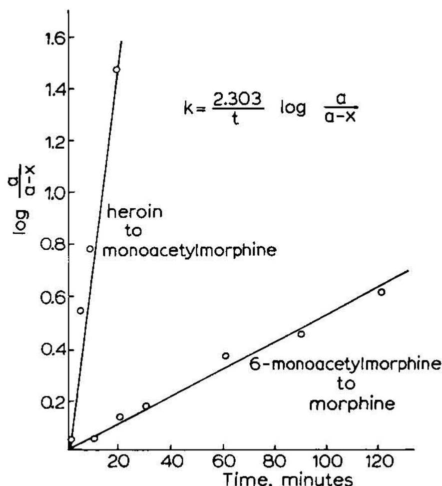


Fig. 5. Concentration *vs.* time plot for series degradation of heroin in 1 *M* Na_2CO_3.

Fig. 6. Linear plots of data in Fig. 4 as a first-order decomposition.

where a is the initial concentration of reactant and x is the product, k is the rate constant, and t is the time of reaction.

The rate constant k of heroin conversion to monoacetylmorphine was 16.6×10^{-2} min^{-1} and that of monoacetylmorphine to morphine was 1.25×10^{-2} min^{-1}. The relative stability of monoacetylmorphine in the aqueous media was inferred. In a separate experiment, it was found that 6-monoacetylmorphine did not completely degrade in hot water (98°) until after 114 h.

The differences in the rate of heroin hydrolysis in whole blood and in serum was studied by using GC and TLC methods. In the serum, as the thin-layer chromatogram of Fig. 2 shows, no cleavage of 6-monoacetylmorphine was observed, while in the whole blood both acetyl groups of heroin were cleaved, resulting in morphine being exhibited on the chromatogram (Fig. 3). The concentration *vs.* time plots of Figs. 7 and 8 demonstrate the rapid deacetylation of heroin as shown by the decrease in heroin concentration with a corresponding increase of monoacetylmorphine. Only for whole blood (Fig. 8) is morphine production apparent as soon as heroin is completely deacetylated. In both serum and whole blood, the amount of monoacetylmorphine remaining in the hydrolysate was relatively large. This finding suggests the advisability of examining *post mortem* blood specimens for the presence of monoacetylmorphine in acute heroin poisoning.

Hydrolysis proceeds at about twice the rate in the whole blood as in the serum; the biological half-life was observed to be 9 min in blood and 22 min in serum. The rate constant k for whole blood was 5.5×10^{-2} min^{-1} while that for the serum was 3.5×10^{-2} min^{-1} as calculated from the slopes shown in Fig. 9. Vincent[11] has characterized blood cells as having an esterase activity and Harthon and Hedstrom[12] demonstrated that whole blood was more vigorous than plasma in deacetylating acetylsalicylic acid.

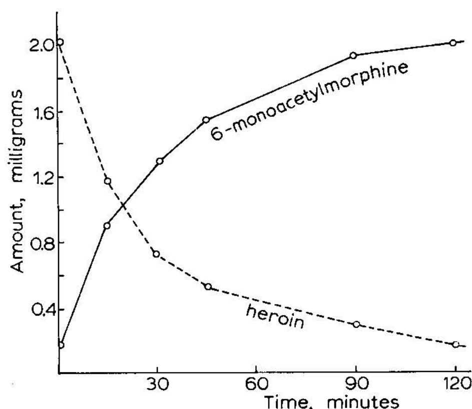


Fig. 7. Concentration *vs.* time plot for hydrolysis of heroin in human serum.

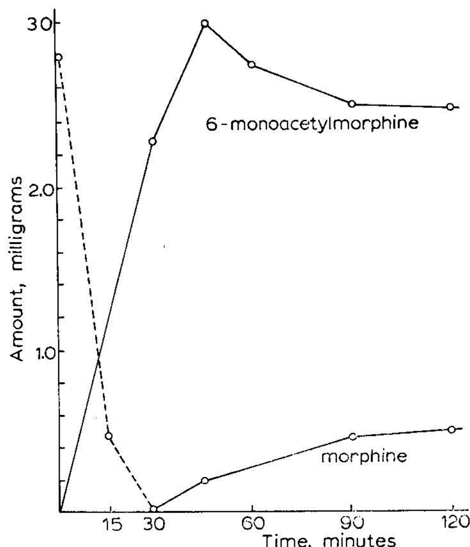


Fig. 8. Concentration *vs.* time plot for hydrolysis of heroin in human whole blood.

Whether 6-monoacetylmorphine can be detected in blood collected from man is left to conjecture, since the presence of this metabolite in human blood has not been reported. It is not infeasible that this could occur, however, since unchanged heroin itself has been detected in human urine samples by Elliott *et al.*[13] and Advenier *et al.*[14]. In an *in vivo* situation, the deacetylation of monoacetylmorphine may proceed at a much faster rate than in an *in vitro* system.

The spontaneous hydrolysis of heroin can be dismissed since a control test conducted in a non-enzymatic system at physiological pH showed that heroin was virtually left undegraded when incubated in phosphate buffer at pH 7.3. To prevent *post mortem* hydrolysis or bacterial action on heroin and 6-monoacetylmorphine in blood specimens, coroner's samples are usually collected in 1% sodium fluoride.

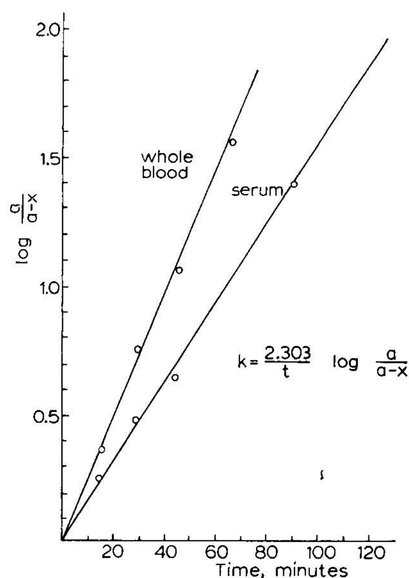


Fig. 9. Linear plots of data in Figs. 7 and 8 as a first-order decomposition.

Plueckhahn and Ballard[15] established that a concentration of more than 1.0% sodium fluoride was necessary to inhibit microbiological formation of ethanol from carbohydrates in stored blood samples.

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EXAMINATION OF THE DIPHENYLPROPANOIDS OF NUTMEG AS THEIR TRIMETHYLSILYL, TRIETHYLSILYL AND TRI-*n*-PROPYLSILYL DERIVATIVES USING COMBINED GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

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SUMMARY

Ethyl acetate extracts of thirteen samples of nutmeg and one sample of mace were examined as trimethylsilyl, triethylsilyl and tri-*n*-propylsilyl derivatives by gas chromatography and mass spectrometry. Eleven compounds derived from two types of diphenylpropanoid were identified; the relative proportions and quantities of these compounds varied considerably in the different samples. Single ion chromatograms were used to obtain the relative concentration of one series of compounds. Preparation of the triethylsilyl and tri-*n*-propylsilyl derivatives resulted in greatly increased separation of the diphenylpropanoids over trimethylsilyl derivatives, which tended to produce a group of compounds with many unresolved peaks.

INTRODUCTION

Nutmeg and mace, both obtained from the plant *Myristica fragrans* Houtt, have long been known to contain a hallucinogenic principle, once thought to be myristicin[1]. Recent work by Forrest and Heacock[2] has shown a similarity in the thin-layer chromatographic properties of both nutmeg extracts and organic extracts of *Cannabis sativa* L. Both plants give positive results with Fast Blue B dye, although few plants are known which do this. Previous investigations of organic extracts of nutmeg[3–9] and mace[10] have led to the identification of many components, including terpenes, phenyl propanoids and, recently, two sets of compounds derived from di-(phenylpropanoids)[11,12]. We have recently examined the constituents of a number of samples of *Cannabis sativa* L.[13] and, because of the apparent similarities in the chromatographic properties between extracts of this plant and those of nutmeg, have now extended this work to include *Myristica fragrans* H.

EXPERIMENTAL

Whole nutmeg and ground nutmeg and mace samples were obtained from local supermarkets.

Extraction and preparation of derivatives

100-mg samples of nutmeg were ground as finely as possible and allowed to stand in ethyl acetate for 1 h with occasional shaking. The solid material was filtered off and washed well with ethyl acetate. The combined extracts were then concentrated to about 1 ml and kept at 0° to complete precipitation of triglycerides (chromatograms of the complete extracts indicated that the diphenylpropanoids remained in solution). The ethyl acetate was removed from the filtered solution with a stream of nitrogen and derivatives were prepared by the addition of 200 μl of the appropriate reagents (described below) and allowing the mixture to stand overnight at room temperature.

Trimethylsilyl derivatives. Trimethylsilyl (TMS) derivatives were prepared by the addition of a pre-prepared mixture of N,O-bis(trimethylsilyl)trifluoroacetamide (80 μl), acetonitrile (80 μl) and trimethylchlorosilane (40 μl).

d_9-TMS derivatives[14]. d_9-TMS derivatives were prepared from 0.01 ml of solution with d_9-N,O-bis-trimethylsilylacetamide (10 μl), acetonitrile (10 μl) and a trace of trimethylchlorosilane as a catalyst.

Triethylsilyl derivatives[15]. The reagent was prepared by mixing triethylchlorosilane (1 ml), pyridine (2 ml) and diethylamine (0.5 ml) in a centrifuge tube, cooling the resulting mixture in ice water and centrifuging to remove the white precipitate. The clear supernatant was removed with a pipette and used for derivative preparation.

Tri-n-propylsilyl derivatives. The reagent was prepared as above with tri-*n*-propylchlorosilane.

For the estimation of the quantity of the diphenylpropanoids in each sample, 0.5 ml of a solution of cholesterol (1 mg/ml) was added to each sample following the extraction and the compounds were converted into TMS derivatives as described above. Response factors were not determined as the individual diphenylpropanoids were not isolated. However, because of the similarity in the composition of the cannabinoids and diphenylpropanoids, these compounds were assumed to have similar response factors. Cannabinol response factors were determined previously[13]. Gas chromatographic peak areas were obtained by the weighing technique. Removal of the triglycerides by prior crystallization was not carried out with these samples.

Gas chromatography

Gas chromatography was carried out with a Varian 2400 gas chromatograph fitted with flame ionization detectors and two 6 ft. × 2 mm glass columns packed with 3% SE-30 on 100–120 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). Nitrogen at 30 ml/min was used as the carrier gas and the column oven was programmed from 150–330° at 4°/min. The temperature was then kept at 330° for about 30 min to complete the elution of the triglycerides. Injector and detector temperatures were maintained at 270°.

Mass spectrometry

Mass spectra were recorded at 22.5 eV with a VG-Micromass 12B mass spectrometer, interfaced via a glass jet separator to a single-column gas chromatographic system similar to that described above. The ion source was maintained at 220° and the spectra were obtained for each chromatographic peak using a 3-sec scan and an accelerating voltage of 2.5 kV. For the single-ion chromatograms, the acce-

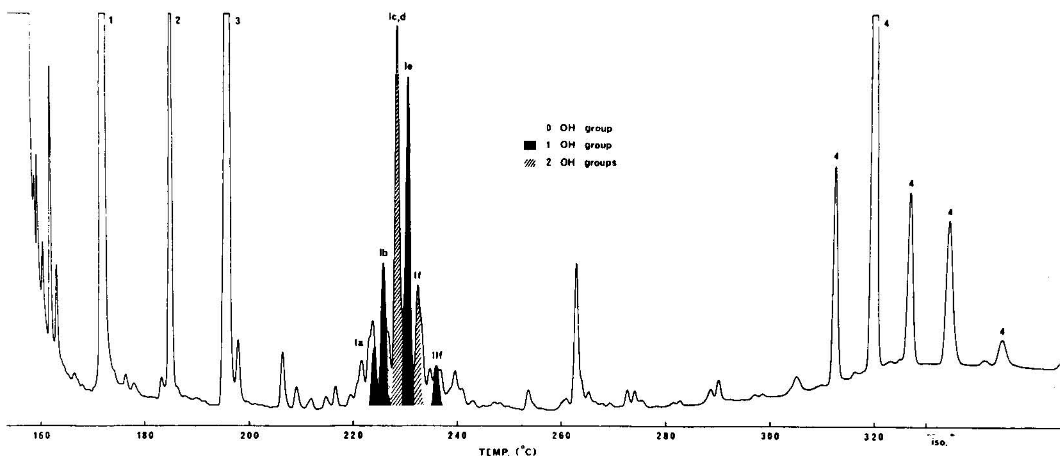


Fig. 1. Separation of the ethyl acetate extract of nutmeg (sample 2) as TMS derivatives on a 6 ft. × 2 mm glass column packed with 3% SE-30 on Gas-Chrom Q and temperature programmed at 4°/min from 150°. Compounds identified include myristic acid (1), palmitic acid (2), oleic and linoleic acids (3), several triglycerides (4) and the diphenylpropanoids (compounds I and II).

lerating voltage was held at 2.5 kV and the magnetic field adjusted to focus the ion in question. The resolving slit was opened to give flat-top peaks and the output was recorded on a Servoscribe 1S single-channel flat-bed recorder.

RESULTS AND DISCUSSION

The lower-boiling constituents of the nutmeg extracts, consisting mainly of monoterpenes and phenylpropanoids, were not examined as the gas chromatograms of this fraction have already been reported[4,5,8,9]. Fig. 1 shows the gas chromatogram on 3% SE-30 of an ethyl acetate extract of a typical sample of nutmeg over the range

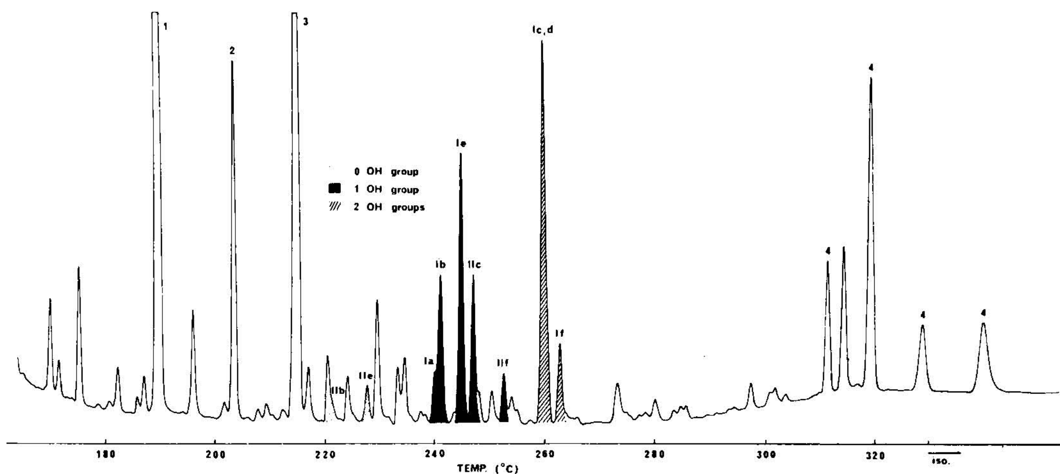


Fig. 2. Separation of the ethyl acetate extract of nutmeg (sample 2) as triethylsilyl derivatives. Conditions, as for Fig. 1.

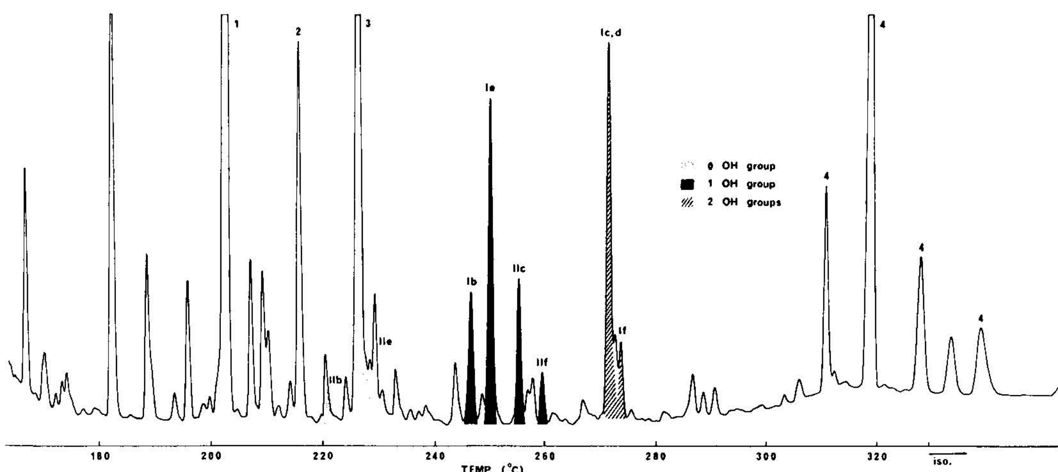


Fig. 3. Separation of the ethyl acetate extract of nutmeg (sample 2) as tripropylsilyl derivatives. Conditions as for Fig. 1.

150–330°. Three groups of compounds were identified: fatty acids, mainly myristic, palmitic and oleic acids, all known constituents of nutmeg[3,8,9]; triglycerides, also known constituents; and a group of compounds derived from diphenylpropanoids. Different samples of nutmeg and mace (fourteen samples were examined) showed the same general pattern, but the proportions and quantities of the various diphenylpropanoids were observed to vary considerably (Figs. 4 and 5). These compounds were present to the extent of 0.4–2% of the total weight of the nutmeg depending on the sample. Table I lists the samples examined, the amount of each sample left after extraction with ethyl acetate, and the per cent of diphenylpropanoid found. The major components of the organic extracts were triglycerides.

Mass spectrometry showed that most of the gas chromatographic peaks in the diphenylpropanoid region were produced by several compounds. Two sets of com-

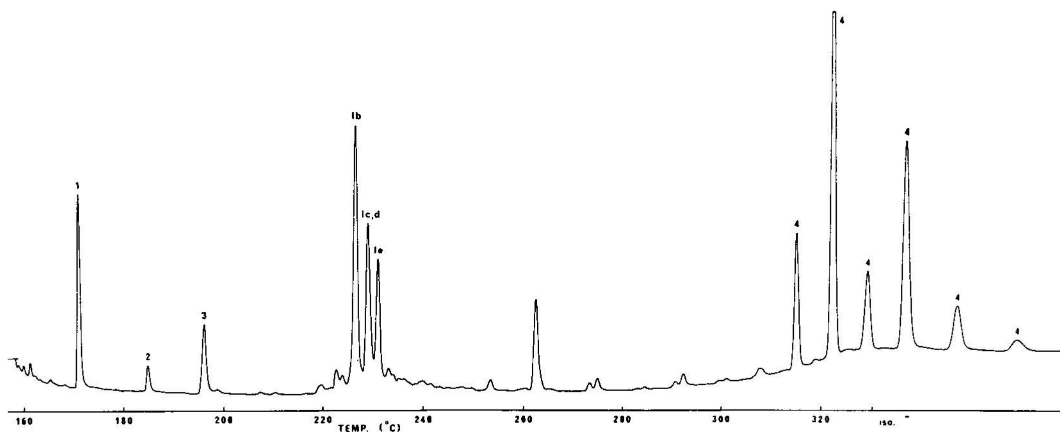


Fig. 4. Separation of the TMS derivatives of the diphenylpropanoids of nutmeg sample 1. Conditions, as for Fig. 1.

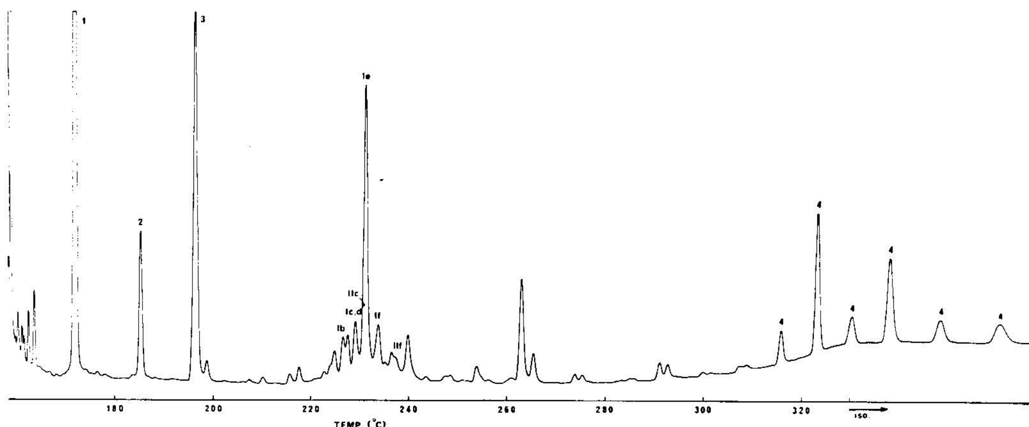
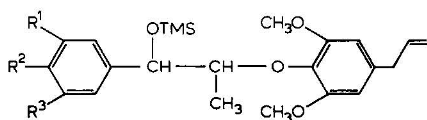


Fig. 5. Separation of the TMS derivatives of the diphenylpropanoids of nutmeg sample 7. Conditions, as for Fig. 1.

pounds were distinguished, and identified by their mass spectral properties as belonging to the two groups of diphenylpropanoids I and II isolated by Forrest *et al.*[12].



I

II

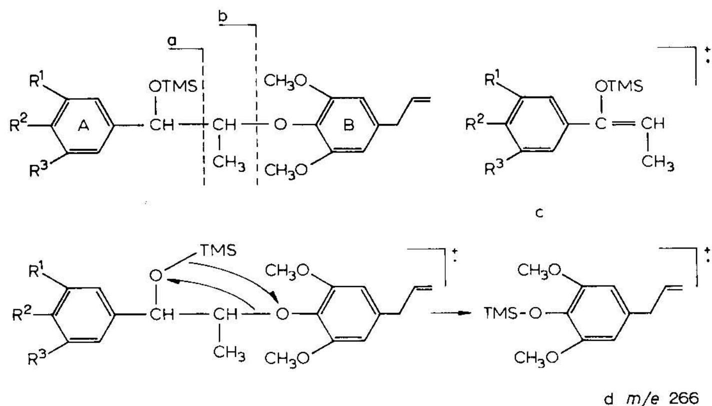
Compound no	R^1	R^2	R^3
Ia	—O—CH$_2$—O—		H
Ib	OCH$_3$	OCH$_3$	H
Ic	OCH$_3$	OH	H
Id	OH	OCH$_3$	H
Ie	OCH$_3$	OCH$_3$	OCH$_3$
If	OCH$_3$	OH	OCH$_3$

Compound no	R^1	R^2	R^3
IIa	—O—CH$_2$—O—		H
IIb	OCH$_3$	OCH$_3$	H
IIc	OCH$_3$	OH	H
IIe	OCH$_3$	OCH$_3$	OCH$_3$
IIf	OCH$_3$	OH	OCH$_3$

The mass spectra of the compounds of type I (Table II) were dominated by four fragment ions, a–d. Molecular and, in most cases, $[M-15]^+$ ions were also present, but in low abundance. Ions a, b and c contained only one of the aromatic rings (termed ring A). The position of these ions in the spectra of the various compounds therefore indicated the change of substitution in this ring. Also the observed shift in the position of these ions in the spectra of the d_9-derivatives was indicative of the number of hydroxyl groups present.

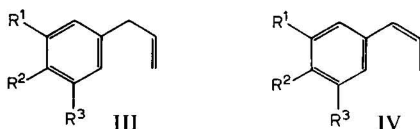
Ions a and b were the result of simple cleavage of the molecule, whereas ion c was produced by a hydrogen migration to the neutral fragment.

Ion d was a rearrangement ion produced by migration of the aliphatic TMS group and expulsion of ring A, presumably as a substituted styrene oxide type of



neutral fragment. All of the compounds of type I found in the nutmeg sample gave ion d at *m/e* 266, thus indicating no change in the substitution pattern of its aromatic ring (termed ring B). All of these compounds therefore had the same basic structure and differed only in the substitution on ring A.

The compounds of type I found in the various samples are given below the scheme of the type I diphenylpropanoids. The positions of the substituents around the aromatic ring were based on reported syntheses[12] and on biochemical considerations; the corresponding phenylpropanoids, for example eugenol (III, R^1 = OMe, R^2 = OH, R^3 = H), have previously been reported as constituents of the essential oil of nutmeg[10]. Compounds of type I may be regarded as derivatives of the isophenylpropanoids, *e.g.* isoeugenol (IV, R^1 = OMe, R^2 = OH, R^3 = H), and isophenylpropanoids corresponding to the substitution present in Ib, Ic and Id have also been reported as constituents of nutmeg oil[10]. Isomyristicin (IV, R^1, R^2 = –O–CH_2–O–, R^3 = OMe) has not been reported although it had been looked for[6], and this may be related to the fact that although myristicin (III, R^1, R^2 = –O–CH_2–O–, R^3 = OMe) is the major phenylpropanoid in most nutmeg samples examined[9], its corresponding diphenylpropanoid (type I) was not found. Again, compound Ia, derived from isosafrole (IV, R^1, R^2 = –O–CH_2–O–, R^3 = H), was usually present in only small amounts, possibly reflecting the apparent absence of a report of the detection of isosafrole in nutmeg.



Compounds Ic and Id had very similar retention indices and mass spectra and were thus probably isomeric around ring A. Compound Ic has been reported previously[12]. No phenylpropanoid containing the substitution pattern of Id has been reported from nutmeg although it (chavibetol) is known in other species. Another possible structure for Id could be the diastereoisomer of the *erythro*[12] compound Ic, but this is unlikely in view of the apparent absence of corresponding isomers of the other compounds.

TABLE I

CHARACTERISTICS OF SAMPLES

Sample No.	*Type*	*State*	*Dry weight** (%)	*Diphenylpropanoids*** (%)
1	Nutmeg	Whole	44.0	2.1
2	Nutmeg	Ground	48.8	2.2
3	Mace	Ground	59.5	1.7
4	Nutmeg	Ground	49.0	0.9
5	Nutmeg	Ground	50.0	2.1
6	Nutmeg	Ground	53.2	1.9
7	Nutmeg	Ground	51.6	1.1
8	Nutmeg	Whole	42.0	0.8
9	Nutmeg	Whole	45.0	0.4
10	Nutmeg	Whole	44.5	0.9
11	Nutmeg	Whole	41.1	0.6
12	Nutmeg	Whole	39.65	0.8
13	Nutmeg	Whole	44.5	1.8
14	Nutmeg	Whole	45.3	1.4

* After separation by ethyl acetate.
** % of total sample.

No isomer of If was observed; this compound also has mixed substituents in ring A. Several of these compounds, in particular Ib ($R^1 = R^2 =$ OMe, $R^3 =$ H) have not been reported previously. It was interesting that compound Ib was the major constituent of sample 1.

Because ion d (*m/e* 266) was produced by all of the compounds of this type and not by the other components, single ion chromatograms of *m/e* 266 were used to examine the proportion of compounds of type I in each sample. Very clear chromatograms consisting essentially of only compounds of type I were produced. The con-

TABLE II

PARTIAL MASS SPECTRA OF COMPOUNDS OF TYPE I (TMS DERIVATIVES)

Ion	*Ia*	*Ib*	*Ic*	*Id*	*Ie*	*If*
M^+	*m/e* 444 (1.4)*	*m/e* 460 (2.1)	*m/e* 518 (1.3)	*m/e* 518 (0.7)	*m/e* 490 (5)	*m/e* 548 (2.1)
$[M-15]^+$	*m/e* 429 (0.25)	*m/e* 445 —	*m/e* 503 (0.9)	*m/e* 503 (0.7)	*m/e* 475 (0.15)	*m/e* 533 (1.25)
a	*m/e* 223 (100)	*m/e* 239 (100)	*m/e* 297 (100)	*m/e* 297 (100)	*m/e* 269 (100)	*m/e* 327 (100)
b	*m/e* 251 (80.5)	*m/e* 267 (65)**	*m/e* 325 (44)	*m/e* 325 (26)	*m/e* 297 (71)	*m/e* 355 (47)
c	*m/e* 250 (38)	*m/e* 266 ***	*m/e* 324 (19.5)	*m/e* 324 (11)	*m/e* 296 (30)	*m/e* 354 (21)
d	*m/e* 266 (75)	*m/e* 266 (86)§	*m/e* 266 (73)	*m/e* 266 (41.5)	*m/e* 266 (88)	*m/e* 266 (85)

* Relative abundances are given in parentheses.
** Isotope contribution from ion d.
*** Ions c and d.
§ Contains contribution from ion c.

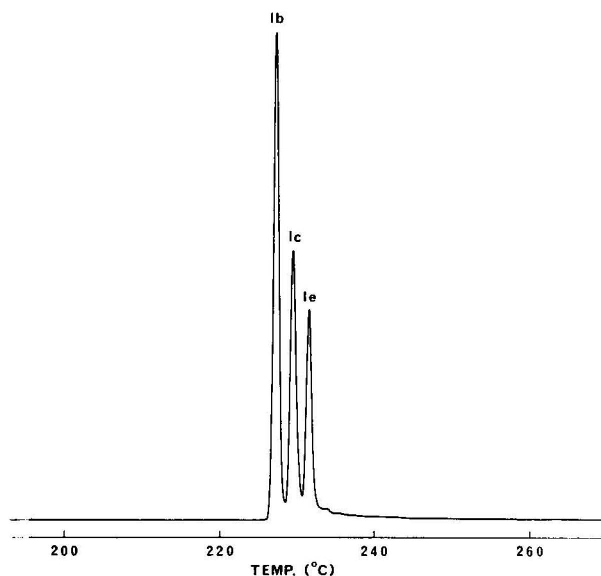


Fig. 6. Single-ion chromatogram of *m/e* 266 (ion d) for nutmeg sample 1. Conditions, as for Fig. 1.

siderable variation in the relative concentration of each component is apparent by comparing Figs. 6–8 and the relative proportions given in Table III. As the percentage of the total ion current carried by ion d was similar for each compound, comparison of concentration could be made directly.

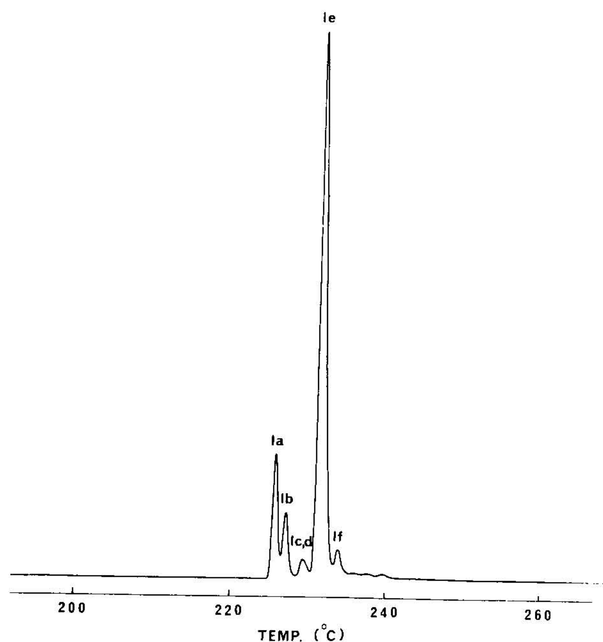


Fig. 7. Single-ion chromatogram of *m/e* 266 (ion d) for nutmeg sample 12. Conditions, as for Fig. 1.

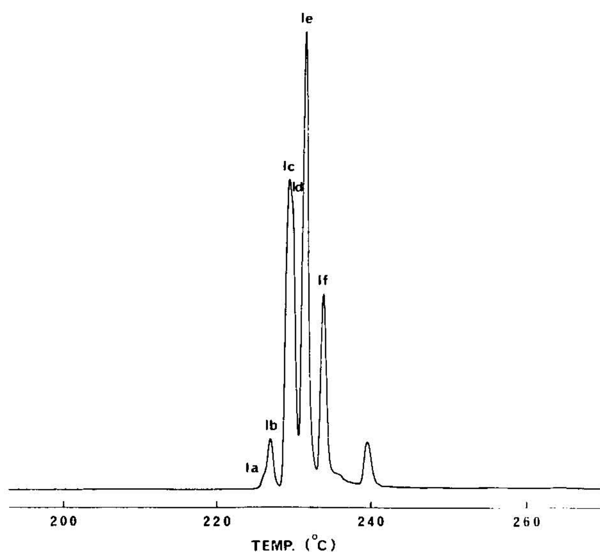


Fig. 8. Single-ion chromatogram of *m/e* 266 (ion d) for nutmeg sample 14. Conditions, as for Fig. 1.

Compounds of type II, related to diisophenylpropanoids, were present in smaller quantities than those of type I, but no abundant characteristic ion was found which was suitable for single-ion monitoring of the group as a whole. Five compounds, three of which (IIa, IIb and IIe) have not been reported previously, were identified by mass spectrometry. These contained the same aromatic substituents as the type I compounds with the exception of Id. Single-ion chromatograms of the molecular ion

TABLE III

PROPORTIONS* OF THE COMPOUNDS OF TYPE I FOUND IN EACH SAMPLE

Sample	*Ia*	*Ib*	*Ic, Id***	*Ie*	*If*
1	–	100	55	46	–
2	7.5	40	100	77	19
3	11	26	19	100	15
4	11	21	18	100	20
5	2	30	100	37	10.5
6	3	35	100	56	14.5
7	9.5	22	22.5	100	21
8	6.5	18.5	7.5	100	13.5
9	–	48	25	100	4.5
10	27	5	36	100	63.5
11	4	12	3	100	5
12	25	14	5	100	6.5
13	3.5	10	0.5	100	–
14	0.05	11	77	100	45

* Expressed as a percentage of the major component. Quantitation is based on peak area measurement.

** The gas–liquid chromatographic peak contained both components. Compound Id was much less abundant in all cases.

(the base peak in all cases) in addition to complete mass spectral scans were used to confirm the presence of these compounds. Again, considerable variation in the relative abundances of these compounds was noted for the different samples, and again the derivative of myristicin was absent.

The presence, in these compounds, of various degrees of hydroxylation (zero, one or two hydroxyl groups) offered a second method of deconvoluting the group of poorly resolved gas chromatographic peaks produced by the TMS derivatives. Preparation of the triethylsilyl or tri-*n*-propylsilyl derivatives[15] introduced a larger retention increment than the TMS derivatives and this was multiplied where several hydroxyl groups were present. Separation of these compounds into three groups according to their degree of hydroxylation could thus be made. This is shown in Figs. 2 and 3, which show the gas chromatograms of the triethylsilyl and tri-*n*-propylsilyl derivatives, respectively, of nutmeg samples whose TMS derivatives are shown in Fig. 1. The underivatized compounds IIb and IIe did not shift their position but the mono- and dihydroxy derivatives separated into two distinct groups at higher retention index values. Retention indices are listed in Table IV. The largest change in retention increment was observed between the TMS and triethylsilyl derivatives, a feature shared with the same derivatives of other compounds such as the cannabinoids.[15] In addition, some improvement in the separation between compounds of type I and II was obtained, for example between Ie and IIc (Figs. 1 and 2). A slightly larger increase in relative retention was produced with compounds of type II. This is shown graphically in Fig. 9.

TABLE IV

RETENTION INDICES OF THE DIPHENYLPROPANOIDS

Compound	*Free*	*TMS*	Et_3Si	Pr_3Si
Ia	—	2630	2940	—*
Ib	—	2655	2955	3065
Ic	—	2700	3300	3540
Id	—	2710	3310	*(Ic)
Ie	—	2740	3020	3125
If	—	2760	3360	3595
IIa	2623	—	—	—
IIb	*(Ib)	—	—	—
IIc	—	2725	3060	3215
IIe	2770	—	—	—
IIf	—	2825	3160	3310

* Peak not resolved: interfering compound in parentheses.

As the geographical origin and the age of the sample were unknown, it was not possible to determine the reason for the variation in the abundance of the diphenylpropanoids. Geographical dependence has previously been demonstrated for nutmeg constituents[5,9]. Similar changes are found in samples of cannabis depending to a large extent on geographical origin. Aging of cannabis samples produces an increase in the proportion of fully aromatic compounds such as cannabinol[16]. It is possible that a similar situation occurs in aged nutmeg samples, but although aromatic compounds, particularly those derived from the type II diphenylpropanoids, were

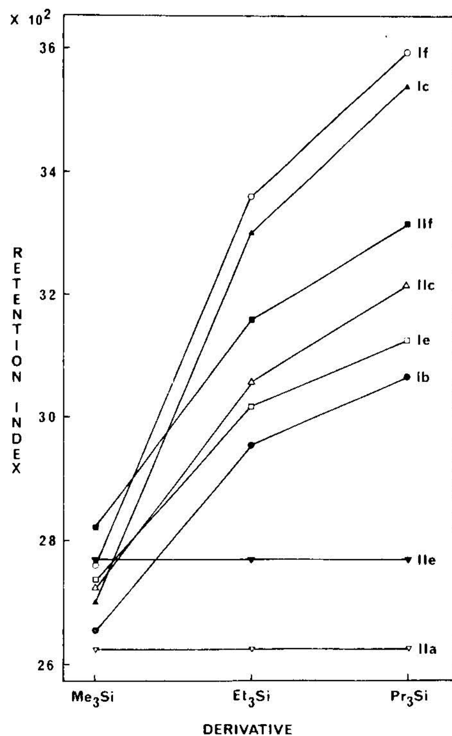


Fig. 9. Retention index plotted as a function of the derivative for the diphenylpropanoids. Three populations are present (ignoring compounds IIa and IIe which are not derivatized), the monohydroxy compounds of type I, those of type II which have a slightly greater change in retention index, and the dihydroxy compounds Ic, Id and If.

looked for, none were found. Aromatic compounds of this type do, however, occur in other species[17].

One sample of mace (sample 3) was examined, but its chromatogram showed no significant differences in diphenylpropanoid content from a number of the nutmeg samples (Table III). Greater differences were found between the individual nutmeg samples themselves.

ACKNOWLEDGEMENTS

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SEPARATION OF TETRACYCLINES BY HIGH-SPEED LIQUID CHROMATOGRAPHY

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SUMMARY

The separation of tetracycline and its four common impurities has been studied by high-speed liquid chromatography. A preliminary study of the effectiveness of ion-exchange, adsorption, liquid–liquid partition and reversed-phase ion-pair chromatography indicated that only the last method showed promise. By developing special hydrocarbon-bonded stationary phases a rapid and complete resolution of all five tetracyclines has been obtained within 10 min. Plate heights using a derivatised 18-μm Partisil are in the range 0.15–0.3 mm. The method can be used to quantify the impurities in tetracycline at around the 1% level.

