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JOURNAL *of* CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

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DERIVATION OF THE DIFFERENTIAL HEAT OF DISSOLUTION OR ADSORPTION FROM GAS–LIQUID CHROMATOGRAPHIC MEASUREMENTS

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(Received March 5th, 1970)

SUMMARY

Taking into account the non-ideality of the gas phase, this paper develops a theory for the determination of the differential heat of dissolution, ΔH_S, from gas–liquid chromatography measurements. When ΔH_S is determined over a small interval of temperature, the calculation of ΔH_S can be made from measurements of retention times only without using the retention volumes. Using a carefully designed equipment, an accuracy in ΔH_S of better than 1% could be reached when ΔH_S is measured over an interval of temperature of one degree.

INTRODUCTION

The activity coefficient, γ, is the primary point of comparison between solution thermodynamics theory and experimental results in gas–liquid chromatography (GLC). However, it is exceedingly difficult to measure γ with a good accuracy because of the many experimental parameters which have to be determined. For most of them an accuracy better than 0.5% is difficult to achieve, whereas retention times could be measured within 0.01%. Consequently, the direct accurate measurement of the differential heat of dissolution, ΔH_S, at infinite dilution from only measurements of retention times, could be interesting.

As is so often the case when a particular physical property is being measured, interference from other effects in the system under study must be taken into account. This applies to GLC because the retention is additionally affected by interactions in the gas phase so that a correction must be used. The error is otherwise in the range 1–5% for most systems encountered in GLC.

Usually, the excess enthalpy of mixing, ΔH_e, is derived from measurements of γ at two or more different temperatures. Extrapolation of the observed retention volumes to zero flow rate is required to determine the absolute retention volume; furthermore, a precise value of the amount of stationary phase is necessary to calculate

the specific retention volume. Instead of this delicate method, this paper gives an accurate method for determining ΔH_S from only the retention times which are much easier to determine with a great accuracy.

THEORY

When the non-ideality of the gas phase is taken into account, it has been shown[1,2] that the actual partition coefficient, k_0, extrapolated to zero pressure from static measurements of vapour pressure, can be expressed by the relationship

$$\ln k_0 = \ln k_0^* - \frac{P_2^0}{RT}(B_{22} - V_2') = \ln k_0^* + \varphi \tag{1}$$

where k_0^* is the partition coefficient between the liquid phase and an ideal carrier gas:

$$k_0^* = \frac{n_3' RT}{\gamma_2^0 P_2^0 V_3'} \tag{2}$$

T = the column temperature (°K);
P_2^0 = the vapour pressure of the pure liquid solute at T;
B_{22} = the second virial coefficient of the pure gas solute at T;
V_2' = the molar volume of the pure liquid solute at T;
V_3' = the molar volume of the stationary phase at T;
γ_2^0 = the activity coefficient of the solute in the liquid phase extrapolated to zero pressure;
R = the ideal gas constant;
n_3' = the number of moles of stationary phase in the column.

In all the following, the subscripts 1, 2 and 3 correspond to the carrier gas, the solute and the stationary phase, respectively.

Thermodynamic theory[3] requires that

$$\frac{\mathrm{d}[\ln(\gamma_2^0 P_2^0)]}{\mathrm{d}T} = \frac{\Delta H_S}{RT^2} \tag{3}$$

so that

$$\frac{\mathrm{d}\left[\ln\left(\frac{k_0^*}{T}\right)\right]}{\mathrm{d}\left[\frac{1}{T}\right]} = \frac{\Delta H_S}{R} - \frac{\mathrm{d}[\ln V_3']}{\mathrm{d}\left[\frac{1}{T}\right]} \tag{4}$$

It has also been shown[1,2] that k_0 can be determined from chromatographic measurements by means of the relationship

$$k_0 = \frac{t_R' \cdot D_o \cdot j}{V_3'(1+\beta)} \tag{5}$$

with

$$\beta = \frac{2B_{12} - V_2'}{RT} P_o J\left(\frac{4}{3}\right)$$

t_R' = the corrected retention time of the solute or difference between the solute and the air retention times;

D_o = the flow rate of the carrier gas measured at the outlet pressure P_o of the column and at the temperature T of the system;

j = James and Martin's coefficient;

$J\left(\frac{4}{3}\right)$ = the pure number $\frac{3[(P_i/P_o)^4 - 1]}{4[(P_i/P_o)^3 - 1]}$;

B_{12} = the second virial coefficient of the gas mixture. As a first approximation, $2\,B_{12} \simeq B_{11} + B_{22}$.

In the following, we shall assume that the column inlet and outlet pressures are carefully controlled. Accordingly, j is constant and does not change with variations in column temperature. Thus, from eqn. 5

$$\frac{d\left[\ln\left(\frac{k_0}{T}\right)\right]}{d\left[\frac{1}{T}\right]} = \frac{d\left[\ln\left(\frac{t_R' \cdot D_o}{T}\right)\right]}{d\left[\frac{1}{T}\right]} - \frac{d[\ln(1+\beta)]}{d\left[\frac{1}{T}\right]} - \frac{d[\ln V_3']}{d\left[\frac{1}{T}\right]} \tag{6}$$

Since usually P_o is 1 atm and the maximum value of β is $3\,P_o J\left(\frac{4}{3}\right) \cdot 10^{-2}$, for the extreme case of carbon dioxide used as the carrier gas[1,2], it can be written from eqns. 1 to 6 that

$$R\frac{d\left[\ln\left(\frac{t_R' \cdot D_o}{T}\right)\right]}{d\left[\frac{1}{T}\right]} = \Delta H_S + R\frac{d\varphi}{d\left[\frac{1}{T}\right]} + R\frac{d\beta}{d\left[\frac{1}{T}\right]} = \Delta H_S + C_1 + C_2 \tag{7}$$

We shall now calculate these two correction factors C_1 and C_2.

Estimation of $C_1 = R\frac{d\varphi}{d\left[\frac{1}{T}\right]}$

φ has been defined in eqn. 1. The solute vapour pressure is usually well fitted by a Clapeyron's relationship in a narrow temperature range.

$$P_2^0 = \exp\left(-\frac{\Delta H_v}{RT} + A\right) \tag{8}$$

so that

$$\frac{dP_2^0}{d\left[\frac{1}{T}\right]} = -\frac{\Delta H_v}{R}P_2^0 \tag{9}$$

To calculate a correction term, eqn. 8 is convenient. For a more precise calculation the use of an Antoine's relationship may become necessary. This would introduce a term $T^2/(T + C)^2$ in eqn. 9. From Berthelot's state equation[4]

$$B_{22} = \frac{9\,RT_c}{128\,P_c}\left\{1 - 6\left(\frac{T_c}{T}\right)^2\right\} \tag{10}$$

so that

$$\frac{dB_{22}}{d\left[\frac{1}{T}\right]} = -\frac{27}{32}\frac{RT_c^3}{P_c T} \tag{11}$$

The variation in density of the pure liquid solute with temperature can be written[5]

$$\varrho = A - BT^* - \frac{C}{E - T^*} \tag{12}$$

(In ref. 5 the numerical values of A, B, C, E are given for T^* in °F and ϱ in g/cm³. In this paper T is always in °K). Thus

$$\frac{dV_2}{d\left[\frac{1}{T}\right]} = -\frac{9}{5} M \frac{T^2}{\varrho 2} \left(B + \frac{C}{(E - T^*)^2}\right) \tag{13}$$

Thus from eqns. 1 and 8–13

$$C_1 = R \frac{d\varphi}{d\left[\frac{1}{T}\right]} = P_2^0 (B_{22} - V_2') \left(\frac{\Delta H_v}{RT} - 1\right)$$

$$+ \frac{27}{32} \frac{P_2^0}{P_{c,2}} RT_{c,2} \left[\frac{T_{c,2}}{T}\right]^2 - P_2^0 \frac{9\,MT}{5\,\varrho^2} \left[B + \frac{C}{(E - T^*)^2}\right] \tag{14}$$

$$C_1 = K + L - N$$

Example: For hexane at $T = 69$°C, the vapour pressure derived from eqn. 8 is 1 atm.