INTRODUCTION

Pharmaceutical preparations of tetracycline (compound I, TC) contain small quantities of related compounds as impurities. The most important are 4-epitetracycline (II, ETC), 7-chlorotetracycline (III, ClTC), anhydrotetracycline (IV, ATC) and 4-epianhydrotetracycline (V, EATC). Their permitted concentrations are listed in the British Pharmacopoeia[1]. The need for an improved assay method for tetracycline and its main impurities prompted us to investigate, in detail, conditions under which these components could be separated and quantitated by high-speed liquid chromatography (HSLC).

Several chromatographic techniques have in the past been applied to the analysis of the tetracyclines. Thin-layer chromatography[2,3], paper chromatography[4] and column chromatography followed by UV spectrophotometric assay[5,6] have proved laborious, often require sample concentration[5], and are generally not sufficiently sensitive or precise. A recent gas chromatographic method[7] requires prior formation of the trimethylsilyl derivative under carefully controlled conditions, while a method using HSLC[8] with a hydrocarbon stationary phase gave poor resolution and plate height. The last method did, however, suggest that reverse-phase methods might offer the most promising approach.

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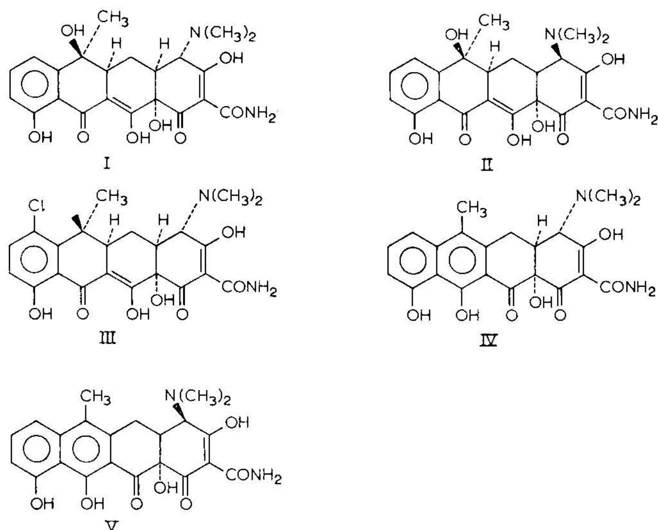

This was confirmed (see below) by a wide-ranging study using adsorption, ion exchange, liquid–liquid partition and reversed-phase ion-pair partition chromatography, of which the last proved the most satisfactory. However, in contrast to Tsuji *et al.*[8], we used hydrocarbons chemically bonded to silica instead of a hydrocarbon polymer coated on to silica. We thereby hoped to avoid many of the undesirable features commonly associated with polymeric stationary phases deposited on a silica surface, *viz.* residual adsorption due to the presence of silanol groups and slow mass transfer in the stationary phase. By studying a number of different bonded hydrocarbon supports, prepared in the Wolfson Liquid Chromatography Unit (WLCU), we have established that the best material for the separation of the five tetracyclines is a short-chain hydrocarbon bonded to a high-quality commercial silica gel such as Partisil 20 (Reeve Angel, London, Great Britain).

EXPERIMENTAL

The high-speed liquid chromatograph comprised the pump of a Model 830 liquid chromatograph (DuPont) operated at 500–3000 p.s.i., a variable wavelength UV photometer (Cecil Instruments, Cambridge, Great Britain, Type 212) fitted with an 8-μl flow cell and operated at 280 nm. Columns were either 2 mm bore and 500 mm long (Column A) or 5 mm bore and 125 mm long (Column B) of internally polished 316S stainless steel with fittings made according to a design described elsewhere[9]. They were terminated by 6-μm porosity frits (B.S.A., Birmingham, Great Britain). Columns were packed by the rotate, bounce-and-tap method[10] and were operated at ambient temperature.

The packing materials used in the preliminary study were: DuPont Zipax strong cation exchanger (SCX), Waters Ass. Corasil (both 37–44-μm particle size), Reeve Angel Partisil (18 μm), Perkin-Elmer Sil-X (13 μm), Zipax (37–44 μm) coated with 1 % polyethylene glycol (PEG) 400 and Partisil (18 μm) reacted with octadecyltrichlorosilane to give an octadecyl-bonded silica (ODS-silica, WLCU No. 171).

In the subsequent more detailed study two further bonded support materials were examined. The first was ODS-silica which had been further treated to substitute any residual ≡Si–OH groups with short-chain trialkylsilyl groups (ODS/TAS silica WLCU No. 263). The second was a sample of silica gel (Partisil) which had been completely silanized to replace the highest possible proportions of ≡Si–OH groups by ≡Si–O–SiR_3 groups where R was a short-chain alkyl group (SC-TAS-silica, WLCU No. 264).

The specimens of tetracyclines, kindly gifted by Harris Pharmaceuticals (London, Great Britain) were: tetracycline·HCl, 7-chlorotetracycline·HCl, anhydrotetracycline·HCl, the ammonium salt of 4-epitetracycline and 4-epianhydrotetracycline·HCl.

Specimens were dissolved in the mobile phase or in water immediately before use to give solutions containing 1–5 mg/ml of each component. 0.1–1 μl of these solutions were injected into the chromatograph through an EPR septum (Waters Ass., Stockport, Great Britain) using a 1-μl Type B syringe (SGE, London, Great Britain).

RESULTS

Presentation of data

The chromatographic behaviour of individual components is expressed by means of two main parameters, the capacity ratio, and the plate height. The capacity ratio, k', which measures the degree of retention of a solute, is obtained from the elution chromatogram by

$$k' = (t_R - t_0)/t_0 \quad (1)$$

where t_R and t_0 are the elution times of a retained and unretained solute respectively. In our study acetone was used as "unretained solute".

The plate height, H, which is a measure of the dispersive capacity of the column, is obtained from the elution chromatogram by

$$H = (L/16)\,(w_t/t_R)^2 \quad (2)$$

where L is the column length, w_t is the peak width at the baseline.

Chromatographic behaviour of tetracyclines using different chromatographic methods

The results of the initial study using different chromatographic methods are summarised in Table I, and show that none of the classical methods gave efficient resolution of tetracyclines. With ion-exchange chromatography using Zipax SCX, tetracyclines could be eluted with alkaline, neutral or acidic solvent, the last giving slowest elution; anhydrotetracyclines were consistently eluted before the corresponding tetracyclines but the epi- and normal forms could not be separated. In all cases column efficiencies and peak shapes were poor. With adsorption chromatography using Partisil (18 μm) the tetracyclines could be eluted with aqueous acidic solvents, but peaks were broad, badly tailed and overlapping. Peak shape was particularly poor for the anhydro forms. The addition of solvating agents such as acetonitrile accelerated elution without improving efficiency. Somewhat better results were ob-

TABLE I

CHROMATOGRAPHIC PARAMETERS FOR TETRACYCLINES IN VARIOUS CHROMATOGRAPHIC SYSTEMS USED IN HSLC

*Chromatographic system and column**	*Support*	*Mobile phase*	*k′*					*H (mm) at 1000 p.s.i.*	*Comments*
			ETC	*TC*	*ClTC*	*EATC*	*TC*		
Ion exchange A	Zipax SCX	0.4 *M* $K_2B_4O_7$ 0.01 *M* EDTA 1% isopropyl alcohol (pH 9.8)	0.25	0.22	0.19	0.07	0.05	0.5 – 1.4	Poor column efficiency, only ATC/TC separation feasible
		0.1 *M* citric acid 0.001 *M* EDTA pH adjusted to 5.2	6.8	6.3	8.7	2.9	1.2	1.2 –15	Very poor column efficiency and peak shape
Adsorption chromatography B	Partisil (18 μm)	Aqueous 0.08 *M* $HClO_4$	1.8	3.8	4.3	4.3	7.5		Very broad overlapping peaks
	Sil-X (13 μm)	0.1 *M* $HClO_4$ 1.9 *M* $NaClO_4$ 0.005 *M* citric acid–acetonitrile (2:1)	0.2	0.2	1.3	1.8	1.8	0.35	Good separation of TC, ClTC and ATC
Liquid–liquid partition chromatography A	Corasil coated with PEG 400	Dioxane–pentane (1.5:1)	11.0	8.0	unknown	1.6	0.6	0.62– 3.0	Poor column efficiency poor reproducibility
Reversed-phase partition chromatography B	ODS chemically bonded on to Partisil (18 μm)	0.1 *M* $HClO_4$ 0.4 *M* $NaClO_4$ 0.0025 *M* citric acid–acetonitrile (85:15 v/v)	3.56	6.0	17.0	long adsorption		0.8	Possible separation of ETC, TC, ClTC but with poor efficiency

* A and B refer respectively to 2 × 500 mm and 5 × 125 mm columns.

tained on Sil-X (13 μm), and with 33% acetonitrile in an aqueous perchlorate buffer a good separation of TC, ClTC and ATC could be obtained within a few minutes (Fig. 1). However, the epiforms were not resolved from the normal forms.

With liquid–liquid partition chromatography on Corasil coated with PEG 400 more symmetrical peaks were obtained but retention times were irreproducible. Here the anhydrotetracyclines were consistently eluted before the tetracyclines. The irreproducibility was thought to arise from the dissociation of hydrochlorides and retention of HCl by the column packing. The effect was similar to that previously observed in the analysis of tricyclic antidepressants[11].

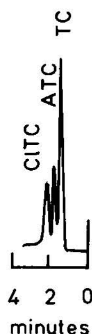

Fig. 1. Separation of TC, ATC and ClTC on Perkin-Elmer Sil-X (13 μm). Column, 5 × 125 mm. Eluent, (0.1 *M* $HClO_4$ + 1.9 *M* $NaClO_4$ + 0.005 *M* citric acid)–acetonitrile (2:1) (v/v). Eluent velocity, 2.6 mm/sec. Detector, UV photometer, wavelength 280 nm, sensitivity 0.2 absorbance units full scale deflection (a.u.f.s.). Sample about 100 ng of each component.

Using ODS-silica and aqueous acetonitrile containing perchloric acid as eluent, separations of ETC and ClTC from TC (Fig. 2) could be achieved with low percentages of acetonitrile in mobile phase but the anhydro compounds were strongly retained and gave very broad peaks. Their retention could be reduced and their peaks sharpened by addition of higher concentrations of acetonitrile but then the separation of the first three components disappeared. Nevertheless the last system was the only one which gave separation of epitetracycline from tetracycline and encouraged the

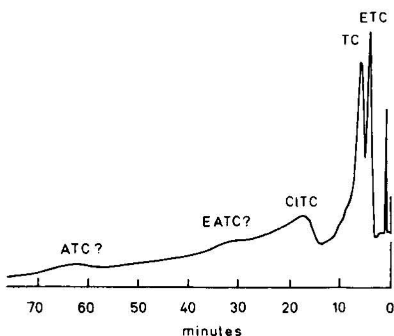

Fig. 2. Separation of ETC, TC and ClTC on Partisil (18 μm) reacted with trichlorooctadecylsilane. Column, 5 × 125 mm. Eluent, (0.1 *M* $HClO_4$ + 0.4 *M* $NaClO_4$ + 0.0025 *M* citric acid)–acetonitrile (85:15). Velocity, 2.1 mm/sec. Detector as in Fig. 1, sensitivity 0.1 a.u.f.s.

belief that a bonded hydrocarbon stationary phase could be developed to give a really satisfactory separation of the tetracyclines.

From the preliminary survey the following conclusions may be drawn:

(1) The poor peak shape obtained in conventional ion-exchange, adsorption and liquid–liquid partition chromatography (particularly for well-retained tetracyclines) seems to be associated with the presence of free Si–OH groups on the support surface. Even in the presence of water, which must be very strongly adsorbed, there apparently remain a small number of sites at which the polar multifunctional tetracycline molecules can displace water and become strongly adsorbed so that even slightly retained tetracyclines give badly tailed peaks.

(2) Coating a siliceous support with hydrocarbon either by physical deposition as in Zipax HCP[8] or by chemical bonding produces increased retention of the tetracyclines but does not change the order of retention. The order of retention is just the opposite of that found with ion exchange and normal phase liquid–liquid partition chromatography. Peak tailing while still present in the reverse phase system is reduced particularly when the surface of a silica is reacted with octadecyl trichlorosilane followed by hydrolysis. We attribute the residual tailing to those original Si–OH groups which remain unreacted or, for silanized silicas, to new Si–OH groups produced by hydrolysis of the Si–Cl groups remaining after silylation.

Since both silica gel and octadecyl-silica (ODS-silica) give the same order of retention, we appear to be dealing with the same form of chromatography in both cases. Since peak shape is apparently adversely affected by the heterogeneity of Si–OH adsorption sites, a procedure for removing all such hydroxyl groups should improve both retention and peak shape.

(3) While adsorption chromatography gives a reasonable spread of k'-values but very badly tailed peaks, chromatography with similar eluents on ODS-silica gives a very wide range of k'-values, *i.e.* high selectivity. To reduce the selectivity of the bonded silica without producing peak tailing it may therefore be useful to reduce the chain-length of the hydrocarbon radicals bonded to the surface.

Chromatographic behaviour of tetracyclines on bonded hydrocarbon stationary phases

To test these ideas we have compared the retention of tetracyclines on the following four supports using the same eluent with each:

(1) Untreated silica gel (Partisil).

(2) Partisil exhaustively treated with octadecyltrichloro silane and then hydrolyzed (ODS-silica, WLCU No. 171).

(3) The material of (2) further treated in order to trialkylsilylate the remaining Si–OH groups (ODS/TAS-silica, WLCU No. 263).

(4) Partisil exhaustively silanized to substitute all Si–OH groups with short-chain trialkylsilyl groups (SC-TAS-silica, WLCU No. 264).

The results of these experiments are presented in Table II. They may be summarised as follows:

(1) Using untreated Partisil and an aqueous eluent 0.08 M in perchloric acid containing 20% acetonitrile, retentions were lower than on any other support while peaks were more badly tailed.

(2) The packings in order of increasing retention were: untreated silica gel, SC-TAS-silica, ODS-silica, ODS/TAS-silica.

TABLE II

CHROMATOGRAPHIC PARAMETERS OF TETRACYCLINES USING DIFFERENT CHEMICALLY BONDED STATIONARY PHASES

Mobile phase, water–acetonitrile (80:20, v/v), 0.08 *M* overall in $HClO_4$; support, Partisil (18 μm); columns, 5 × 125 mm.

Column number	*Stationary phase*	*k'*					*H (mm) at 1000 p.s.i.*	*Comments*
		ETC	*TC*	*ClTC*	*EATC*	*ATC*		
1	None	0	0	0	0	0	Peaks broad and tailed	All components emerge essentially unretained
2	ODS only	2.80	5.00	16.0	31.0	61.0	1.0 –1.5	Poor efficiency particularly of the well retained anhydrotetracyclines
3	ODS/TAS	5.26	7.45	31.0	44.0	68.0	0.25–0.60	Good separation of first three components
4	SC-TAS	2.50	3.80	11.0	23.0	35.0	0.20–0.30	Good separation of all five components

(3) The packings in order of increasing peak symmetry and decreasing plate height were: Silica, ODS-silica, ODS/TAS-silica, SC-TAS-silica, with the last two being roughly equivalent at the same *k'* value.

These results confirm our previous conclusions. With 20% aqueous acetonitrile as eluent, tetracyclines are more strongly retained by hydrocarbon-like groups than by Si–OH groups. This is presumably because the Si–OH groups are highly hydrated. Nevertheless, whenever Si–OH groups are present, a proportion can apparently still adsorb the tetracyclines strongly in spite of hydration, and it is these groups, with their range of affinity for TC's, which cause tailing even when their total number is small. Once the residual Si–OH groups are completely removed by vigorous silanization, as in the ODS/TAS and SC-TAS-silica's, their effect is more or less eliminated and good peak shape and retention are obtained. Strong retention by a hydrocarbon phase is, of course, quite unexpected since tetracyclines either as bases or simple salts are virtually insoluble in hydrocarbon solvents. As argued in the Discussion we believe this is explained if the partitioning phase is acetonitrile dissolved in the bonded hydrocarbon and if the species being extracted are perchlorate ion-pairs which are solvated by the acetonitrile associated with the support surface. It then follows that the greater the loading of alkyl groups the greater the retention.

Fig. 3A shows the excellent resolution and peak shape attainable with the ODS/TAS material for the separation of ETC, TC and ClTC. With this material, however, the anhydro forms are very strongly retained. Fig. 3B shows a typical chromatogram in which all five tetracyclines are resolved in under 10 min on the SC-TAS-silica. Both columns have efficiencies of around 500 theoretical plates in a 125-mm column giving values of *H* between 0.25 and 0.35 mm.

Comparative experiments in the ODS/TAS and SC-TAS-silica show that the latter is less selective and so more suitable for separation in a single run of all five tetracyclines. Subsequent experiments on the effects of eluent composition were therefore carried with SC-TAS-silica.

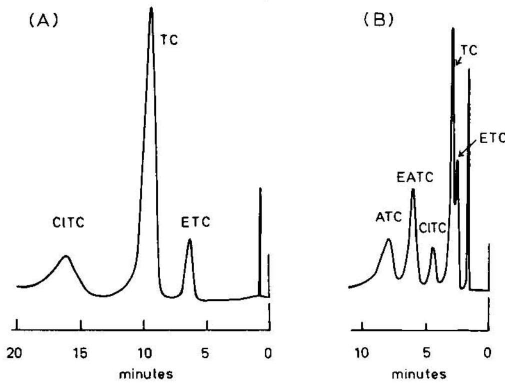

Fig. 3. Separations of tetracyclines on ODS/TAS and SC-TAS-silica's. (A) Packing, ODS/TAS-silica. Column, eluent and detector as for Fig. 2. Velocity, 2.4 mm/sec. (B) Packing, SC-TAS-silica. Column, 5 × 125 mm. Eluent, water–acetonitrile (3:1), 0.1 M overall in $HClO_4$. Velocity 1.3 mm/sec. Detector as in Fig. 2.

Effects of eluent composition on the performance of SC-TAS-silica

Experiments were carried out using (a) different solvating agents, (b) different acids (Table III), and (c) different concentrations of acetonitrile, the preferred solvating agent (Fig. 4). The main conclusions were as follows:

(a) Of the four solvating agents examined, *viz.* methanol, propanol, dioxane and acetonitrile, acetonitrile gave the lowest plate heights and the best resolution.

(b) Up to threefold changes of acid concentrations, as shown by Table III, had little effect on k' values although there was a slight tendency for k' to increase with acid concentration. Changes in the nature of the acid as shown in Table III had much more effect. The greatest retention was obtained with perchloric acid. Nitric acid at the same concentration gave about half the retention and sulphuric acid about a quar-

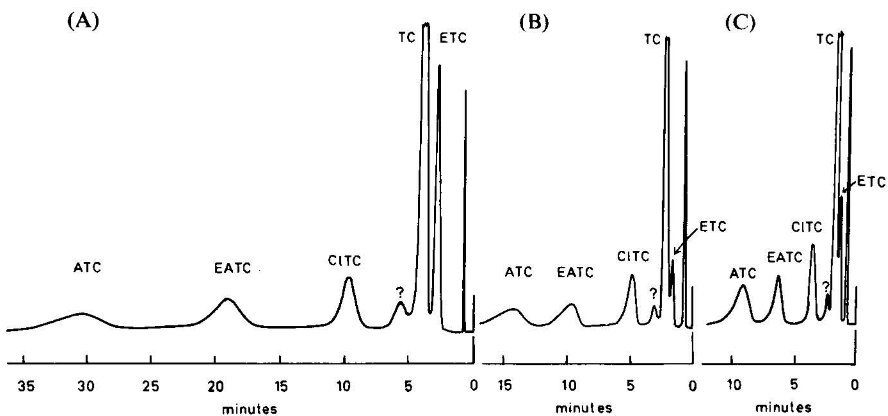

Fig. 4. Effect of acid on retention of tetracyclines on SC-TAS-silica. Column, 5 × 125 mm. Eluent, aqueous acid–acetonitrile (85:15). Overall acid concentrations: (A), 0.1 M $HClO_4$; (B), 0.1 M H_2SO_4; (C), 0.05 M HNO_3. Velocity 2.6 mm/sec. Detector as in Fig. 1, sensitivity 0.1 a.u.f.s. Unknown peak is impurity in ClTC formed on standing.

TABLE III

CHROMATOGRAPHIC PARAMETERS OF TETRACYCLINES ON SC-TAS-SILICA USING DIFFERENT ACIDS IN THE MOBILE PHASE

Acid	*Overall concentration of acid*	*Concentration of acetonitrile (%, v/v)*	*k'*					*H (mm) at 1000 p.s.i.*
			ETC	*TC*	*CITC*	*EATC*	*ATC*	
H_2SO_4	0.1 *M*	15	0.33	0.70	2.30	5.70	8.70	
	0.2 *M*	15	0.33	0.78	2.80	5.90	9.0	0.3
HNO_3	0.05 *M*	15	1.00	1.50	5.00	10.7	16.5	0.3
	0.1 *M*	15	1.30	1.90	6.90	14.0	19.5	0.3
$HClO_4$	0.03 *M*	15	2.00	3.30	9.00	18.2	29.0	0.4–0.8
	0.06 *M*	15	2.70	3.50	8.60	20.5	33.0	0.30
	0.1 *M*	15	2.50	3.80	11.20	23.1	36.5	0.30
	0.1 *M*	25	1.50	1.80	6.20	11.5	17.5	0.25
Citric acid	0.05 *M*	25	0.22	0.43	1.38	2.40	3.40	0.40
	0.01 *M*	25	0.22	0.44	1.33	2.33	3.34	0.40
	0.15 *M*	25	0.20	0.41	1.30	2.30	3.50	
Formic acid	0.3 *M*	25	0.35	0.40	1.00	1.40	3.30	0.5

ter to a fifth of the retention. Citric and formic acids gave about one fifth of the retention obtained with perchloric acid.

The addition of sodium perchlorate to perchloric acid gave somewhat poorer resolution and higher plate heights, and was discontinued.

(c) Increasing acetonitrile concentration from 15% to 35%, as shown in Fig. 5, reduced retention between 5 and 8.5 times, the decrease being greatest for the most retained solutes. With the lowest concentration of acetonitrile the separation of the first three components was optimum but the anhydro forms were then very strongly retained.

The experiments whose results are shown in Fig. 5 were carried out in a single series. After washing the column overnight for approximately 16 h with water, each

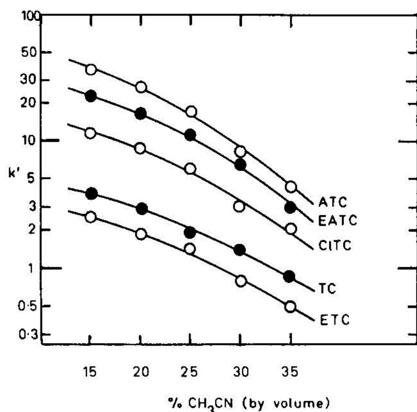

Fig. 5. Effect of proportion of acetonitrile on column capacity ratio of tetracyclines on SC-TAS-silica. All eluents 0.1 *M* in $HClO_4$ overall.

solvent was run for 30 min before determining column parameters starting with that containing 15% acetonitrile. In this time approximately 30 column volumes of eluent were passed. Later experiments suggested that this procedure may not have resulted in complete column equilibration. Thus in the long series of calibration experiments in which a 25% acetonitrile solution was used the k' values were similar to those obtained in the present series with 35% content of acetonitrile. The reason for this slow equilibration of the column, if indeed equilibration was slow, is not clear at this stage.

Quantitative analysis of tetracyclines

According to the British Pharmacopoeia[1] the allowed impurity limits in tetracycline are as follows, ETC 4%, ClTC 2%, EATC 0.5%, ATC 0.5%.

While separation of the five tetracyclines using a single solvent (for example 25% aqueous acetonitrile 0.1 *M* in perchloric acid) is readily achieved using the SC-TAS column, if the five components are present in comparable amounts (Fig. 3), analysis of the four main impurities at the 1% level requires two solvents or gradient elution. For the least retained components, ETC, TC and ClTC 10–16% aqueous acetonitrile is required to obtain adequate resolution of ETC and ClTC from the massive TC peak, but under these conditions EATC and ATC are so strongly retained that they can hardly be detected at the trace level. It is necessary for their elution and determination to use a richer eluent containing 25–35% acetonitrile. Typical calibration curves for the five tetracyclines under such conditions are shown in Fig. 6. Chromatograms of a TC sample to which small amounts of impurities had been added are shown in Fig. 7.

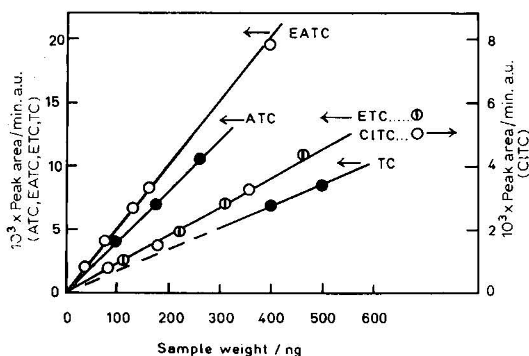

Fig. 6. Calibration curves for determination of tetracyclines. Column and detector conditions as for Fig. 1. Eluent for EATC and ETC in TC, water–acetonitrile (3:1), 0.1 *M* overall in $HClO_4$; for ETC and ClTC in TC, previous eluent diluted with half its volume of water.

DISCUSSION

The results of the present work show that tetracyclines can be rapidly and precisely determined by HSLC on siliceous supports whose surface hydroxyl groups have been completely substituted with trialkylsilyl groups. Such materials give excellent peak symmetry and good retention. The plate heights using 18-μm Partisil were around 0.2–0.3 mm at linear velocities in the region of 5 mm/sec. Considering

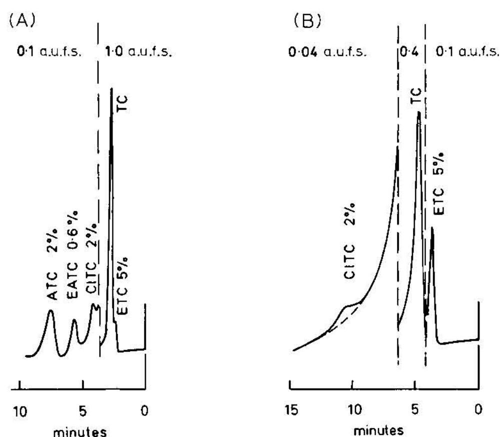

Fig. 7. Chromatograms of 10 μg of a TC sample with impurity contents made up to values shown. Chromatographic conditions as for Fig. 6. Eluents: (A) water–acetonitrile (3:1) 0.1 *M* overall in $HClO_4$; (B) previous eluent diluted with half its volume of water.

the viscosity of the solvent and the low diffusion coefficient of the tetracyclines or their derived ion pairs, these values are reasonably good, but better and faster resolution nevertheless might be obtained using smaller particles although the pressure capability of the available equipment might then become limiting.

Our results show clearly that the degree of retention is dependent upon the hydrocarbon content of the bonded packing material ($\approx$15% for the ODS/TAS silica and $\approx$5% for the SC-TAS-silica). As already noted it is surprising that substances such as tetracyclines which are virtually insoluble in hydrocarbon solvents can be retained by a hydrocarbon stationary phase. We believe that the retaining phase is in fact acetonitrile which is extracted from the aqueous phase into the bonded hydrocarbon phase to give a thin surface layer which contains a high mole fraction of acetonitrile and a low mole fraction of water. In this way the hydrocarbon layer generates what amounts to a liquid–liquid partition system in which partitioning occurs between an aqueous mobile phase and a predominantly organic, but nevertheless polar, stationary phase. Acetonitrile may be particularly suitable substance for this self-partitioning role since it cannot form hydrogen bonds with itself yet is both hydrophilic and organophilic. Because the hydrocarbon layer is saturated with acetonitrile, increasing the proportion of acetonitrile in the eluent improves the solubility of the tetracycline in the eluent but has little or no effect upon its solubility in the stationary phase. Increasing the acetonitrile content of the eluent therefore reduces retention.

We believe that the species which are partitioned between the mobile and stationary phase are almost certainly tetracycline–perchlorate ion-pairs and not unionized tetracycline molecules. This is indicated by a number of observations. In the first place chromatography is only possible when the aqueous phase is acidified to a pH of 1.5–2.0. Under these conditions the tetracyclines exist in their ammonium forms. The widely differing effects of different acids and the very slight effect of hydrogen ion concentration strongly suggests that the nature of the anion is the key to retention for if undissociated amine molecules were being partitioned then pH would have a major effect on retention and the nature of the anion little effect. Since it is exceedingly

improbable that the ammonium ions themselves could be extracted it is almost certain that ion pairs are the important species in the partitioning. Accordingly, we believe that the mode of chromatography which we have employed is best termed "reverse phase ion-pair partition chromatography". This conclusion is supported by the results of Wachlund and Groningsson[12] who have shown that the extraction coefficients of perchlorate ion pairs of some hydrophilic amines are several times greater than those of sulphate ion pairs in accord with our observations on retention.

The order of retention in general correlates with the structure of the main part of the TC molecules and with their polarity. The anhydro forms which possess two aromatic rings are more strongly retained than TC and ETC which possess only one, and ClTC possessing a polar Cl group is retained more than the unsubstituted TC. Such polar effects will determine the affinity of molecules or ion pairs for the stationary phase which is rich in acetonitrile. By contrast they will have little effect in the aqueous phase where the major solvating forces arise from hydrogen bonding of water to the oxygen and nitrogen atoms. Thus the elution order follows the polarity of the parent molecules. The opposite order of retention is obtained in liquid–liquid partition chromatography on Corasil where hydrogen bonding determines the affinity for the stationary phase (PEG 400) and polarity the affinity for the mobile phase.

The extraction of ammonium compounds as ion-pairs with inorganic anions is now well documented[13–25] both in batch extraction and in partition chromatography. Recently this principle of separation has been applied successfully to the HSLC of catecholamines[26] and tricyclic antidepressants[11] using an organic eluent and an aqueous stationary phase. Although reversed-phase ion-pair systems are now beginning to be used, the systems published to date[12,27] have all used stationary phases containing hydrophobic aliphatic alcohols. Hydrocarbon stationary phases on the other hand have been mostly applied to reversed-phase partition chromatography of unionized compounds[28] although in a few cases they have been used for ionizable bases such as the ergot[29] and hashish alkaloids[28] and librium[29]. Our work and that of Tsuji *et al.*[8] appear to be the only cases where a hydrocarbon stationary phase has been used to separate strongly hydrophilic amines as ion pairs and in this context it seems that the presence of an acid in the aqueous phase is essential for the successful elution.

An important advantage of reversed-phase ion-pair partition chromatography over normal ion-pair partition chromatography is that the counter anion is continually replaced rather than being gradually removed from the stationary phase by successive samples, as occurs in normal phase ion-pair systems, and can also be varied much more readily. Thus the reversed-phase mode has much greater flexibility under practical operating conditions.

CONCLUSIONS

The results of the present study demonstrate that bonded hydrocarbon stationary phases can be used with great effect in the reversed-phase ion-pair partition chromatography of strongly hydrophilic bases such as the tetracyclines.

The SC-TAS-silica developed especially for this particular problem has been shown to be a high quality column packing for HSLC which can be used for quantitative micro-analysis. An important aspect of the present technique is that no pre-

conditioning of the column and fittings was required such as the washing with EDTA which was considered essential by previous authors[8]. In the present work there was no evidence of loss of tetracyclines from the formation of metal derivatives. We believe this is because the pH of the mobile phase was kept low (pH 1.5–2).

The technique can be used for the separation and accurate quantitation at the sub-microgram level of the tetracycline group, and has considerable potential as a basis for the development of a standard method for the assay of pharmaceutical preparations of tetracyclines and for the determination of tetracyclines in biological specimens.

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CHROM. 8272

PREPARATION AND THIN-LAYER CHROMATOGRAPHY OF BROMO-DERIVATIVES OF UNSATURATED FATTY ACID ESTERS

A SIMPLE AND RAPID PROCEDURE FOR FATTY ACID ANALYSIS

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SUMMARY

Bromo-addition products of unsaturated long-chain fatty acid esters have been prepared and chromatographed on thin layers of unmodified silica gel. The polarity of these derivatives was found to be directly related to the number of double bonds of the parent fatty acid from which they were derived. This has been made the basis of a simple method for assessing the relative proportions of the main fatty acid classes in a mixture.

INTRODUCTION

Bromine addition to the double bonds of unsaturated fatty acids has been widely used in the past as a means of achieving the separation of various fatty acid classes[1,2]; the method, however, has been little used since the inception of gas–liquid chromatographic techniques. Although reversed-phase partition thin-layer chromatography (TLC), as well as argentation TLC, have become popular tools for fatty acids analysis, few attempts have been made to separate the bromo-derivatives of unsaturated fatty acids by conventional TLC. Nevertheless, the work of Kaufmann *et al.*[3] demonstrated that addition of bromine to the double bonds of unsaturated fatty acids can be used advantageously to improve the resolution of certain critical pairs of fatty acids on a thin layer of kieselguhr impregnated with undecane.

Recently, we have reported that mild treatment of long-chain fatty acids with bromine in chloroform solution results in the production of non-polar derivatives and that the polarity of these derivatives is directly related to the number of double bonds in the fatty acids from which they are derived[4]. As will be shown here, these non-polar fatty acid derivatives are, in fact, bromine-containing ethyl esters of the acids. The observed excellent chromatographic resolution of the bromo-derivatives on layers of unmodified silica gel prompted us to devise the simple method for fatty acid analysis that will be detailed here.

EXPERIMENTAL

Preparation of fatty ethyl (or methyl) esters

Ethylation (or methylation) of the free fatty acids and trans-esterification of the glycerolipids were performed in 2–3 ml of 2% sulphuric acid in ethanol (or methanol)–light petroleum (4:1) at 70° for 1 h (for fatty acids) or 4 h (for glycerolipids).

Thin-layer chromatography

Ready-coated plates of silica gel (0.25 mm thick) supplied by Merck were used throughout this work, without prior activation. The substances were applied with a micropipette as a line 1 cm long and 1.5 cm from the lower edge of the plate. The chromatoplates were developed in unequilibrated tanks to a height of 12.5 cm, at room temperature, with light petroleum (60–80°)–diethyl ether (9:1, v/v) as the solvent system. Detection was achieved by using copper acetate reagent as described by Fewster *et al.*[5].

Thin-layer densitometry

The optical density of the charred spots was measured with a Vitatron TLD-100 flying-spot photodensitometer (Vitatron, Dieren, The Netherlands); peak areas were determined by scanning in the direction of the solvent flow and calculated by triangulation.

Gas–liquid chromatography (GLC)

Analysis of the fatty acid methyl esters was carried out on a Packard 824 chromatograph using a 6-ft. glass column packed with 20% of DEGS coated on Chromosorb W HMDS (60–80 mesh) (Varian, Fife, Scotland); nitrogen was the carrier gas at a flow-rate of 25 ml/min. The area under each fatty acid peak was estimated by triangulation.

Mass spectrometry

The mass spectra were obtained on an LKB 9000 S instrument with gas chromatograph and direct inlets. Recording conditions were as follows: ionising energy, 70 eV; trap current, 60 μA; and ion-source temperature, 270°. The temperature of the direct inlet to the mass spectrometer was set at approximately 100°. The column (2 m × 3 mm I.D.) used for GLC was 1% of OV-1 on Gas-Chrom P AW DMCS (100–120 mesh) prepared according to Horning *et al.*[6]. The flash heater, column and separator temperatures were, respectively, 260, 220 and 265°, and the carrier gas (helium) flow-rate was set at 30 ml/min.

Products

The following commercially available fatty acids were used: palmitic and stearic acids (BDH, Poole, Great Britain), arachidic and oleic acid (Fluka, Buchs, Switzerland), linoleic acid (Merck, Darmstadt, G.F.R.), arachidonic acid (Sigma, St. Louis, Mo., U.S.A.) and docosa-4,7,10,13,16,19-hexaenoic acid (Applied Science Labs., State College, Pa., U.S.A.). [1-^{14}C]Palmitic acid (57.5 mCi/mmole) certified to be 99% pure was purchased from the Radiochemical Centre (Amersham, Great Britain). All the organic solvents used were of analytical grade.

RESULTS AND DISCUSSION

Reaction of the fatty acids with bromine in chloroform solution

Sample of various fatty acids dissolved in chloroform (2 mg/ml) were allowed to react with bromine (40 μl/ml) in a PTFE screw-capped phial at room temperature for 16 h. Such treatment results in quantitative conversion of the fatty acids into less polar compounds, as was shown by TLC of radioactive palmitic acid that had been treated with bromine (see Fig. 1); no change in the R_F values of the different fatty acids was observed after brief treatment with bromine.

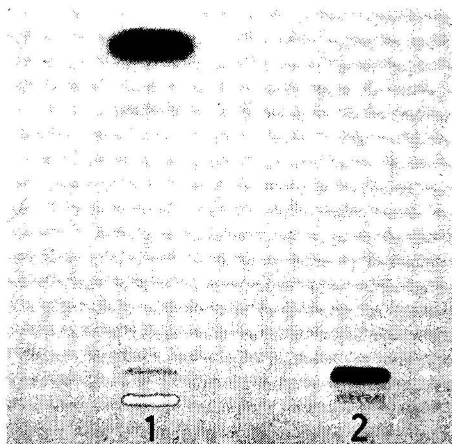

Fig. 1. Production of a non-polar derivative from palmitic acid treated with bromine in chloroform. 1, 1.08 nmole of [1-^{14}C]palmitic acid (0.062 μCi) treated with bromine; 2, the same amount of untreated labelled palmitic acid. Detection by autoradiography (Ferrania No-Screen film; exposure time, 5 days).

The polarities of these unknown derivatives were proportional to the number of double bonds of the parent fatty acids (see Fig. 2); in the solvent system light petroleum–diethyl ether (9:1), R_F values of 0.73, 0.61, 0.53, 0.37 and 0.31 were found for the derivatives of palmitic, oleic, linoleic, arachidonic and docosa-4,7,10,13,16,19-hexaenoic acid, respectively. However, the non-polar derivatives of several long-chain saturated fatty acids (*e.g.*, palmitic, stearic and arachidic acids) could not be resolved in this system.

From the experiments with [1-^{14}C]palmitate (see Fig. 1), it can be inferred that no decarboxylation occurs during the reaction, as there was no loss of radioactivity when this acid was converted into a non-polar substance.

The mass-spectrometric fragmentation of the substance derived from palmitic acid (see Fig. 3) shows a molecular ion at m/e 284, and the ion intensities relative to the base peak were similar to those quoted for the ethyl esters of palmitic acid[7]; moreover, the fragmentation pattern obtained with the palmitoyl ethyl ester was identical with that shown in Fig. 3. Further evidence for the identity of the unknown non-polar derivative with a palmitoyl ethyl ester was gained by GLC and TLC, which showed that both substances exhibited identical retention times and were characterized by the same R_F values.

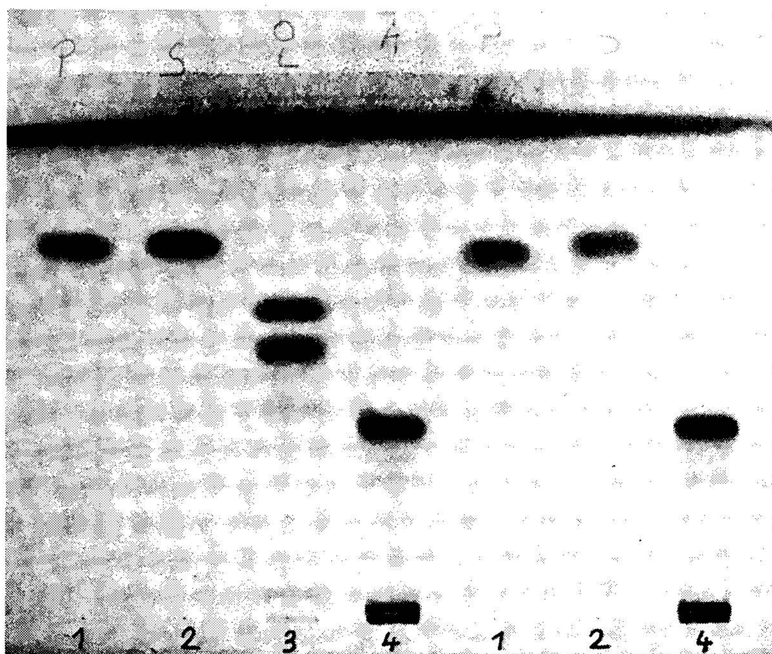

Fig. 2. TLC of the non-polar substances derived from various fatty acids treated with bromine. 1, Palmitic acid; 2, stearic acid; 3, mixture of oleic and linoleic acids; 4, arachidonic acid. Detection by the copper acetate reagent.

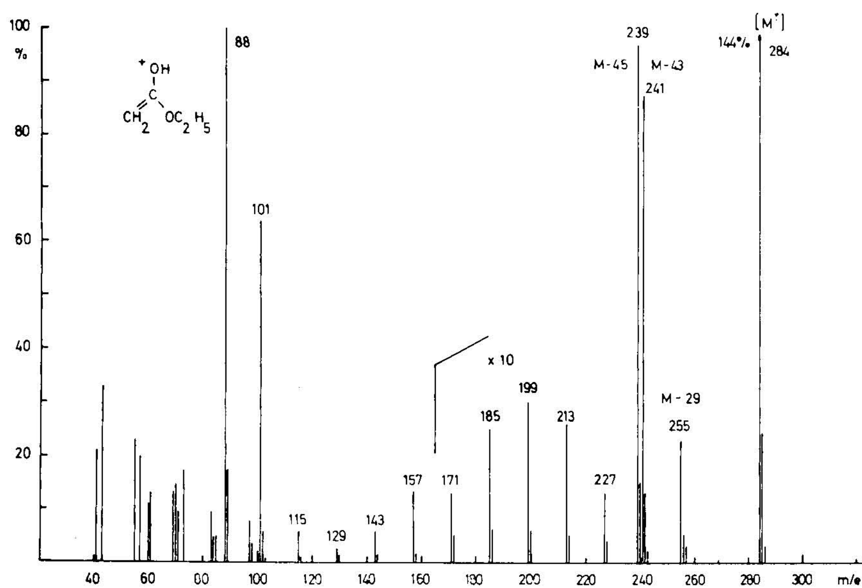

Fig. 3. Mass spectrum of the unknown substance derived from palmitic acid treated with bromine; the spectrum was recorded in the gas chromatograph inlet. An identical pattern was obtained by using the direct inlet.

The mass spectrum of the substance derived from linoleic acid treated with bromine (as recorded by direct inlet mass spectrometry) is shown in Fig. 4A and compared with that of the linoleyl ethyl ester (Fig. 4B); the isotopic-abundance ratio indicates the presence of four bromine atoms in the unknown derivative. It should be noted that the fragmentation pattern recorded via the gas chromatograph inlet differed from that in Fig. 4A and was similar to that of the linoleyl ester (Fig. 4B), probably owing to loss of halogen during GLC.

On TLC, the linoleyl ester proved to be less polar than the substance derived from linoleic acid after bromine treatment; however, within a few seconds of the reaction with bromine, the linoleyl ethyl ester could be converted into a more polar compound having a R_F value identical with that of the derivative of linoleic acid

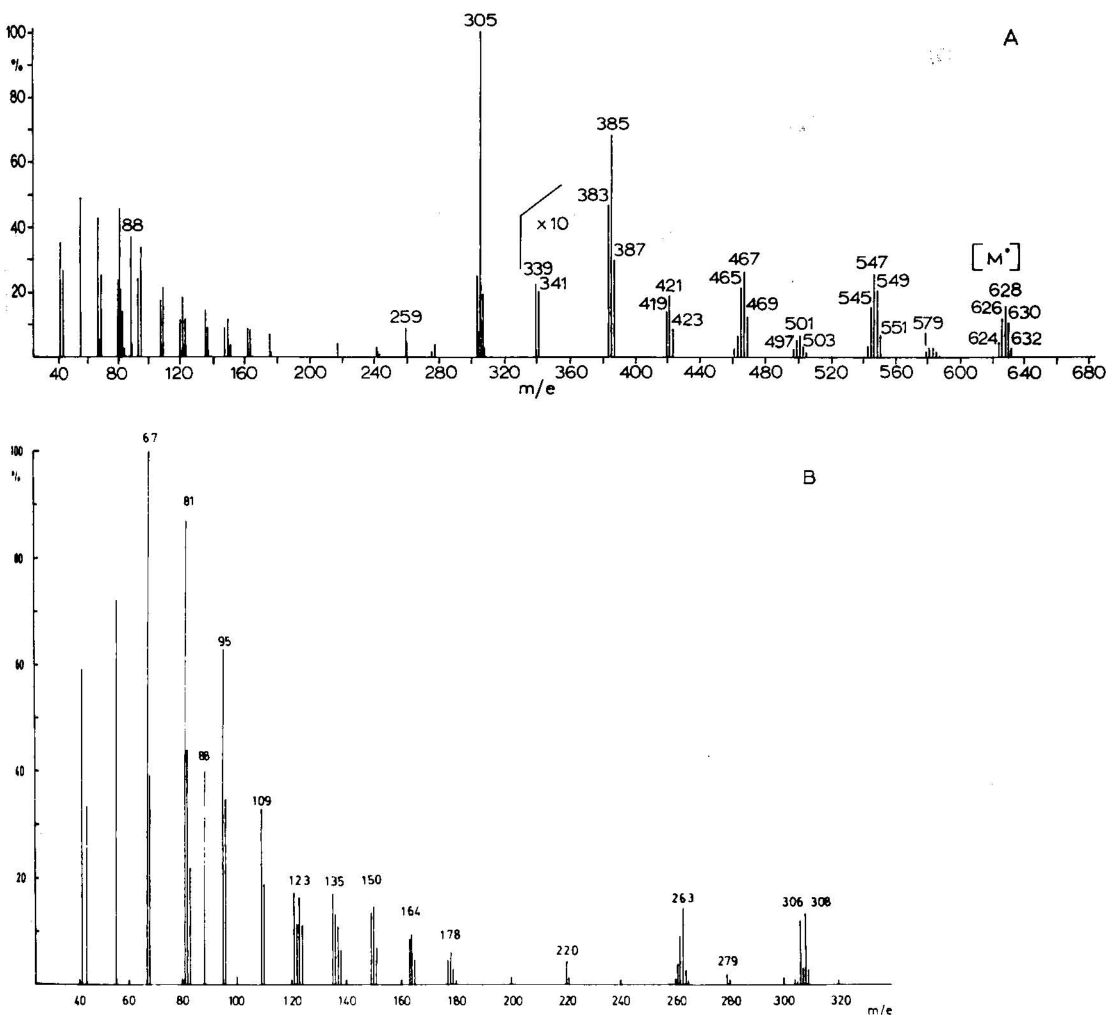

Fig. 4. Mass spectra of the unknown derivative of linoleic acid (A) and of linoleyl ethyl ester (B); the spectra were recorded either in the direct inlet (A) or through the gas chromatograph inlet (B).

(see Fig. 5). These observations suggest that the latter compound is a polybromo-derivative of linoleyl ethyl ester, which is in agreement with the mass-spectrometric fragmentation studies.

It thus appears that bromine treatment of fatty acids in chloroform medium results not only in addition of bromine to the double bonds, but also in esterification of the carboxyl group. Although the mechanism of the latter reaction is not yet known, esterification would depend on the presence of small amounts of ethanol in the chloroform: indeed, the formation of the non-polar derivatives did not occur when the reaction with bromine was carried out in other solvents, such as carbon tetrachloride or benzene, or in chloroform that had been freed of ethanol by passage through an alumina column. Formation of a reactive acid bromide intermediate may be a possible mechanism.

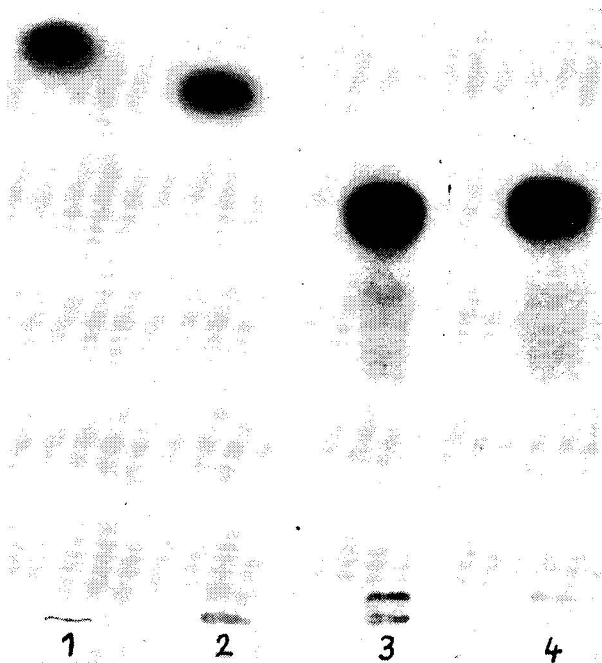

Fig. 5. Chromatographic evidence for identity of the unknown linoleyl derivative with the bromo-derivative of linoleyl ethyl ester. 1, Palmitoyl ethyl ester; 2, linoleyl ethyl ester; 3, linoleyl ethyl ester treated with bromine; 4, unknown substance derived from linoleic acid treated with bromine. Detection by the copper acetate reagent.

While the bromine-catalyzed formation of fatty acid ethyl esters is a rather slow reaction, addition of bromine to the double bonds of unsaturated fatty acid ethyl esters takes place immediately, so that a kinetic study of this latter reaction could not be undertaken.

Application to fatty acid analysis

The above-mentioned findings form the basis of a simple method for the rapid estimation of the distribution of various fatty acid classes in a mixture.

The bromo-derivatives of fatty acid ethyl esters can be prepared in two ways depending on the starting material. For unesterified fatty acids, the sample is simply dissolved in a known amount of chloroform containing 4% of bromine and the solution is set aside overnight at room temperature. When starting from phospholipids, the fatty acid ethyl esters are first prepared by trans-esterification as described in Experimental; an appropriate sample is then evaporated under nitrogen in a conical tube, and the residue is taken up in 4% bromine solution in chloroform and used for TLC in the system light petroleum–diethyl ether (9:1). The TLC can be carried out on a small amount of material, preferably 20–40 μg of total fatty acids (corresponding to 1–2 μg of lipid phosphorus for analyses of phospholipids).

The validity of our method was assessed by comparing the fatty acid distribution as measured by the optical scanning of the TLC plate with the percentage distribution determined by GLC of the methyl esters. Table I shows that the percentage distribution obtained by our method closely approximates to the value calculated from the GLC results.

The bromo-derivatives of fatty acid methyl esters were found to be slightly more polar than those of the corresponding ethyl esters; the former substances, how-

TABLE I

PERCENTAGE DISTRIBUTION OF THE VARIOUS FATTY ACID CLASSES. COMPARISON OF THE TLC TECHNIQUE WITH GLC ANALYSIS

Sample No.	*Acid structure*	*Distribution (%)*		*Sample No.*	*Acid structure*	*Distribution (%)*	
		*TLC**	*GLC***			*TLC**	*GLC***
Fatty acid mixtures				*Rat-liver phosphatidylcholine*			
1	16:0	27	28	1	16:0	–	28
	18:1	23	22		18:0	–	11
	18:2	26	24		Total saturated	42	39
	20:4	24	25		16:1	–	4
					18:1	–	12
2	16:0	45	48		Total monoenes	14	16
	18:1	10	8		18:2	26	26
	18:2	28	24		20:4	18	19
	20:4	17	20				
				Rat-liver phosphatidylethanolamine			
3	16:0	43	55	1	16:0	–	29
	18:1	12	10		18:0	–	24
	18:2	36	28		Total saturated	47	53
	20:4	9	7		16:1	–	–
					18:1	–	9
4	16:0	11	12		Total monoenes	9	9
	18:1	28	28		18:2	21	14
	18:2	42	39		20:4	23	24
	20:4	18	20				
5	16:0	27	23				
	18:1	31	27				
	18:2	12	10				
	20:4	30	39				

* Results obtained by TLC of bromo-derivatives of fatty acid ethyl esters.
** Results obtained by GLC of fatty acid methyl esters.

ever, may also be used for chromatographic separation according to the degree of unsaturation. Small additional spots have occasionally been detected after TLC analysis of the polybromo-derivatives of arachidonyl methyl esters, which suggests that these esters may exhibit some degree of instability. On the other hand, the bromine-addition compound of arachidonyl ethyl ester never gave more than one spot during TLC.

The heating period after spraying with the copper acetate reagent should be sufficiently long (around 2 h at 180°) to allow complete detection of the saturated fatty acid esters; reduction in the heating time could lead to erroneous estimation of the percentage distribution of each fatty acid class.

CONCLUSIONS

The analytical method described in this paper can be used to estimate the relative proportions of the various fatty acid classes in a mixture and therefore offers an alternative to argentation chromatography; its main advantage over the latter method lies in its lower cost and greater simplicity. In view of the increasing use of photodensitometric techniques, it may also be noted that our method can be used as an alternative to GLC, at least to some extent; however, no separation of the different saturated fatty acid ethyl esters could be achieved on layers of unmodified silica gel.

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CHROM. 8295

FLUORIMETRISCHE TESTOSTERONBESTIMMUNG AUF Al_2O_3 DURCH DÜNNSCHICHTCHROMATOGRAPHISCHE TRENNUNG DER TRIMETHYLSILYLÄTHER

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(Eingegangen am 19. November 1974; geänderte Fassung eingegangen am 4. März 1975)

SUMMARY

Fluorimetric determination of testosterone on Al_2O_3 by thin-layer chromatographic separation of the trimethylsilyl ether derivatives

Small quantities (parts per 10^9) of testosterone were determined in the presence of large amounts of other steroids in urine samples obtained during pregnancy by subjecting their trimethylsilyl derivatives to spectrofluorimetry on alumina after purification by thin-layer chromatography on silica gel. The activity of the alumina and the fluorescence reactions are discussed.

EINLEITUNG

Chromatographische Bestimmungen von Spurenbestandteilen neben einem grossen Überschuss anderer Substanzen werden oft durch Überlagerungen gestört. Dieses Problem ist besonders aktuell bei der Hormonbestimmung aus biologischem Material, die meistens erst nach einer Vorfraktionierung (Vorreinigung), Derivatisierung und dem Einsatz spezifischer Nachweisverfahren ermöglicht wird. Während in der Gaschromatographie (GC) die Derivatisierungstechnik weit verbreitet ist, ist sie in der Dünnschichtchromatographie (DC) erst vereinzelt angewandt worden, wie z.B. bei der Eluierung der Corticosteroide als Acetate auf Al_2O_3 zur Vermeidung von Nebenreaktionen[1,2]. Die Derivatisierung ist für die DC aber auch im Hinblick spezifischer Trennungen interessant. So konnte nach dieser Arbeit Testosteron im ppb-Bereich aus dem Urin während der Schwangerschaft, bei der es zu einem starken Anstieg der ausgeschiedenen Steroidmetabolite kommt, erst nach Silylierung des dünnschichtchromatographisch vorgereinigten Extraktes dünnschichtchromatographisch bestimmt werden. Entwickelt wurde auf Al_2O_3-Platten mit nachfolgender Al_2O_3-Fluoreszenzdetektion für 3-Keto-Δ^4-steroide, die erstmals zur Testosteronbestimmung aus dem Urin[3,4] und inzwischen auch zur Progesteronbestimmung aus dem Plasma[5] eingesetzt wurde.

Voruntersuchungen zeigten, dass auch die 17-Hydroxycorticosteroide Cortisol und Cortison nach Abspaltung der polaren Seitenkette durch Wismutatoxydation

(Cortisol → 4-Androsten-11β-ol-3,17-dion, Cortison → 4-Androsten-3,11,17-trion) oder Chromsäureoxydation (Cortisol, Cortison → 4-Androsten-3,11,17-trion)[6] und die 20-Dihydrocorticosteroide nach Perjodsäureoxydation der Glycerolseitenkette[7], als weitere von der GC her bekannte Derivatisierungstechniken, auf Al_2O_3 bestimmt werden können.

EXPERIMENTELLES

Dünnschichtchromatographische Trennung der Trimethylsilyläther auf Al_2O_3

Bei den Trimethylsilyl (TMS)-äthern ist die Wechselwirkung der Hydroxylgruppen mit dem Adsorbens aufgehoben. Eine Folge davon ist, dass nach Tabelle I die R_F-Werte der Steroide von der Anzahl der Ketogruppen bestimmt wird (wie in der GC bei polaren Phasen!). Gelegentlich wurden mit dem Laufmittel Dichlormethan zu hohe R_F-Werte festgestellt. Zur Testosteronbestimmung bewährte sich dann das Laufmittelgemisch Dichlormethan–Cyclohexan (70:30). Beim TMS-Testosteron war die Nachweisempfindlichkeit gegenüber freiem Testosteron auf Al_2O_3, Typ T (Merck) um 34% erhöht (beim Acetat um 65%, gleiche Laufzeiten vorausgesetzt). Dagegen wurden die mit * bezeichneten Derivate der Tabelle I unter Schweifbildung zersetzt, nicht aber auf dem stärker basischem Typ E, der bei faktisch unveränderten R_F-Werten eine wesentlich kürzere Laufzeit aufwies. Im Falle der Testosteronbestimmung wirkte sich die Zersetzung einiger TMS-Derivate günstig aus, da dadurch Überlagerungen vermieden wurden. Diese Zersetzung trat nicht auf, wenn zuvor die Lewis-Säurezentren durch Pyridindämpfe desaktiviert worden waren, was jedoch mit einer Verminderung der Trennleistung erkauft werden musste. Fig. 1 zeigt ein Chromatogram von TMS-5β-Pregnen-3β-ol-20-on mit ausgeprägtem Tailing zwischen den Peaks von Derivat und freiem Steroid (ein Beispiel für Reaktionschromatographie[8–10] bzw. "Simultan-Reaktionschromatographie"[11]).

TABELLE I

R_F-WERTE EINIGER DURCH FLUORESZENZ ANGEZEIGTER STEROIDE NACH SILYLIERUNG AUF Al_2O_3, TYP T (MERCK)

Laufmittel: Dichlormethan.

Verbindungen	*R_F-Werte*
4-Androsten-3,11,17-trion	0.18
4-Androsten-3,17-dion	0.34
TMS-4-Pregnen-11α-ol-3,20-dion*	0.40
Progesteron	0.44
TMS-Epitestosteron*	0.54
TMS-Testosteron	0.58
TMS-5β-Pregnen-3β-ol-20-on*	0.71
TMS-Dehydroepiandrosteron*	0.72
TMS-Cholesterin*	0.84

MATERIAL UND METHODE

Sämtliche Reagenzien (p.a.), Kieselgel 60 GF_{254}, Al_2O_3-DC-Platten 150 F_{254}, Typ T und 60 F_{254}, Typ E waren von Merck. Ein Camag-Chromatocharger, ein Camag-

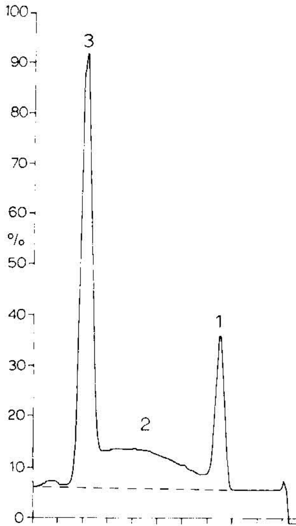

Fig. 1. Chromatogramm von TMS-5β-Pregnen-3β-ol-20-on auf Al_2O_3 (Typ T) durch Fluoreszenzdetektion. Laufmittel: Dichlormethan. 1 = Freies Steroid; 2 = Tailing durch Zersetzung während der Eluierung; 3 = TMS-Derivat.

Z-Scanner zum Zeiss Spektralphotometer PMQ II und ein Desaga-DC-Streichgerät wurden verwendet. Bezüglich der allgemeinen methodischen Ausführungen (Aufarbeitung des Urins, Fluoreszenzreaktion und DC-Direktauswertung) wird auf die vorausgehende Arbeit[3] Bezug genommen. Zur zusätzlichen DC Reinigung des Rohextraktes und anschliessender Silylierung wurde der Trockenrückstand des Rohextraktes in 0.3 ml Aceton gelöst und mittels des Chromatochargers bei einer Strichlänge von 14 cm auf eine selbstgefertigte und bei Raumtemperatur getrocknete Kieselgelplatte aufgetragen und mit 0.2 ml Aceton nachgespült. Nach der Eluierung mit Chloroform–Essigsäureäthylester–Cyclohexan (30:20:10) wurde die durch einen seitlich, punktförmig aufgetragenen Testosteronstandard und unter kurzwelligem UV markierte Zone auf eine Breite von 20 mm abgekratzt, mit 3 × 2 ml Aceton über einer Glasfritte in ein Spitzkölbchen eluiert, und das Aceton im Wasserbad von 50° abgeblasen. Letzte Reste von Wasser wurden nach Zugabe von 0.5 ml Äthanol beim nochmaligen Abdampfen entfernt. Silyliert wurde mit 0.5 ml Hexamethyldisilazan und fünf Tropfen Trimethylsilylchlorid während 1 h bei 60°. Anschliessend wurden die überschüssigen Reagenzien mittels einer Wasserstrahlpumpe abgesaugt. Die durchströmende Luft wurde durch je eine Waschflasche mit konz. H_2SO_4 und NaOH-Plätzchen getrocknet. Der Rückstand wurde in 100 µl Äthanol gelöst, wovon 2 × 5 µl (eingesetzte Harnmenge 50 ml) unter Zwischentrocknung (kalter Föhn!) punktförmig auf eine Al_2O_3-Platte, Typ T, zusammen mit einer Probe mit 0.2 µg/10 µl Testosteron als innerer Standard aufgetragen wurden. Unmittelbar danach (TMS-Testosteron auf Al_2O_3 zersetzt sich allmählich beim Lagern!) wurde mit Dichlormethan, bzw. bei zu hohen R_F-Werten mit Dichlormethan–Cyclohexan (70:30) eluiert, 20 min auf 170–180° erhitzt und mittels Chromatogramm-Spektralphotometer ausgewertet.

ERGEBNISSE

Fig. 2 zeigt ein Chromatogramm mit 63 ng Testosteron entsprechend einer Ausscheidung von 10 μg/24 h-Harn. TMS-Epitestosteron, welches nach Tabelle I getrennt angezeigt werden sollte, wurde durch Zersetzung restlos unterdrückt. Vergleichsweise wurden nach GC-Bestimmungen bei Frauen Normalwerte zwischen 2.5 und 9.2 μg/24 h Testosteron (freies + Glucuronid) gefunden[12]. Die nach dieser Methode gewonnenen Werte lagen bei zwanzig verschiedenen Bestimmungen ebenfalls in diesem Bereich. Die relative Standardabweichung der Einzelmessung, die mit abnehmender Menge zunimmt[3], betrug $\pm$ 10% bei 25 ng Probenaufgabe, entsprechend 5 μg/1000 ml. Da zur Detektion keine Sprühreagenzien benötigt werden, ist der durch Untergrundschwankungen bedingte Fehler relativ klein.

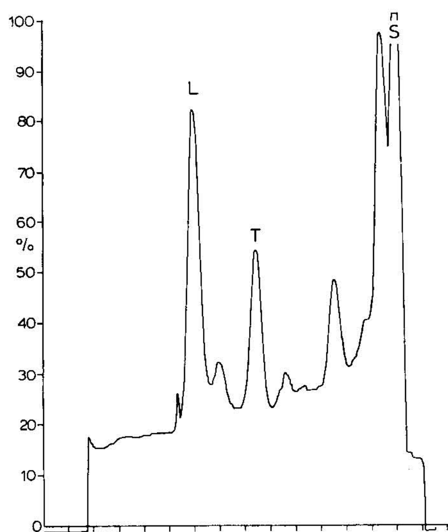

Fig. 2. Dünnschichtchromatographische Fluoreszenzdetektion von Testosteron eines dünnschichtchromatographisch vorgereinigten Urinextraktes auf Al_2O_3 (Typ T). Laufmittel: Dichlormethan. S = Start; T = TMS-Testosteron (63 ng); L = Laufmittelfront.

Acetate führten im vorliegenden Fall zu weniger guten Trennungen. Sie eignen sich jedoch zu Kontrollbestimmungen bei erhöhten Werten (R_F-Werte unter den Bedingungen der Tabelle I: Testosteron-, 0.37; Epitestosteronacetat, 0.49). Eine GC-Bestimmung von TMS-Testosteron mittels XE-60 war bei nur einer einzigen Vorreinigungsstufe nicht möglich, sodass der Vorzug des geringeren Arbeitsaufwandes dieser Methode (ev. auch gegenüber der radioimmunologischen Testosteronbestimmung aus dem Urin) bestehen bleibt.

DISKUSSION

Reaktionen der Steroide auf Al_2O_3

Beim Einsatz von Al_2O_3 als Adsorbens muss seine katalytische Aktivität berücksichtigt werden. Durch die heterogene Zusammensetzung der Oberfläche, die als

aktive Zentren Hydroxylgruppen, Oxidionen und elektrophile Al^{3+}-Ionen (Lewis-Säure) enthält[13–15], ergeben sich verschiedene Reaktionsmöglichkeiten. Die Al^{3+}-Ionen als aktivste Zentren sind verantwortlich für die unerwünschten Reaktionen bei der Chromatographie von Steroiden wie Verseifung, Dehydratisierung und Isomerisierung von Alkoholen[16,17], Abspaltung der polaren Seitenkette der 17-Hydroxycorticosteroide (von der Auftragungsstelle wurden die entsprechenden 17-Ketosteroide eluiert, die sich durch Al_2O_3-Fluoreszenz nachweisen liessen) und die Zersetzung einiger Silyläther.

Basische Katalyse liegt bei der Fluoreszenzreaktion der 3-Keto-Δ^4-steroide vor. Mit zunehmender Basizität des Al_2O_3 konnte die Reaktionstemperatur herabgesetzt werden (180° Typ T, 150° Typ E, Heizdauer 20 min). Der parallele Reaktionsverlauf in alkalischer Lösung und auf Al_2O_3 legt nahe, den Kornblum–De la Mare-Mechanismus[18] für die Oxydation von gelösten Ketonen mit Luftsauerstoff auch der heterogenen Reaktion zu Grunde zu legen. Wie zu erwarten, reagieren die synthetischen 3-Keto-Δ^5-steroide mit derselben Empfindlichkeit wie die 3-Keto-Δ^4-steroide, da sich nach basenkatalysierter Eliminierung von H^+ von der betreffenden aktivierten Methylengruppe dieselbe mesomere Zwischenstufe ergibt.

Nebenreaktionen traten durch eine teilweise Oxydation von Hydroxy- zu Ketosteroiden auf, z.B. der 3-OH-Δ^5-steroide zu 3-Keto-Δ^4-steroiden, die somit ebenfalls angezeigt wurden. Zusätzliche Nebenreaktionen wurden mit nicht vollständig abgedampften Eluierungsmitteln beobachtet. Durch Äthanol wurden Ketosteroide zu den entsprechenden α,β-isomeren Alkoholen reduziert und durch Aceton umgekehrt Steroidalkohole zu Ketonen oxydiert, die sich durch GC nachweisen liessen (vgl. Meerwein–Pondorf–Verley-Reduktion und Oppenauer-Oxydation).

Photochemische Aspekte der Fluoreszenz

Als Chromophor war eine Oberflächenverbindung von I der Fig. 3 mit Al_2O_3 angesehen worden[4]. Da dieselbe Fluoreszenz auch in Lösung mit Al^{3+}-Ionen unter Chelatbildung auftritt, kann auch der Oberflächenverbindung der Al-Chelatkomplex (II) als chromophore Gruppe zugeordnet werden.

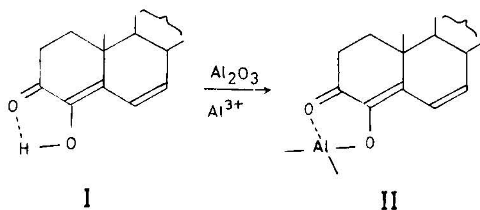

Fig. 3. Der Al-Chelatkomplex (II) der 3-Keto-4-hydroxy-$\Delta^{4,6}$-steroide (I) als Fluorochrom der Fluoreszenzreaktion der 3-Keto-Δ^4-steroide.

Sowohl I als auch II haben in Äthanol ein Absorptionsmaximum bei 317 nm, welches einem $\pi \rightarrow \pi^*$-Übergang entspricht[4]. Die λ_{max}-Werte von Anregung und Emission der sehr schwachen Fluoreszenz von I in Äthanol betrugen 372/458 nm. Mit Alkali trat unter Bildung des hellgelben Enolates eine Zunahme der Fluoreszenzintensität auf das Dreifache auf. Die Bildung des Al-Chelatkomplexes (II) in Äthanol

nach Zugabe einiger Tropfen einer Al-Salzlösung erhöhte dagegen die Fluoreszenz auf das 55-fache mit den λ_{max}-Werten 380/466 nm. Auf Al_2O_3 als stärker polares Medium wurden λ_{max}-Werte im Bereich von 370–380/450–460 nm gemessen, die eine Blauverschiebung anzeigen. Nach semiempirischen Kriterien (geringe absolute Absorptionsintensität, grössere Wellenlänge relativ zum $\pi \rightarrow \pi^*$-Übergang und Blauverschiebung der Absorptionsbande in polareren Medien)[19] liegt bei dieser Anregung ein $n \rightarrow \pi^*$-Übergang am Carbonylsauerstoff vor (nicht aber, wie ursprünglich gefolgert wurde, eine Rotverschiebung des $\pi \rightarrow \pi^*$-Übergangs von 317 auf 370–380 nm[4], die in diesem Ausmass auch viel zu gross wäre).

Die Frage, ob die Emission von einem Singulett- (Fluoreszenz) oder Triplettzustand (Phosphoreszenz) ausgeht, wurde nicht experimentell entschieden. Es ist jedoch sehr wahrscheinlich, dass bei diesen Wellenlängen wie bei anderen Carbonylverbindungen[19,20] eine Singulett–Singulett-Absorption und eine Triplett–Singulett-Emission erfolgt.

Für die Erhöhung der Bandenintensität der Lumineszenz durch Komplexbildung kommen prinzipiell zwei Möglichkeiten in Betracht: Die Erhöhung der Übergangswahrscheinlichkeit der Absorption (bzw. der Dipol- oder Oszillatorenstärke) als eine Folge eines gelockerten Übergangsverbotes durch den störenden Einfluss von Al^{3+} oder/und durch eine Erhöhung der Quantenausbeute der Emission bei einer Verminderung strahlungsloser Desaktivierung durch intermolekulare Prozesse (Abschirmung der chromophoren Gruppe). Das letztere scheint überwiegend der Fall zu sein, da die Lumineszenz der Al-freien Verbindung in Alkohol–Äther–Glas bei 77°K stark zunimmt, beim Al-Chelatkomplex auf Al_2O_3 dagegen konstant bleibt. Mit einer Koordinationszahl 6 des Al^{3+} können maximal drei Liganden gebunden werden. Ob die Übertragung elektronischer Energie zwischen zwei symmetrisch angeordneten, gleichen Liganden (Resonanzfall) bei der Intensitätszunahme ebenfalls eine Rolle spielt (im Sinne eines "Käfig-Effektes" mit einer verhinderten Übergabe elektronischer Energie an einen Quencher) bleibt dahingestellt.

ZUSAMMENFASSUNG

Die niedrigen Testosteronmengen im ppb-Bereich neben einem grossen Überschuss anderer Steroide im Schwangerschaftsharn wurden nach dünnschichtchromatographischer Vorreinigung auf Kieselgel als Trimethylsilyläther durch Chromatogramm-Spektralfluorimetrie auf Aluminiumoxid bestimmt. Die Aktivität des Aluminiumoxides und die Fluoreszenzreaktion werden diskutiert.

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IONENAUSTAUSCH BEI HOHEN KONZENTRATIONEN DER LÖSUNG

IV. MITT. SORPTION VON THALLIUM(III) AUS HBr AN VERSCHIEDENEN IONENAUSTAUSCHERN

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SUMMARY

Ion exchange at high concentrations of the mobile phase. IV. Sorption of thallium(III) from HBr on various ion exchangers

The influence of the structure of the matrix and the functional groups of various ion exchangers on the sorption of $TlBr_4^-$-complexes has been investigated. Sorption of Tl(III) from HBr solution was found with all the sorbents studied. The highest degree of sorption can be observed on exchangers of aromatic structure by studying the matrix effect, *i.e.* the interaction between the anions of the complex and the matrix of the resin. Here the sequence anion exchangers > chelon exchangers > cation exchangers is valid. A substantially smaller degree of sorption was found for anion exchangers with an aliphatic matrix, sorption being due only to the interaction of the $TlBr_4^-$ complexes and the functional groups of the resin.

EINLEITUNG

Dreiwertiges Thallium wird aus HBr bei Konzentrationen > 0.5 Mol/l durch verschiedene feste Sorbenzien, wie Anionenaustauscher und sulfonsaure Kationenaustauscher, sowie makroporöse Copolymerisate auf Basis Polystyrol–Divinylbenzol ohne funktionelle Gruppe mit hohen Verteilungskoeffizienten sorbiert[1–4]. Einen Ionenaustauschmechanismus kann man im Fall der Kationenaustauscher und der Copolymerisate ausschliessen, da Tl(III) unter diesen Bedingungen in beiden Phasen in Form komplexer $TlBr_4^-$-Ionen vorliegt[5]. Die Sorption wird vielmehr durch die Wechselwirkung der nicht hydratisierten komplexen Anionen mit den Molekülen des Harzgerüstes bedingt (Matrixeffekt). Ausserdem wird dieser Effekt durch die Verdrängung der $TlBr_4^-$-Ionen infolge ihrer negativen Hydratation in die Phase mit stark gestörter Wasserstruktur, d.h. die Harzphase begünstigt[6]. Bei Vorliegen eines solchen Mechanismus sollte prinzipiell jeder Austauscher mit polymerer organischer Matrix komplexe Anionen dieses Typs aufnehmen.

In der vorliegenden Arbeit wurde zur Prüfung dieser Hypothese die Sorption von Tl(III) in Abhängigkeit von der HBr-Konzentration durch eine Reihe kommerzieller Austauscher bestimmt, um sowohl den Einfluss der Struktur des Harzgerüstes als auch den der funktionellen Gruppe zu untersuchen. In Tabelle I sind die verwendeten Austauscher zusammengestellt.