$P_2^0 = 1$ bar; $P_{c,2} = 30.3$ bars; $T_{c,2} = 507$°K; $A = 0.7198$; $B = 46.10^{-5}$; $C = 12.6$; $E = 516.2$; $K \# -296$ cal; $L \# 62.5$ cal; $N \# 1.68$ cal.
$C_1 \# -235$ cal/mole, *i.e.* about 3% of ΔH_S.

Numerical values calculated from data in ref. 5 are given in Table I for other compounds.

TABLE I

CORRECTIONS[a] FOR NON-IDEAL BEHAVIOUR IN THE GAS PHASE[b]

Compounds	*Boiling point (°C)*	K	L	N	C_1	C_2	$\frac{dC_1}{dT}$	$\frac{dC_2}{dT}$
n-Hexane	69	296	62.5	1.68	−235	−102	−4.7	0.6
n-Nonane	150.7	417	85.1	2.99	−334	−134	−5.6	0.6
Cyclopentane	49.1	125	25.2	1.03	−101	− 47.5	−2.2	0.3
Cyclohexane	80.6	268	56.1	1.27	−213	− 92.1	−4.1	0.5
Benzene	80.0	238	48.9	1.01	−191	− 81.1	−3.9	0.5
o-Xylene	144.3	386	77.6	1.63	−310	−122	−5.5	0.6

[a] K, L, N, C_1, C_2 in cal/moles; dC_1/dT, dC_2/dT in cal/mole × °K.
[b] The corrections are calculated at the boiling point of each compound.

Estimation of $C_2 = R \frac{d\beta}{d\left[\frac{1}{T}\right]}$

As it has been proved by the numerical example above, the contribution of the volume term is negligible so that

$$C_2 = P_o J\left(\tfrac{4}{3}\right) \left[B_{11} + B_{22} - V_2' - \frac{27\,R}{32\,T^2} \left\{\left(\frac{T_c^3}{P_c}\right)_1 + \left(\frac{T_c^3}{P_c}\right)_2\right\}\right] \tag{15}$$

Example: For hexane at $T = 69°C$ and $P_o J(\frac{4}{3}) = 1$ bar, with carbon dioxide as carrier gas, which is the most unfavourable case. $C_2 \# -102$ cal/mole, *i.e.* about 1% in ΔH_S. Numerical values are given in Table I for other compounds.

CALCULATION OF ΔH_S FROM RETENTION TIMES

Eqn. 7 shows that we could calculate ΔH_S from simple measurements of corrected retention times at various temperatures if we could assume that in an interval of temperature (T_1,T_2), very small compared to T, the RHS terms of eqn. 7 are constant. Let

$$\delta T = T_2 - T_1 \tag{16}$$

This will be proved below. Then, integrating this equation between temperatures T_1 and T_2

$$R \ln \frac{t_R'(T_1) \cdot D_o(T_2) \cdot T_1}{t_R'(T_2) \cdot D_o(T_1) \cdot T_2} = \left(\frac{1}{T_2} - \frac{1}{T_1}\right)(\Delta H_S + C_1 + C_2) \tag{17}$$

Over this small temperature interval, we can assume that the variation of viscosity with temperature is given by

$$\eta = K T^{5/6} \tag{18}$$

Among other types of equations that have been proposed, this equation gives the best results to take into account the change in viscosity with respect to temperature[6]. Over a small interval of temperature, the validity of this equation might be better than 0.1%.

From Darcy's law[6], it can be written

$$D_o(T) = \frac{\lambda}{2\eta L} \frac{P_i^2 - P_o^2}{P_o} \tag{19}$$

in which λ depends on the column permeability and gas crosssection. As we assume that the inlet and outlet pressures of the column are controlled at values independent of the column temperature, we have

$$\frac{D_o(T_2)}{D_o(T_1)} = \frac{\eta(T_1)}{\eta(T_2)} = \left(\frac{T_1}{T_2}\right)^{5/6} \tag{20}$$

and noting that

$$\ln\left(\frac{T_1}{T_2}\right) \# -\frac{\delta T}{T} \tag{21}$$

with an error of less than 0.15%. Then eqn. 17 becomes

$$\Delta H_S = \frac{-T_2^2}{\delta T} R \cdot \ln \frac{t_R'(T_1)}{t_R'(T_2)} + \frac{11}{6} RT_2 - (C_1 + C_2) \tag{22}$$

Whenever it is possible with a great precision to measure retention times at very close temperatures, eqn. 22 allows the calculation of ΔH_S. Moreover, if δT is sufficiently small, it becomes possible to carry out measurements of ΔH_S for different such small temperature intervals, and, thus, the variation of ΔH_S with respect to temperature can be studied.

We shall now discuss the validity of the assumption that the *RHS* terms of eqn. 7 are not temperature dependent in a small temperature range.

TEMPERATURE DEPENDENCE OF THE *RHS* TERMS OF EQN. 7

This is best studied by calculating the temperature coefficients of C_1, C_2 and ΔH_S.

Variation of C_1 with T

As shown above the variation of the volume term with T, in eqn. 14, can be neglected. We can write

$$C_1 = P_2^0 (B_{22} - V_2') \left(\frac{\Delta H_v}{RT_2} - 1\right) + \frac{27}{32} \frac{P_2^0}{P_{c,2}} RT_{c,2} \left(\frac{T_c}{T_2}\right)^2 \quad (23)$$

Differentiation of eqn. 23 gives

$$\frac{dC_1}{dT} = P_2^0 (B_{22} - V_2') \frac{\Delta H_v}{RT_2^2} \left(\frac{\Delta H_v}{RT_2} - 2\right) + \frac{27}{32} \frac{P_2^0}{P_{c,2}} R \left(\frac{T_{c,2}}{T_2}\right)^3 \left(\frac{2\Delta H_v}{RT_2} - 3\right) \quad (24)$$

This coefficient is usually small as shown by the following example.

Example: For hexane at $T_2 = 69°C$

$$\left|\frac{dC_1}{dT}\right| \# 4.6 \text{ cal/degree}$$

For $\delta T = 1°C$, this value results in a variation of about 2% of C_1 and less than 0.1% of ΔH_S. Other numerical results are given in Table I.

Variation of C_2 with T

Differentiation of eqn. 15 gives

$$\frac{dC_2}{dT} = \frac{81}{32} J(\tfrac{4}{3}) R \left\{\left(\frac{T_c}{T_2}\right)_1^3 \left(\frac{P_o}{P_c}\right)_1 + \left(\frac{T_c}{T_2}\right)_2^3 \left(\frac{P_o}{P_c}\right)_2\right\} \quad (25)$$

This coefficient is small because P_o is much smaller than P_c in most cases, whereas T_c/T_2 is most often smaller than 2.

Example: For hexane at $T_2 = 69°C$ and $P_o J(\frac{4}{3}) = 1$ bar with CO_2 as carrier gas

$$\left|\frac{dC_2}{dT}\right| = 0.6 \text{ cal/degree}$$

This is only 0.6% of C_2 and less than 0.01% of ΔH_S for $\delta T = 1$. With the more conventional carrier gases such as H_2, He, N_2 and A, this coefficient would be still smaller.

Variation of ΔH_S with T

Generally, over a large temperature range, ΔH_S decreases with increasing temperature at a rate of about 5 to 10 cal/degree. (From ref. 5 it results that the variations in ΔH_v are of this magnitude.) Thus the variation of the *RHS* of eqn. 7 is smaller

than 20 cal, about 0.2% in ΔH_S for a temperature variation of 1°C. It will be shown in the next section that the error in the determination of ΔH_S which can be expected from a carefully prepared chromatographic apparatus is much larger than 0.2%.

Validity of Berthelot's state equation

Berthelot's state equation is a relationship with two constants. These constants can be determined for most components by applying the law of corresponding states which postulates that the ratio $P_c V_c / RT_c$ is constant for all compounds. This however is not true, and the ratio may vary in the range 5–10% for most hydrocarbons and carrier gases encountered in GLC (*cf.* Table II). Consequently an error of this order of magnitude on B_{11} or B_{22} may be expected.

TABLE II

COMPARISON BETWEEN THE EXPERIMENTAL AND CALCULATED VALUES OF THE SECOND VIRIAL COEFFICIENT[a]

Compound	*T (°K)*	B_{22} *(cm³)*	B_{22}' *(cm³)*	$\frac{B_{22} - B_{22}'}{B_{22}}$ *(%)*
		Experimental	*Calculated*	
Benzene	366.5	1046.6	945.2	4.5
	422.0	662.8	646.0	2.5
n-Hexane	366.5	1144.9	1029.9	11.2
	399.8	1039.2	849.5	18.9
Propylene	310.9	307.6	334.9	−8.4
	333.1	278.1	257.0	7.9
Acetylene	277.6	199.4	185.4	7.3
	294.3	167.9	161.8	3.6

[a] The calculated value is obtained from eqn. 10 and critical data from ref. 5. The observed value is derived from experimental data taken in ref. 9; $\log f/p$ is plotted *vs.* P and B_{22} calculated from the slope of the straight line obtained.

Table II shows a comparison between values of the second virial coefficient calculated by eqn. 10 for various compounds and values derived from experimental data on the compressibility of their vapour[9]. The accuracy of these experimental values may be estimated to 8%. The deviation is important only for *n*-hexane at 126°C, *i.e.* at temperatures much above the boiling point.

The data in Table II are in agreement with a possible error of 5 to 10% on B_{22}, arising from the use of eqn. 10. Since the correction terms C_1 and C_2 amount to about 4% of ΔH_S and depend mainly on B_{11} and B_{22}, the use of an approximative state equation introduces a systematic error of less than 0.4% in the measurement of ΔH_S.

PRECISION OF THE DETERMINATION OF ΔH_S

The temperature interval T_2–T_1 is very small; consequently $t_R'(T_1)$ and $t_R'(T_2)$ are very near. To derive the error on ΔH_S calculated from experimental data using eqn. 22, we shall let

$$t_R'(T_1) = t_R'(T_2) + \delta\tau \tag{26}$$

Expanding the logarithm in eqn. 22, we obtain

$$\Delta H_S \# \frac{-RT_2^2}{t_R'(T_2)} \cdot \frac{\delta\tau}{\delta T} + \frac{11}{6} RT_2 - (C_1 + C_2) \tag{27}$$

It is obvious that the preponderant error in ΔH_S comes from the $\delta\tau/\delta T$ terms which are both small differences between large quantities. If θ denotes absolute errors:

$$\frac{\theta(\Delta H_S)}{\Delta H_S} \# \frac{\theta\left(\frac{\delta\tau}{\delta T}\right)}{\frac{\delta\tau}{\delta T}} = \frac{\theta(\delta T)}{\delta T} + \frac{\theta(\delta\tau)}{\delta\tau} \tag{28}$$

The error on the absolute temperatures T_1 and T_2 may be 0.1°K because it is difficult to calibrate better a thermometer in the thermodynamical scale[7]. It is easier, however, to measure temperature differences, since the calibration error is then of the second order and the error on δT will be smaller than 0.01°C. The contribution to the error of temperature measurement to the error on ΔH_S could thus be smaller than 1%, as far as precise measurements of temperature differences are possible.

TABLE III

CRITICAL VALUE OF PV/RT FOR VARIOUS COMPOUNDS[8]

Methane	0.288	2,3-Dimethylbutane	0.269	Phenol	0.24
Nitrogen	0.292	Cyclopentane	0.276	Xylenol	0.38
Oxygen	0.300	1-Butene	0.277	Water	0.228
Hydrogen	0.305	Benzene	0.771	Methanol	0.224
Helium	0.305	Toluene	0.264	Ethanol	0.248
Argon	0.292	Ethylbenzene	0.263	1-Butanol	0.258
n-Hexane	0.264	*o*-Xylene	0.263	2-Propanone	0.236
n-Decane	0.246	*m,p*-Xylene	0.260	Ethyl acetate	0.253
3-Methylpentane	0.273	Methylchloride	0.276	Diethyl ether	0.259

With a carefully controlled precision equipment, it is possible to measure retention times with an accuracy of a few parts in 10^{-4} (ref. 7). If the relative error on t_R' is 3×10^{-4}, the absolute error on $\delta\tau$ will be $\theta(\delta\tau) = 6 \times 10^{-4} t_R'$. The absolute magnitude of $\delta\tau$ may be estimated from the conventional relationship

$$t_R' = k' t_m \tag{29}$$

where k' is the column partition coefficient and t_m the air retention time. If temperature varies slightly we shall assume that t_m remains constant in eqn. 29. This approximation is valid in an error calculation. Then

$$\delta\tau \simeq \frac{\delta t_R'}{\delta T}\delta T = \frac{\Delta H_S}{RT^2} k' t_m \delta T \tag{30}$$

Combining eqns. 29 and 30 gives

$$\frac{\theta(\delta\tau)}{\delta\tau} = 6 \times 10^{-4} \frac{RT^2}{\Delta H_S \delta T} \tag{31}$$

If $T = 350$°K, $\delta T = 1$°K and $\Delta H_S = 10^4$ cal/mole, we obtain a relative error on $\delta\tau$ of 0.75%. This precision could be improved by refining the methods of temperature

control, temperature measurements and retention time measurements. Furthermore, systematic measurements of t_R *vs.* T at very small temperature intervals and statistical analysis of the data will still reduce the error. As shown by eqn. 31, however, a very good accuracy in the measurement of retention times is necessary if the value of ΔH_S is to be measured at various temperatures.

CONCLUSION

The method we have described here gives access to the value of the differential heat of dissolution, ΔH_S, from GLC measurements taking account of the effects of the gas phase non-ideality. The apparatus that is suggested should be able to reach an accuracy in ΔH_S better than 1% when ΔH_S is determined over a temperature interval of about one degree.

From the point of view of chromatography theory, one very interesting conclusion is that the method ought to be extended to the gas–solid chromatographic measurements as well. With respect to the precision achieved, it can be expected that the variation of the differential heat of adsorption with temperature should be significant enough to give access to the value of the partial molar heat capacity. In this way, information on the degree of freedom of the adsorbed molecule can be obtained.

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CHROM. 4753

COMPARISON OF ACTIVITY COEFFICIENTS MEASURED BY A STATIC AND BY A DYNAMIC METHOD

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SUMMARY

Activity coefficients at infinite dilution were determined for various chlorinated hydrocarbons in squalane and dinonyl phthalate by two distinct chromatographic methods. The first, and well known method, involved the measurement of specific retention volumes; the second, and less common method, involved measurements obtained from the diffuse edge of a single chromatographic peak. In the latter case the injection of very large samples was necessary. The results are compared with activity coefficients obtained by the static method that uses a McBain balance. Generally, agreement between the dynamic and static methods is good. A conclusion reached is that non-linearity of the partition isotherm may be an important factor when measuring activity coefficients even at infinite dilution.

INTRODUCTION

The measurement of the activity coefficients of some chlorinated hydrocarbons in squalane and in dinonylphthalate by a static method (McBain balance) have been described elsewhere[1,2]. The object of this paper is to compare results obtained by gas chromatographic techniques with those obtained by the static method. Two distinct chromatographic methods were used to obtain values of γ_2^∞, the activity coefficient of the solute at infinite dilution. In the first, small samples of the pure solutes were injected onto a chromatographic column in which either squalane or dinonylphthalate was used as the stationary phase and, from the elution peaks obtained, γ_2^∞ was calculated using the expression:

$$\gamma_2^\infty = \frac{1.704 \times 10^7}{M p_2^0 V_g^0} - \frac{1}{RT}\{p_2^0 B_{22} - \bar{p}(2B_{23} + B_{33})\}$$

where V_g^0 is the specific retention volume, that is, the net retention volume per gram of stationary phase, at 0°; p_2^0 is the saturated vapour pressure; M is the molecular weight, and $\bar{p}$ is the mean column pressure[7]. The second term on the right hand side corrects for imperfections in the gas phase. Values of the second virial coefficients B_{22} (solute) and B_{33} (carrier gas) were calculated from the Berthelot equation and the cross term, B_{23}, from the theory of corresponding states[3]. A third term involving the molar volumes was neglected. Measurements of V_g^0 were also made from the chro-

matogram in two ways; firstly from the "air peak" to the solute peak maximum, and secondly from the "air peak" to the solute peak front. The position of the peak front was established by drawing the tangent to the point of inflection in the peak front and taking the point where it intersected the base line.