TABELLE I

ZUSAMMENSTELLUNG DER VERWENDETEN IONENAUSTAUSCHER

Nr.	*Harz*	*Matrix*	*Funktionelle Gruppe*	*Quellung* *q (ml H_2O/g)*	*Kapazität* *(mäquiv./g)*	*Kanalstruktur*
1	Wof SBW	Styrol–Divinylbenzol	$-\overset{+}{N}(CH_3)_3\ Cl^-$	1.52	3.5	
2	Wof SBT	Styrol–Divinylbenzol	$-\overset{+}{N}(CH_3)_3\ Cl^-$	1.14	3.0	
3	Wof EA 60	Styrol–Divinylbenzol	$-\overset{+}{N}(CH_3)_3\ Cl^-$	2.05	3.8	+
4	Wof ES	Styrol–Divinylbenzol	$-\overset{+}{N}(CH_3)_3\ Cl^-$	2.31	3.5	
5	Wof SBK	Styrol–Divinylbenzol	$-\overset{+}{N}C_2H_4OH(CH_3)_2\ Cl^-$	0.89	3.0	
6	Wof SBU	Styrol–Divinylbenzol	$p\text{-}C_5H_4\overset{+}{N}R\ Cl^-$	1.19	3.1	
7	Wof AD 41	Styrol–Divinylbenzol	$-N(CH_3)_2$	1.0	4.2	+
8	Wof AK 40	Styrol–Divinylbenzol	$-NH_2$, $-NHR$	1.18	5.5	+
9	Wof L 150	Polyalkylenpolyamin	$>NH$, $>N-$, $-\overset{+}{N}R_3$	0.94	9.63	
10	Wof L 165	Polyalkylenpolyamin	$>N-$, $-\overset{+}{N}R_3$	0.87	7.87	
11	Wof N	Polykondensat aus aromatischem Amin + Formaldehyd	$-NH_2$ oder $>NH$ (aromatisch)	0.41	4.3	
12	Wof E	Polykondensat aus aromatischem Amin + Formaldehyd	$-NH_2$ oder $>NH$ (aromatisch)	0.43	2.34	
13	Wof EZ	Polykondensat aus aromatischem Amin + Formaldehyd	$-NH_2$ oder $>NH$ (aromatisch)	0.602	1.72	
14	Chelex 100	Styrol–Divinylbenzol	$-N(CH_2COOH)_2$	0.48	5.82	
15	Wof MC 50	Styrol–Divinylbenzol	$-N(CH_2COOH)_2$	0.58	5.24	+
16	Wof Y 11	Styrol–Divinylbenzol	$-\overset{+}{N}(CH_3)_3\ Cl^-$	1.84	1.41	+
17	Wof Y 17	Acrylamid	$-N(CH_3)_2$	1.55	4.9	
18	Wof P	Phenol–Formaldehyd	$-SO_3H$, $-OH$	0.55	1.38; 4.66	
19	Wof F	Phenol–Formaldehyd	$-SO_3H$, $-OH$	1.10	2.55; 4.03	
20	Wof KPS	Styrol–Divinylbenzol	$-SO_3H$	1.13	5.01	
21	Wof KS 10	Styrol–Divinylbenzol	$-SO_3H$	1.12	4.80	+
22	Wof Y 15	Styrol–Divinylbenzol	$-SO_3H$	1.50	4.96	+
23	Wof CV	Phenol–Formaldehyd	$-COOH$, $-OH$	0.34	5.93	
24	Wof CN	Aromatische Carbonsäuren–Formaldehyd	$-COOH$	0.205	1.83	
25	Wof CP	Acrylsäure–Divinylbenzol	$-COOH$	1.10	10.6	
26	Wof CA 20	Acrylsäure–Divinylbenzol	$-COOH$	1.13	9.9	+

EXPERIMENTELLES

Die Ionenaustauscher wurden zur Entfernung von Metallspuren sorgfältig mit 6 *N* HCl gewaschen und anschliessend in der üblichen Weise mit 2 *N* NH_4OH und 6 *N* HCl eingefahren. Die Kationenaustauscher wurden in der H^+-Form, die starkbasischen Anionenaustauscher in der Cl^--Form und die mittelstark- bzw. schwachbasischen Anionenaustauscher in der OH^--Form eingesetzt.

Zur Bestimmung der Verteilungskoeffizienten diente die batch-Technik mit ^{204}Tl als Indikator[2]. Es wurden Harzmengen von 20–200 mg eingesetzt; das Lösungsvolumen betrug 6 ml. Zur Bestimmung der Quellung wurden 200 mg des lufttrockenen Harzes auf einer Mikrofritte G 3 mit Wasser, bzw. HBr der entsprechenden Konzentration gewaschen. Nach vier Tagen wurde von der überstehenden HBr abzentrifugiert (10 min bei 2300 *g*), das Gesamtgewicht bestimmt, die HBr ausgewaschen und mit 0.1 *N* NaOH titriert. Der auf der Oberfläche des Harzes und der Mikrofritte verbleibende Lösungsrest wurde korrigiert.

ERGEBNISSE UND DISKUSSION

In der Fig. 1 sind die Verteilungskoeffizienten für Tl(III) in Abhängigkeit von der HBr-Konzentration im Bereich 0.1–8.4 Mol/l für die verschiedenen Sorbenzien graphisch dargestellt. Man sieht, dass Tl(III) von allen untersuchten Harzen, unabhängig von der Art der funktionellen Gruppe, sorbiert wird (Befund 1).

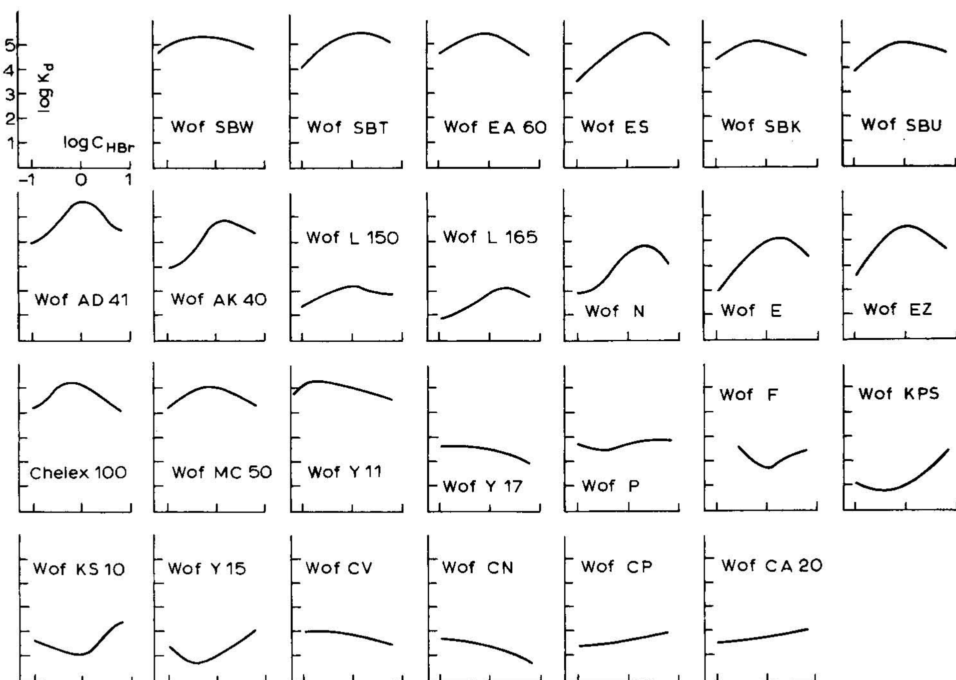

Fig. 1. Sorption von Tl(III) an verschiedenen Ionenaustauschern in Abhängigkeit von der HBr-Konzentration.

Ähnliche Ergebnisse wurden bei der Untersuchung der Sorption von Au(III) aus HCl an einer Reihe von Austauscherpapieren unterschiedlicher chemischer Natur erhalten[7]. Die an sulfonsauren Kationenaustauschern beobachtete beträchtliche Sorption anionischer Komplexe von Fe(III), Tl(III), Au(III) und Ga(III) bei hohen HCl- und HBr-Konzentrationen ist deshalb keine für diesen Austauschertyp charakteristische Anomalie[3,4,8].

Für die Mehrzahl der hier untersuchten Austauscher, d.h. die Anionenaustauscher, Chelonharze und Adsorberharze, hat die Verteilungskurve bei einer HBr-Konzentration von 2 Mol/l ein Maximum (Befund 2).

Die gleiche Abhängigkeit wurde für die makroporösen Copolymerisate ohne funktionelle Gruppe gefunden[1]. Im Gegensatz dazu hat die Verteilungskurve für die sulfonsauren Kationenaustauscher bei einer HBr-Konzentration von 0.5–1 Mol/l ein Minimum; bei höheren HBr-Konzentrationen steigen die K_d-Werte erneut an (Befund 3).

Bei den schwachsauren Kationenaustauschern Wof CN und Wof CV fällt der Verteilungskoeffizient mit der HBr-Konzentration kontinuierlich ab, während man für Wof CP und Wof CA 20 eine geringe Zunahme der Tl(III)-Sorption mit der HBr-Konzentration beobachtet.

In Tabelle II sind die K_d-Werte für die HBr-Konzentrationen 2 Mol/l und 6 Mol/l, sowie die entsprechenden Quellungsdaten, zusammengestellt. Daraus ergibt sich folgende Sorptionsreihe: Anionenaustauscher auf Basis Polystyrol–DVB $>$ Chelonharze $>$ Adsorberharze auf Basis aromatisches Amin $+$ Formaldehyd $>$ Anionenaustauscher mit aliphatischer Matrix $\approx$ Kationenaustauscher (Befund 4).

Bei 6 N HBr ist die Sorption an den Kationenaustauschern auf Basis Polystyrol–DVB (KPS, KS 10, Y 15) bzw. Acrylsäure–DVB (CP, CA 20) grösser als die an den Anionenaustauschern mit aliphatischer Matrix (L 150, L 165, Y 17). Innerhalb der Gruppe der Anionenaustauscher auf Basis Polystyrol–DVB hat die Basizität der funktionellen Gruppe nur einen geringen Einfluss (Befund 5). So unterscheiden sich die Verteilungskoeffizienten an den Standardtypen SBW, SBT, EA 60, ES und Y 11 mit Trimethylammoniumgruppen, an SBU mit p-$C_5H_4N^+R$-Gruppen, sowie an AD 41 und AK 40 mit $N(CH_3)_2$- bzw. NH_2- und NHR-Gruppen nur unwesentlich, besonders wenn man die Unterschiede in der Vernetzung und Porosität der verschiedenen Sorbenzien berücksichtigt.

Bezieht man die K_d-Werte auf die Quellung des Harzes, die mit Ausnahme der makroporösen Produkte im wesentlichen vom Vernetzungsgrad des Harzes abhängig ist, so erhält man die in Tabelle II für die HBr-Konzentrationen 2 Mol/l und 6 Mol/l angegebenen Werte. Auch in diesem Fall findet man keinen signifikanten Unterschied für die verschiedenen Anionenaustauscher und Chelonharze. Das bestätigt Befund 5.

Die dargelegten Ergebnisse zeigen die Rolle der Matrix bei der Aufnahme von $TlBr_4^-$-Komplexen durch Ionenaustauscher. Entsprechend Befund 1 werden $TlBr_4^-$-Ionen durch Anionenaustauscher, Chelonharze, Adsorberharze und Kationenaustauscher aufgenommen. Im Fall der Anionenaustauscher können die $TlBr_4^-$-Ionen sowohl mit den positiven Ankergruppen, als auch mit den Molekülen des Harzgerüstes in Wechselwirkung treten. So tragen bei einer Kapazität von 3–3.5 Mäquiv./g an den Standardtypen, wie z.B. SBW nur ungefähr 60% der aromatischen Kerne des Harzes eine funktionelle Gruppe.

TABELLE II

EINFLUSS DER HBr-KONZENTRATION AUF DIE SORPTION VON Tl(III) AN VERSCHIEDENEN SORBENZIEN

Nr.	*Austauscher*	*2 N HBr*			*6 N HBr*		
		log K_d (ml/g)	*q (ml/g)*	*log (K_d/q)*	*log K_d (ml/g)*	*q (ml/g)*	*log (K_d/q)*
1	SBW	5.14	0.67	5.31	4.82	0.81	4.91
2	SBT	5.40	0.48	5.72	5.06	0.55	5.32
3	EA 60	5.15	1.16	5.094	4.52	1.17	4.45
4	ES	5.39	0.78	5.50	4.97	0.93	5.00
5	SBK	4.90	0.35	5.36	4.52	0.40	4.92
6	SBU	4.84	0.44	5.20	4.57	0.57	4.816
7	AD 41	5.36	0.79	5.46	4.54	0.73	4.675
8	AK 40	4.84	0.87	4.90	4.42	0.77	4.53
9	L 150	2.072	0.52	2.356	1.967	0.51	2.26
10	L 165	2.068	0.58	2.305	1.732	0.52	2.02
11	N	3.78	0.40	4.18	3.14	0.41	3.53
12	E	4.05	0.44	4.406	3.42	0.46	3.76
13	EZ	4.39	0.46	4.535	3.62	0.53	3.90
14	Chelex 100	4.78	1.24	4.69	4.03	0.98	4.04
15	MC 50	4.84	1.15	4.78	4.33	1.00	4.33
16	Y 11	4.89	1.21	4.81	4.56	1.12	4.51
17	Y 17	2.42	0.96	2.44	2.037	0.85	2.11
18	P	2.86	0.43	3.226	2.88	0.35	3.34
19	F	2.10	0.79	2.203	2.45	0.50	2.75
20	KPS	1.60	0.93	1.632	2.40	0.64	2.59
21	KS 10	1.548	1.00	1.548	2.43	0.79	2.53
22	Y 15	1.428	1.44	1.27	2.118	1.23	2.03
23	CV	1.79	0.29	2.33	1.57	0.23	2.21
24	CN	1.26	0.16	2.06	0.909	0.17	1.68
25	CP	1.88	0.68	2.05	2.09	0.29	2.63
26	CA 20	1.97	0.85	2.04	2.176	0.34	2.67

Der Unterschied von drei Grössenordnungen in den K_d-Werten der Anionenaustauscher auf Basis Polystyrol–DVB (SBW, SBT, SBU, SBK, ES, AD 41) und der auf Basis aliphatischer Polyamine (L 150, L 165, Y 17), die z.T. eine wesentlich höhere Austauschkapazität besitzen, beweist den Einfluss der Matrix bei Anionenaustauschern mit aromatischer Struktur auf die Sorption anionischer Komplexe des Typs $TlBr_4^-$, denn entsprechend Befund 5 geben die Unterschiede in der Basizität der funktionellen Gruppe dafür keine hinreichende Erklärung. Das gleiche gilt für die Adsorberharze Wof N, E und EZ, die Polykondensate aus aromatischem Amin und Formaldehyd sind und deren Verteilungskoeffizienten zwei Grössenordnungen über denen von Wof L 150 und Wof L 165 liegen.

Die Abnahme der Sorption bei höheren HBr-Konzentrationen an den Anionenaustauschern, Chelonharzen und Adsorberharzen (Befund 2) ist eine Folge der dabei zunehmenden Zerstörung der Wasserstruktur in der äusseren Lösung, wodurch der Einbau der nicht hydratisierten $TlBr_4^-$-Ionen in diese Phase begünstigt wird, sowie der Konkurrenz der Br^--Gegenionen.

Die Aufnahme der $TlBr_4^-$-Komplexe durch Kationenaustauscher zeigt die Rolle des Matrixeffektes auch bei diesem Austauschertyp[2,9,10]. Bei Konzentrationen > 0.5 *N* HBr beginnt mit der Bildung der $TlBr_4^-$-Komplexe der Wiederanstieg der

Verteilungskoeffizienten. Der gefundene Kurvenverlauf zeigt, dass die Wechselwirkung der $TlBr_4^-$-Ionen mit der unpolaren Matrix durch deren negative Aufladung infolge der Dissoziation der funktionellen Gruppe insbesondere bei sulfonsauren Austauschern behindert wird. Erst bei höheren HBr-Konzentrationen wird die funktionelle Gruppe infolge der ansteigenden Harzinvasion und der abnehmenden Wasseraktivität in beiden Phasen zunehmend abgeschirmt und die Wechselwirkung mit der Matrix spielt eine wesentliche Rolle (Befund 3). Besonders charakteristisch ist dieser Effekt für die sulfonsauren Produkte KPS, KS 10, Y 15, F und P. In Übereinstimmung damit steht auch die grössere Sorption an Wof P in Vergleich mit Wof F, die beide Polykondensationsprodukte auf Basis Phenol–Formaldehyd sind, infolge der geringeren Kapazität und Acidität der SO_3H-Gruppe von Wof P[11]. Zur Deutung der Verteilungskurve an den schwachsauren Kationenaustauschern wurde die Quellung und Harzinvasion in Abhängigkeit von der HBr-Konzentration untersucht. In den Fig. 2 und 3 sind die Ergebnisse dargestellt.

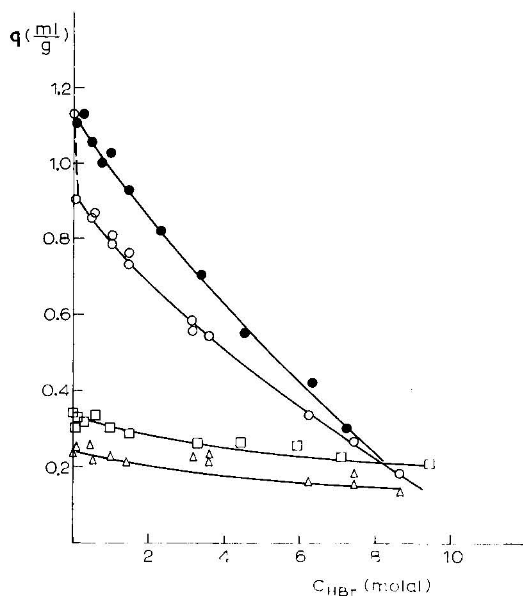

Fig. 2. Quellung verschiedener schwachsaurer Kationenaustauscher in HBr. ●, CA 20; ○, CP; □, CV; △, CN.

Die Quellungskurven beweisen die geringe Vernetzung von Wof CP und Wof CA 20, im Gegensatz zu Wof CN und Wof CV, die durch eine stark vernetzte Matrix und sehr geringe Quellfähigkeit charakterisiert sind. Die gefundenen Donnankoeffizienten sind im gesamten Konzentrationsbereich < 1. Der Elektrolytausschluss nimmt in der Reihenfolge CV $<$ CN $<$ CP zu. Alle vier untersuchten Harze zeigen damit trotz der geringen Acidität ihrer funktionellen Gruppe ein für Kationenaustauscher typisches Verhalten. Der schwache Anstieg der K_d-Werte für Tl(III) an Wof CP und Wof CA 20 mit der HBr-Konzentration, $K_d\ (2\ N)/K_d\ (6\ N) = 0.6$, ist deshalb wie bei den sulfonsauren Kationenaustauschern, $K_d\ (2\ N)/K_d\ (6\ N) = 0.13$–

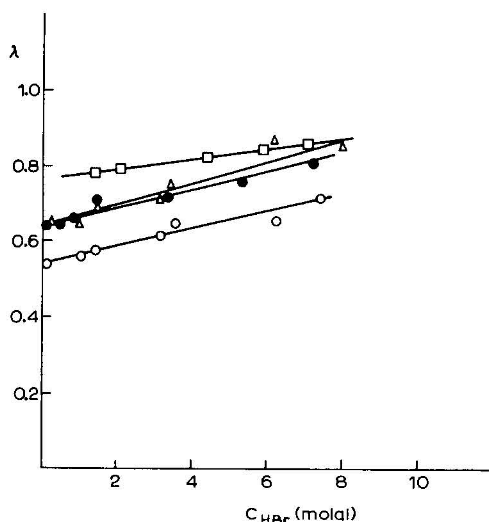

Fig. 3. Aufnahme von HBr durch verschiedene schwachsaure Kationenaustauscher. ●, CA 20; ○, CP; □, CV; △, CN.

0.20, durch die Abschirmung der funktionellen Gruppe durch den Grundelektrolyten HBr bedingt. Allerdings ist dieser Effekt bei den schwachsauren Harzen, wie die obigen Werte zeigen, bedeutend weniger ausgeprägt. Demgegenüber wird der Verlauf der Sorptionskurve im Fall von Wof CN und Wof CV insbesondere durch die sterische Behinderung der Sorption infolge der geringen Porosität und Quellung der Harze bestimmt, die sich z.B. auch in der schlechten Sorptionskinetik dieser Harze (Gleichgewichtseinstellung nach vierzehn Tagen) äussert.

Zusammenfassend ergibt sich aus den hier dargestellten Ergebnissen, dass für die Sorption von $TlBr_4^-$-Komplexen durch verschiedene Sorbenzien zwei Prozesse charakteristisch sind, einmal die Wechselwirkung der Komplexionen mit den Molekülen des Harzgerüstes bei Sorbenzien mit aromatischer Struktur unabhängig von der Art der funktionellen Gruppe und im Fall der Anionenaustauscher ein Ionenaustauschprozess mit den Gegenionen der funktionellen Gruppe.

DANK

Der Autor dankt Herrn Dr. H. Schelhorn für anregende Diskussionen und Frau S. Wittmar für experimentelle Mitarbeit.

ZUSAMMENFASSUNG

Der Einfluss der Struktur der Matrix und der funktionellen Gruppe verschiedener Ionenaustauscher auf die Sorption von $TlBr_4^-$-Komplexen wird untersucht. Es wurde Sorption von Tl(III) aus HBr an allen betrachteten Sorbenzien gefunden. Die höchste Sorption beobachtet man auf Grund des Matrixeffektes, d.h. der Wechselwirkung der komplexen Anionen mit dem Harzgerüst, an Austauschern mit aromatischer Struktur. Es gilt dabei die Sorptionsreihe Anionenaustauscher $>$ Chelon-

austauscher > Kationenaustauscher. Für Anionenaustauscher mit aliphatischer Matrix wurde eine wesentlich geringere Sorption gefunden, die allein durch die Wechselwirkung der $TlBr_4^-$-Komplexe mit der funktionellen Gruppe des Harzes bedingt ist.

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CHROM. 8307

QUANTITATIVE DETERMINATION OF COCAINE AND ITS METABOLITES BENZOYLECGONINE AND ECGONINE BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and reliable method was developed to determine quantitatively cocaine and its metabolites benzoylecgonine and ecgonine. The method involved the formation of fluoro derivatives which were separated on 3% and 5% OV-1 columns and detected in picomole quantities using an electron capture detector. Ecgonine and benzoylecgonine were derivatized with a mixture of hexafluoroisopropanol–heptafluorobutyric anhydride (1:2). Cocaine was first reduced by $LiAlH_4$ and then acylated by pentafluoropropionic anhydride. Benzoylecgonine, but not ecgonine, could also be determined by reduction and subsequent acylation. This provided the basis for the determination of cocaine, benzoylecgonine and ecgonine from the same sample. Cocaine could be determined in urine and plasma by this method.

INTRODUCTION

It has been suggested that cocaine is extensively metabolized[1–3]. However, little is known about its absorption, distribution and excretion. It is thought to be metabolized through the hydrolysis of two ester bonds, first to benzoylecgonine and finally to ecgonine. In recent years the emphasis has been on the development of methodology for the detection of cocaine in drug abuse programs for screening urines and, to date, there is no method available to determine cocaine and its metabolites together from the same sample. A number of thin-layer chromatography (TLC) and gas–liquid chromatography (GLC) methods are available for the detection of cocaine or benzoylecgonine[4–10]. However, most of these methods are nonquantitative and are not very sensitive. An enzyme multiplied immunoassay technique[11] (EMIT) has been developed for the detection of urinary benzoylecgonine. Although the method is rapid and generally reliable it does not determine unchanged cocaine and does not differentiate between benzoylecgonine and ecgonine. Also, the method is not applicable to plasma because of high protein concentration and lysozyme activity in plasma. Because of the suspected low amounts of ecgonine there is no report for its determination, although recently Bastos *et al.*[9] have described a TLC method for the detection of ecgonine.

This communication describes a sensitive and reliable GLC method for the quantitative determination of cocaine, benzoylecgonine, and ecgonine and has the potential of being useful in studying the metabolism, distribution and excretion of cocaine.

EXPERIMENTAL

Materials and methods

All chemicals used were reagent grade. Cocaine hydrochloride and nanograde cyclohexane were obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). Benzoylecgonine (Lot No. 81-24-D) and ecgonine (Lot No. 70-220-D) were purchased from Technam (Park Forest South, Ill., U.S.A.). $LiAlH_4$, hexafluoroisopropanol, heptafluorobutyric anhydride and pentafluoropropionic anhydride were from Chemical Procurement Labs. (College Point, N.Y., U.S.A.). All samples were run on a Packard Model 824 gas chromatograph equipped with a ^3H-electron capture detector. The derivatives were separated on a coiled column (6 ft. × 2 mm I.D.) packed with 3% or 5% OV-1 on 80–100 mesh GHP (Supelco, Bellefonte, Pa., U.S.A.). Nitrogen was used as carrier and purge gas.

Procedures

Determination of cocaine. Cocaine was determined by the method of Blake *et al.*[10] with a slight modification in chromatographic conditions. Briefly, cocaine hydrochloride solution (0.1–2.0 μg in methanol) was taken in Kimax culture tubes (125 × 6 mm). The methanol was evaporated under nitrogen and 2 ml of cyclohexane added to the tube. After mixing, 50 μl of saturated $LiAlH_4$ solution in ether were added. The solution was mixed and left at room temperature for 10 min. Then 50 μl of water were added to destroy the excess of $LiAlH_4$. The solution was mixed vigorously and 50 μl of pentafluoropropionic anhydride were added. The mixture was mixed thoroughly after 3 min and left at room temperature for another 2 min. Then 6 ml of saturated sodium borate solution were added and the solution was mixed for 4 min on a shaker. The tubes were centrifuged, if necessary, to separate the organic and aqueous phases and 1 μl of the cyclohexane phase was injected. The chromatographic conditions for separation are summarized in Table I.

TABLE I

CHROMATOGRAPHIC CONDITIONS AND RETENTION TIMES FOR COCAINE, ECGONINE AND BENZOYLECGONINE DERIVATIVES

	Reduced cocaine and benzoylecgonine	*Ecgonine*	*Benzoylecgonine*
Column temperature*	110°	130°	190°
Injection port temperature	160°	160°	200°
Detector temperature	160°	160°	200°
Gas flow-rate (ml/min)	20	30	50
Range	1×10^{-9}	1×10^{-9}	1×10^{-9}
Retention time (min)**	4.2	12.5	15.6

* The column was operated at isothermal temperatures.

** These retention times are for the 5% OV-1 column. For the 3% OV-1 column the retention time for cocaine, ecgonine and benzoylecgonine under the above conditions is 1.8 min, 6.0 min and 5.8 min, respectively.

Determination of ecgonine. Ecgonine solution (0.05–1.0 μg in methanol) was taken in 1.5 ml capacity Miniaktor tubes (Applied Science Labs., State College, Pa., U.S.A.). After the methanol was evaporated under nitrogen, 100 μl of the derivatizing mixture hexafluoroisopropanol–heptafluorobutyric anhydride (1:2, v/v) were added and the tubes were heated for 30 min at 75°. The excess of the reagents was then removed by evaporation under nitrogen until there was no odor of heptafluorobutyric anhydride (3–5 min). Then 1 ml of cyclohexane was added and 1 μl of the sample was injected. The operation conditions for chromatography are given in Table I.

Determination of benzoylecgonine. Benzoylecgonine solution (1–15 μg in methanol) was derivatized exactly as described for ecgonine and determined under the conditions given in Table I.

Benzoylecgonine was also determined after reduction and acylation as in the cocaine determination.

RESULTS AND DISCUSSION

The use of acyl derivatives in GLC has been extensively investigated and is commonly used for the determination of compounds containing hydroxyl groups[12–19]. Although cocaine (benzoyl methyl ecgonine) does not have any free functional groups, recently Blake *et al.*[10] were able to reduce cocaine to 2-hydroxymethyl tropine. The reduced product was then O-acylated by treating with pentafluoropropionic anhydride or heptafluorobutyric anhydride and the derivative was detected by an electron capture detector with greatly increased sensitivity. We have extended this method to determine cocaine quantitatively. Fig. 1B shows a typical chromatogram of reduced cocaine acylated with pentafluoropropionic anhydride. Under the conditions given in Table I a single symmetrical peak was obtained in which the peak height was proportional to the concentration of the sample injected. Fig. 1A is a reagent blank. The arrow points to the position where reduced cocaine was eluted. Fig. 4C shows the linearity of the plot of peak heights with increasing concentrations of cocaine. Since the detector response depends on the specific sensitivity setting, the linearity range should be checked with each instrument. Under the conditions described here 50–750 pg of the injected cocaine derivative gave a linear response. It was found that the best reproducible results were obtained at concentrations lower than 0.5 μg/ml. Irrespective of the original concentration, the sample could be diluted to desired concentration with cyclohexane. The derivatives were stable for more than 12 h. Since cocaine is a benzoylmethyl ester of ecgonine one should be able to reduce both ecgonine and benzoylecgonine to 2-hydroxymethyl tropine. Reduction and subsequent acylation of benzoylecgonine under the conditions used for cocaine resulted in a single symmetrical peak identical to that obtained from cocaine (Fig. 1C). Although the derivative obtained from benzoylecgonine was not identified by mass spectroscopic analysis in these studies, we assume from the gas chromatographic behavior that it was identical to pentafluoropropyl 1,2-difluoro-2-hydroxymethyl tropine obtained from cocaine and identified by mass spectroscopic analysis[10]. This assumption was further supported by the fact that cocaine and benzoylecgonine gave a similar response after reduction and acylation when separated on 3% and 5% OV-1 columns. Also, when equimoles of cocaine and benzoylecgonine were reduced and derivatized

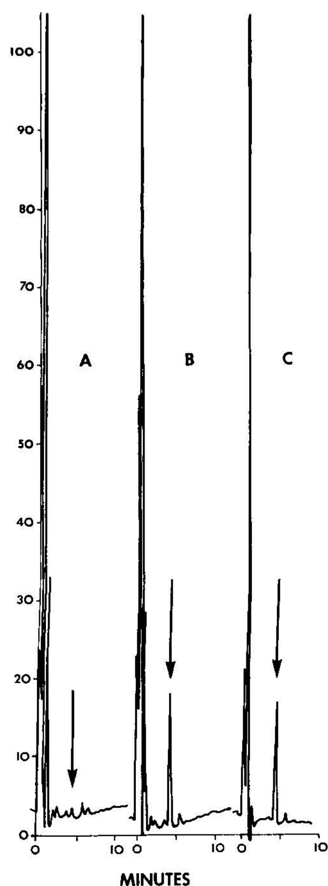


Fig. 1. Typical chromatograms of (A) reduced and acylated reagent blank, (B) reduced and acylated cocaine, (C) reduced and acylated benzoylecgonine. The derivatives were separated on a 5% OV-1 column under the conditions described in Table I.

the response was additive. Ecgonine, however, was not reduced under these conditions. The reason for this is not clear.

For the determination of ecgonine and benzoylecgonine, a variety of derivatization conditions were tried. Optimal derivatization for both was achieved by heating with a mixture of hexafluoroisopropanol–heptafluorobutyric anhydride (1:2, v/v). Fig. 2B shows a single peak obtained from ecgonine and Fig. 3B shows a typical chromatogram of benzoylecgonine. The two were separated under different chromatographic conditions (Table I). The presence of one did not interfere with the analysis of the other. The linearity range for ecgonine and benzoylecgonine under the conditions described here is shown in Figs. 4A and 4B, respectively. Ecgonine gave a linear response from 50–500 pg whereas benzoylecgonine was linear from 1–10 ng. The sensitivity of the method can be increased by increasing the sensitivity of the instrument or by decreasing the final volume of the sample.

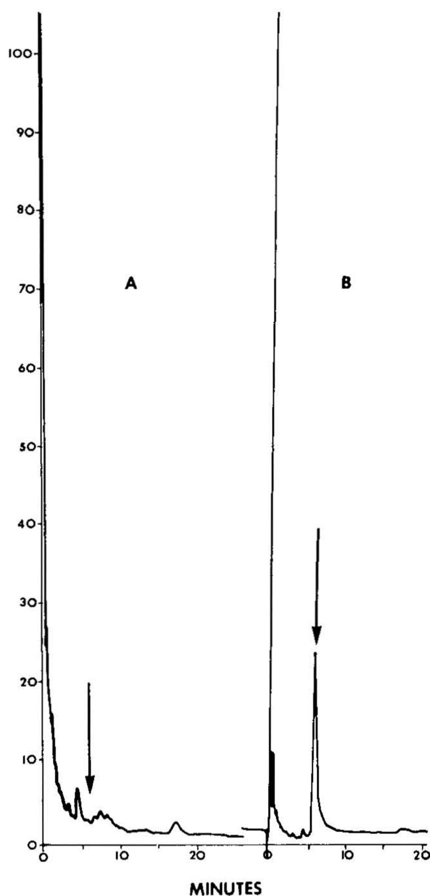
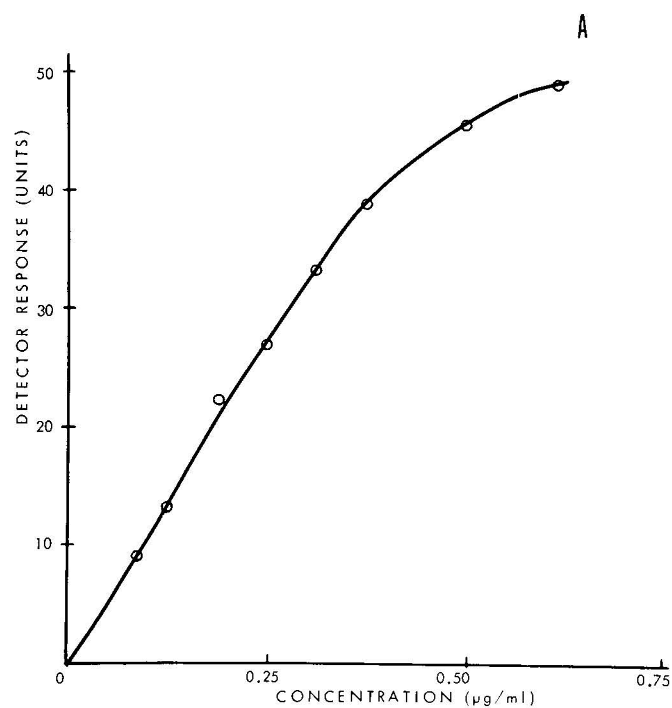


Fig. 2. Typical chromatogram of (A) derivatizing reagent blank (hexafluoroisopropanol–heptafluorobutyric anhydride, 1:2), the position of the arrow indicates where ecgonine was eluted; (B) derivatized ecgonine. Column used was 3% OV-1. Chromatographic conditions are given in Table I.

Fig. 3. Typical chromatogram of (A) derivatizing reagent blank, (B) derivatized benzoylecgonine. Column used was 5% OV-1. Chromatographic conditions are given in Table I.

Simultaneous determination of cocaine, ecgonine and benzoylecgonine

The method described here was used to determine cocaine, ecgonine and benzoylecgonine in a given sample simultaneously. A methanol solution containing known amounts of cocaine, ecgonine and benzoylecgonine was prepared. An aliquot was taken to determine ecgonine and benzoylecgonine. Since benzoylecgonine has only one free carboxyl group with benzoyl ester at the hydroxyl position, it gave a less volatile fluoro derivative than ecgonine, in which carboxyl as well as hydroxyl groups were acylated. Ecgonine and benzoylecgonine derivatives were separated on 3% and 5% OV-1 columns under different conditions (Table I). At an isothermal temperature of 130°, ecgonine had a retention time of 6.0 min (3% OV-1) whereas benzoylecgonine was eluted after 40 min as a very broad peak. However, at 190° benzoylecgonine gave a narrow, symmetrical peak with a retention time of 5.8 min whereas ecgonine was eluted with the solvent front. Cocaine and benzoylecgonine were determined in



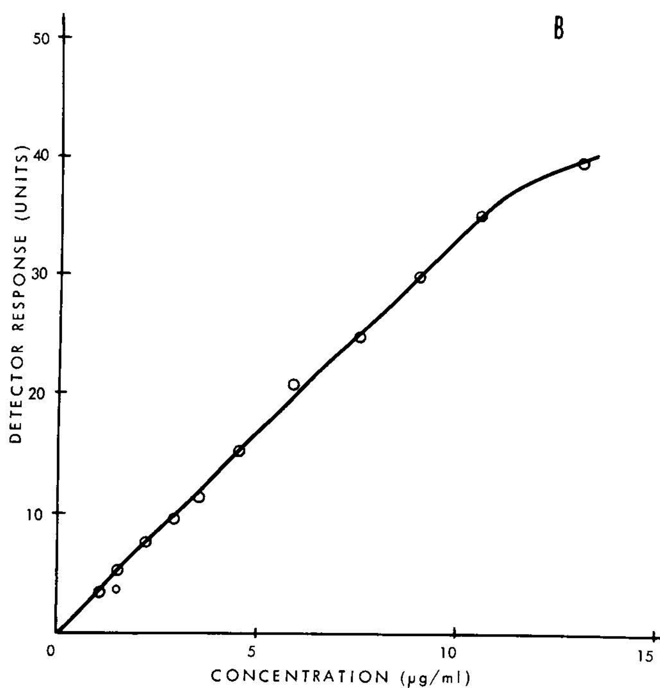


Fig. 4.

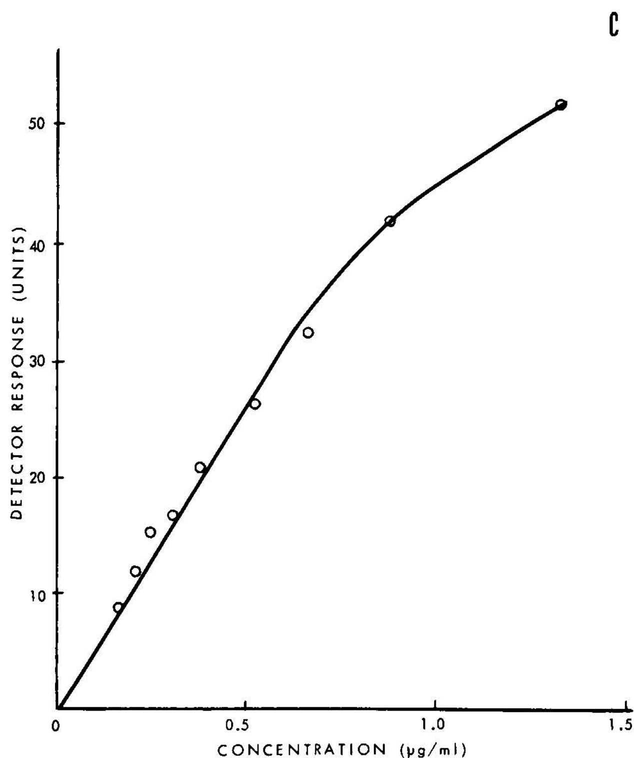


Fig. 4. Standard curves for (A) derivatized ecgonine, (B) benzoylecgonine, (C) reduced and acylated cocaine.

another aliquot for the sample after reduction and acylation. Knowing the amount of benzoylecgonine the amount of cocaine was calculated. These results are given in Table II. The method appears to be reliable and reproducible.

Extraction of cocaine from urine and plasma

To check whether the method could be extended to biological materials, cocaine was added to normal human urine (0.2 µg/ml urine). 5 ml of the urine were extracted with 2 ml of cyclohexane as described by Blake *et al.*[10] Cocaine was determined after

TABLE II

SIMULTANEOUS DETERMINATION OF COCAINE, ECGONINE AND BENZOYLECGONINE IN THE SAME SAMPLE

	Concentration (µg/ml)	
	Calculated	*Determined**
Cocaine	0.25	0.21
Ecgonine	0.1	0.12
Benzoylecgonine	3.0	2.9

* These values represent average of three determinations.