In the second method, large samples—up to 250 μl—were injected onto the column and absorption isotherms calculated from the diffuse fronts of the chromatograms, following the method of CREMER AND HUBER[4], HUBER AND KEULEMANS[5] and KNOZINGER AND SPANNHEIMER[6] as applied to the determination of adsorption isotherms from gas–solid chromatography. In the present work the term "isotherm" refers to the plot of the amount (a) of solute absorbed against the vapour pressure (p) of the solute, per gram of stationary phase (the solvent). These quantities are given by:

$$a = \frac{nW}{A} \cdot \int_{h=0}^{h=p} \lambda \mathrm{d}h$$

$$p = \frac{h}{S_p} = \frac{nRT_m x h}{AjF_m}$$

where n is the number of mole of absorbate (solute); T_m and F_m are flow meter temperature and flow rate of carrier gas respectively; x is chart recorder speed; h and A are peak height and peak area, respectively; j is the compressibility factor; W is weight of absorbent (stationary phase), S_p is detector sensitivity; and λ is given by xt_r, where t_r is the retention time.

The integration is carried out graphically from the chromatogram by measuring the area from the air peak to the peak maximum within the limits $h = 0$ to $h = p$. Both this area and the peak area were determined by cutting out the chart and weighing, the weights being compared with the weight of a known area of chart paper taken from a region close to the chromatographic peak.

HUBER AND KEULEMANS[5] have shown that the effect of sample size on the position of the peak front is small, and the present work confirmed this observation. It was therefore considered permissible to calculate an isotherm from the diffuse edge of a single large peak by taking points corresponding to different concentrations, rather than using a number of peaks of different height. A more accurate estimate of the isotherm can, however, be made from the maxima of a number of peaks of different height, provided that the runs are carried out under identical conditions, by superimposing all the chromatograms and drawing a curve through the peak maxima. This curve is then used as if it were a diffuse front boundary. In this way the effects of diffusion, convective mixing and mass transfer are minimised.

Values of $\ln\gamma_2^{\infty}(T,0)$ may be calculated from the expression:

$$RT\ln\gamma_2^{\infty}(T,0) = RT\ln\gamma_2^{\infty *} - (B_{22} - v_2^0)p_2^0 + 2(B_{23} - B_{33} - v_2^{\infty})p$$

where

$$\gamma_2^{\infty *} = \lim x^{\mathrm{L}} \to 0 \; (p_2/p_2^0 \cdot x_2^{\mathrm{L}})$$

$\ln\gamma_2^{\infty *}$ is obtained by plotting $\ln(p_2/p_2^0 \cdot x_2^{\mathrm{L}})$ against the mole fraction x_2, where x_2^{L} is the mole fraction of the solute dissolved in the absorbate, and extrapolating to $x_2 = 0$. The virial correction can then be made to give $\ln\gamma_2^{\infty}(T,0)$. Values of the virial

TABLE I

VALUES OF THE MIXED SECOND VIRIAL COEFFICIENTS OF THE ABSORBATES WITH HYDROGEN AT DIFFERENT TEMPERATURES

Calculated from corresponding states theory.
1,1-DCE = 1,1-dichloroethane; 1,2-DCE = 1,2-dichloroethane; 1,1,1-TCE = 1,1,1-trichloroethane; *cis*-1,2-DCEth = *cis*-1,2-dichloroethylene; *trans*-1,2-DCEth = *trans*-1,2-dichloroethylene; 1,1,2,2-TCE = 1,1,2,2-tetrachloroethane; PCE = pentachloroethane.

Absorbate	*Mixed virial coefficient* $-B_{12}$ *cm³/mole (°C)*			
	30	*40*	*50*	*60*
1,1-DCE	28	25	21	18
1,2-DCE	32	28	24	21
1,1,1-TCE	35	30	26	22
cis-1,2-DCEth	22	19	17	14
trans-1,2-DCEth	27	23	20	16
CCl_4	34	30	26	22
CH_2Cl_2	23	20	17	14
$CHCl_3$	27	23	20	17
	90	*100*	*110*	*120*
1,1,2,2-TCE	22	19	16	12

coefficient B_{22} have been given elsewhere[2]; values for the cross term B_{23} and the virial corrections are given in Tables I and II.

EXPERIMENTAL

The apparatus was based on a Griffin Mark III katharometer chromatograph. During any given determination the oven temperature remained within $\pm 0.05°$, or better, of the measured temperature, and the variation along the column length was

TABLE II

VALUES OF THE VIRIAL CORRECTION TERM USED IN CORRECTING $\log p^r$ FOR GAS IMPERFECTIONS

Absorbate	*Virial correction (°C)*			
	30	*40*	*50*	*60*
1,1-DCE	0.005	0.007	0.010	0.013
1,2-DCE	0.000	0.001	0.003	0.005
1,1,1-TCE	0.002	0.005	0.007	0.011
cis-1,2-DCEth	0.004	0.006	0.009	0.012
trans-1,2-DCEth	0.006	0.009	0.012	0.016
CCl_4	0.001	0.003	0.006	0.008
CH_2Cl_2	0.003	0.006	0.008	0.012
$CHCl_3$	0.009	0.012	0.016	0.021
	90	*100*	*110*	*120*
1,1,2,2-TCE	0.001	0.002	0.003	0.004
PCE	0.001	0.002	0.003	0.004

less than 0.1°. Hydrogen was used as carrier gas and the flow rate was measured with a soap film flow meter. Outlet pressure was atmospheric, and the column inlet pressure was measured to the nearest millimetre on a mercury manometer. Carrier gas flow rates were chosen to give acceptable retention times and near optimum column efficiency (minimum HETP). In the first method, 0.2 μl samples were injected with a Hamilton syringe and five injections were made for each determination of the solute retention volume, and each determination was done in duplicate. In the second method, the size of the sample injected was varied according to the vapour pressure of the solute, but usually it was about 250 μl. The apparatus "dead" volume was determined by injecting air and recording the retention time by means of a stop watch. Other retention times were obtained from the recorder chart.

Columns (4 ft. $\times$ $\frac{1}{4}$ in. O.D. copper) were packed with 44–60 mesh acid-washed Embacel (May & Baker) loaded with 20% (w/w) squalane or dinonylphthalate. Silanised and non-silanised supports were tried. Neither showed any significant tailing with the solutes used, but results obtained with silanised supports showed better agreement with the static results and were used henceforth.

RESULTS AND DISCUSSION

Values of $\log\gamma^{\infty}$ determined at various temperatures are shown in Tables III(a) and III(b). The values given include those obtained from retention volume measurements—both from the peak front (PF) and from the peak maximum (PM)—together with the values from the isotherms obtained by the chromatographic method (GCI), and those previously reported from the static isotherms (SI).

Some workers[7,8] have found that the initial retention volume (V_{RI}) gives better agreement with the results obtained by the static method, others[9], that retention data calculated from the peak maxima give better agreement. In the present work no definite trend was observed, but the peak front usually gave the better agreement when squalane was used as the solvent. With dinonylphthalate, on the other hand, peak maximum values are slightly favoured. It is well known that in non-linear chromatography with isotherms of the "anti-Langmuir" type the peak will tend to show the phenomenon of fronting and the retention volume measured to the peak maximum will increase as the sample size increases. It is usually assumed that if the sample size is small enough the concentrations of absorbate involved will lie on that part of the isotherm that is effectively linear, and that within this range the retention volume will be independent of the sample size. With strongly curved isotherms, however, peak distortion will still tend to be present even at very low solute concentration, consequently the retention volume, if measured to the peak maximum will be too large and the value of γ^{∞} thus derived will be too small. In many cases the value of γ^{∞} from the peak maximum is smaller than the value from the static measurements, and this is particularly marked in the case of solutions of 1,1,1-trichloroethane in both squalane and dinonylphthalate, and it is significant that the curvature of the isotherms for these systems is greater than that of any other system studied in the present work. Thus there seems to be a third effect, in addition to the two mentioned by CRUICKSHANK *et al.*[9], that may be important even at low concentrations of solute, namely, non-linearity of the isotherm.

In Fig. 1(a) and 1(b) isotherms obtained by the chromatographic method are

TABLE III(a)

VALUES OF $\log \gamma^{\infty}$ FROM GLC MEASUREMENTS AND COMPARISON WITH STATIC RESULTS.

PF = peak front; PM = peak maximum; GCI = gas chromatographic isotherm; SI = static isotherm.
Solutions in squalane.

Solute	*30°*				*40°*				*50°*				*60°*			
	PF	*PM*	*GCI*	*SI*	*PF*	*PM*	*GCI*	*SI*	*PF*	*PM*	*GCI*	*SI*	*PF*	*PM*	*GCI*	*SI*
1,1-DCE	$\bar{1}$.986	$\bar{1}$.949	$\bar{1}$.968	$\bar{1}$.961	$\bar{1}$.961	$\bar{1}$.914	–	$\bar{1}$.938	$\bar{1}$.955	$\bar{1}$.919	$\bar{1}$.938	$\bar{1}$.930	$\bar{1}$.927	$\bar{1}$.886		
1,2-DCE	0.120	0.090	0.102	0.114	0.088	0.058		0.076	0.052	0.027	0.029	0.047	0.033	$\bar{1}$.997		
1,1,1-TCE	$\bar{1}$.819	$\bar{1}$.787	$\bar{1}$.802	$\bar{1}$.840	$\bar{1}$.800	$\bar{1}$.768	$\bar{1}$.777	$\bar{1}$.830	$\bar{1}$.781	$\bar{1}$.752	$\bar{1}$.767	$\bar{1}$.814	$\bar{1}$.767	$\bar{1}$.734		
1,1,2,2-TCE					$\bar{1}$.842	$\bar{1}$.812		~0.06	$\bar{1}$.842	$\bar{1}$.812		~0.06				
PCE	$\bar{1}$.710	$\bar{1}$.695			$\bar{1}$.723	$\bar{1}$.705			$\bar{1}$.734	$\bar{1}$.714						
cis-1,2-DCEth	$\bar{1}$.913	$\bar{1}$.880	$\bar{1}$.910	$\bar{1}$.902	$\bar{1}$.903	$\bar{1}$.868	$\bar{1}$.893	$\bar{1}$.892	$\bar{1}$.880	$\bar{1}$.847	$\bar{1}$.883	$\bar{1}$.885	$\bar{1}$.864	$\bar{1}$.827		
trans-1,2-DCEth	$\bar{1}$.805	$\bar{1}$.773	$\bar{1}$.809	$\bar{1}$.793	$\bar{1}$.744	$\bar{1}$.713	$\bar{1}$.710	$\bar{1}$.741	$\bar{1}$.743	$\bar{1}$.712	$\bar{1}$.716	$\bar{1}$.733	$\bar{1}$.738	$\bar{1}$.706		
CCl_4	$\bar{1}$.753	$\bar{1}$.722	$\bar{1}$.736	$\bar{1}$.733	$\bar{1}$.744	$\bar{1}$.713	$\bar{1}$.710	$\bar{1}$.741	$\bar{1}$.743	$\bar{1}$.712	$\bar{1}$.716	$\bar{1}$.733	$\bar{1}$.738	$\bar{1}$.706		
CH_2Cl_2[a]	$\bar{1}$.992	$\bar{1}$.956	$\bar{1}$.959	0.032	$\bar{1}$.968	$\bar{1}$.919		0.022	$\bar{1}$.943	$\bar{1}$.897	–	$\bar{1}$.980	$\bar{1}$.918	$\bar{1}$.866		
$CHCl_3$[a]	$\bar{1}$.843	$\bar{1}$.811	$\bar{1}$.833	$\bar{1}$.815	$\bar{1}$.833	$\bar{1}$.797	$\bar{1}$.800	$\bar{1}$.806	$\bar{1}$.820	$\bar{1}$.784	$\bar{1}$.796		$\bar{1}$.796	$\bar{1}$.760		

[a] Static results due to FREEGUARD.

TABLE III(b)

VALUES OF $\log \gamma^{\infty}$ FROM GLC MEASUREMENTS AND COMPARISON WITH STATIC RESULTS
Solutions in dinonylphthalate.

Solute	*30°*				*40°*				*50°*				*60°*			
	PF	*PM*	*GCI*	*SI*	*PF*	*PM*	*GCI*	*SI*	*PF*	*PM*	*GCI*	*SI*	*PF*	*PM*	*GCI*	*SI*
1,1-DCE	$\bar{1}$.682	$\bar{1}$.639	$\bar{1}$.611	$\bar{1}$.636	$\bar{1}$.700	$\bar{1}$.656	$\bar{1}$.628	$\bar{1}$.656	$\bar{1}$.694	$\bar{1}$.650	$\bar{1}$.641	$\bar{1}$.649	$\bar{1}$.696	$\bar{1}$.649		
1,2-DCE	$\bar{1}$.688	$\bar{1}$.653	$\bar{1}$.663	$\bar{1}$.668	$\bar{1}$.698	$\bar{1}$.663	$\bar{1}$.628	$\bar{1}$.656	$\bar{1}$.694	$\bar{1}$.658	$\bar{1}$.637	$\bar{1}$.679	$\bar{1}$.697	$\bar{1}$.658	$\bar{1}$.668	$\bar{1}$.744
1,1,1-TCE	$\bar{1}$.732	$\bar{1}$.686	$\bar{1}$.710	$\bar{1}$.723	$\bar{1}$.733	$\bar{1}$.690	$\bar{1}$.682	$\bar{1}$.728	$\bar{1}$.721	$\bar{1}$.675	$\bar{1}$.693	$\bar{1}$.728	$\bar{1}$.718	$\bar{1}$.672	$\bar{1}$.693	
1,1,2,2-TCE					$\bar{1}$.381	$\bar{1}$.360		$\bar{1}$.542	$\bar{1}$.413	$\bar{1}$.392		$\bar{1}$.545				
PCE	$\bar{1}$.516	$\bar{1}$.486			$\bar{1}$.544	$\bar{1}$.514			$\bar{1}$.569	$\bar{1}$.639						
cis-1,2-DCEth	$\bar{1}$.497	$\bar{1}$.461	$\bar{1}$.458	$\bar{1}$.476	$\bar{1}$.515	$\bar{1}$.479	$\bar{1}$.458	$\bar{1}$.508	$\bar{1}$.530	$\bar{1}$.494		$\bar{1}$.533	$\bar{1}$.544	$\bar{1}$.502		
trans-1,2-DCEth	$\bar{1}$.678	$\bar{1}$.640	$\bar{1}$.694	$\bar{1}$.611	$\bar{1}$.689	$\bar{1}$.659		$\bar{1}$.624	$\bar{1}$.690	$\bar{1}$.646		$\bar{1}$.639	$\bar{1}$.698	$\bar{1}$.648		
CCl_4[a]	$\bar{1}$.810	$\bar{1}$.767	$\bar{1}$.746	$\bar{1}$.778	$\bar{1}$.805	$\bar{1}$.764	$\bar{1}$.740	$\bar{1}$.774								
CH_2Cl_2[a]	$\bar{1}$.559	$\bar{1}$.517		$\bar{1}$.579	$\bar{1}$.567	$\bar{1}$.522		$\bar{1}$.580	$\bar{1}$.564	$\bar{1}$.516						
$CHCl_3$[a]	$\bar{1}$.445	$\bar{1}$.410	$\bar{1}$.407	$\bar{1}$.400	$\bar{1}$.468	$\bar{1}$.430	$\bar{1}$.435	$\bar{1}$.425								

[a] Static results due to FREEGUARD.