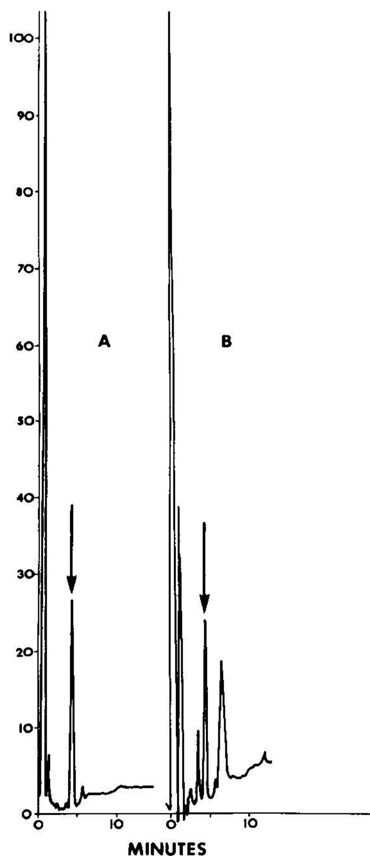


Fig. 5. Chromatograms of (A) cocaine added to normal human urine (0.2 μg/ml) and determined after extraction with cyclohexane as described in the text, (B) cocaine from rat plasma determined after extraction with cyclohexane as described in the text.

reduction. Fig. 5A shows that cocaine gave a symmetrical peak in urine without any interfering peaks. Under these conditions ecgonine and benzoylecgonine were not extracted.

In another experiment a rat was injected with cocaine hydrochloride solution in physiological saline (20 mg per kg body weight). The rat was sacrificed after 15 min and 5 ml of blood were collected in a beaker containing 0.5 ml of 0.15 *M* EDTA. Blood was centrifuged for 10 min at 1000 *g* and 0.1 ml of the plasma were diluted to 5 ml with water and extracted with 2 ml of cyclohexane as above. Fig. 5B shows the cocaine peak obtained after reduction and acylation.

These results indicate that the method described here would be useful in the determination of cocaine and its metabolites in biological samples.

ACKNOWLEDGEMENT

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CHROM. 8247

SEPARATION OF TRITON X-100 AND SIMILAR MIXTURES INTO COMPONENT OLIGOMERS BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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(Received February 10th, 1975)

SUMMARY

A relatively simple liquid chromatographic method for the isolation of individual oligomers of Triton X-100 (Reg. No. 9004-88-0) and similar polyoxyethylene-based surfactants has been developed. The method has been used for separations of mixtures containing between a few milligrams and a few grams of oligomers with up to eighteen oxyethylene subunits on silicic acid type adsorbent using acetic acid–water–ethyl acetate eluents.

INTRODUCTION

A number of biological studies have been carried out using Triton X-100 and other commercial surfactants which are mixed oligomers of polyoxyethylated phenols or alcohols. In some cases it has been of interest to determine whether individual oligomers of these or other surfactants with modified hydrophobic groups might be more effective or display altered activity in these studies. In this paper a procedure is described for separating individual oligomers from such surfactant mixtures (and their tritiated derivatives) by high-pressure liquid chromatography (HPLC).

Triton X-100 is predominantly a mixture of *p-tert.*-octylphenoxy-polyethoxy-ethanol oligomers (α-[4-(1,1,3,3-tetramethylbutyl)phenyl]-ω-hydroxy poly[oxy-1,2-ethanediyl]) which are abbreviated here as *p-tert.*-OPE$_n$, where n refers to the number of ethylene oxide units attached to the alkylphenol during polymerization. The oligomers are present in approximately Poisson distribution, with $n_{av.}$ of about 9.5. Similar products with other $n_{av.}$ values are commercially available or can be readily prepared by reaction of an appropriate phenol or alcohol with ethylene oxide[1].

Individual oligomers of ^{3}H-labeled Triton X-100, with n up to sixteen, were required for biological studies. Mansfield and Locke[2] have synthesized multi-gram amounts of the first ten members of the *p-tert.*-OPE$_n$ series, but for isolation of small amounts of several oligomers, chromatographic fractionation of Triton preparations offers the multiple advantage of conserving radioactive material and requiring less effort, especially for higher members of the series which are especially tedious to synthesize.

By the use of HPLC on silicic acid adsorbents it has been possible to isolate individual oligomers of Triton with *n* up to sixteen and more without the use of large columns, and more conveniently than by chromatographic methods previously reported[3–5].

EXPERIMENTAL

Chromatography was carried out in an ALC 201 liquid chromatograph (Waters Ass., Milford, Mass., U.S.A.) fitted with sapphire pistons resistant to corrosive effects of aqueous acetic acid. The adsorbent was Porasil A(60), 37–75 μm (Waters Ass.). Preparative columns ($^3/_8$ in. $\times$ 8 ft.) were loaded with up to 4 g of Triton, and analytical columns ($^1/_8$ in. $\times$ 2 ft.) were used with up to about 30 mg of Triton.

A linear gradient of ethyl acetate–acetic acid–water (100:32:30) (solution A) against ethyl acetate was used as eluent in the preliminary fractionation of Triton mixtures, and various ratios of ethyl acetate, acetic acid, and water were used for purification of individual *p-tert.*-OPE_n oligomers. Triton concentration in the eluate was followed by absorption at 280 nm using a Model UA-2 ultraviolet (UV) analyzer (Instrument Specialties, Lincoln, Nebr., U.S.A.) with a 2-mm flow cell, or by use of a Techtron 635 Series UV–visible spectrophotometer with an 8 μl flow cell (Varian, Palo Alto, Calif., U.S.A.). A Waters differential refractometer was also used in preparative work, or with compounds which do not absorb adequately in the UV range. The solvent flow-rate was 6 ml/min for the larger diameter column and 0.5 ml/min for the analytical column. The temperature was ambient. Ethyl acetate (99% pure, from Ashland, Santa Fe Springs, Calif., U.S.A.) and acetic acid, reagent grade, were distilled before use. Columns were washed between runs with a 1:1 mixture of acetic acid and water deaerated by boiling and cooled to room temperature before use.

Small-scale reversed-phase separations were carried out on a $^1/_8$-in. $\times$ 2-ft. column of Vydac Reverse Phase, 30–44 μm (Applied Science Labs., State College, Pa., U.S.A.).

Thin-layer chromatography (TLC) was carried out on pre-coated silica gel G plates without fluorescent indicator (E. Merck, Darmstadt, G.F.R.) with varying ratios of ethyl acetate, acetic acid and water[6] depending on the polarity of the substrates (140:32:30, 2 cycles, for $n \geqslant 13$; 140:32:30, 1 cycle, for $n = 7$–12; and 140:8:8, for $n \leqslant 7$). The *p-tert.*-OPE_n's were visualized under UV light after spraying with a 0.003% solution of rhodamine 6G in 1 *M* NaOH (ref. 7) or with Dragendorff reagent.

Triton X-100 ($n_{av.} = 9.5$), Triton X-114 ($n_{av.} = 7$–8), and Triton X-165 ($n_{av.} = 16$) (Rohm & Haas, Philadelphia, Pa., U.S.A.) were used, as appropriate, for sources of unlabeled oligomers. Triton X-100 was labeled in the aromatic ring to a specific activity of 472 μCi/mg by the tritium gas exposure method of Wilzbach[8] (New England Nuclear, Boston, Mass., U.S.A.) and used for separation of labeled oligomers.

Authentic *p-tert.*-OPE_4 was prepared by condensation of recrystallized *p-tert.*-octylphenol with tetraethylene glycol monochloride as described by Mansfield and Locke[2].

RESULTS

Preliminary fractionation of Triton X-100, using gradient elution, resolved 22 peaks (Fig. 1). TLC of some of the fractions of such an experiment is shown in Fig. 2. TLC was used to detect impurities in individual fractions and to identify the n values of oligomers by comparing their R_F values to that of the synthetic standard oligomer of $n = 4$. Purified fractions obtained from Triton could then be used as standards, since oligomers with increasing n appeared in sequence with decreasing R_F values.

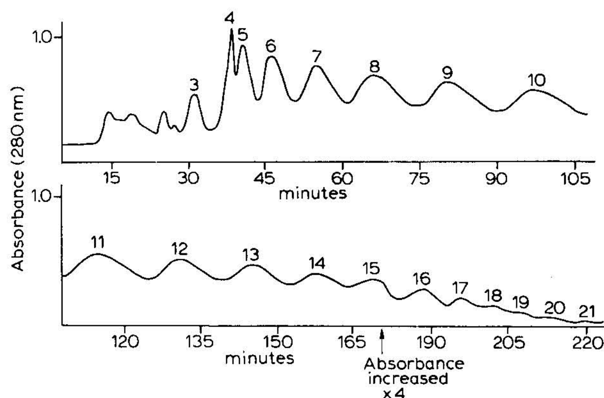

Fig. 1. Liquid chromatography of Triton X-100 (1 g) on 3/8-in. Porasil column. Solvent, linear gradient of ethyl acetate–acetic acid–water (100:32:30) (600 ml) against ethyl acetate (600 ml). Flow-rate, 6 ml/min. Numbers above peaks indicate the n-values of oligomers.

Acetate esters of Triton oligomers are slowly formed via transesterification in the acidic eluents, and prolonged exposure of the oligomers to these solvents should be avoided. The esters have a mobility in the TLC system used similar to the oligomer with three fewer oxyethylene units. (Traces of such esters are present in samples 7, 9, 10, and 11 in Fig. 2).

Fractions from several preliminary chromatograms of 1–4 g of Triton were combined following comparison by TLC and re-chromatographed on the preparative Porasil column to separate the major component from adjacent oligomers. Solvents used were as follows: Ethyl acetate (n = 3–5), ethyl acetate–solution A (3:1) (n = 6–8), ethyl acetate–solution A (2:1) (n = 9–10), ethyl acetate–solution A (1:1) (n = 11–13), and solution A (n = 14–18).

Successive stages in the purification of the $n = 7$ oligomer are shown in Fig. 3. As this figure indicates, a significant amount of material is eluted from the Porasil only after extensive washing of the column with polar eluents. As a result, two cycles of re-chromatography were necessary to bring homogeneity of a given oligomer to $> 97\%$ as judged by relative peak areas and TLC.

The isomeric homogeneity of the oligomers separated by chromatography will reflect the composition of the phenol used in its preparation. Thus, preparation of the pure *p-tert.*-octyl isomer would require starting with pure *p-tert.*-octylphenol. Commercial Tritons contain small amounts of other isomers and perhaps some

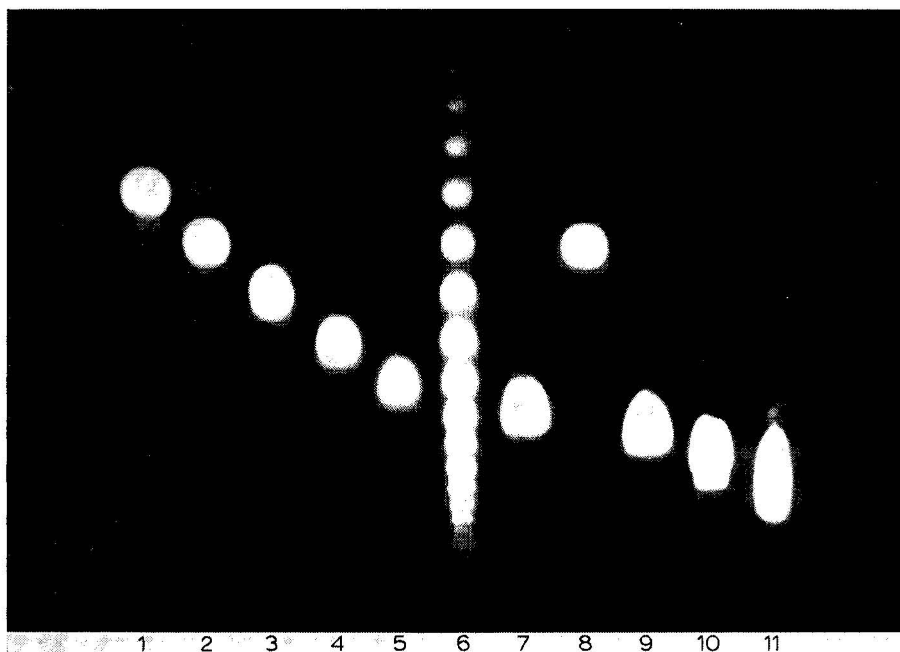

Fig. 2. Thin-layer chromatogram of fractions from a separation of Triton X-100 oligomers as in Fig. 1. Samples: 1–5, n = 7–11 oligomers, respectively; 6, Triton X-100; 7, n = 12 oligomer; 8, n = 8 standard oligomer; 9–11, n = 13–15 oligomers. Solvent, ethyl acetate–acetic acid–water (140:32:30).

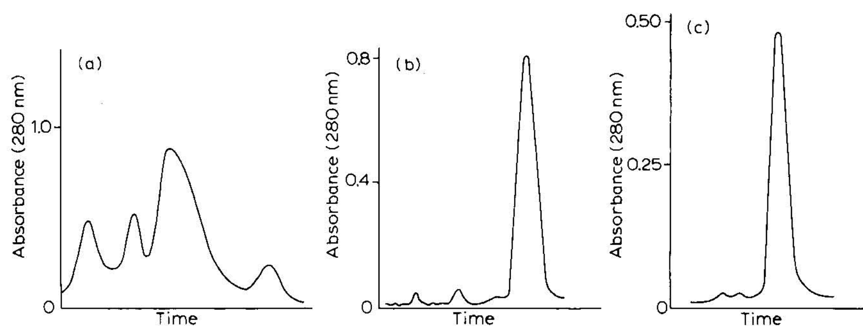

Fig. 3. Purification of n = 7 oligomer by two-stage re-chromatography on Porasil A(60) with ethyl acetate–solution A (2:1). (a) n = 7 oligomer as prepared in Fig. 1; (b) major peak in Fig. 3a; (c) major peak in Fig. 3b.

species with other than eight carbons in the aliphatic residues. Polyethylene glycols may also be present in small amounts.

Reversed-phase chromatography of the purified n = 7 oligomer using 55% methanol as eluent further resolved the isomeric mixture as expected. Three peaks preceding the major component contained in total less than 0.5% of the mixture.

Duplicate elemental analyses of the n = 7 product (Fig. 3c) indicate the composition to be 63.16% C, 9.58% H (calculated values for $C_{28}H_{50}O_8$: 65.30% C,

9.79% H). Nuclear magnetic resonance analysis[9] in $CDCl_3$ solution with a 220 MHz spectrophotometer at 20° indicated a ratio for *tert.*-butyl protons:methyl groups on benzylic carbon:*n* of 9.0:1.97:7.08 (theory, 9:2:7).

The above method has been used on similar mixtures of oligomers prepared in our laboratory from chemically pure alcohols and phenols. This approach is more convenient for obtaining structurally homogeneous oligomers than is separation of individual isomers from a given oligomer derived from impure starting materials.

ACKNOWLEDGEMENTS

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DOSAGE DE GLYCÉRIDES PAR DENSITOMÉTRIE

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SUMMARY

Quantitative determination of glycerides by densitometry

After determination of charring conditions and the measurement by densitometry of lipids separated by thin-layer chromatography, a statistical analysis was performed to determine the significance of the results. Calibration curves were studied and response factors calculated for some saturated and unsaturated triglycerides.

INTRODUCTION

Les mélanges de triglycérides, diglycérides, monoglycérides et acides gras libres, isolés à partir de milieux naturels ou synthétiques et séparables par diverses techniques chromatographiques, peuvent être dosés par différentes méthodes.

Pour des échantillons importants, les glycérides peuvent être séparés sur colonne et les fractions recueillies après élution estimées soit par pesée, soit par titrage iodométrique du glycérol libéré par hydrolyse[1]. Pour des échantillons de l'ordre de 10 mg, les glycérides sont avantageusement séparés par chromatographie sur couches minces préparatives. Après grattage des plaques et extraction au chloroforme, les fractions peuvent être dosées par colorimétrie[2] ou par titrage du glycérol libéré par hydrolyse[1]. Les quantités plus faibles de glycérides séparés sur plaques de couches minces peuvent être déterminées par densitométrie. Cette technique est appliquée au dosage de produits colorés[3], de sucres après carbonisation[4–6] et d'amines après révélation par le réactif de Dragendorff[7]. Vioque et Holman[8] et Kaufmann et Mukherjee[9] ont étudié le dosage d'esters gras. Privett *et al.*[10], Privett et Blank[11], Naudet[12] et Wolff[13] abordent le problème du dosage de lipides par densitométrie.

L'objet de ce travail est l'étude approfondie du dosage densitométrique de petites quantités de glycérides séparés par chromatographie sur couches minces (CCM). Nous déterminons les meilleures conditions de carbonisation en faisant varier le temps de chauffage des plaques placées dans une étuve à 187°. Nous effectuons une analyse de la variance pour connaître la variabilité des résultats obtenus en fonction du nombre de plaques utilisées et du nombre de mesures prises par plaque. Sur la base de ces données, nous construisons les courbes d'étalonnage de la tripalmitine, de l'acide palmitique, des dipalmitines 1/3 et 1/2 et de la monopalmitine. Enfin, nous déterminons les différences de carbonisation qui existent entre triglycérides saturés et insaturés.

PARTIE EXPÉRIMENTALE

Séparation des glycérides

Les tri-, di- et monopalmitines synthétisées au laboratoire et les autres lipides fournis par Applied Science Labs. (Palo Alto, Calif., États-Unis) ou Fluka (Buchs, Suisse) sont séparés par CCM sur des plaques de Kieselgel G (Merck, No. 5721) de 0.25 mm d'épaisseur. Ces plaques finies offrent divers avantages par rapport aux plaques préparées au laboratoire: Elles réduisent le bruit de fond par leur composition et leur épaisseur plus constantes, augmentent la sensibilité en présentant des traits plus condensés et se détériorent plus difficilement au chauffage.

Après élution des lipides, dans des cuves Desaga, au moyen du mélange solvant: éther de pétrole–éther éthylique–acide formique (60:40:1.5), et révélation par carbonisation, le dosage des produits est effectué par densitométrie au moyen du spectromètre chromatographique Zeiss dérivé du PMQ II. La mesure des surfaces des pics est réalisée par planimétrie (Planimètre Amsler 2002/948).

Dosage des glycérides

Les lipides neutres saturés, monoinsaturés ou polyinsaturés non conjugués absorbent peu les rayons UV ou visibles; c'est pourquoi, nous les révélons par carbonisation en vaporisant les plaques sous hotte avec une solution 50:50 de sulfate d'ammonium à 20% et de $(NH_4)_2SO_4$ à 20% acidifié par H_2SO_4 (4%) au moment de l'utilisation[14]. Ces plaques sont ensuite placées dans une étuve à 187° pendant un temps que nous déterminons expérimentalement. (Voir détermination du temps de chauffage des plaques.) Les lipides, étalés sous forme d'un trait continu au moyen d'un applicateur Camag et d'une seringue Hamilton, sont dosés par densitométrie (longueur d'onde: 370 λ). La fente du densitomètre ayant une largeur de 14 mm et balayant les plaques perpendiculairement aux traits à une vitesse de 20 mm/min. Il est aisé de réaliser plusieurs mesures différentes par ligne.

RÉSULTATS

Détermination du temps de chauffage des plaques

Nous utilisons une même solution initiale contenant par 10 ml 83.3 mg de tripalmitine (Tri) et d'acide palmitique (Ac), 77.3 mg de dipalmitine 1/3 (Di 1/3), 6.05 mg de dipalmitine 1/2 (Di 1/2) et 26.2 mg de monopalmitine (Mo).

Après séparation de ces composés par CCM et pulvérisation de la solution de

TABLEAU I

ÉTUDE DES DENSITÉS DE NOIRCISSEMENT EN FONCTION DU TEMPS DE CHAUFFAGE DES PLAQUES

Surfaces en mm². Moyennes de dix mesures.

Temps (min)	*Glycéride*				
	Tri (47) *	*Ac (47)*	*Di 1/3 (43.65)*	*Di 1/2 (3.42)*	*Mo (14.79)*
30	1622	0	1896	522	298
60	3596	686	3639	1065	961
120	4963	3615	3709	1098	996
240	5021	4316	3649	1011	819
360	4529	4023	3622	939	752

* Concentration par 14 mm de trait (μg).

sulfate d'ammonium acidifié, nous plaçons les plaques dans une étuve à 187° pendant des temps variables (30–360 min) en vue de déterminer la durée du chauffage qui donne le meilleur noircissement. La température de l'étuve a été choisie en fonction d'essais préliminaires et du matériel mis à notre disposition.

Le Tableau I et la Fig. 1 indiquent que les différents glycérides ne noircissent pas avec la même vitesse. Il semble intéressant de chauffer les plaques pendant au moins 120 min.

Détermination du nombre de plaques et de passages par plaque

Une analyse statistique des résultats sert à déterminer le nombre de plaques et de passages nécessaires pour une variabilité fixée et permet l'étude d'une relation entre la concentration et la surface mesurée.

A partir de la même solution de lipides et après séparation par couches minces, des lots de cinq plaques sont révélés, après vaporisation de la solution de sulfate d'ammonium, dans l'étuve chauffée à 187° pendant 120, 240 et 360 min (temps de chauffage déterminés précédemment). Chaque essai est répété trois fois à raison d'un par jour. Cinq mesures densitométriques sont réalisées par plaque et par substance, ce qui donne un total de 75 données par produit et pour un même temps de chauffage.

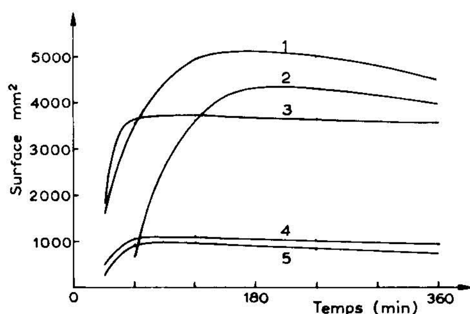

Fig. 1. Evolution de la carbonisation des lipides en fonction du temps de chauffage à 187°. 1 = tripalmitine (47 μg/14 mm); 2 = acide palmitique (47 μg/14 mm); 3 = dipalmitine 1/3 (43.65 μg/14 mm); 4 = dipalmitine 1/2 (3.42 μg/14 mm); 5 = monopalmitine (14.79 μg/14 mm).

TABLEAU II

ANALYSE DE LA VARIANCE RÉALISÉE À PARTIR DES MESURES EFFECTUÉES AUX TEMPS 120, 240 ET 360 MIN

D°L = Degrés de liberté; SCE = somme des carrés des écarts; CM = carré moyen; F = variable de Snedecor; CV = coefficient de variation.

Source de variation		*D°L*	*SCE*	*CM*	*F*
Tri	Temps	2	10884184	5442092	61.77
	Jours/temps	6	299450	49908	<1
	Plaques/jour	36	3171744	88104	21.00
	Passages	180	755160	4195	
	Total	224	15110538	5584299	
Moyenne générale: 4838; ecart-type résiduel: 64.8; CV résiduel: 1.3					
Ac	Temps	2	18582912	9291456	87.96
	Jours/temps	6	776296	129383	1.22
	Plaques/jour	36	3802832	105634	6.95
	Passages	180	2735160	15195	
	Total	224	25897200	9541668	
Moyenne générale: 3985; écart-type résiduel: 123.3; CV résiduel: 3.1.					
Di 1/3	Temps	2	298497	149248	1.42
	Jours/temps	6	56488	9415	<1
	Plaques/jour	36	3780528	105014	26.91
	Passages	180	702480	3902	
	Total	224	4837993	267579	
Moyenne générale: 3660; écart-type résiduel: 62.5; CV résiduel: 1.7.					
Di 1/2	Temps	2	948696	474348	50.00
	Jours/temps	6	16408	2735	<1
	Plaques/jour	36	341542	9487	4.86
	Passages	180	351440	1952	
	Total	224	1658086	488522	
Moyenne générale: 1016; écart-type résiduel: 44.2; CV résiduel: 4.3.					
Mo	Temps	2	2383484	1191742	140.04
	Jours/temps	6	11273	1879	<1
	Plaques/jour	36	306378	8510	7.77
	Passages	180	197280	1096	
	Total	224	2898415	1203227	
Moyenne générale: 856; écart-type résiduel: 33.1; CV résiduel: 3.9.					

Nous nous sommes limités à des lots de cinq plaques pour des raisons essentiellement matérielles. Par contre, le nombre de passages est fonction de la largeur de fente du densitomètre et de la longueur du dépôt.

Au schéma espérimental décrit ci-dessus correspond un modèle d'analyse de la variance hiérarchisé à deux critères de classification[15]. Chaque jour de l'expérience constiue une entité. Les plaques sont subordonnées aux jours et les passages ou répétitions dépendent des plaques. Les analyses effectuées aux trois temps différents (Tableau II) permettent la détermination des composantes de variances pour les plaques ($\hat{\sigma}_p^2$) et pour les passages ($\hat{\sigma}_N^2$). À partir de ces estimations, on calcule pour

des nombres de plaques (q) et de passages (n) la variance de la moyenne selon la relation

$$\hat{\sigma}_{\bar{x}}^2 = \frac{\hat{\sigma}_p^2}{q} + \frac{\hat{\sigma}_N^2}{qn}$$

ou

$$\hat{\sigma}_{\bar{x}}^2 = \frac{1}{q}\left(\hat{\sigma}_p^2 + \frac{\hat{\sigma}_N^2}{n}\right)$$

La variabilité relative au nombre de plaques et de passages est exprimée dans le Tableau III en terme de coefficients de variation. On constate que la variabilité entre les plaques est plus importante que la variabilité entre les mesures effectuées sur une même plaque.

De plus, à concentrations égales, le coefficient de variation de l'acide est nettement plus élevé que le coefficient de variation de la tripalmitine. Ce fait peut être expliqué par la difficulté qu'il y a à définir correctement la ligne de base des pics de l'acide.

TABLEAU III

COEFFICIENTS DE VARIATION PAR RAPPORT À LA MOYENNE CALCULÉS À PARTIR DE L'ENSEMBLE DES MESURES

Produit	*n*	*q*				
		1	*2*	*3*	*4*	*5*
Tri	1	2.99	2.12	1.73	1.50	1.34
	2	2.84	2.01	1.64	1.42	1.27
	3	2.79	1.97	1.61	1.39	1.25
	4	2.76	1.95	1.59	1.38	1.23
	5	2.74	1.94	1.58	1.37	1.23
Ac	1	4.58	3.24	2.64	2.29	2.05
	2	4.02	2.84	2.32	2.01	1.80
	3	3.82	2.70	2.20	1.91	1.71
	4	3.71	2.63	2.14	1.86	1.66
	5	3.65	2.58	2.11	1.82	1.63
Di 1/3	1	4.24	3.00	2.45	2.12	1.90
	2	4.07	2.88	2.35	2.03	1.82
	3	4.01	2.83	2.31	2.00	1.79
	4	3.98	2.81	2.30	1.99	1.78
	5	3.96	2.80	2.29	1.98	1.77
Di 1/2	1	5.79	4.09	3.34	2.89	2.59
	2	4.90	3.47	2.83	2.45	2.19
	3	4.57	3.23	2.64	2.29	2.04
	4	4.40	3.11	2.54	2.20	1.97
	5	4.29	3.03	2.48	2.14	1.92
Mo	1	5.93	4.19	3.43	2.97	2.65
	2	5.26	3.72	3.04	2.63	2.35
	3	5.02	3.55	2.90	2.51	2.25
	4	4.90	3.46	2.83	2.45	2.19
	5	4.82	3.41	2.78	2.41	2.16

TABLEAU IV

COEFFICIENTS DE VARIATION ($n=q=1$)

Temps (min)	*Tri*	*Ac*	*Di 1/3*	*Di 1/2*	*Mo*
120	4.76	7.19	6.63	7.31	8.03
240	1.28	3.11	2.09	5.25	2.63
360	1.22	2.96	2.17	3.55	3.91

L'examen des résultats obtenus pour des temps différents (Tableau IV), tout en confirmant les remarques précédentes, fait apparaître la nécessité de toujours travailler avec le même temps de chauffage des plaques. Les coefficients de variation au temps 120 min sont nettement plus élevés. Cela pourrait être provoqué par une stabilisation lente de l'étuve et une hétérogénéité dans le noircissement. Nous conseillons donc de chauffer les plaques pendant 240 min. On constate également qu'il n'y a pas de différences significatives entre les jours. Compte tenu de ces résultats, nous utiliserons trois plaques par essai et effectuerons trois mesures densitométriques par plaque et par composé.

Courbes d'étalonnage

En vue d'établir la relation liant la concentration Y (μg/14 mm de trait) en lipide à la surface de réponse X (mm^2), nous préparons des solutions de concentrations différentes. Les plaques, après élution, sont placées dans une étuve à 187° pendant 240 min. Pour chaque concentration et pour chaque produit, nous effectuons neuf mesures (trois plaques par jour, trois mesures par plaque).

La représentation graphique des résultats a orienté le choix des ajustements au sens des moindres carrés vers les fonctions de puissances. Une transformation logarithmique des données a permis la réduction de la variation résiduelle, la linéarisation de la relation et une correcte dispersion des observations. Dès lors, l'équation liant la concentration à la surface de réponse s'écrit

$$Y = aX^b$$

ou

$$\log Y = \log a + b \log X$$

TABLEAU V

PARAMÈTRES DES ÉQUATIONS DE RÉGRESSION

	Tri	*Ac*	*Di 1/3*	*Di 1/2*	*Mo*
$\hat{m}_Y$	53.2559	53.2474	54.8823	6.3933	33.1138
$\hat{\sigma}_Y$	84.4178	84.4068	80.8102	6.6827	30.0057
$Y = aX^b$					
a	$0.2032 \cdot 10^{-5}$	$0.2382 \cdot 10^{-5}$	$0.3069 \cdot 10^{-5}$	$0.2270 \cdot 10^{-5}$	$0.1726 \cdot 10^{-4}$
b	$0.1992 \cdot 10^{1}$	$0.2009 \cdot 10^{1}$	$0.2012 \cdot 10^{1}$	$0.2056 \cdot 10^{1}$	$0.2047 \cdot 10^{1}$
$\hat{\sigma}_{Y \cdot X}$	0.4400	0.4993	0.1828	0.0834	0.5831
$\hat{V}_{Y \cdot X}$	0.0083	0.0094	0.0033	0.0128	0.0176

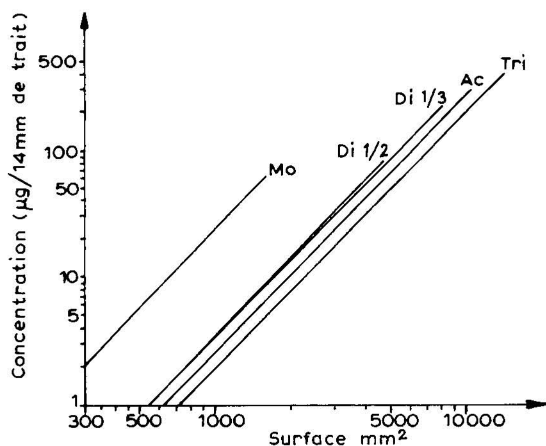

Fig. 2. Relations entre la concentration Y et la surface du pic X. Tri = tripalmitine; Ac = acide palmitique; Di = dipalmitine; Mo = monopalmitine.

Le Tableau V reprend pour chaque produit la moyenne ($\hat{m}_Y$) et l'écart-type estimé ($\hat{\sigma}_Y$) des concentrations, les valeurs des paramètres a et b, l'écart-type $\hat{\sigma}_{Y.X}$ et le coefficient de variation $\hat{V}_{Y.X}$ des résidus. La Fig. 2 donne une représentation graphique des équations en échelles logarithmiques.

Différences de carbonisation entre triglycérides

Comme les lipides étudiés précédemment ne se carbonisent pas avec la même vitesse, il nous semble intéressant d'étudier pour terminer les réponses de différents triglycérides. Si C est la concentration effective en triglycéride et Y la concentration calculée au moyen de l'équation de la droite de régression trouvée pour la tripalmitine en fonction de la surface du pic (X) mesurée par densitométrie, nous obtenons des coefficients de réponse (f_w) dont les valeurs sont colligées dans le Tableau VI.

TABLEAU VI

COEFFICIENTS DE RÉPONSE DES TRIGLYCÉRIDES

Triglycéride	R_F	*% de carbone*	f_w	$\frac{1}{f_w}$
Tributyrine	0.50	59.6	—	—
Triheptanoine	0.67	67.3	3.783	0.264
Tricapryline	0.71	68.9	2.383	0.420
Tricaprine	0.77	71.5	1.627	0.615
Trilaurine	0.80	73.4	1.294	0.773
Trimyristine	0.83	74.8	1.136	0.880
Tripalmitine	0.87	75.9	1.000	1.000
Tristéarine	0.90	76.9	0.936	1.068
Tripalmitoléine	0.90	76.5	0.918	1.089
Trioléine	0.91	77.4	0.846	1.182
Trilinoléine	0.92	77.9	0.703	1.422
Trilinolénine	0.92	78.4	0.545	1.835

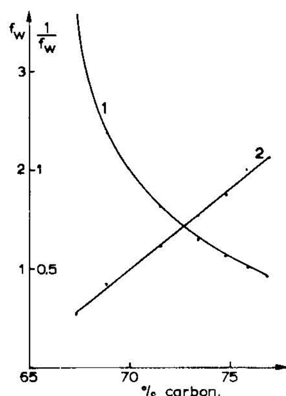

Fig. 3. Relation entre $1/f_w$ (2) et le pourcentage de carbone.

$$f_w = \frac{Y(\text{tripalmitine})}{Y(\text{triglycéride})} \times \frac{C(\text{triglycéride})}{C(\text{tripalmitine})}$$

On constate que la relation entre $1/f_w$ et le pourcentage en carbone est pratiquement linéaire (Fig. 3). De plus les doubles liaisons accentuent le noircissement.

CONCLUSIONS

L'analyse de lipides par densitométrie est une méthode sensible si on révèle les plaques en pulvérisant une solution de sulfate d'ammonium acidifié par H_2SO_4 et en les chauffant pendant 240 min à 187°. En examinant les résultats d'une analyse complète de la variance et en nous fixant une limite de variabilité, nous estimons qu'il est nécessaire d'utiliser pour chaque essai trois plaques et de répéter par plaque trois mesures à des endroits différents.

Par la méthode des droites de régression, nous montrons également que la relation entre la concentration (Y) et la surface du pic (X) est du type $\log Y = \log b + a \log X$. Les différences de noircissement entre triglycérides nous obligent à émettre des réserves quant au dosage de lipides issus de milieux biologiques. En effet, dans pareils cas, le facteur de réponse ne peut être calculé car il dépend des concentrations des produits de même R_F. Cette méthode n'est valable que lorsqu'il n'y a qu'une substance par trait.

RÉSUMÉ

Les glycérides séparés par chromatographie sur couches minces et révélés par carbonisation sont dosés par densitométrie. Après la mise au point des conditions de carbonisation, l'analyse statistique détermine la variabilité des résultats; des courbes de calibrage sont proposées et des facteurs de réponse sont calculés pour plusieurs triglycérides saturés et insaturés.

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CHROM. 8293

Note

Thin-layer chromatography on a chromatosheet coated with resin in different ionic forms for the separation of amino acid mixtures containing asparagine and glutamine

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Chromatography on serially connected thin layers combines the chromatographic features of the component layers, and this paper reports on chromatography on an ion-exchange chromatosheet coated with two different ionic forms of a resin on two areas of the sheet for the resolution of substances that are not separated by the individual ionic forms, and the determination of the ratio of the two ionic forms of the resin needed for the optimal separation of the mixture.

This approach was applied to the one-dimensional separation of asparagine and glutamine in amino acid mixtures, which is of great importance in sequence analysis and in the investigation of biological fluids.

The two-dimensional separation of asparagine and glutamine on a cellulose thin layer is possible[1], but in the one-dimensional separation of amino acids by ion-exchange thin-layer chromatography on resin-coated chromatosheets in the Na^+ form[2] the separation of asparagine and glutamine could not be achieved. This separation problem has been resolved by using an automatic analyzer technique with lithium citrate buffers[3,4].

EXPERIMENTAL

Strongly acidic cation-exchange resin-coated chromatosheets (20 × 20 cm; Fixion 50X8, Chinoin-Nagytétény, Budapest, Hungary, and Ionex 25SA-Na, Macherey, Nagel and Co., Düren, G.F.R.) were used. The chromatosheets were treated as follows in order to have one area of the resin in the Na^+ form and the remainder in the Li^+ form.

(a) The chromatosheets, which are available in Na^+ form, were converted completely into the Li^+ form by continuous development for 24 h with 1 *M* lithium chloride solution at room temperature.

(b) The sheets were then dried and the excess of lithium chloride was removed by continuous development with deionized water for 24 h at room temperature.

(c) The lower part of each thin layer was converted from the Li^+ form back into the Na^+ form by developing the lower 4 cm of the sheet with 1 *M* sodium chloride solution (2.5 cm above the starting line).

(d) The final equilibration of the chromatosheets was achieved by continuous development for 24 h with eluting buffer (see below) diluted 10-fold.

Sheets completely in the Li^+ form were prepared by treatments (a), (b) and (d), while thin layers completely in the Na^+ form were used after treatment (d). These sheets with resins in a single ionic form were used for comparative purposes.

The eluting buffer was Li^+ (0.20 *M*)–citrate (0.05 *M*)–formate (0.05 *M*), of pH 2.80 $\pm$ 0.02. It was prepared by mixing lithium citrate tetrahydrate (14.10 g), lithium chloride (2.12 g), 85% formic acid (2.30 ml) and 37% hydrochloric acid (8.0 ml), and making the volume up to 1000 ml with deionized water.

The chromatograms were developed with the eluting buffer at 37°. The sheets were fitted on to a glass plate and were developed for 4 h until the solvent front migrated into the filter-paper pad attached to the top of the sheets.

Ninhydrin spray reagent containing cadmium acetate and collidine was used for staining the chromatograms.