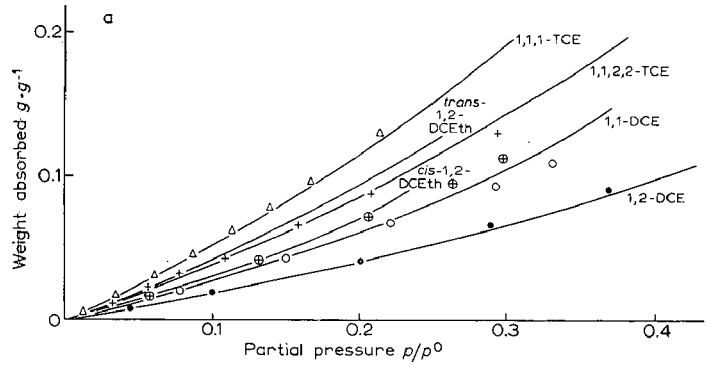


Fig. 1(a). Comparison of static and gas chromatographic isotherms. Solutions in squalane at 30°. ○ = 1,1-DCE; ● = 1,2-DCE; △ = 1,1,1-TCE; ⊕ = *cis*-1,2-DCEth; + = *trans*-1,2-DCEth. Static isotherms are shown as full lines.

compared with those from the static method. The chromatographic measurements tend to give lower values of the absorption, especially at higher relative pressures. At lower relative pressures agreement is surprisingly good and this is reflected in the agreement between the activity coefficients obtained by the two methods.

In order to obtain data over as wide a pressure range as possible in the chromatographic method it was necessary to use very large samples. When the sample concentration is high, non-ideality, non-linearity and sorption effects should be taken into account as shown by HAARHOFF AND VAN DER LINDE[10]. Under such circumstances the sign of an empirical parameter, called by these authors the "characteristic number", determines whether the chromatogram shall have a diffuse front or a diffuse rear boundary. It is further predicted that when the distribution coefficient is relatively large, tailing peaks should be found above a certain column temperature, and

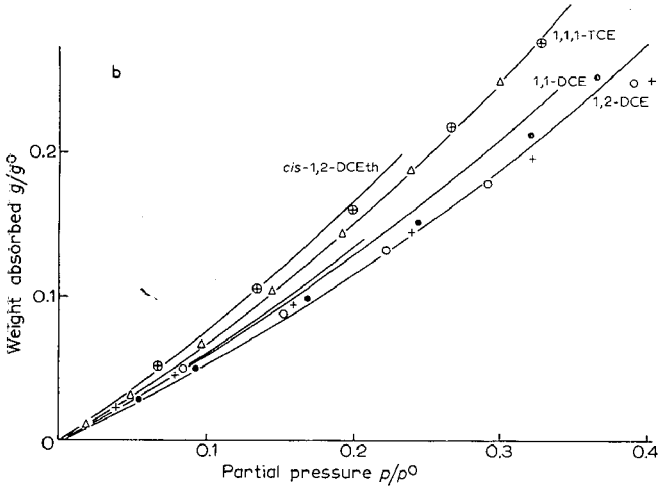


Fig. 1(b). Comparison of static and gas chromatographic isotherms. Solutions in dinonylphthalate at 30°. For key to GLC points, see Fig. 1(a).

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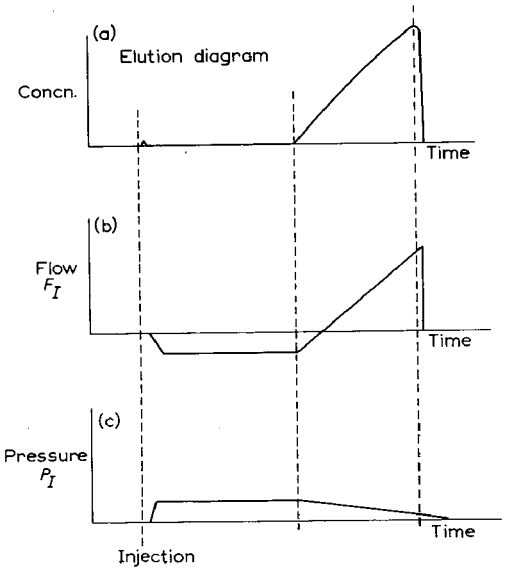


Fig. 2. Effect of high solute concentration on flow rate and inlet pressure. F_I = flow of pure carrier gas; P_I = pressure of pure carrier gas.

fronting peaks below this temperature. Such behaviour was in fact observed in the systems: dichloromethane–squalane and *trans*-1,2–dichloroethylene–squalane, as well as dichloromethane–dinonylphthalate and *cis*- and *trans*-1,2-dichloroethylene–dinonylphthalate. When a tailing peak was found it was not possible to calculate an isotherm from it.

It was observed that during the elution of a large peak from the column there was a change in the flow rate of the carrier gas as measured at the column outlet, and there was a corresponding increase in the column inlet pressure. Pressure changes caused by variations in the viscosity and flow rate when an increase in flow velocity is observed have been discussed by several workers[11–13]. In the present work the flow was monitored at the column outlet and the pressure drop across the column was observed from the change in inlet pressure. Results for 1,1,1-trichloroethane on a dinonylphthalate column maintained at 50° are shown in Fig. 2. Fig. 2(a) shows the chromatogram and Fig. 2(b) and 2(c) the corresponding flow and pressure changes. Readings were taken after the initial pressure fluctuations caused by sample injection had subsided. The sharp increase in flow as the peak is eluted is the so-called "surge effect" described by VAN DER CRAATS[11] and is caused by the fraction of the component in the stationary phase being transferred into the gaseous phase at the end of the column. The size of the change naturally depends on the size of the sample, but is relatively insensitive to the nature of the solute and the column temperature. Pressure changes were about 0.5 to 1.0% of the column pressure, and flow changes about 6.0 to 10.0% of the normal flow rate, according to the sample size (50–150 μl). An explanation is that a back pressure is produced causing a reduction in gas flow; as the solute is eluted the back pressure drops and the normal surge effect is then observed. The most important consequence of this is on the calculation of the column pressure and hence of the concentration, of the solute. For example, if the flow rate used in

the calculation is the one taken with pure carrier gas only through the column, then the calculated pressure term is too low. The change in the column pressure also has an effect on the solute pressure term and in the same sense as the flow rate change.

BECHTOLD[14] has suggested a graphical method for obtaining a correction for the effect of diffusion. The shapes of the elution peaks obtained indicate, however, that diffusion of the solute band was slight except with the chloroform–dinonylphthalate system, even so the results obtained for this system are in good agreement with the static measurements. In those cases where a change from fronting to tailing occurs the absence of diffusion is only established when a vertical edge to the peak is obtained. When a symmetrical, or near-symmetrical peak is obtained the two effects, that is, diffusion and the change from a fronting to a tailing peak, are inseparable as they both tend to enhance peak symmetry.

Values for activity coefficients taken from the literature are given in Table IV. The results of MARTIRE AND POLLARA[15] were obtained at temperatures higher than those used in the present work, hence the values given were obtained by extrapolating the graph of $\log \gamma^{\infty}$ against $1/T$ to values of T corresponding to those used in the present work. MARTIRE AND POLLARA's results were obtained from specific, corrected retention volumes, that is, using peak maxima. YOUNG's[16] values were obtained from

TABLE IV

COMPARISON OF VALUES OF $\log \gamma^{\infty}$ WITH RESULTS FROM OTHER SOURCES

a = Results of this work; b = results of MARTIRE AND POLLARA; c = results of YOUNG; d = results of HARDY. SQ = squalane; DNP = dinonyl phthalate.