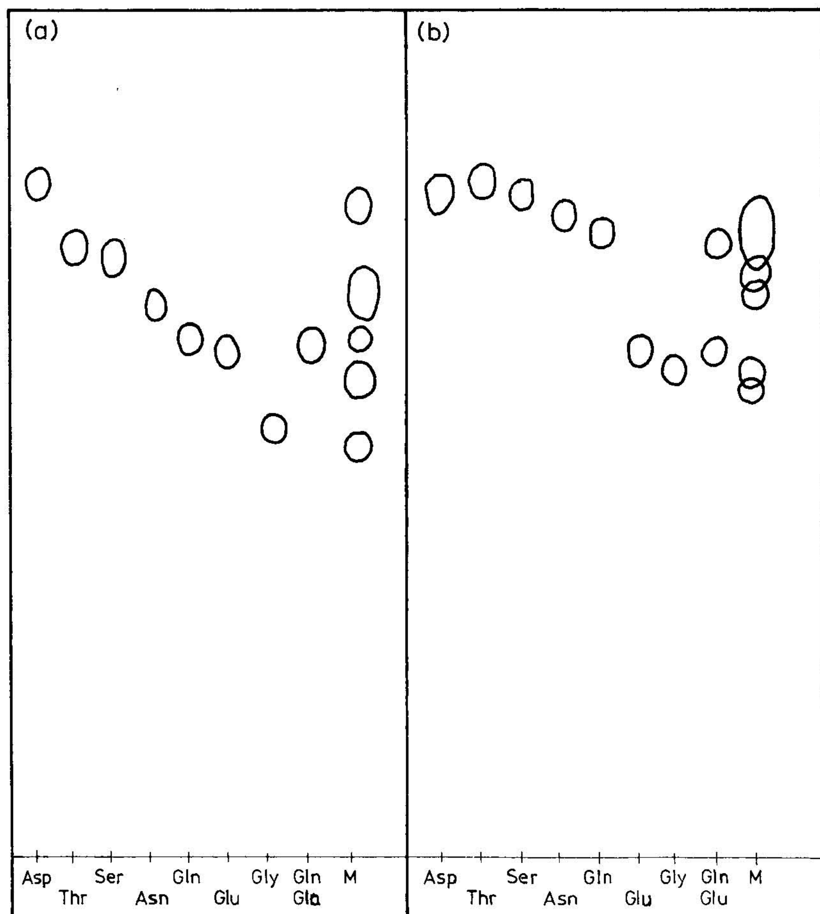

Fig. 1. Separation of amino acids on individual ion-exchange layers: (a) resin in Li^+ form; (b) resin in Na^+ form.

RESULTS AND DISCUSSION

For comparison, Figs. 1a and b show chromatograms obtained on sheets coated with resin completely in the Li^+ and Na^+ form, respectively. While in the case of the layer in the Li^+ form the amino acids are well separated with the exception of glutamine and glutamic acid, the reverse is true for the layer in the Na^+ form.

In general, it could be anticipated that if R_F values of three given compounds, (1), (2) and (3), on two different thin layers, (A) and (B), are:

$$R_{F(A)}^{(1)} = R_{F(A)}^{(2)} \neq R_{F(A)}^{(3)} \tag{1}$$

and

$$R_{F(B)}^{(1)} \neq R_{F(B)}^{(2)} = R_{F(B)}^{(3)} \tag{2}$$

then the separation of these three compounds might be achieved by the connection of (A) and (B).

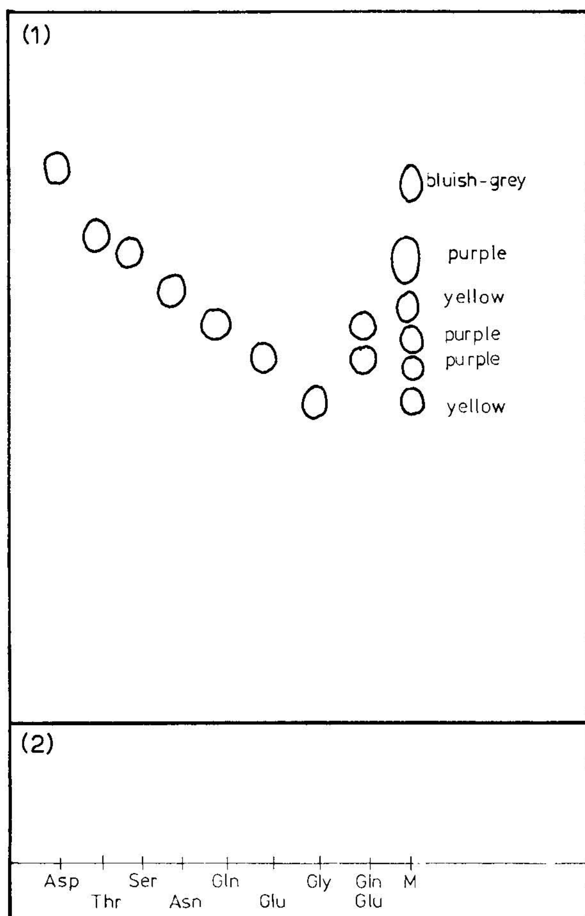

Fig. 2. Separation of amino acids on a layer of ion-exchange resin in the Na^+ and Li^+ forms in the areas (2) (2.5 cm) and (1) (16 cm), respectively.

Clearly, the R_F values of asparagine, glutamine and glutamic acid satisfy the conditions described in eqns. 1 and 2. Therefore, chromatography on a layer partly in the Na^+ and partly in the Li^+ form, combining the features of the two different ionic forms, might resolve this separation problem.

Fig. 2 shows a typical chromatogram on a layer consisting of resin in the Na^+ and Li^+ forms in different areas. Aspartic acid, threonine + serine, asparagine, glutamine, glutamic acid and glycine are separated from each other. The R_F values of other amino acids are much lower than the R_F values of glycine and therefore they do not interfere in the separation.

The optimal ratio of the heights of the Na^+ and Li^+ forms of the resin layer was determined by a simple procedure in which the height of the Na^+ form of the layer was varied from 0.5 to 5 cm. Fig. 3 shows that the best separation was achieved when the height of the Na^+ form of the layer was 2.5 cm.

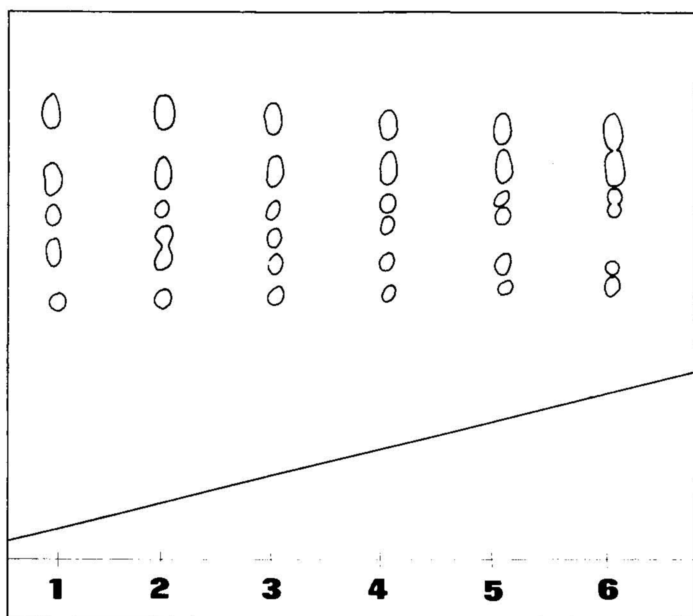

Fig. 3. Experimental determination of the ratio of the heights of the Na^+ and Li^+ forms of the resin layer required for the optimal separation of the amino acids listed in Figs. 1 and 2. The height of the Na^+ form of the layer varies from 0.5 to 5 cm. The best separation was achieved at position 3, where the height of Na^+ form of the layer is 2.5 cm.

The optimal ratio of the heights of A and B in the layer can be calculated as follows. The R_F value of a given compound (1) on the A + B layer, to a first approximation, is

$$R_{F(A+B)}^{(1)} = \frac{a}{SF} + R_{F(B)}^{(1)} - \frac{a}{SF} \cdot \frac{R_{F(B)}^{(1)}}{R_{F(A)}^{(1)}} \tag{3}$$

where SF is the height of the solvent front, a is the height of the layer A in the A + B layer and A refers to the lower layer.

The ratio expressed in terms of the pre-determined relative R_F value of two compounds ($r_{1,2}$) follows from eqn. 3:

$$\frac{a}{SF} = \frac{R_{F(B)}^{(1)} - r_{1,2}\, R_{F(B)}^{(2)}}{\left(1 - \dfrac{R_{F(B)}^{(2)}}{R_{F(A)}^{(2)}}\right) r_{1,2} + \dfrac{R_{F(B)}^{(1)}}{R_{F(A)}^{(1)}} + 1} \tag{4}$$

ACKNOWLEDGEMENT

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CHROM. 8304

Note

Separation of isomeric pentitols and hexitols by paper and thin-layer chromatography

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The metabolism of the polyhydric alcohols is a particularly important aspect of carbohydrate biochemistry. Numerous organisms, including many plant species from a variety of taxa, contain one or several polyols[1,2]. Qualitative and quantitative analysis of such compounds, as well as their metabolism in any organism, has hitherto been difficult, as successful chromatographic methods for the reliable and rapid separation or identification of isomeric pentitols and hexitols were scarce. Some procedures[3–7] that have been described in the past few years for the chromatographic analysis of native or synthetic polyols seem to be impractical or to achieve relatively incomplete and unsatisfactory separation of the isomers.

In this paper, some solvent systems are proposed for the identification and characterization of the naturally occurring pentitols (xylitol, arabitol and adonitol) and hexitols (mannitol, dulcitol and sorbitol) by paper (PC) or thin-layer chromatography (TLC). These systems have similar properties and can be applied for analytical as well as preparative purposes.

EXPERIMENTAL

Materials

Paper. Schleicher & Schüll 2043 b Mgl.

Adsorbent. Cellulose MN 300.

Solvents. (A) Ethyl methyl ketone–acetic acid–0.75 *M* boric acid (40:10:9),
(B) 1-Butanol–0.75 *M* boric acid (85:15),
(C) 2-Propanol–acetic acid–0.75 *M* boric acid (70:10:20),
(D) Ethyl methyl ketone–acetic acid–0.7 *M* boric acid (100:10:5),
(E) 1-Butanol–0.75 *M* boric acid (85:10), and
(F) 1-Butanol–distilled water (90:10).

Detection reagents. Ammonium cerium(IV) nitrate–N,N-dimethyl-*p*-phenylenediamine dihydrochloride[7] or sodium periodate–benzidine[8].

Method. Paper chromatograms (solvents D–F) were prepared by the descending techniques using 60-cm × 15-cm sheets; thin-layer chromatography was carried out on 20-cm × 20-cm plates at room temperature (solvents A–C).

RESULTS AND DISCUSSION

On the basis of Boeseken's findings that polyols form borate complexes in solutions of boric acid[9], Rees and Reynolds devised a solvent system[10] that provides a good separation of polyols from their corresponding sugars; our solvents A and D are modifications of this system. These solvents, and mixtures of 1-butanol with aqueous boric acid (solvents B and E), achieve a clear distinction of the naturally occurring polyols and polyol isomers either by TLC (see Table I) or by PC (see Table II). However, the solvent composition used in TLC may not also be in PC if the same efficiency is desired.

TABLE I

MOBILITIES OF SEVERAL POLYOLS ON THIN-LAYER CHROMATOGRAMS

Polyol	$R_{mannitol} \times$ *100 values*		
	Solvent A	*Solvent B*	*Solvent C*
Volemitol*	75	81	78
Mannitol	100	100	100
Dulcitol	90	84	90
Sorbitol	110	100	97
Xylitol	140	123	110
Arabitol	135	134	115
Adonitol	156	180	128
Erythritol	170	200	135
Glycerol	207	280	145

* Prepared as [^{14}C]volemitol from *Pelvetia canaliculata*[12].

As can be seen from Tables I and II, sorbitol and adonitol (ribitol) are clearly separated from the other hexitol and pentitol isomers, whereas mannitol and dulcitol (galactitol) or xylitol and arabitol, respectively, show closer mobilities. These latter polyols can be better separated in solvents B and C. The other C_3, C_4 and C_7 polyols are clearly separable without any problems.

Although a one-dimensional development of the chromatograms is sufficient

TABLE II

MOBILITIES OF SEVERAL POLYOLS ON PAPER CHROMATOGRAMS

Polyol	$R_{mannitol} \times$ *100 values*		
	Solvent D	*Solvent E*	*Solvent F*
Volemitol	65	—	—
Mannitol	100	100	100
Dulcitol	104	83	81
Sorbitol	131	108	81
Xylitol	172	142	139
Arabitol	160	153	157
Adonitol	180	196	157
Erythritol	207	189	236
Glycerol	290	295	380

for a reliable identification of and distinction between the polyols, solvents A and D, and B and E, respectively, can be employed for two-dimensional chromatography. Of course, PC requires longer development times, *e.g.*, 24 h in the first (solvent D) and 48 h in the second (solvent E) direction. Two-dimensional TLC of the polyols in solvents A and B gives satisfactory results with development for 3–4 h in each direction.

For a very sharp separation, especially of mannitol from dulcitol or of xylitol from arabitol, and in particular for preparative purposes, the use of solvent C (TLC) or F (PC) is recommended.

The new solvent systems presented here may be very useful in investigations on the biochemistry and physiology of fungi, algae and lichens. One or more polyols are involved in the metabolism of these organisms, and are probably inter-converted to each other[11]. Rapid chromatography and reliable identification of products and substrates will help in elucidating such metabolic pathways.

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Note

A rapid and sensitive detection of proteolytic enzymes after electrophoresis

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(Received January 15th, 1975)

N-Substituted amino acid nitroanilides are widely used substrates for the quantitative and qualitative determination of proteolytic enzymes[1,2]. Recently, Gertler *et al.*[3] have published a qualitative method for the detection of trypsin and chymotrypsin after cellulose acetate electrophoresis using benzoyl-DL-arginine- and acetyl-L-tyrosine-*p*-nitroanilide, respectively, as substrate. However, no staining of the strips could be achieved and therefore no quantitative determination of the enzymes could be made. In our experiments on insect proteases, usually only obtainable in milligram amounts, we found that the sensitivity of this method can be further enhanced, the results better documented, and a quantitative determination of the enzymes made, if the nitroaniline produced during the enzymatic reaction is diazotized and then coupled with naphthylethylenediamine, as described by Bratton and Marshall[4].

This method is applicable not only for cellulose acetate electrophoresis but also for acrylamide and agarose gels normally used in disc and immunoelectrophoretic experiments.

METHODS

Cellulose acetate electrophoresis was performed on Macherey and Nagel (Düren, G.F.R.) membranes (25.5 × 145 mm) using 24 m*M* Veronal–HCl buffer (pH 8.6). The proteins were separated at 200 V for 30 min. Protein was stained with Coomassie Brilliant Blue. For clearing and scanning, the strips were treated as described by Perl and Voggel.[5] Disc electrophoresis was carried out in 40 × 1 mm capillaries at pH 4.3. The method of Reisfeld *et al.*[6] was slightly modified in so far as the separation gel contained 1% Triton X-100 and the stacking gel 0.5%, and the ammonium peroxydisulfate concentration was reduced to 90 mg/100 ml water. The gels were kept in a moist chamber for 12 h before use. For electrophoresis, 1 μl of the protein solution and 0.2 μl of methylene blue (0.2%) were applied to the gel, and the separation was carried out with a current of 0.1 mA/gel for 1 h. Scanning of the gels was performed with a Gilford spectrometer at 578 nm.

Substrate-staining solution

In all the experiments, the substrates (benzoyl-L-arginine- and acetyl-L-tyro-

sine-*p*-nitroanilide (E. Merck, Darmstadt, G.F.R.) were used in a concentration of 5 m*M* (final concentration) dissolved in 0.2 *N* Tris–HCl buffer (pH 8.4) containing 5% (v/v) dimethylformamide. Before use, a solution of $NaNO_2$ and N-α-naphthylethylenediamine (Serva, Heidelberg, G.F.R.) was added to the substrate solution to give final concentrations of 0.1% (w/v) and 0.5% (w/v), respectively.

RESULTS AND DISCUSSION

Disc electrophoresis gels were incubated directly in the substrate-staining solution at 35° for 10–30 min. After incubation the gels were washed with water for 1 min to remove the excess of substrate and then treated with an aqueous solution of 12.5% trichloroacetic acid (TCA) (w/v) for 30 sec, during which time a purple colour became visible. (A longer treatment with TCA causes precipitation of the substrate in the gel, and no quantitative determination is then possible.) The substrate which precipitated on the surface of the gel was washed out with 0.2 *N* Tris–HCl buffer (pH 8.4) until the gel became transparent again. Typical staining patterns of bovine chymotrypsin, trypsin (Worthington), hornet chymotrypsin, and honey-bee trypsin are shown in Fig. 1. Under our conditions with a 30-min incubation time at 35°, as little as 20 ng of trypsin were detectable after staining with benzoyl-L-arginine-*p*-nitroanilide; however, the best results were achieved by applying 60–80 ng trypsin/gel. Honey-bee trypsin, which has a higher specific activity than bovine trypsin, can be determined in a concentration of 6 ng/gel. Using acetyl-L-tyrosine-*p*-nitroanilide as substrate it was possible to stain 120 ng of bovine chymotrypsin and 30 ng of hornet chymotryp-

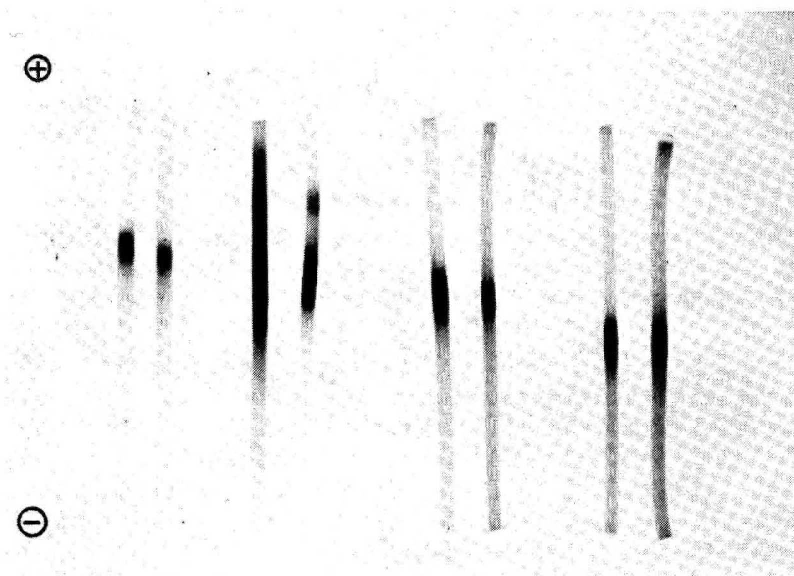

Fig. 1. Demonstration of proteolytic activities. Electrophoresis at pH 4.3, 20% gels, incubation at 35° for 30 min. From left to right: 426 and 213 ng bovine chymotrypsin; 184 and 94 ng hornet chymotrypsin (acetyl-L-tyrosine-*p*-nitroanilide used as substrate); 178 and 92 ng bovine trypsin; 38 ng and 76 ng honey-bee trypsin (benzoyl-L-arginine-*p*-nitroanilide used as substrate).

sin. For a comparison of the sensitivity of this method with that formerly used in these laboratories, *i.e.* staining proteolytic enzymes with glutaryl-L-phenylalanine-β-naphthylamide[7], two series of tests were performed. In the first experiment glutaryl-L-phenylalanine-*p*-nitroanilide was used as substrate, and in the second the naphthylamide substrate (50 m*M* in 0.2 *N* Tris–HCl buffer (pH 8.4)) was used. After 30 min of incubation in the nitroanilide substrate, 0.5 μg of bovine chymotrypsin was detectable, whereas 2.6 μg was the minimum detectable amount of the enzyme after a 2-h incubation in the naphthylamide substrate.

The quantitative determination of bovine chymotrypsin activity in electrophoresis gels is shown in Fig. 2. The enzyme was subjected to electrophoresis and scanned at 578 nm after staining with acetyl-L-tyrosine-*p*-nitroanilide. The linear dependence of the integrated peak area on the incubation time and the quantity of the enzyme applied indicates that only very small amounts of the diazotized nitroaniline were washed out during the washing processes.

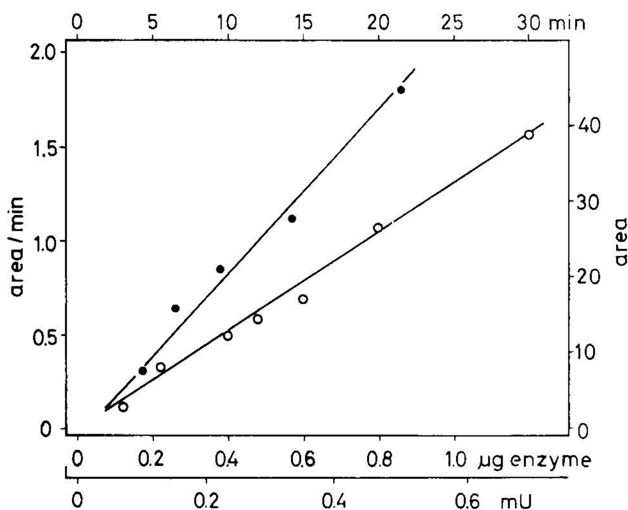

Fig. 2. Determination of the bovine chymotrypsin activity using acetyl-L-tyrosine-*p*-nitroanilide as substrate after electrophoresis and incubation at 35°. ○, Time dependent activity, 0.63 μg chymotrypsin; ●, activity dependent on enzyme concentration, 30 min incubation.

This method can also be performed on a macroscale, but then, because of the acidity of the gel buffer, it is necessary to neutralize the gels before incubation to prevent precipitation of the substrate. Slight diffusion of the bands during this procedure is inevitable. As staining the cellulose acetate strips by incubating them in the substrate-staining solution was unsuccessful, the substrate-staining solution was dissolved in 0.1% agarose and then poured out as a thin layer onto a microscopic slide. After electrophoresis the strips were laid on the agarose layer and incubated at 35° for 10 min. The cellulose acetate membranes were then dipped into 12.5% TCA until the yellow nitroaniline had changed to a bright purple colour. Excess of substrate and acid were removed by washing with water. Fig. 3 shows an electrophoretic pattern of 14 μg of porcine pancreatic crude extract (autolyzed) stained with Coomassie Brilliant

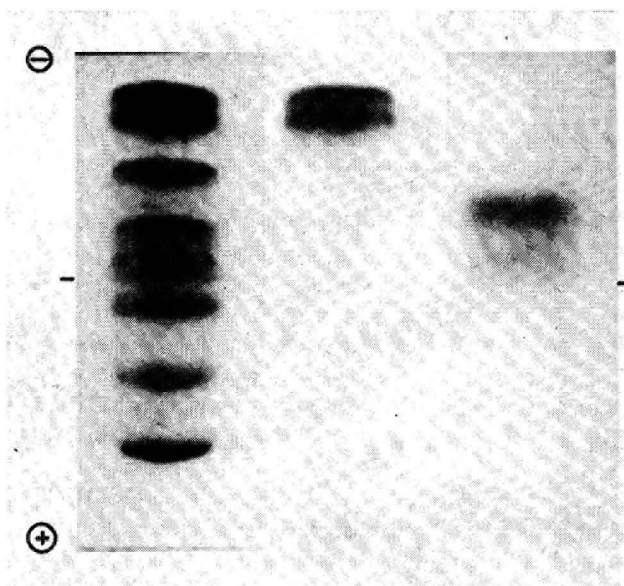

Fig. 3. Cellulose acetate electrophoresis of a crude porcine pancreas extract at pH 8.6. From left to right: protein staining; staining for trypsin activity with benzoyl-L-arginine-*p*-nitroanilide; staining for chymotrypsin activity with acetyl-L-tyrosine-*p*-nitroanilide.

Blue, and of acetyl-L-tyrosine- and benzoyl-L-arginine-*p*-nitroanilide. Clearing the stained strips permits absorptiometric scanning at 578 nm.

We have shown, therefore, that incubating the cellulose acetate membranes (as well as all kinds of gels used for electrophoresis) in an amino acid nitroanilide medium, and finally diazotizing and coupling the nitroaniline with naphthylethylenediamine, is a very useful method for identifying and determining proteolytic enzymes (endopeptidases as well as exopeptidases) in the applied sample. Not the least advantage of this simple and rapid staining procedure is its high sensitivity: the purple colour appears even when no yellow nitroaniline is visible on the gel with the naked eye.

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Note

Gas chromatographic–mass spectrometric investigation of the photo-epoxidation of vitamin K_3^*

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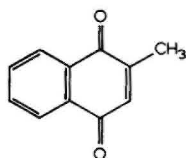
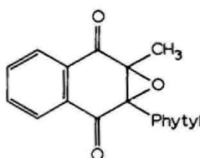
Vitamin K is widely distributed in plants, animal tissues and in the intestinal tract of most animals and man. However, the vitamin K content as well as its active location in the animal body is not fully understood[1]. Vitamin K has been reported to be affected by sunlight[2], irradiation[3], or radioactivity[4]. But it is also not clear what the significance of these effects on the vitamin K structure might be.

Structurally, vitamin K refers to a group of related compounds which are derivatives of 2-methylnaphthoquinone (I), or vitamin K_3, a synthetic vitamin available in pharmaceutic preparations. Vitamin K_1 oxide (II) has been made chemically by H_2O_2 oxidation, under alkaline conditions, of the parent vitamin also for pharmaceutical purposes[5–7]. Recently, vitamin K_1 oxide has been found as a metabolite of the parent vitamin in a system which forms the 2,3-epoxide and regenerates the vitamin in a cyclic fashion[8,9], the metabolism playing an important role in the physiological regulations of prothrombin synthesis[10].



I



II

Gas chromatography (GC) and mass spectrometry (MS) have been found to be useful for analysis[11–14] and characterization[15] of vitamin K. In the course of studies on the availability of vitamin K by the GC technique[16], it was found that a second GC peak appears at the longer retention time which is distinct from the vitamin K_3 peak during the routine calibration check of the standard solution stored under normal indoor lighting conditions. The studies here indicate that vitamin K_3 is readily converted to vitamin K_3 oxide with sunlight in the presence of oxygen, and suggest that the photo-epoxidation reaction involves a singlet molecule oxygen receiving electrons

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from the vitamin K_3 molecule in a simple system containing vitamin K_3, ethanol and exogenous oxygen.

EXPERIMENTAL

Pure menadione, vitamin K_3 (N.B.C., Cleveland, Ohio, U.S.A.) was dissolved in 95% ethanol to make up a final concentration of 0.1%. The gas chromatograph used in this study was an MT-220 (Tracor, Austin, Texas, U.S.A.), with a four-column oven, and equipped with a dual-flame ionization detector. A 6 ft. × $\frac{1}{4}$ in. I.D. U-glass column packed with 5% (w/w) Silar-5CP (Applied Science Labs., State College, Pa., U.S.A.) on 80–100 mesh Gas-Chrom Q was used for the analysis of vitamin K and its derivative. The column temperature was 180° and the inlet temperature 220°. The carrier gas (helium) flow-rate was 50 ml/min. A linear calibration curve between the vitamin K_3 (menadione) concentration and the detector response was established for the standard vitamin (5–30 μg).

Samples of menadione crystal and standard ethanolic menadione were used for mass spectrometry. The instruments used were a Varian-Aerograph 1400 gas chromatograph interfaced via a Finnigan 3000 quadrupole mass spectrometer. The probe containing the crystal was placed into the ionization chamber at ambient temperature and 70 eV and the electron multiplier voltage was 2.0 kV. The ethanolic vitamin mixture was injected into a 3 ft. × 0.079 in. I.D. glass column of the same packing material as that stated above. The column temperature was 140° and the inlet temperature 210°. The helium flow-rate was 25 ml/min.

Chemical oxidation was carried out by reacting 1 ml of menadione standard (0.01% in ethanol) with 0.1 ml of H_2O_2 (30%) and 0.1 ml of Na_2CO_3 (10%). Photo-oxidation was conducted under the sunlight exposure at noon with or without the enclosure of air, oxygen or nitrogen.

RESULTS AND DISCUSSION

Fig. 1 shows the GC curves of the separation and the patterns of menadione, menadione oxide and the derivative of the vitamin under the various experimental conditions. The results show that over 50% of the menadione (peak a) is converted into the oxide (peak b) and the derivative (peak c) after sunlight reaction (1 min, noon) in the presence of air (Fig. 1B). The retention times of peaks a, b, and c are 4, 5.8 and 8 min, respectively. Samples of menadione mixture under the nitrogen atmosphere failed to show peaks b and c even under the prolonged exposure to sun-light (Fig. 1c). These observations suggest the possibility that in this case the photo-induced oxidation only occurred when exogenous oxygen was available. For example, 95% or more of the menadione (0.01% in ethanol) was converted into the oxide form when exposed to sunlight at noon for 10 min in the presence of oxygen (Fig. 1C). The chemical conversion of vitamin K_3 to vitamin K_3 oxide was achieved with alkaline H_2O_2. The chemically induced oxides elute at the same retention time as do the photo-induced oxides on a the GC column under the same operation conditions. The effect of acid on the oxide resulting from both sunlight- and H_2O_2-treated samples is shown in Fig. 1D. The poor recovery of the peak c component upon the disappearance of peak b demonstrates the destructive effect of acid on the oxide and the increase in

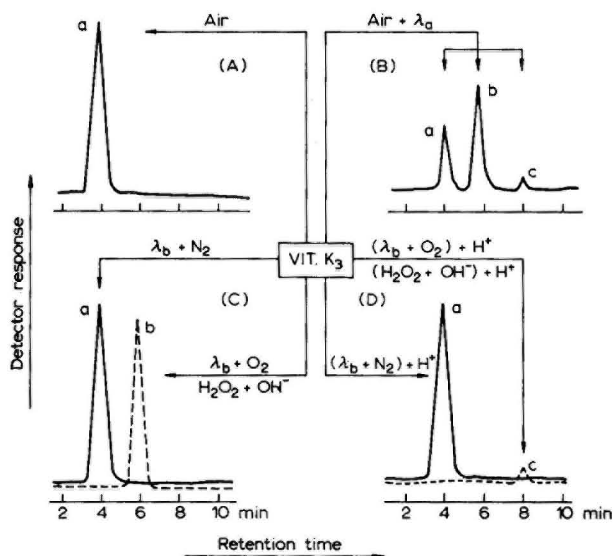

Fig. 1. GC curves showing the elution, the retention, and the patterns of menadione (a), menadione oxide (b) and derivative (c) as influenced by sunlight in the presence of air, oxygen, nitrogen, and acid. 5% (w/w) Silar-5CP on 80–100 mesh Gas-Chrom Q, 6 ft. × ¼ in. I.D. U-glass. Column temperature, 180°, inlet temperature, 220°. Helium flow-rate, 50 ml/min. λ_a = 1 min and λ_b = 10 min sunlight at noon.

polarity of the degraded product(s). Application of light to vitamin K_3 or oxygen alone and immediate recombination of the two reactants failed to show any sign of photo-induced epoxidation of the vitamin. It is suggested, therefore, that the photo-epoxidation involves a singlet molecule oxygen (excited state) receiving electrons from the vitamin K_3 molecule to form a peroxy anion (O_2^{2-}). The peroxy anion has been known to have the O–O bond lengthened to such an extent that the oxygen molecule splits into two atoms, and photo-epoxidation takes one single oxygen to complete.

The mass spectra of vitamin K_3 and its oxide regarding fragmentation of the molecule are shown in Table I. The relative abundance of the ten most prominent ions in the mass spectra indicates that the molecular ion of vitamin K_3 is 172 for both crystal (solid probe) and mixture (GC–MS), and that that of vitamin K_3 oxide is 188 for both photo-induced and chemically induced products. Addition of one oxygen atom to the vitamin K_3 molecule is therefore characterized by GC–MS. It is noted that the characteristic fragmentations between *m/e* 172 and *m/e* 188 show several different patterns. The masses of vitamin K_3 (in solution) and that of crystal (solid probe) and those of their subsequent fragments are identical, suggesting the high stability of vitamin K_3 solution during GC analysis. On the other hand, the characteristic fragmentation pattern of the photo-induced oxide corresponds essentially to that of the chemically induced oxide. Thus, there is a structural similarity between the photo-oxygenated menadione and the chemically oxygenated menadione —both combine with one oxygen atom to yield an oxide or epoxide. The application of these studies should be useful in attempts to unravel the intricacies of the physiological actions of vitamin K.

TABLE I

RELATIVE ABUNDANCE OF TEN MOST PROMINENT IONS IN MASS SPECTRA OF VITAMIN K_3 AND ITS OXIDE

Vitamin K_3				*Vitamin K_3 oxide*			
Solid probe (crystal)		*GC–MS (N_2-λ)*		*GC–MS (O_2-λ)*		*GC–MS (H_2O_2)*	
m/e	*RA**	*m/e*	*RA**	*m/e*	*RA**	*m/e*	*RA**
172**	100	172**	100	188**	52	188**	55
144	24	144	22	173	86	173	81
116	40	116	43	160	69	160	68
115	46	115	51	131	60	131	52
105	16	105	20	105	62	105	52
104	47	104	62	104	52	104	45
76	49	76	59	89	100	89	100
74	13	74	13	76	95	76	82
50	31	50	35	50	81	50	87
39	21	—	—	43	95	43	87

* Relative abundance with *m/e* in each case.

** Molecular ion.

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Note

Gas chromatography and characterization of tetraethyl derivatives of uric acid

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We are interested in assaying uric acid in serum with high accuracy by a similar principle to that described for serum calcium[1] using stable isotope dilution–mass spectrometry[2]. To facilitate both purification and introduction of samples into the mass spectrometer, it was desirable to find a derivative of uric acid which would be easily prepared and stable; soluble in common organic solvents; volatile enough for gas chromatography (GC); and give a suitable fragmentation pattern in the mass spectrometer. One or other of the methylated derivatives[3–6] of uric acid might meet these requirements, but to achieve specificity, it seemed preferable to use an ethyl derivative since methylated purine compounds are commonly found in biological materials. The synthesis of tetraethyl derivatives of uric acid has been attempted[7]; the present paper describes a simple method for the direct ethylation of uric acid, and the GC and characterization of the principal products.

EXPERIMENTAL

Materials

Diethyl sulphate (BDH, Poole, Great Britain), was used as received. Organic solvents and reagents were all of the analytical grade quality. [^{14}C]-Uric acid (specific activity 56.7 mCi/mmole) was purchased from the Radiochemical Centre (Amersham, Great Britain).

Preparation of derivatives

Solutions of uric acid were prepared by dissolution in 1 *M* KOH, with subsequent dilution, or addition of alkali, as required.

A solution of uric acid (2.5 mg in 5 ml of 5 *M* KOH) was mechanically stirred with diethyl sulphate (5 ml) in a flask kept at 65°. The pH of the reaction mixture was monitored by a Pye Autotitrator and was kept above pH 11 by adding 10 *M* KOH (approx. 1 ml) during 30 min. More diethyl sulphate (5 ml) was then added, together with more 10 *M* KOH (5 ml), and the reaction was allowed to continue as before for a further 30 min. A clear solution was then obtained, indicating utilisation of all the diethyl sulphate, and it was then extracted three times with 2-ml portions of diethyl ether. The ether extracts were combined and aliquots were taken for the GC.

Gas chromatography

Pye 104 and Becker 409 gas chromatographs were used, both with flame ionization detectors. The former was fitted with a stream splitter (1:10) and a preparative column (15 ft.) coated with 1% OV-17 on Gas-Chrom Q, 80–100 mesh, and was run isothermally at 220°; the Becker chromatograph was equipped with a 20 m open-tubular capillary column coated with OV-101, and was run with temperature programming from 100 to 200°, the rate of change being 2°/min. Liquid injection was used for the preparative chromatography, with caffeine as internal standard; solid injection was carried out on the Becker chromatograph.

RESULTS AND DISCUSSION

Gas chromatography of uric acid derivatives

Ethylation of uric acid gave two major ether-soluble volatile compounds, which, when chromatographed on OV-17, showed relative retention times (RRT) of 1.25 and 1.79 respectively, using caffeine as the internal standard (Fig. 1A). Although there are 12 possible isomers of tetraethyl uric acid, only five minor —quantitatively insignificant— peaks were noted. Using the stream splitter, the two major fractions were trapped for further characterization.

Each fraction was a white crystalline substance, readily soluble in water and the common polar solvents. Re-chromatography of the two fractions using the capillary column, with much higher resolution, showed that each gave only a single peak (Fig. 1B), so that each fraction may be regarded as a single compound (I and II respectively).

Spectroscopic characterization of the principal products

The mass spectra of compounds I and II were identical (Fig. 1C) and showed a prominent molecular ion at *m/e* 280, with four successive losses of 28 mass units (Fig. 2) giving peaks at *m/e* 252, 224, 196 and 168. The additional ions at *m/e* 265, 237, 209 and 181 corresponded to losses of 15 mass units (methyl) and were considered to arise from the ethyl substituents.

Although the mass spectrum is consistent with each compound being a tetraethyl derivative of uric acid, the fragmentation pattern does not permit differentiation between N-ethyl or O-ethyl substitution, since in both cases McLafferty γ-hydrogen rearrangement[8] could occur, to give rise to the loss of 28 mass units as shown (Fig. 2).

To differentiate the structure of the two isomers, infrared (IR) and nuclear magnetic resonance (NMR) analyses were carried out (Figs. 1D and E, respectively). The IR spectrum of compound II showed two strong bands in the carbonyl region at 1682 and 1712 cm^{-1} respectively. This indicates that only two of the three oxygen atoms are in the lactam form, and the third in a lactim associated with an ethyl; thus it is likely that compound I has three N-ethyl substituents and one O-ethyl substituent (Fig. 3).

By contrast, the IR spectrum of compound I showed three intense absorption bands in the carbonyl region at 1610, 1654 and 1700 cm^{-1}, corresponding unequivocally to a triketone structure, and showing that compound I is a tetra-N-ethyl derivative with the oxygen atoms at positions 2, 6, 8 in the lactam form.

The NMR spectrum confirmed that compound I corresponds to a structure

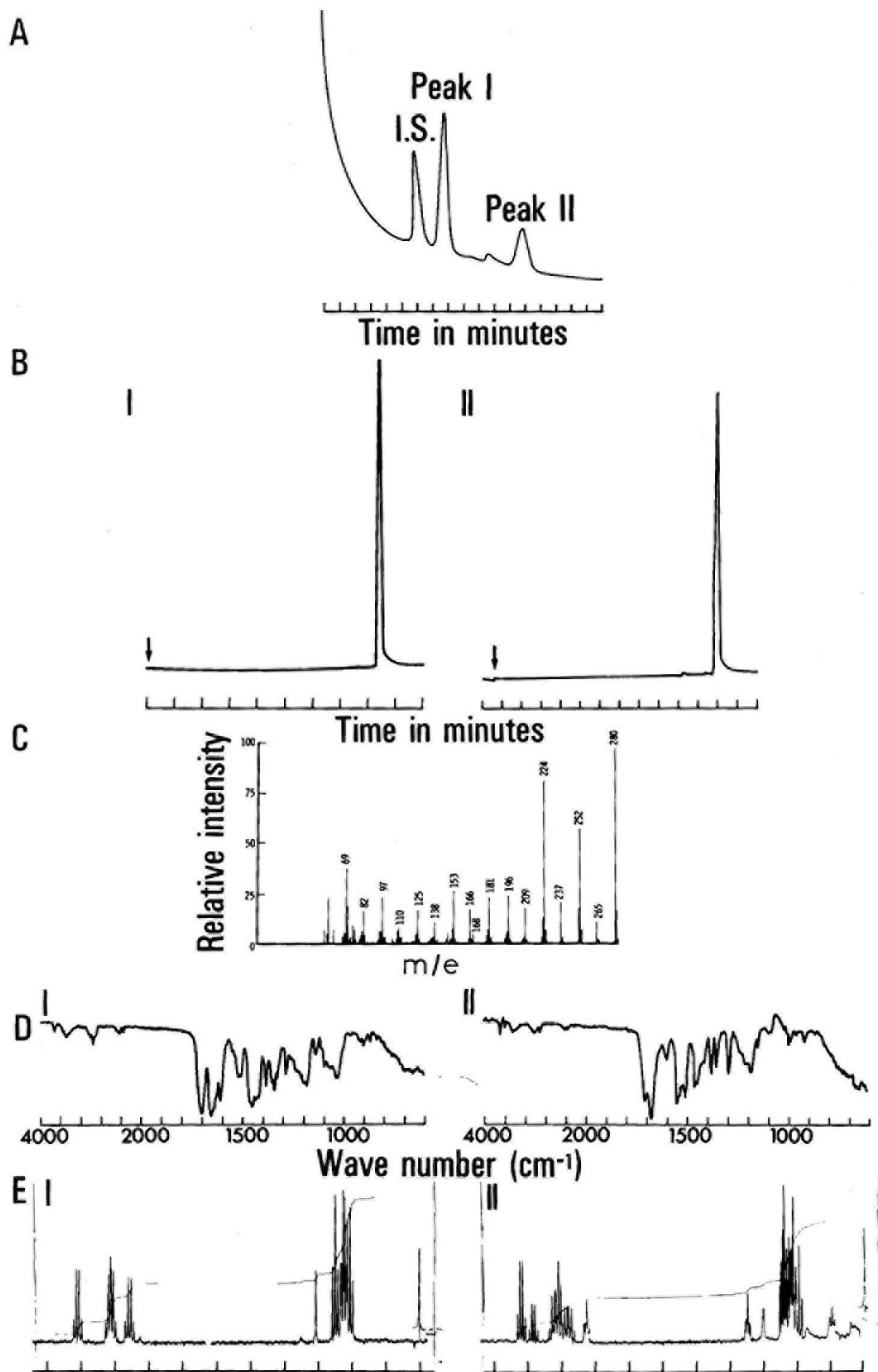

Fig. 1. (A), GC of ethylated derivatives of uric acid with caffeine as the internal standard (I.S.); (B), re-chromatography of trapped peaks I and II on the capillary columns; (C), mass spectrum of the two tetraethyl uric acid derivatives; peaks I and II gave identical spectra; (D), IR spectra of peak I and of peak II, both in chloroform solution; (E), NMR spectra of peak I and of peak II, both in chloroform solution.

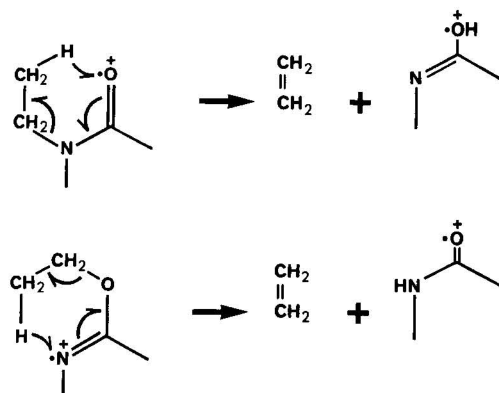

Fig. 2. Scheme of McLafferty γ-hydrogen rearrangement.

containing four distinct ethyl groups. With compound II however, the NMR spectrum suggested that one ethyl group is attached to an oxygen, (probably that of the pyrazine ring), two are attached to N-1 and N-3 of the pyrimidine ring, while the position of the fourth ethyl substituent is uncertain, being either at N-7 or N-9, or both, in tautomeric equilibrium (Fig. 3).

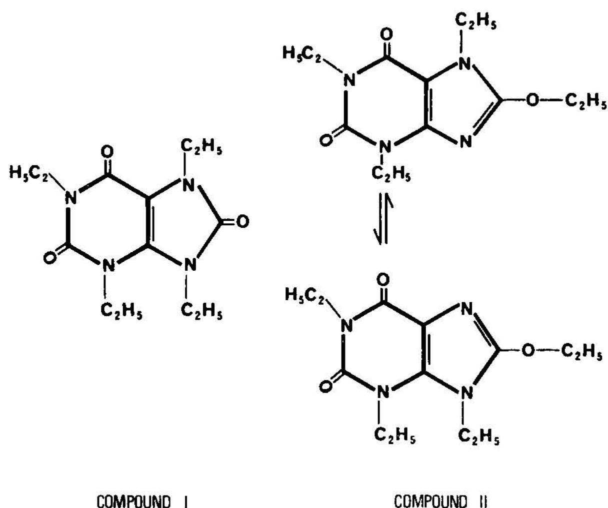

Fig. 3. Structural formula of compounds I and II.

CONCLUSIONS

With the reaction conditions described, compound I was produced in approximately twice the yield of compound II, as judged by the relative peak areas on the gas chromatogram. The sum of the two isomers accounted for an overall yield of 50–70%, as estimated by using [^{14}C]-uric acid as an internal standard, and by comparison with caffeine as the internal chromatographic standard, and correcting for the molecular response.

The two tetraethyl derivatives of uric acid were found to be stable in diethyl ether or chloroform solution for up to 7 days at room temperature, and stable in a dry form for at least one month.

These derivatives of uric acid are simple to synthesise, stable, can be gas chromatographed readily, and afford a characteristic mass fragmentation pattern. These properties make them very suitable for use in the assay of uric acid in biological matrices, and for stable isotope-dilution studies.

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Note

Elution sequence as a function of temperature in the gas–liquid chromatography of monoterpene hydrocarbons

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The chromatographic behaviour of the main monoterpene hydrocarbons in essential oils has been investigated and, in attempts to find the best conditions for their quantitative assay, these compounds were examined by gas–liquid chromatography (GLC) on different columns at different column temperatures. It was noticed that the Kováts retention indices (I) behaved in a different manner for the aliphatic and the alicyclic hydrocarbons when the temperature of the GLC column was changed. This effect was particularly evident when polyethyleneglycol was used as the stationary phase.

The influence of temperature on the retention parameters has been reported earlier[1–5] and effects on terpenoid compounds have been observed by Roberts[6] and Andersen and Falcone[7].

The variation of I values with temperature (T) can be used to distinguish between different chemical groups in a complex mixture such as essential oils. The $\Delta I/\Delta T$ values may also give a clue as to the identity of a compound. When capillary gas chromatography is applied to the analysis of terpene mixtures, the $\Delta I/\Delta T$ value gives additional information on the compounds. We have investigated several essential oils[8] and the identities of myrcene, *cis*-β-ocimene and *trans*-β-ocimene in the hydrocarbon fraction could well be indicated by this method.

Compounds that belong to other groups, *e.g.*, oxygenated terpenes, can be identified satisfactorily by the procedure described here. 1,8-Cineol, a compound that very often elutes together with the hydrocarbon fraction in a normal column preseparation of the essential oil[9], is identified by its $\Delta I/\Delta T$ value.

A typical series of analyses is shown in Fig. 1. The monoterpene hydrocarbon fraction of the essential oil of *Juniperus communis* L. spiked with 1,8-cineol and *trans*-β-ocimene was analyzed at four different temperatures. 1,8-Cineol (peak 23), myrcene (8) and *trans*-β-ocimene (18) change positions in the sequence of peaks and will thereby be indicated as compounds belonging to different chemical groups.

If the temperature at which such a group of compounds is analyzed is chosen at random, the total separation may be greatly influenced. We therefore would like

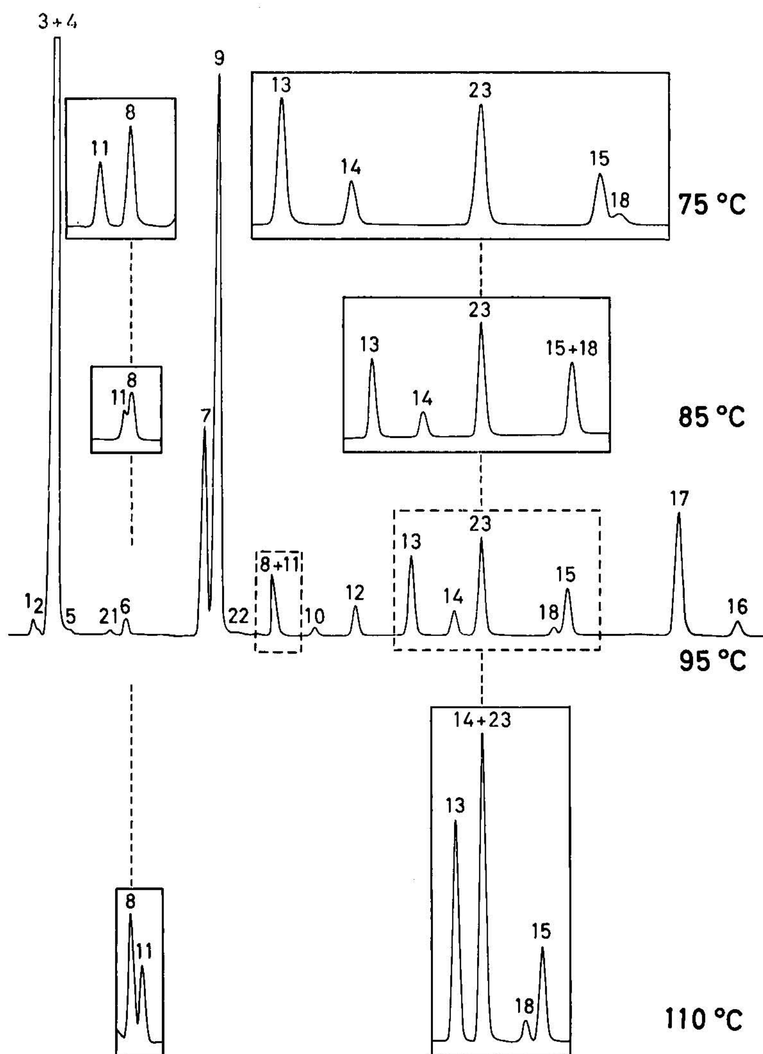

Fig. 1. Chromatograms of a terpene mixture obtained on a PEG 1540 column at 75°, 85°, 95° and 110°. Peaks: 1, tricyclene; 2, unidentified; 3, β-pinene; 4, α-tnujene; 5, unidentified; 6, camphene; 7, β-pinene; 8, myrcene; 9, sabinene; 10, α-phellandrene; 11, Δ_3-carene; 12, α-terpinene; 13, limonene; 14, β-phellandrene; 15, γ-terpinene; 16, terpinolene; 17, p-cymene; 18, *trans*-β-ocimene; 21, α-fenchene; 22, Δ_4-carene; 23, 1,8-cineol.

to stress the importance of using different column temperatures when complex mixtures are to be analyzed, as this technique increases the information given by GLC analysis.

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Note

2-Thiobarbituric acid as a reagent for the detection of meconic acid by thin-layer chromatography

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Poisoning by morphine is not uncommon in India and, in forensic toxicological work, it often becomes necessary to establish the source of morphine in such cases. A number of morphine formulations are in use in medicine and opium may also be acquired.

Certain 9-N-phenanthrene alkaloids are closely related to the morphine group in structure, *e.g.*, sinomenine[1] from *Sinomenium diversifolius* and *Sinomenine actum*, the Japanese plant belonging to the menispermaceae. Another interesting variant with a structure similar to morphine is hasubanonine from *Stephania japonica*[2]. On the other hand, thebaine, an alkaloid of the morphine group, has also been found in *Rauwolfia serpentina* Benth and in oriental papaver[3]. Opium is described in the Indian Opium Act, 1878, as capsules or coagulated juice of such capsules of *Papaver somniferum*. A study of different species of papaver in India showed that only *Papaver somniferum* contains all five of the alkaloids morphine, codeine, thebaine, papaverine and narcotine[4]. Most of the alkaloids from opium are present in the form of meconates and lactates, and meconic acid is strictly peculiar to opium. Consequently, in a case of poisoning, the detection of meconic acid together with morphine and the above opium alkaloids in the test samples is necessary in order to establish the source of morphine as opium.

Meconic acid is usually detected by its colour reaction with iron(III) chloride. However, in toxicological work, tissue extracts, particularly from a decomposed body, may show trace amounts of phenolic groups by the iron(III) chloride test. There are certain morphine preparations, such as elixir of dimorphine and terpene, camphorated linctus of dimorphine and camphorated linctus of opium, that contain pine and/or borneol (terpene)[5], which also react with iron(III) chloride. In a search for an alternative reagent, we found that 2-thiobarbituric acid (2-TBA) in dimethyl sulphoxide gives a cherry red colour with meconic acid.

EXPERIMENTAL AND RESULTS

Preparation of plates

Thin-layer chromatographic (TLC) plates were prepared with silica gel G (E. Merck, Darmstadt, G.F.R.) as adsorbent and were activated by heating for half an hour at 110°. The thickness of the layer was 250 μm.

Solvent system

Methanol–benzene–acetone–perchloric acid (7:2:1:0.2) was found to be the best solvent system.

Spray reagent

2-TBA (7.2 g) was dissolved in about 75 ml of dimethyl sulphoxide and the volume made up to 100 ml with the same solvent, giving a concentration of 0.5 *M*.

Procedure

Meconic acid extracted from an aqueous acidic solution of opium with chloroform–ethanol (4:6) and an authentic sample of pure meconic acid were spotted on a TLC plate and a chromatogram was developed by the ascending technique using the above solvent system. After a 10-cm run, the plate was taken out of the chromatographic chamber and allowed to dry, then sprayed with 0.5 *M* 2-TBA. The plate was then heated in a vacuum oven at 140° for about 30 min, and cherry red spots (R_F 0.64) were observed on very faint yellow background.

Several workers have used 2-TBA as a reagent for TLC with some prior treatment. Copius-Peereboom and Beekes[6] and Pinella *et al.*[7] used this reagent for the detection of sorbic acid after spraying with potassium dichromate. Nisbet[8] described a TLC method for the detection of polyhydric alcohols after treatment with potassium dichromate. Feigl and Libergott[9] converted sugars into aldehydes before treatment with 2-TBA. Pyrimidine derivatives, after hydrolysis, form coloured compounds with 2-TBA[10]. Feigl *et al.*[11] also reported a spot test for sulphadiazine using 2-TBA. A quantitative method has been described for the determination of chloral hydrate[12].

In our work, 2-TBA in dimethyl sulphoxide is directly sprayed on to the thin-layer plate. Under the above conditions, phenol and phenolic compounds such as salicylic acid derivatives and 2,4-dichlorophenol did not show any coloured spots. Lactic acid gave a yellow spot slightly more intense than the background yellow colour (R_F 0.45). The sensitivity of 2-TBA in dimethyl sulphoxide for meconic acid was found to be *ca.* 10 µg, compared with *ca.* 20 µg for iron(III) chloride.

When the test was carried out in a test-tube, adding the 2-TBA reagent to an authentic sample of meconic acid, a clear cherry red solution with an absorption maximum at 529 nm was obtained. This reaction can be used for the quantitative determination of meconic acid.

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Note

Detection of inhibitors of *Erwinia carotovora* and *E. herbicola* on thin-layer chromatograms

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Published methods for detecting antibacterial compounds on TLC plates[1–4] have usually involved placing the chromatogram in contact with a seeded agar medium to allow diffusion of the compound, with subsequent incubation of the agar medium and observation of zones of inhibition of the bacterium. These zones may be intensified by several methods, particularly by the use of tetrazolium salts, of which *p*-iodonitrotetrazolium violet (INT) has been reported as being one of the most sensitive[5].

This note describes methods for detecting inhibitors of *Erwinia carotovora* and *E. herbicola* directly on TLC plates. Good results have been obtained with a method that is based on the ability of the organism to hydrolyze aesculin[6], and this method may be applicable to a wide range of bacteria as an alternative to the use of tetrazolium salts.

MATERIALS AND METHODS

Bacteria

Three strains of *E. carotovora* var. *atroseptica* (*E. atroseptica*) (G120, BL/CL 30/101, BL/CL 30/120), three strains of *E. carotovora* var. *carotovora* (*E. carotovora*) (BL/M 46/1, BL/CL 30/104, BL/CL 30/135), two strains of *E. herbicola* var. *herbicola* (708, G142) and *Bacillus cereus* M8 were from a collection maintained in this laboratory.

Extraction of garlic (Allium sativum)

Tissue (20 g) from fresh garlic bulbs was extracted with 200 ml of distilled water by treatment in an MSE microhomogeniser. The extract was centrifuged (30,000 *g*; 30 min), the supernatant liquid was filtered, evaporated to dryness under reduced pressure at 40°, and the residue was extracted with 2 ml of methanol (fraction 1) and then with 2 ml of water (fraction 2).

Culture media

Cultures were grown in Trypticase-soy-broth (TSB) (Baltimore Biological Labs., Baltimore, Md., U.S.A.) (20 ml of medium in 100-ml flasks) at 25° for 24–72 h on a shaker with a 1-in. rotary movement at a speed of 250 rpm.

Detection of antibacterial activity on agar plates

Plates of Trypticase-soy-agar (BBL) were surface-seeded with a culture of the appropriate bacterium in TSB. A measured volume of the solution to be tested was placed on a sterile filter-paper pad 6 mm in diameter (Antibiotic Assay Discs, Whatman), which was placed on the seeded plate. After incubation at 25° for 24 h, the diameter of the zones of inhibition were recorded. Control tests with solvent gave no zones of inhibition.

Thin-layer chromatography

Prepared TLC plates (silica gel 60 or silica gel 60, F254, Merck) were washed by ascending development with methanol before use. The chromatograms were developed with cyclohexane–ethyl acetate (1:1, v/v).

Detection of antibacterial activity on TLC plates

The antibacterial compounds used were coumarin (BDH, Poole, Great Britain), streptomycin sulphate (Sigma, St. Louis, Mo., U.S.A.) and constituents present in the extracts of garlic. After development, the plates were dried to remove solvent. Cultures of bacteria in TSB were centrifuged (3,000 *g* for 20 min), the supernatant liquid was discarded, and the sedimented bacteria were re-suspended in fresh TSB to give an absorption of 0.84 at 560 nm (equivalent to approx. 10^9 bacteria per ml). This suspension was sprayed on to the TLC plates using a fine spray (7CR, Quickfit); approximately 18 ml of suspension were used for a 20 $\times$ 20 cm plate. The plates were then dried in a stream of cold air, just sufficiently to remove any film of water and give a translucent appearance, before being incubated at 25° overnight in boxes lined with wet filter paper to maintain high humidity. After incubation, the plates were dried until opaque and immediately sprayed with one of the following solutions: (1) aesculin spray [aesculin (BDH), 0.2% w/v; ammonium ferric citrate, 0.1% w/v; yeast extract (Difco), 0.5% w/v, in distilled water]; (2) an aqueous solution of INT (Grade 1, Sigma), 2 $mg \cdot ml^{-1}$ (see ref. 5) (3) an aqueous solution of 2,3,5-triphenyltetrazolium chloride (TTC) (BDH), 20 $mg \cdot ml^{-1}$ (see ref. 5). The plates were then again incubated at 25° in the lined boxes to allow hydrolysis of aesculin or reduction of tetrazolium salts to occur.

In the conditions used, aesculin sprayed on to TLC plates appeared colourless in tungsten light, strongly fluorescent in UV radiation (366 or 254 nm) and was not significant affected by the iron salt. A similar quantity of aesculetin gave a yellow colour in tungsten light and was much less fluorescent than aesculin in UV radiation[7], the iron salt gave a brown colour with aesculetin in tungsten light and a decrease in fluorescence in UV radiation. On plates sprayed with the bacteria and subsequently with the aesculin spray, hydrolysis of aesculin resulted in development of a brown colour, the zones of inhibition of bacteria remaining colourless. Under UV radiation of 366 nm (or 254 nm for the plates without a fluorescent additive), zones of inhibition were fluorescent against a dark background.

On plates sprayed with the bacteria and subsequently with INT or TTC, zones of inhibition appeared colourless against a violet or a red background, respectively.

RESULTS AND DISCUSSION

Tests of antibacterial activity against *E. atroseptica* G 120 on agar plates showed that 2 mg of coumarin, 100 μg of streptomycin sulphate and 20 μl of the extracts of garlic produced zones of inhibition of 13, 23 and 17–18 mm in diameter, respectively.

When attempts were made to detect antibacterial activity of these compounds on TLC plates, none of the strains of *Erwinia* reduced TTC during incubation for 24 h; in contrast, *B. cereus* M8 reduced this compound in 2 h. All the strains of *Erwinia* hydrolyzed aesculin and reduced INT in 2–24 h, and both these sprays could be used to detect inhibition of bacterial growth. The tests with streptomycin sulphate, which was spotted on to plates, but not developed with a solvent mixture, showed that, with this water-soluble compound, careful spraying resulted in well-defined zones of inhibition, without distortion due to the aqueous sprays.

The times required for hydrolysis of the aesculin and for reduction of the INT differed according to the strain of *Erwinia* used. Generally, the reaction occurred within 2–7 h at 25°, but incubation overnight was sometimes necessary. Clear results could be obtained with both sprays, but with some strains of *Erwinia* slight inhibition by coumarin was revealed more clearly by the aesculin spray than by the INT. Examination under UV radiation intensified the zones of inhibition on plates sprayed with aesculin (see Fig. 1).

The sensitivity of detection of inhibitors on the TLC plates was assessed by comparing the areas of zones of inhibition with those obtained on agar plates. Tests

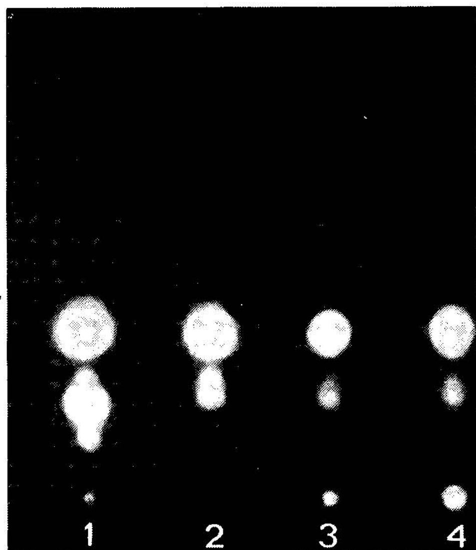

Fig. 1. Zones of inhibition of *E. carotovora* var. *atroseptica* G120 produced after chromatography of extracts of garlic on a silica gel 60, F254 plate in cyclohexane–ethyl acetate (1:1, v/v). The plate was then sprayed with bacteria and subsequently with aesculin spray. Photographed under UV radiation (366 nm). 1,2 = 10 μl and 5 μl (respectively) of fraction 1; 3,4 = 10 μl and 5 μl (respectively) of fraction 2 (see Materials and methods). The photograph was taken on High-Speed Ektachrome Type B film (Kodak), a black-and-white internegative being made on Plus X film (Kodak).

were made with coumarin (1–2 mg) against two strains each of *E. atroseptica* and *E. carotovora* and with garlic extracts (20 μl) against one strain of *E. atroseptica*. In each instance, larger areas of inhibition were formed on the TLC plates than in tests on the agar plates.

The results with extracts of garlic showed that these methods could be applied successfully to compounds in a crude extract from plant tissue. The aesculin spray has also been used to demonstrate inhibition of *E. atroseptica* by rishitin in extracts from potato tissue[8].

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Note

Chromatographie en phase gazeuse de dérivés cyclaniques et aromatiques substitués par les groupements ou atomes C=O, OH, Cl, Br, F et CH_3

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(Reçu le 9 décembre 1974; manuscrit modifié reçu le 28 février 1975)

Dans le cadre général d'une étude de dérivés substitués par différents groupements (Cl, Br, F, CH_3, OH, C=O) en série cyclanique[1–3] nous avons été amenés à synthétiser des composés dont les structures ont été établies par différentes techniques (chromatographie gas–liquide, UV, IR, résonance magnétique nucléaire, RX)[2–6]. En chromatographie gaz–liquide afin d'identifier les composés et de connaître l'influence des substituants suivant sa position dans le cycle (α, β, γ), nous avons déterminé les indices de rétention de Kováts[7,8] de quatre-vingts composés présentant de un à quatre substituants et ayant suivant les composés différentes positions et orientations.

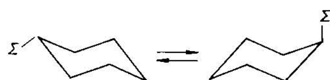
CONDITIONS EXPÉRIMENTALES

Chromatographie gaz–liquide

Le remplissage chromatographique utilisé est constitué de 10% de phtalate de diisodecyle (Applied Science Labs., State College, Pa., États-Unis) déposé sur du Chromosorb G de granulométrie 0.12–0.16 mm. Trois colonnes en inox ont été employées pour notre étude: une colonne de 1/4 in. (6 m de longueur) et deux colonnes de 1/8 in. (4 m de longueur). L'appareil utilisé est un Varian 1521 à détection catharométrique. Le gaz vecteur est l'hydrogène.

Origine des produits

Certains produits sont commerciaux (*cf.* Tableau I), cependant nous avons synthétisé la plus grande majorité, inconnus jusqu'alors, selon des méthodes originales. La particularité de la série cyclohexanique est de présenter un équilibre conformationnel.



* Attachée de Recherches au CNRS.

Les propriétés physicochimiques de ces équilibres sont étudiées dans les références 1–4. Ces travaux ont exigé des produits de très haute pureté et ont tous été testés par chromatographie. Outre l'aspect purement analytique, il nous a semblé que cette étude contribue à l'avancement et la connaissance des interactions soluté–solvant en chromatographie en phase gazeuse, précédemment étudié par nous sur diverses autres séries: aromatiques, alcools et cétones linéaires[9–12].

Pureté des produits utilisés

Les produits commerciaux (pureté 99%) ont été utilisés directement. Dans le cas contraire, leur pureté a été assurée par chromatographie préparative sur un appareil Hupe Bush APG 402. Les produits synthétisés ont été obtenus à un degré de pureté supérieur à 99% soit par cristallisations successives, soit par chromatographie préparative.

PHASE STATIONNAIRE

Le phtalate de diisodécyle de masse moléculaire 446.67 a un point d'ébullition de 255° sous 5 mm. Nous avons déterminé sa densité à différentes températures. On trouve: —0.973 à 30°, 0.956 à 50.4° et 0.940 à 75°. Selon notre échelle[7–11], la polarité 29 à 140° classe ce composé parmi les solvants peu polaires. La classification de Brown[13] lui confère une forte valeur de la fraction de rétention.

Ce solvant chromatographique solubilise bien les dérivés apolaires, donneurs d'électrons ou possédant un hydrogène actif. Ces qualités nous l'ont fait choisir pour l'étude des divers composés hydroxylés et halogénés à effectuer.

L'efficacité d'une colonne chromatographique est liée à la valeur k de la pente des droites représentatives de la fonction $\log d'_R \rightarrow F(I)$. La différence d'indice de rétention, ΔI, nécessaire entre deux solutés pour obtenir la séparation théorique limite est:

$$\Delta I = \frac{0.8686}{k\sqrt{N_{\text{eff}}}}$$

où N_{eff} est le nombre de plateaux théoriques effectifs.

L'efficacité théorique maximum est trouvée pour une colonne sur laquelle tous les solutés auraient un coefficient d'activité égal à un. La valeur de k à 140° est alors de 3.10^{-3}; pour une colonne de 4 m possédant 4000 plateaux (valeur moyenne trouvée sur le Chromosorb G), on peut calculer $\Delta I = 4.5$ unités d'indice.

Sur le phtalate de diisodécyle on détermine à 140° une valeur de k égale à $2.56 \cdot 10^{-3}$ ce qui permet de calculer une valeur de ΔI de 5.4 unités d'indice, relativement proche de la valeur maximum théorique.

INDICES DE RETENTION I°

Les indices de rétention I°[7–12] sont calculés dans le cas de l'idéalité pour lequel tous les solutés ont un coefficient d'activité de 1. On a

$$I^{\circ} = 100\,\frac{\log \overset{\circ}{P_z} - \log \overset{\circ}{P_x}}{\log P^{\circ}_{z+1} - \log P^{\circ}_z} + 100\,Z$$

TABLEAU I

VALEURS DE RÉTENTION

No.	*Composés*	*Origine**	*I° à 160°*	*∂I°/∂T*	*Indices de rétention*		
					140°	*160°*	*170°*
1	Cyclopentane	a	540.8	0.0114	592	598	
2	Cyclohexane	a	644.1	0.0567	694	709	
3	Cyclohexène	b	650.5	0.0444	723	735	
4	Benzène	a	636.0	−0.0200	740	745	
5	Fluoro benzène	a	649.0		758		
6	Méthyl cyclohexane	b	716.9	0.133	761	767	
7	Fluoro cyclohexane	c (2)			803	808	
8	Cyclopentanol	a			905	903	
9	Cyclopentanone	a			906	920	
10	Méthyl-2 cyclopentanone	a			947	953	
11	Chloro benzène	a	825.7	0.077	958	966	
12	Méthyl-3 cyclopentanone	b			964	971	
13	Cyclopentène-2 one	b			979	984	
14	Chloro cyclohexane	a				994	
15	Cyclohexanol	a	944.0		1010	1013	
16	Méthyl-2 fluoro-2 cyclohexanone	c (2)			1026	1029	
17	Cyclohexanone	a	920.4	0.100	1015	1028	1033
18	Diméthyl-3,4 cyclopentanone	b			1031	1033	
19	Anisole	a					1034
20	*tert.*-Butyl cyclohexane	a			1027	1036	
21	Bromo benzène	a	922.6	0.140	1056	1068	
22	Méthyl-2 cyclohexanone	b			1065	1076	
23	*cis*-Méthyl-4 cyclohexanol	b			1072	1083	
24	*trans*-Méthyl-4 cyclohexanol	b			1079	1083	
25	Cyclohexénone	b			1082	1090	
26	Méthyl-4 cyclohexanone	b			1078	1092	1091
27	Méthyl-3 cyclohexanone	b			1086	1100	
28	Diméthyl-2,6 cyclohexanone	b			1111	1117	
29	Difluoro-1,1 bromo-4 cyclohexane	c (2)					1124
30	Méthyl-2 cyclohexènone	c (2)			1124	1130	
31	Méthyl-4 cyclohexènone	c (2)			1136	1144	
32	Diméthyl-2,5 cyclohexanol	b			1142	1151	
33	Diméthyl-4,4 cyclohexanone	c (2)			1147	1156	
34	Diméthyl-4,4 deutéro-2,2,6,6 cyclohexanone	c (2)			1143		
35	Diméthyl-4,4 cyclohexénone	c (2)			1167	1176	
36	Triméthyl-3,3,5 cyclohexanone	b			1160	1170	
37	Acétophénone	a	1129.4	0.060		1230	
38	Méthyl-2 chloro-2 cyclohexanone	c (2)			1178	1188	
39	*o*-Chloro phénol	a				1208	
40	Hydroxy-2 cyclohexanone	a				1225	
41	Tétraméthyl-2,2,4,4 cyclohexanone	c (2)			1206	1225	
42	Tétraméthyl-3,3,5,5 cyclohexanone	b			1218	1230	
43	Alcool benzylique	a	1165.0	−0.327			1234
44	*trans*-Dichloro-1,2 cyclohexane	b				1207	1235
45	*cis*-Dichloro-1,4 cyclohexane	b					1245
46	Chloro-2 cyclohexanone	c (2)					1245
47	Phénol	a	1044.6	−0.468		1237	1247
48	Dichloro-1,2 benzène	a	1026	0.137	1168	1178	

(Continué sur la p. 200)

TABLE I *(continué)*

No.	*Composés*	*Origine**	*I° à 160°*	*∂I°/∂T*	*Indices de rétention*		
					140°	*160°*	*170°*
49	*trans*-Dichloro-1,4 cyclohexane	b					1268
50	Chloro-4 cyclohexanone	c (1, 2)				1284	
51	Fluoro-4 phénol	b				1295	1302
52	Diméthyl-2,6 phénol	b					1320
53	Phényl éthyl cétone	a	1206	0.029			1325
54	Fluoro-3 phénol	b					1325
55	Cyclohexane dione-1,4	a				1324	1328
56	*p*-Cresol	a	1145.5	−0.344			1338
57	*cis-tert.*-Butyl-4 cyclohexanol	c (2)					1336
58	*m*-Cresol	a	1145.7	−0.343			1344
59	*cis*-Cyclohexane diol-1,2	b				1275	1286
60	*trans*-Cyclohexane diol-1,2	b				1289	1296
61	*cis*-Cyclohexane diol-1,3	b					1346
62	*cis*-Cyclohexane diol-1,4	b				1350–1356	1354
63	*trans*-Cyclohexane diol-1,3	b					1360
64	*trans-tert.*-Butyl-4 cyclohexanol	c (2)					1362
65	*tert.*-Butyl-4 cyclohexanone	a				1374	
66	*trans*-Dibromo-1,2 cyclohexane	a				1384	
67	Méthyl-4 hydroxy-4 cyclohexanone	c (2)				1385	
68	Diméthyl-2,5 phénol	a	1180.1	−0.161		1387	
69	Hydroxy-4 cyclohexanone	c (1, 2)				1388	
70	*tert.*-Butyl-4 cyclohexénone	c (2)					1418
71	Bromo-4 cyclohexanol	c (2)					
72	Diméthyl-2,3 phénol	a	1220.9	−0.136			1425
73	*cis*-Dibromo-1,4 cyclohexane	c (2)					1433
74	Dichloro-2,4 phénol	a					1437
75	Diméthyl-3,5 phénol	a	1247.6	−0.485			1440
76	Dichloro-2,5 phénol	a					1445
77	Diméthyl-3,4 phénol	a	1278.8	−0.470			1464
78	*trans*-Dibromo-1,4 cyclohexane	c (2)					1496
79	*p*-Chloro phénol	a					1535
80	Bromo-4 phénol	b					1650
81	Bromo-4 cyclohexanone	c (2)					1425

* (a) Produit commercial pureté $> 99\%$. (b) Produit commercial purifié par chromatographie préparative (appareil Hupe Bush). (c) Produit nouveau synthétisé (bibl.).

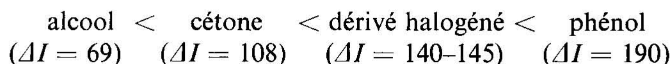
où P° représente les pressions de vapeur du soluté x et de deux alcanes z et $z+1$ dont les pics chromatographiques encadrent celui du soluté x.

L'écart entre l'indice de rétention expérimental I_x et l'indice I_x° marque la différence de comportement du soluté x par rapport aux alcanes normaux. Si $I_x - I_x^\circ$ est négatif le coefficient d'activité du soluté est supérieur à celui de l'alcane de même indice. La solubilité du composé x est alors moins bonne que celle de l'alcane. Si au contraire $I_x - I_x^\circ$ est positif, le coefficient d'activité de x est inférieur (meilleure solubilité) à celui de l'alcane. On démontre en effet que

$$\log \frac{\overset{\circ}{P_x}}{P_z^\circ} = k^\circ (I_x^\circ - 100z) - k\,(I_x - 100z)$$

Le coefficient d'activité du *n*-nonane a été déterminé par une méthode d'analyse frontale. Sa valeur est de: 1.30 à 80° —1.24 à 100° — 1.19 à 120°.

Le Tableau I montre pour les composés étudiés que la différence $I - I°$ est toujours positive. Pour les fonctions dans l'ordre croissant de retard d'élution on trouve



alcool $<$ cétone $<$ dérivé halogéné $<$ phénol
($\Delta I = 69$) ($\Delta I = 108$) ($\Delta I = 140$–145) ($\Delta I = 190$)

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Note

The chromatographic preparation of high-purity solutions of tetrasulfur dinitride

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The study of the chemistry of tetrasulfur dinitride, S_4N_2, has been severely hindered by the difficulties associated with obtaining pure S_4N_2. Known reactions deal mainly with hydrolysis or oxidation of contained sulfur[1–4].

The red compound, which melts at 23° (ref. 2), decomposes quite readily to sulfur and nitrogen in the solid state at 0° (ref. 5). However, S_4N_2 is more stable when kept as a solution in organic solvents[5]. This is further evidenced by the fact that the correct molecular formula was not determined until 1951 by Meuwsen[3] although the compound was probably first prepared in 1850[6].

Recent work by Nelson and Heal[7], as well as work in this laboratory[8], has shown that the compound is probably cyclic with the nitrogens in the one and three positions.

Presently, two methods are commonly employed for the synthesis of S_4N_2. The first method is a three-step synthesis involving the use of the salt $Hg_5(NS)_8$ (refs. 3 and 9) while the second entails the heating of tetrasulfur tetranitride, S_4N_4, and sulfur in CS_2 in an autoclave[10]. Both methods yield a complex mixture of products but are capable of producing pure S_4N_2 (ref. 7).