System	*Temperature (°C)*			*Reference*
	30	*40*	*50*	
1,1-DCE/SQ		$\bar{1}.914$	$\bar{1}.919$	a
		$\bar{1}.922$	$\bar{1}.911$	b
1,2-DCE/SQ		0.058	0.027	a
		0.037	0.023	b
cis-1,2-DCEth		$\bar{1}.868$	$\bar{1}.847$	a
		$\bar{1}.887$	$\bar{1}.877$	b
trans-1,2-DCEth		$\bar{1}.776$	$\bar{1}.751$	a
		$\bar{1}.774$	$\bar{1}.765$	b
CCl_4–SQ	$\bar{1}.722$	$\bar{1}.713$	$\bar{1}.712$	a
		$\bar{1}.742$	$\bar{1}.742$	b
	$\bar{1}.732$			c
CH_2Cl_2–SQ	$\bar{1}.956$	$\bar{1}.919$	$\bar{1}.897$	a
		$\bar{1}.917$	$\bar{1}.901$	b
	$\bar{1}.860$			c
$CHCl_3$–SQ	$\bar{1}.811$	$\bar{1}.797$	$\bar{1}.784$	a
		$\bar{1}.818$	$\bar{1}.813$	b
	$\bar{1}.723$			c
CCl_4–DNP	$\bar{1}.767$	$\bar{1}.764$		a
	$\bar{1}.776$			c
		$\bar{1}.851$		d
CH_2Cl_2–DNP	$\bar{1}.517$	$\bar{1}.522$		a
	$\bar{1}.513$			c
		$\bar{1}.623$		d
$CHCl_3$–DNP	$\bar{1}.410$	$\bar{1}.430$		a
	$\bar{1}.407$			c
		$\bar{1}.519$		d

retention volumes measured to peak maxima at a series of pressures (2–12 atmospheres) and the values extrapolated to zero pressure. YOUNG's results for chloroform and dichloromethane in squalane would appear to be low, and in a series of experiments using longer columns, from 6 ft. upwards in length, he has obtained values approaching those found in this work. He also used a McBain balance to determine $\log \gamma^{\infty}$ for dichloromethane in squalane at 30° and obtained a value of 1.99. Finally, it would appear that the value obtained by FREEGUARD[1] for dichloromethane in squalane is too high and that this system should have an activity coefficient less than 1.0 at all temperatures in the range studied. The early results of HARDY[17] would also appear to be in error.

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AN ELECTRICAL MODEL FOR THE OPTICAL BEHAVIOUR OF THIN-MEDIA CHROMATOGRAMS

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SUMMARY

Described is an electrical transmission line with resistive parameters as a model simulating the optical behaviour of chromatographic media as described by the KUBELKA AND MUNK equation. The model is characterised by its attenuation constant γ, its characteristic impedance ζ_0 and the reflection coefficient ϱ. The significance of the latter is derived from Ohm's Law, in order to make the treatment understandable to persons not possessing a background in electrical engineering. A concentrated parameter multi-section representation of the transmission line model is described covering the whole range over which the KUBELKA AND MUNK equations are valid. In order to reduce the complexity of the model, the number of sections may be reduced making the simulation valid only for a restricted range of optical parameters of the medium.

(1) INTRODUCTION

In two earlier papers[1,2] we discussed the quantitative evaluation of paper and thin-layer chromatograms by spectrophotometric methods mainly with regard to the limitations imposed by the optical "noise" upon the obtainable accuracy and sensitivity. The optical noise is caused by irregularities in the transmittance or reflectance of the background material of the chromatogram from one part to another. The discussion was partly based on the simplifying assumption that there is a linear dependence of the transmission decrement upon the concentration of the investigated substance. This assumption is justified, however, only for very low concentrations. In this paper we intend to investigate the relationship between the decrement in transmission or reflection and the concentration of analysed substance for the more general case, where neither linearity nor even logarithmically linear dependence can be assumed without considerable error.

Paper and other thin-media supports used in chromatographic separations have

mostly a rather complex physical structure. As a consequence of this, their optical behaviour is too complicated to be described by simple mathematical relationships. For most practical purposes, however, simplified expressions may be used and these give an adequate picture of the optical performance of the medium. One of these relations, which is extensively used in technical applications, is based upon a simplified theory proposed by KUBELKA AND MUNK[3] and is generally known as the KUBELKA AND MUNK set of equations. It relates to plane parallel isotropic heterogeneous media. The assumption of isotropy is frequently valid only to a limited extent, so that measured results need not always completely agree with the values predicted by the theory. In most practical cases, however, these deviations are relatively insignificant, in which case the KUBELKA AND MUNK theory is perfectly adequate and represents a powerful tool for the solution of many technical problems. The KUBELKA AND MUNK equation can be solved in an easy and straightforward manner. The solutions, however, are rather difficult to interpret for practical purposes. Because of this, graphical representations[4] and simplified solutions valid only for a limited range of parameters of the medium[5] have been developed.

In another paper recently published[6] one of us showed that under these conditions the optical behaviour of the medium may be simulated by an electrical transmission line composed of purely resistive elements. As shown in Fig. 1, a transmission line of this type is characterised by a longitudinal resistivity R and a transversal conductivity G. Both are related to the optical parameters of the medium by the relations:

$$R = 2S + K \qquad (1)$$
$$G = K$$

In the equations K is the absorbance of the medium and S its coefficient of scattering. The electrical length of the model is assumed to be unity and the same applies to the thickness of the medium.

Wheh the medium is illuminated by a light intensity I, part of the incident light I_S is reflected at the surface[7]. This component does not enter the medium at all and does not therefore convey any useful information about its interior. For the purposes envisaged here, this surface or specular reflectance may be disregarded. The amount of light reflected in this way has to be substracted from the total illuminating light flux. The remaining light enters the medium, which is assumed to be made up of homogeneous particles embedded in a heterogeneous environment. At every particle boundary scattering takes place, whilst inside the particles themselves part of the light is absorbed and converted to heat. No energy loss is produced by scattering. According to KUBELKA AND MUNK the result of this multiple scattering and absorption may with good approximation be represented by splitting the entering light flux into two components, *viz.* one travelling in the forward direction and the other travelling in the reverse direction (Fig. 2). For technical purposes we are only interested in these components at the surfaces, where they are entering or leaving the medium.

It has been shown by POLLAK[6] that the forward light component $j(x)$ and the backwards component $r(x)$ at a distance x from the near surface may be expressed by the voltage $v(x)$ and current $i(x)$ at the corresponding point in the transmission line model according to the following relations:

$$j(x) = \tfrac{1}{2}[v(x) + i(x)]$$

$$r(x) = \tfrac{1}{2}[v(x) - i(x)] \tag{2}$$

In order to obtain the corresponding values at the near (illuminated) surface, we have to put $x = 0$, and for the far surface $x = 1$. $v(0)$ and $i(0)$ are then the input values of voltage and current of the model, respectively, while $v(1)$ and $i(1)$ are the respective output values. No optical back scattering of course takes place at the far end, so that $r(1) = 0$. From this we obtain:

$$v(1) = i(1) \tag{3}$$

It therefore follows that the load impedance ζ_L of the model has to be equal to 1.

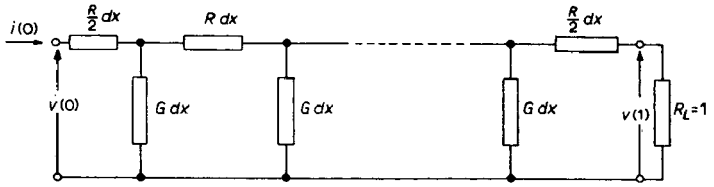


Fig. 1. Basic diagram of a resistive homogeneous transmission line.

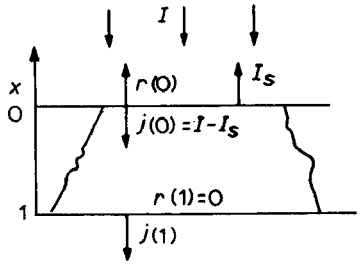


Fig. 2. Schematic diagram of light distribution at the surfaces of a plane parallel scattering medium. I = Illuminating flux; I_S = surface reflected flux; r = diffusely reflected component; j = forward travelling flux.