Since we desired to study the reactions of S_4N_2 with a large number of compounds it was desirable to develop a synthetic method that would lead to pure solutions of S_4N_2 in a relatively efficient and easy manner.

We achieved this goal by utilizing a less common method of S_4N_2 synthesis, that of refluxing tetrasulfur tetranitride and sulfur in toluene[3] with a subsequent use of column chromatography. Analysis of the solution was carried out by spectrophotometry and chemical analysis.

EXPERIMENTAL

Tetrasulfur tetranitride was prepared according to Jolly[11]. However, for further purification the S_4N_4 was washed twice with CS_2 (ref. 12), instead of recrystallization from benzene, taking advantage of differences in solubility of S_4N_4 and S_8 in CS_2

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* Taken in part from a thesis submitted by Ronald R. Adkins to the faculty of the University of Detroit in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

(ref. 13). The purity was determined by infrared (IR) spectroscopy[1] and melting point (182°, with explosion). The sulfur used showed no absorbance in the 400–4000 cm^{-1} region of the IR spectrum and was used without further purification.

The solvents toluene, carbon tetrachloride, and hexanes (ACS reagent chemical) were of reagent grade. They were further purified by refluxing over phosphorus pentoxide for 18 h and then distilled from calcium sulfate.

The silica gel powder, 60–200 mesh (J. T. Baker, Phillipsburgh, N.J., U.S.A.), used for columns was heated at 200° for 18 h prior to use[14].

Thin-layer plates were prepared from a slurry of 70 g silica gel GF-254 (Anspec, Ann Arbor, Mich., U.S.A.) and 200 ml chloroform. The plates were heated at 110° for 12 h prior to use.

Absorbances in the ultraviolet–visible range were read on a Beckman DB spectrophotometer.

The IR spectrum of the hexanes solution of S_4N_2 was observed in the potassium bromide region on a Perkin-Elmer Grating 457 spectrophotometer.

The S_4N_2 was synthesized as follows. Tetrasulfur tetranitride (2 g) and sulfur (4 g) were refluxed in 40 ml toluene. The refluxing was stopped after 18–22 h and the reaction mixture was allowed to cool to room temperature. The toluene was removed by a stream of dry nitrogen gas. The S_4N_2 was then dissolved in 5 ml of carbon tetrachloride and 5 ml of hexanes. The solution was then filtered through a sintered glass funnel and passed over a 0.37-m column (I.D. 2.5 cm) packed with 50 g silica gel powder in hexanes. The hexanes flow-rate was 5 ml/min.

Molecular sulfur was found to elute from the column approx. 8–10 cm ahead of the S_4N_2 band. However, a trace amount of sulfur was still leaving the column when the S_4N_2 began to elute. Therefore, the first 60 ml of the S_4N_2 fractions were discarded. The remaining S_4N_2 fractions were collected and found to be free of all contaminants as evidenced by thin-layer chromatography (TLC).

The collected S_4N_2 was analyzed by observing the absorbance of the solutions at $\lambda_{max.}$ = 377 and 455 nm[7] as a function of concentration (Fig. 1), with $\varepsilon_{max.}$ (377) =

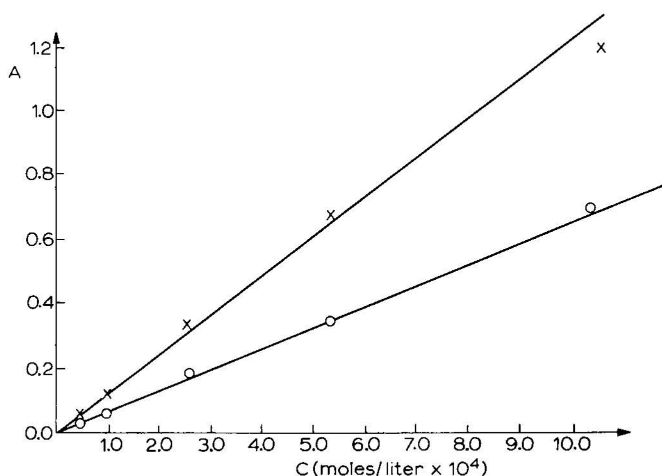

Fig. 1. Lambert-Beer law plot for S_4N_2 in hexanes. ×—×, 377 nm; O—O, 455 nm.

1296 and $\varepsilon_{max.}$ (455) = 670. Concentrations were determined by Kjeldahl analysis of the parent solution. This method produced 180–210 mg of S_4N_2.

RESULTS

TLC of the reaction mixture revealed five products when exposed to iodine vapors (Table I). R_F values were determined for carbon tetrachloride, hexanes and toluene. From Table I one can see that hexanes will effectively separate any S_4N_2 and sulfur from the other compounds.

TABLE I

R_F VALUES OF S_4N_4 AND S_8 REACTION PRODUCTS

Compound	*Solvent*		
	Carbon tetrachloride	*Hexanes*	*Toluene*
S_8	0.78	0.73	0.65
S_4N_2	0.65	0.28	0.62
S_4N_4	0.23	0.09	0.48
Unknown 1	0.45	0.10	0.61
Unknown 2	0.01	0.0	0.10

The absence of sulfur in the collected S_4N_2 was established by TLC. A TLC plate, developed in carbon tetrachloride, indicated the presence of no sulfur upon exposure to iodine vapors for 8 h. To determine the limit of detection of S_8 by the iodine vapor technique a series of sulfur solutions of known concentrations were made. Each solution was analyzed as above. The results showed that the limit of detection for sulfur by this method is 4×10^{-3} g per 250 ml of solution.

The results of the Kjeldahl analysis were 41.3 mg S_4N_2 for both 50-ml aliquots 1 and 2. It was assumed that two moles of NH_3 are produced from one mole of S_4N_2.

CONCLUSIONS

The present method produced S_4N_2 in smaller yields than the two more common methods of synthesis[3,10]. However, no attempts were made to find the conditions which give the maximum yields such as time of refluxing and the weight ratio $(S_4N_4)/(S_8)$.

The procedure obviates the need of autoclaves, sublimation or the synthesis of the salt $Hg_5(NS)_8$ and the compound $S_4N_4H_4$, and thus offers an attractive alternative to existing syntheses.

Quantitative analysis of S_4N_2 is possible as hexanes solutions of this dinitride closely follow the Lambert–Beer law, at wavelengths of 377 and 455 nm. The results demonstrate that the produced S_4N_2 is free of S_8 or any other contaminant.

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CHROM. 8282

Note

Simultaneous assay of methylphenobarbitone and phenobarbitone using gas–liquid chromatography with on-column butylation

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(Received February 6th, 1975)

The methods available for barbiturate analysis have recently been assessed by Kananen *et al.*[1], who advocated the use of a gas–liquid chromatographic (GLC) procedure involving methylation of the barbiturates with trimethylphenylammonium hydroxide[2]. While this technique is excellent for many barbiturates, it is unsuitable for the simultaneous determination of methylphenobarbitone (MPB) and phenobarbitone (PB) since these compounds yield a common 1,3-dimethyl product when methylated[3]. MPB is sometimes used as an anticonvulsant[4], and is known to be converted in the body to PB[5]. Therefore, a simultaneous quantitative determination of the two compounds is desirable. This has been achieved by GLC of the underivatized barbiturates[5–8], but such methods suffer from the widely acknowledged difficulties associated with chromatographing underivatized barbiturates[1,9]. Ethyl derivatives have been used[3], but we have not found them satisfactory for quantitation. Greeley[9] has recently described a technique for the formation of N-alkyl-barbiturates which did not involve on-column alkylation, and has demonstrated its application to the quantitative analysis of barbiturates in blood.

The present report describes a simple and convenient on-column butylation procedure for the assay of MPB and PB in plasma or serum which is based on the work of Kowblansky *et al.*[10] and does not suffer from the difficulties ascribed to on-column alkylation by Greeley[9].

EXPERIMENTAL

Materials

The barbiturates were obtained from the following pharmaceutical companies: phenobarbitone from Drug Houses of Australia, methylphenobarbitone from Sterling Pharmaceuticals (Sydney, Australia), and Alphenal from Analabs (New Haven, Conn., U.S.A.)*. Tetrabutylammonium hydroxide (TBAH) titrant (25% in methanol) was obtained from Eastman-Kodak, Rochester, N.Y., U.S.A. All other reagents were of analytical grade.

GLC was carried out on a Varian Model 2100 instrument, equipped with flame ionization detectors. Columns were 1.8 m × 2 mm I.D. Pyrex U-tubes, packed with 3% OV-101 on Chrom G HP, 100–120 mesh (Varian). The instrument parameters

* Generously donated by Warner-Lambert International, Morris Plains, N.J., U.S.A.

used were: nitrogen, 50 ml/min, hydrogen, 35 ml/min, air, 300 ml/min; the column oven temperature was 195° and the injector and detector temperatures were 275°.

Method

Plasma (1.0 ml), hydrochloric acid (1.0 ml; 0.2 *M*) and chloroform (5.0 ml, containing Alphenal, 3.0 mg/l) were added to a screw-capped Pyrex tube (150 × 20 mm), which was capped and shaken for 2 min. After centrifugation (2 min at 1000 *g*), the aqueous layer was aspirated and discarded, and the chloroform phase poured carefully into a Pyrex test tube with a ground-glass conical joint. The solvent was removed *in vacuo* using a Buchi Rotovapor R, and the tube flushed briefly with a stream of nitrogen to ensure complete removal of chloroform. The residue was taken up in TBAH (50 μl), and 1–2 μl were injected into the gas chromatograph, operated as described above.

Aliquots of MPB and PB were added to samples of Red Cross Blood Bank plasma to give single or mixed standard solutions ranging in drug concentration from 1.0–50.0 μg/ml. These were compared with aqueous standards, similarly prepared.

RESULTS

A typical chromatogram of an aqueous standard (MPB and PB, each 20 μg/ml) is shown in Fig. 1A, and the corresponding plasma standard in Fig. 1B. The retention times for the butylated barbiturates were: MPB, 4.4 min; PB, 8.3 min; Alphenal, 9.8 min. Comparison of plasma standards with aqueous standards gave recoveries for MPB and PB which were greater than 95%.

Calibration curves for plasma standards, using ratios of peak heights to concentration are given in Fig. 2. Linear regression analysis gave coefficients of determination (r^2) values for the two assays as 0.995 (MPB) and 0.996 (PB). The respective regression equations were

$$\text{MPB conc.} = 11.48 \times R_H \frac{\text{MPB}}{\text{Alphenal}}$$

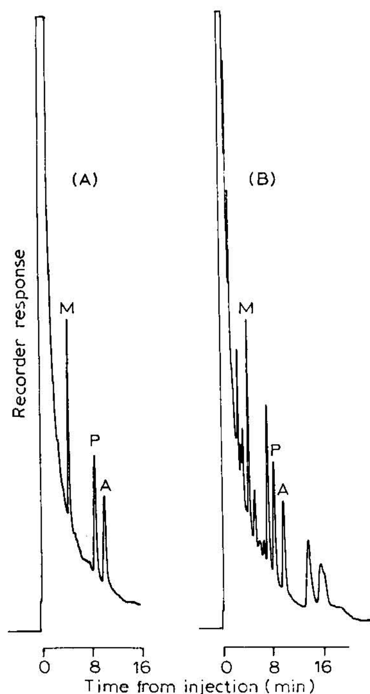
$$\text{PB conc.} = 16.97 \times R_H \frac{\text{PB}}{\text{Alphenal}} + 0.39$$

Table I gives the results of applying this technique to determining both MPB and PB in the plasma of patients taking the doses of MPB indicated. These assays were carried out when each patient had been taking the indicated dosage for at least one month, so that steady-state plasma concentrations had had time to develop.

DISCUSSION

Kowblansky *et al.*[10] showed that on-column N-butylation of xanthines and barbiturates could be effected using tetrabutylammonium hydroxide. They noted that the method offered some advantages over N-methylation, particularly with respect to selectivity. The present report describes one such application, in which simple, rapid quantitative simultaneous determination of MPB and PB is achieved.

Greeley[9] introduced an alkylation procedure which did not involve on-column derivatization. While that procedure has the significant advantage of providing a route to any N-alkyl derivative using the corresponding primary alkyl iodide, the on-



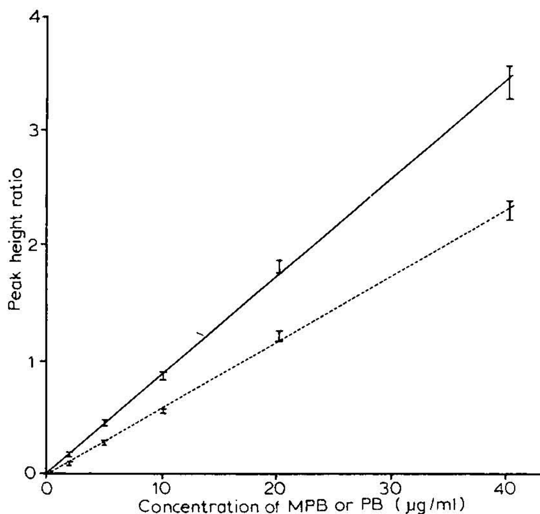


Fig. 1. Chromatograms from aqueous standard (A) and plasma standard (B), each containing MPB (peak M) and PB (peak P) at 20 µg/ml. The internal standard, Alphenal, gives peak A.

Fig. 2. Calibration curves for plasma standards of MPB (continuous line) and PB (broken line). Bars indicate one standard deviation either side of the mean of six determinations.

column procedure offers convenience and simplicity. We have not experienced problems with on-column butylation to the degree which Greeley reported. Although column life is certainly variable and unpredictable, one column has been in continuous use for nine months without loss of performance, and column conditioning with "Silyl 8" (Pierce, Rockford, Ill., U.S.A.) was needed only occasionally.

While we have applied the technique only to the determination of two barbiturates of particular interest, it is apparent that the procedure could be readily extended to include more barbiturates, possibly with the use of temperature programming, and a lower initial temperature than 195°.

Initial attempts at assaying plasma standards were made using expired blood (3–6 weeks' storage at 4°) from the Red Cross Blood Bank. However, all samples of these gave rise to a large peak which coincided with that of PB. It was subsequently shown that a freshly collected bag of blood lacked this peak, but it was present once the blood had been stored in the refrigerator for two weeks or more. Possibly the interfering peak was a plasticizer component which slowly dissolved from the bag[11], since storage for two weeks in glass of an aliquot of the blood removed from a bag immediately after collection produced no corresponding peak. This raised the possibility of false-positive values for PB if blood is collected in plastic tubes, but we have not observed this in the commonly used polypropylene tubes, even after extended storage periods.

The simplicity of the extraction technique used has been obtained at the cost of slowing the analysis due to the late peaks which arise from plasma (Fig. 1B).

TABLE I

STEADY-STATE CONCENTRATIONS OF MPB AND PB IN PLASMA OF PATIENTS TAKING MPB

Patient	*MPB dose (mg/day)*	*MPB (μg/ml)*	*PB (μg/ml)*	*DPH (μg/ml)**
1	90	1.0	18.6	16.8
2	180	1.4	22.6	20.9
3	90	0.7	11.1	20.8
4	120	0	6.0	6.2
5	240	2.2	16.9	27.9
6	600	4.3	35.1	8.5
7	180	0	7.8	8.6
8	120	4.4	6.5	5.4
9	90	0.7	7.2	13.2
10	45	0	1.2	2.4

* DPH = diphenylhydantoin; assayed separately by a minor modification of the method of MacGee[12].

However, samples can be injected at intervals of 15 min, which permits complete elution of these late peaks between successive samples.

Each of the barbiturates tested gave rise to two peaks under the butylation conditions used, but with the chromatographic conditions selected the "early" peaks were eluted in the solvent front. This is similar to the documented behaviour of PB when methylated[13], but does not affect the utility of the assay. We have not noted significant variations in the relative proportions of the two peaks from either MPB or PB when these were allowed to stand at room temperature for up to 3 h, although the corresponding effect has been reported to cause errors in the methylation of PB[14].

The results of determining steady-state plasma levels of MPB and PB in patients taking MPB (Table I) illustrate the utility of the technique. The levels of PB are markedly higher than those of MPB in most cases, and there is not a good correlation between MPB dose and the plasma level of either drug. Further studies on the metabolic fate of MPB are in progress, and will be reported separately.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the assistance of Misses L. M. Cotter and G. A. Smith in setting up the assay for routine use, and obtaining the data for Table I.

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CHROM. 8289

Note

Separation of some 16-androstenes on hydroxyalkoxypropyl-Sephadex (Lipidex™)

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(Received February 28th, 1975)

Interest has been aroused recently in the odoriferous 16-androstenes in view of their effects as pheromones in pigs[1]. In our studies on the metabolism and estimation of this group of steroids, it has become increasingly necessary to achieve a separation of the various compounds from each other. Chromatography on columns of alumina has been used extensively in the past with reasonable success[2] but this method does not resolve 5α-androst-16-en-3α-ol from 4,16-androstadien-3-one. Following the reports[3–5] that oestradiol, cortisol and 17-hydroxyprogesterone can be separated on Sephadex LH-20, similar methods were applied to mixtures of 16-androstenes using non-polar solvents such as benzene and *n*-pentane, but no separations have so far been achieved. Jänne *et al.*[6] have improved on the Sephadex LH-20 separation by using a lipophilic Sephadex derivative, hydroxyalkoxypropyl-Sephadex[7] (Lipidex™), which has allowed relatively non-polar steroids such as testosterone and progesterone to be resolved. This report describes how Lipidex™ can be utilized to separate some of the very non-polar 16-androstenes, such as 5α-androst-16-en-3-one and 4,16-androstadien-3-one.

EXPERIMENTAL

[5α-^{3}H]5α-Androst-16-en-3-one (15.01 Ci/mmole) was generously supplied by Dr. W. Hafferl, Syntex Research, Palo Alto, Calif., U.S.A. [5α-^{3}H]5α-Androst-16-en-3α- and -3β-ols were prepared from [5α-^{3}H]5α-androst-16-en-3-one by reduction with KBH_4 followed by separation by thin-layer chromatography[8]. [7α-^{3}H]Androsta-4,16-dien-3-one (125 Ci/mole) was synthesized by the method of Wilkinson *et al.*[9].

Lipidex™ (obtained from Packard-Becker B. W. Chemical Operations, Groningen, The Netherlands) was allowed to equilibrate for at least 24 h in the same solvent system as was to be used for elution. A glass column (I.D. 9 mm), fitted with a plug of fat-free cotton wool, was then packed with 12.5 g of equilibrated Lipidex™ in *n*-pentane–cyclohexane (99.5:0.5) and the gel allowed to settle by gravitation. The resulting gel column was 500 mm long. The labelled 16-androstenes, first separately and then as mixtures, were applied to the top of the column bed in 0.1 ml of the eluting solvent mixture. Elution was carried out with *n*-pentane–cyclohexane (99.5:0.5) with a flow-rate of 60 ml/h. Fractions (1 ml) were collected, transferred to counting pots and scintillant was added before radioactivity was measured in a liquid scintillation spectrometer.

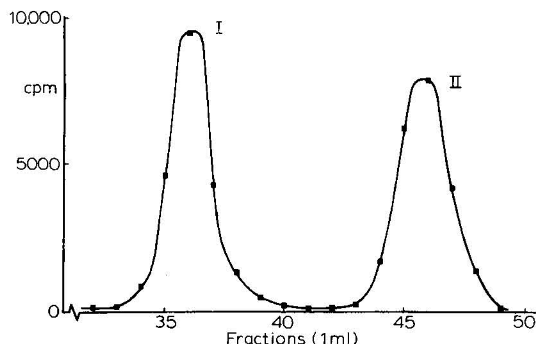

Fig. 1. Separation of [5α-^{3}H]5α-androst-16-en-3-one (I) from [7α-^{3}H]androsta-4,16-dien-3-one (II) on a column (length 500 mm) of Lipidex^{TM}. The eluting solvent used was *n*-pentane–cyclohexane (99.5:0.5).

RESULTS AND DISCUSSION

Fig. 1 illustrates a typical separation of two 16-androstenes, 5α-androst-16-en-3-one and 4,16-androstadien-3-one. Other 16-androstenes have been applied to Lipidex^{TM} using the same conditions as described above, and Table I summarizes the elution peak volumes and the elution fractions containing each steroid.

The column has been useful in separating 5α-androst-16-en-3-one and 4,16-androstadien-3-one extracted from human peripheral blood plasma samples. Approximately 70% recovery was obtained for each steroid, as shown by the addition of tracer amounts of each labelled steroid to the plasma prior to extraction. The resolution of 4,16-androstadienone and 5α-androst-16-en-3α-ol, not possible with alumina chromatography[2], has also been achieved with Lipidex^{TM}. This separation would be a particularly useful step to include in a radioimmunoassay method for each 16-androstene.

TABLE I

SEPARATION OF SOME 16-ANDROSTENES ON LIPIDEX^{TM} WITH *n*-PENTANE–CYCLOHEXANE (99.5:0.5) AS SOLVENT SYSTEM

Column height, 500 mm; flow-rate, 60 ml/h.

Steroid	*Approx. elution peak (ml)*	*Elution fraction (ml)*
5α-Androst-16-en-3-one	36	33– 40
4,16-Androstadien-3-one	46	43– 49
5α-Androst-16-en-3α-ol	99	87–111
5α-Androst-16-en-3β-ol	123	111–135

ACKNOWLEDGEMENTS

We are grateful to Dr. W. Hafferl for a gift of [5α-^{3}H]5α-androst-16-en-3-one and to Packard-Becker, Groningen, The Netherlands, for Lipidex^{TM}. The financial support of The Dunhill Trust is gratefully acknowledged.

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journal of chromatography news section

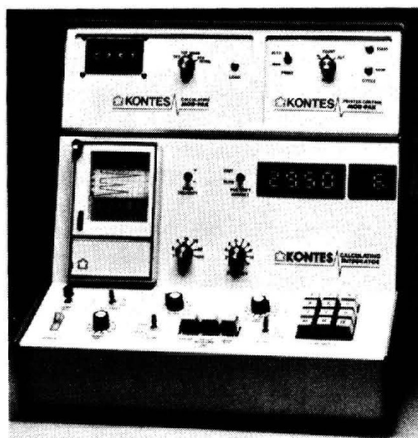
APPARATUS

N-563

TLC CALCULATING INTEGRATOR

The TLC Calculating Integrator from Kontes, is a practical data collection and reduction device designed especially for TLC applications. The solid state circuitry allows selection of any of 12 integration rates to match rapid scanning times. Suitable rates can also be selected for gas or liquid chromatography.

The K-21 Integrator has a built-in strip-chart recorder to provide graphic as well as digital pictures of spot-readings from any TLC scanner, a memory for storage of data for 12 peaks and 12 companion memories with thumbwheel entry for dialing-in constants, standards, or variable input for direct calculation of percentage.



N-568

UV CHROMATOGRAM CAMERA

Alphametrics have introduced an integrated photographic system for use with PC and TLC chromatograms under UV radiation. The Model 33 ultraviolet chromatogram camera is designed for UV fluorescence and absorption photographs. Chromatograms up to 20-cm square can be photographed by either reflected or transmitted UV radiation from selectable 254-nm and 365-nm sources. The camera has a photographic/viewing cabinet designed for convenient access and viewing. Other features include interchangeable 35mm SLR and Polaroid camera backs, quartz lens with transmission down to 200 nm, variable magnification, 6-position barrier filter wheel and electronic exposure control.

N-566

MICROCOLUMN AMINO ACID ANALYZER

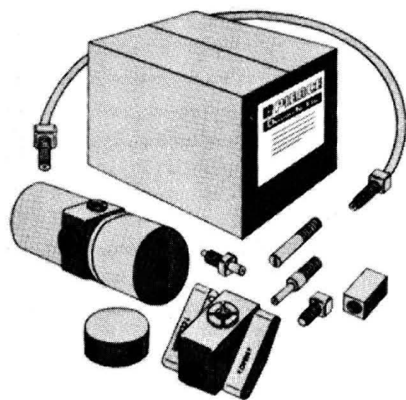
A ten-page Bulletin SB-430 describes Beckman's Microcolumn Model 121M amino acid analyzer which uses 2.8-mm diameter microbore columns. The Model 121M cuts analysis time, requires 1/10 of the normal sample and reagent volumes, and produces 20 times the sensitivity of a conventional amino acid analyzer.

For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

N-651

MINIATURE TUBING SYSTEM

A series of chemically and biologically inert miniature tubing components is available from Pierce. Pressure rated to 500 p.s.i., the system uses standard 1.5 mm–0.3 mm I.D. microbore Teflon® tubing with zero dead volume connectors, couplers and adaptors. Slide valves, sample injection valves, solenoid valves and actuators are available for manual and automatic switching capabilities.



N-636

SPECTRAL SENSING UNIT FOR FLUORESCENCE

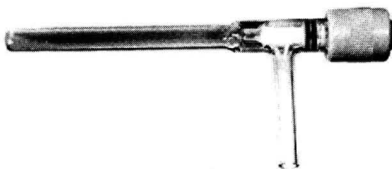
Scans of emission spectra of fluorescing compounds separated chromatographically on thin-layer plates can now be obtained with the Schoeffel SD 3000 spectrodensitometer using an attachable spectral-sensing unit, the SDA 600. Fluorescence excited by monochromatic light beam from the illuminating prism monochromator is focused onto the entrance slit of a grating monochromator equipped with a motor wavelength drive and, optionally, with an analog output to drive the X-axis of an X-Y recorder. The light transmitted by the analyzing monochromator is detected by a side-on S-5 photomultiplier tube (tubes with extended sensitivity range are available).

High-quality mirror and quartz optics, careful focalization, an efficient monochromator, use of blocking filters in the excitation and emission light path and a selected PMT result in a sensitive, low-stray light detecting system which enables recording fluorescence spectra in the wavelength range from short UV to near IR from sample quantities in nanogram range.

N-654

VACUUM REACTION TUBE

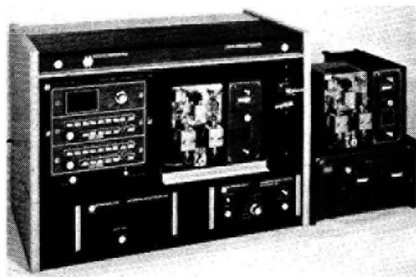
A re-usable vacuum reaction tube is available from Pierce. The tube can be used for protein hydrolysis, lyophilization, hydrazinolysis, and other applications requiring a vacuum environment. It is equipped with a Teflon® plunger which turns into the neck of the tube, and seals against an internal shoulder, thereby providing an inert, efficient vacuum seal. After the desired reaction is complete, the plunger knob can be turned to retract the plunger for sample removal. Because they remain intact, they may be re-used many times. The tubes may be used in ovens to 110° and in heaters to higher temperatures. Three sizes are available.



N-669

ABSORBANCE DETECTOR

Waters Associates' multiwavelength (UV and visible) Model 440 absorbance detector for liquid chromatography offers flow stability (by correcting for transient RI effects) and can be used to monitor flow-programmed LC separations. Available with single or dual channels, the Model 440 also has pushbuttons to allow the chromatographer to choose any of nine sensitivities from 0.005 a.f.s.–2.0 a.f.s. with full linearity over a wide sensitivity range. If the dual-channel model is used, the chromatographer can simultaneously monitor the separation at two different wavelengths and/or two different sensitivities–or measure the difference in absorbance between two wavelengths. This permits quantitative results on both major constituents and trace compounds without attenuation changes. The dual-channel model can also be used as two separate detectors.



N-671

STACK MONITORING SYSTEM

A 6-page brochure is available from Tracor Instruments, describing the state-of-the art techniques in stack gas analysis of sulfur compounds. The brochure describes the origin of predominant sulfur compounds developed by both petroleum and pulp and paper industries and their effect on environmental pollution. Also described is Tracor's stack monitoring system for sulfur compounds including operating principles, column technology, flow schematics, and chromatograms showing separation and analysis of H_2S, SO_2, CH_3SH, DMS, and DMDS at stack gas concentrations.

N-640

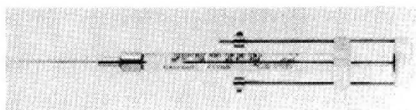
MILLIPORE BULLETIN (IN DUTCH)

"Millipore-nieuws" from Millipore describes their 13-mm (membrane diameter) pressure cell for molecular separations such as virus purification and concentration, protein concentration and de-salting, and deproteinization. The Pellicon molecular filtrate ion cell is designed for use with small samples requiring quick yet careful handling. The cell is autoclaveable and the membrane can undergo chemical sterilization. A magnetic stirrer just above the membrane's surface helps keep this part of the apparatus clean.

N-667

PORTABLE GC

The Model 511 portable flame ionization gas chromatograph from Analytical Instrument Development can accept several new detector and valve options. The electron capture detector option is used primarily for SF_6 tracer studies, freon, and low level chlorine analysis. The thermal conductivity detector is used for general gas chromatographic analysis in addition to the standard Orsat type analysis including methane. The addition of Ni Catalyst system to a standard flame ionization detector provides a system that will analyze sub-ppm levels of carbon monoxide, methane and total hydrocarbons. By adding different valve configurations to detector options increased flexibility of analysis can be achieved.



N-575

SYRINGES FOR HPLC

A series of gas- and liquid-tight syringes has been designed by Hamilton Company, primarily for high pressure liquid chromatography.

Available in 50-µl, 100-µl, 250-µl and 500-µl capacities, these syringes withstand routine inlet pressures from 2000 p.s.i. to 3000 p.s.i.

Special features include a metal hub to use in holding and steadying the syringe during insertion, a plunger guide to prevent bending, and stops to prevent plunger blow-out during injection.

N-569

LONG PATH ELECTROPHORESIS CELL

E-C Apparatus Corp. have introduced a Long Path Vertical Gel Slab electrophoresis cell with a migration path of 44cm. The slab area is 17 cm x 44cm and will accept up to 16 samples per separation. All E-C Vertical Gel Slab Cells are available with 1.5mm to 9mm gel-slab thickness spacing and have intimate cooling of the gel slab. The EC480 Long Path Cell complements the series of E-C Slab Units (Standard EC470, 17cm x 12cm slab area and Survey EC490, 17cm x 24cm). One or a combination of the E-C Vertical Gel Slab Cells permit continuous, discontinuous, two-dimensional, gradient and isoelectric focusing separations.

CHEMICALS

N-564

ANALYSIS OF VINYL CHLORIDE

A column packing for gas chromatography of vinyl chloride monomer in air at the ppm level from Supelco consists of 0.4% Carbowax 1500 on Carbopack A (graphitized carbon black). A technical bulletin (744) is available.

N-562

WALL CHARTS FOR ELECTROPHORESIS

Gelman has prepared a set of four charts showing Electrophoretic Interpretative Patterns in two colors with Normals and Abnormals overlapped. By printing Abnormals in red over the Normal patterns outlined in black, the relationship between the two is effectively and dramatically shown. Four critically important areas of clinical electrophoresis are covered by the charts, including serum proteins (chart E^1), hemoglobins (chart E^2), lipoproteins (chart E^3), and LDH isozymes (chart E^4). The charts are suitable for laboratory posting and regular reference during interpretation of electrophoretic patterns.

N-584

LIPOPROTEIN ELECTROPHORESIS REAGENT SET

Lipoprotein Electrophoresis Reagent Set as well as a series of equipment are available from Gelman. An important part of the procedure is the lipoprotein electrophoresis stain and rinse solution set including an Oil Red 0 solution and a lipoprotein rinse. The procedure is described in Gelman Technical Bulletin 21 on Lipoprotein Electrophoresis.

PROCEDURES

N-586

APPLIED SCIENCE LABS. CATALOG

Catalog 18 (1975) from Applied Science Labs. contains sections dealing with: chromatographic products (GC, LC, TLC) biochemicals and standards (drugs, exotic lipids, fatty acids, pesticides and steroids), isotope-labelled chemicals (14-C and 13-C; also custom synthesized), pilot plant services (also custom synthesized) and reagents (clinical, esterification, silylation).

PUBLICATION SCHEDULE FOR 1975

Journal of Chromatography (incorporating *Chromatographic Reviews*)

MONTH	D 1974	J	F	M	A	M	J	J	A	S	O	N	D
JOURNAL	101/1 101/2 102	103/1 103/2 104/1	104/2 105/1 105/2	106/1 106/2	107/1 107/2	108/1 108/2	109/1 109/2	110/1 110/2	111/1	111/2		112	114/1 114/2
REVIEWS*				113/1					113/2		113/3		

* Volume 113 will consist of *Chromatographic Reviews*. The issues comprising this volume will not be published consecutively, but will appear at various times in the course of the year.

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

Types of Contributions. (a) Original research work not previously published in a generally accessible language in other periodicals (Full-length papers). (b) Review articles. (c) Short communications and Notes. (d) Book reviews; News; Announcements. (e) Bibliography of Paper Chromatography, Thin-Layer Chromatography, Column Chromatography, Gas Chromatography and Electrophoretic Techniques. (f) Chromatographic Data.

Submission of Papers. Three copies of manuscripts in English, French or German should be sent to: Editorial office of the Journal of Chromatography, P.O. Box 681, Amsterdam, The Netherlands. For *Review articles*, an outline of the proposed article should first be forwarded to the Editorial office for preliminary discussion prior to preparation.

Manuscripts. The manuscript should be typed with double spacing on pages of uniform size and should be accompanied by a separate title page. The name and the complete address of the author to whom proofs are to be sent should be given on this page. Authors of papers in French or German are requested to supply an English translation of the title. A short running title of not more than 50 letters (including spaces between the words) is also required for Full-length papers and Review articles. All illustrations, photographs, tables, etc., should be on separate sheets.

Heading. The title of the paper should be concise and informative. The title should be followed by the authors' full names, academic or professional affiliations, and addresses.

Summary. Full-length papers and Review articles should have a summary of 50–100 words. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes will be published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Particular attention should be paid to the size of the lettering to ensure that it does not become unreadable after reduction. Sharp, glossy photographs are required to obtain good halftones. Each illustration should have a legend, all the *legends* being typed together on a *separate sheet*. Coloured illustrations are reproduced at the author's expense.

References. References should be numbered in the order in which they are cited in the text and listed in numerical sequence on a separate sheet at the end of the article. The numbers should appear in the text at the appropriate places using superscript numerals. In the reference list, periodicals[1], books[2], and multi-author books[3] should be cited in accordance with the following examples:

1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 R. D. Marshall and A. Neuberger, in A. Gottschalk (Editor), *Glycoproteins*, Vol. 5, Part A, Elsevier, Amsterdam, 2nd ed., 1972, Ch. 3, p. 251.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*.

Proofs. Two sets of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints of Full-length papers, Short communications and Notes will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

News. News releases of new products and developments, and information leaflets of meetings should be addressed to: The Editor of the News Section, Journal of Chromatography, Elsevier Scientific Publishing Company, P.O. Box 330, Amsterdam, The Netherlands.

Subscription orders. Subscription orders should be sent to: Elsevier Scientific Publishing Company, P.O. Box 211, Amsterdam, The Netherlands.

Publication. The *Journal of Chromatography* (including *Chromatographic Reviews*) appears fortnightly and has 14 volumes in 1975. The subscription price for 1975 [Vols. 101–114 and Supplementary Vol. 4 (Bibliography of Electrophoresis 1968–1972)] is Dfl. 1365.00 plus Dfl. 120.00 (postage) (total US$ 631.91). Subscribers in the U.S.A., Canada and Japan receive their copies by air mail. Additional charges for air mail to other countries are available on request. Back volumes of the *Journal of Chromatography* (Vols. 1 through 100) are available at Dfl. 100.00 (plus postage).

Advertisements. Advertisement rates are available from the publisher on request. The Editor of the journal accepts no responsibility for the content of the advertisements.

DESAGA
Gradient TLC
acc. to STAHL

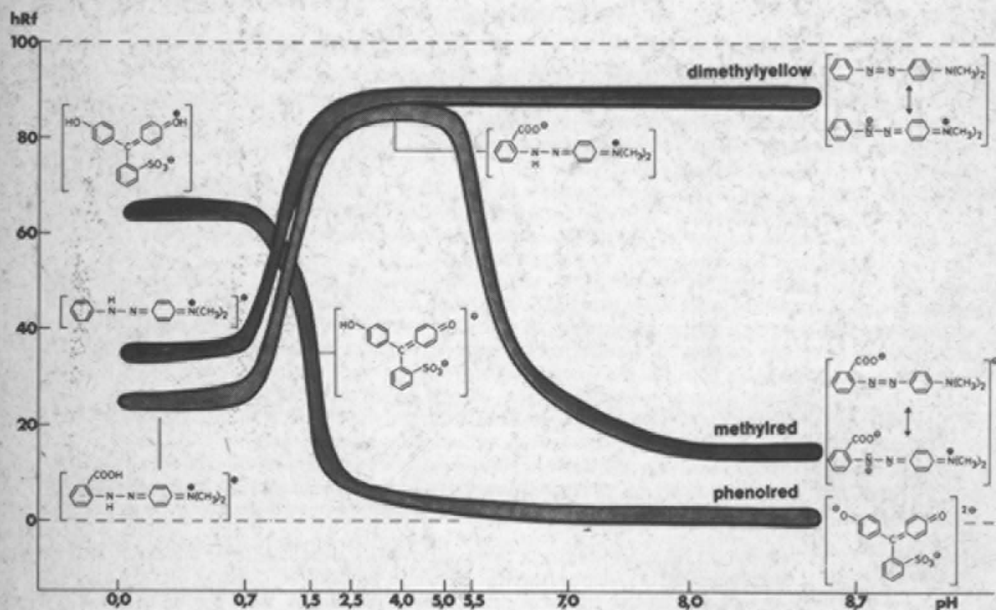


Fig. 1
pH-T-Chromatogram of indicator dyes

Gradient TLC uses a stationary phase with a continuous transition of separation properties.

Gradient layers are easily made by means of the DESAGA Gradient Spreader.

If the chromatography is carried out perpendicular to the gradient, then there are on one plate numerous "open separation columns" lying next to each other, each different in its characteristics.

Each substance is characterized by its own profile. The above separation shows the typical profiles of three different indicator dyes in a pH-gradient.

Some application examples:

pH-gradient for differentiating alkaloids;
silver-nitrate gradient for determining optimal impregnation
agar or gelatine gradient layers in growth retardmend tests.

Please ask for product information!

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