The transmittance of the medium (A_T) is defined as the ratio of the light intensity $j(1)$ leaving the far end of the medium and the incident light $I(0)$. Applying eqns. 2 and 3 we obtain:

$$A_T = \frac{j(1)}{j(0)} = \frac{v(1) + i(1)}{v(0) + i(0)} = \frac{2v(1)}{v(0) + i(0)} \tag{4}$$

In an analogous way we obtain for reflectance (A_R):

$$A_R = \frac{r(0)}{j(0)} = \frac{v(0) - i(0)}{v(0) + i(0)} \tag{5}$$

(2) THE INPUT–OUTPUT REPRESENTATION

A transmission line can be considered as a special case of a three-terminal electrical network. The response of such a network to an applied electrical signal is completely specified by a set of input–output parameters without knowing its internal set-up. The network itself may then be considered as a black box with unknown content.

For a transmission line the most convenient set of parameters specifying the input–output behaviour of the network is: the characteristic impedance ζ_0, the propagation constant γ and the reflection coefficient ϱ. ζ_0 and γ can be expressed in terms of the electrical parameters of the line; in our case, where the line is homogeneous and purely resistive, these reduce to the total longitudinal resistance R and transversal conductance G. It may easily be shown that

$$\zeta_0 = \sqrt{\frac{R}{G}} = \sqrt{\left(\frac{2S + K}{K}\right)} \tag{6}$$

$$\gamma = \sqrt{RG} = \sqrt{\{K(2S + K)\}} = K\zeta_0 \tag{7}$$

ζ_0 is defined as the impedance of a line of infinite length. Another, but equivalent, definition states that ζ_0 is the impedance seen at the one end of a line of finite length if this is terminated at the other end by an impedance equal to ζ_0. The line is then said to be matched. γ is the logarithm of the ratio of output to input voltage under matched conditions. On a resistive line γ determines only the amplitude relations, that is the attenuation of the line. In the general case it would also specify its phase properties.

The coefficient of reflection ϱ is determined by the characteristic impedance ζ_0 and the load impedance ζ_L. Under matched conditions $\varrho = 0$ and no reflection occurs. As is evident from eqn. 3 the load impedance in our case is equal to 1.

$$\varrho = \frac{\zeta_L - \zeta_0}{\zeta_L + \zeta_0} = \frac{1 - \zeta_0}{1 + \zeta_0} \qquad (\zeta_L = 1) \tag{8}$$

The meaning of the reflection coefficient ϱ is best understood and eqn. 8 verified from the simple diagram in Fig. 3.

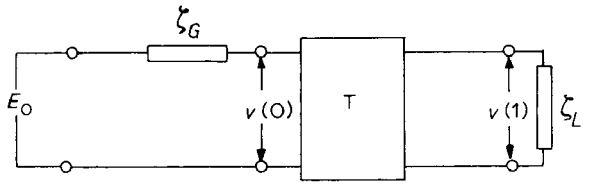


Fig. 3. Explanation of the reflection coefficient. T = network equivalent to the line with attenuation; ζ_G = generator impedance; ζ_L = load impedance.

The characteristic impedance ζ_0 is by definition the impedance seen at the input, if the network is terminated by ζ_0. Since the network considered here is bidirectional, the impedance seen at any terminal equals ζ_0, if the other one is terminated by an

impedance equal to ζ_0. Let us now assume that $\zeta_G = \zeta_L = \zeta_0$. Under these conditions we have evidently $v(0)_M = E_0/2$ and $v(1)_M = v(0) \cdot e^{-\gamma}$, the index M indicating the matched condition.

Let us now consider a mismatch at the load terminal with $\zeta_L \neq \zeta_0$. Looking to the left into the network from the load terminals we see all the time the impedance ζ_0, since the generator terminal is still matched. Putting for the moment $\gamma = 0$, the whole diagram reduces to a simple voltage divider, the output voltage becoming

$$v(1) = E_0 \frac{\zeta_L}{\zeta_L + \zeta_0} \tag{9}$$

This voltage can be considered as the sum of a forward component equal to $v(1)_M$ and a reflected component $v(1)_R$ travelling in the backward direction.

$$v(1) = v(1)_M + v(1)_R = E_0 \frac{\zeta_L}{\zeta_L + \zeta_0} = \frac{E_0}{2}\left(1 + \frac{\zeta_L - \zeta_0}{\zeta_L + \zeta_0}\right) = \frac{E_0}{2}(1+\varrho) \tag{10}$$

Now let us consider the effect of attenuation. For $v(1)$ we obtain evidently:

$$v(1) = \frac{E_0}{2} \cdot e^{-\gamma}(1 + \varrho) \tag{11}$$

The situation is more involved at the input. For $\gamma = 0$ we evidently have $v(0) = v(1)$. With $\gamma \neq 0$, $v(0)$ is again the sum of the forward component $v(0)_M$ and the component reflected at the load terminal. Travelling back to the input, the latter is attenuated by a factor $e^{-\gamma}$. This results in

$$v(0)_R = v(1)_R \cdot e^{-\gamma} = \frac{E_0}{2} \cdot \varrho \cdot e^{-2\gamma}$$

$$v(0) = v(0)_M + v(0)_R = \frac{E_0}{2}(1 + \varrho \cdot e^{-2\gamma}) \tag{12}$$

Similar reasoning applies to the current values. They too may be considered as the sum of a forward and a reflected component. For the matched case we have:

$$i(0)_M = E_0 \frac{1}{2\zeta_0}$$

$$i(1)_M = E_0 \cdot \frac{1}{2\zeta_0} \cdot e^{-\gamma} \tag{13}$$

In the general case we obtain:

$$i(1) = i(1)_M + i(1)_R = \frac{v(1)}{\zeta_L} = E_0 \cdot e^{-\gamma} \cdot \frac{1}{\zeta_L + \zeta_0} = \frac{E_0}{2\zeta_0} \cdot e^{-\gamma}(1 - \varrho) \tag{14}$$

$$i(0) = i(0)_M + i(0)_R = \frac{E_0}{2\zeta_0}(1 - \varrho \cdot e^{-2\gamma}) \tag{15}$$

It should be noted that in the model all reflection takes place at the load terminals, whilst in the optical medium reflection occurs throughout the medium.

It may also be worth mentioning that the relations above are usually derived

in a straightforward way from the differential equations of the general transmission line problem. It is felt, however, that the approach chosen here is more illustrative for the reader, who is not familiar with electrical transmission line theory. It should be kept in mind that the forward component and the reflected wave are not mere mathematical fictions, but real entities. In most cases they are accessible to direct measurements, though this does not apply to the purely resistive line, which represents a degenerate condition.

(3) REFLECTANCE AND TRANSMITTANCE IN TERMS OF LINE PARAMETERS

Following the brief comments above it now remains to express eqns. 4 and 5 in terms of the parameters which were just introduced. First we wish to define the limiting values which ζ_0 may assume. From eqn. 6 it follows that

$$1 \leqslant \zeta_0 \leqslant \infty \tag{16}$$

Remembering that in our case $\zeta_L = 1$, we obtain for ϱ:

$$-1 \leqslant \varrho \leqslant 0 \tag{17}$$

Further we can write

$$v(0) + i(0) = \frac{E_0}{2}\left\{1 + \frac{1}{\zeta_0} + \varrho \cdot e^{-2\gamma}\left(1 - \frac{1}{\zeta_0}\right)\right\}$$

$$= \frac{E_0}{2\zeta_0}(1 + \zeta_0)(1 - \varrho^2 \cdot e^{-2\gamma}) \tag{18}$$

$$v(0) - i(0) = \frac{E_0}{2}\left\{1 - \frac{1}{\zeta_0} + \varrho \cdot e^{-2\gamma}\left(1 + \frac{1}{\zeta_0}\right)\right\}$$

$$= \frac{E_0}{2\zeta_0}(1 + \zeta_0) \cdot \varrho\,(e^{-2\gamma} - 1) \tag{19}$$

$$A_T = \frac{v(1) + i(1)}{v(0) + i(0)} = \frac{e^{-\gamma}(1 + \varrho)}{1 - \varrho^2 \cdot e^{-2\gamma}} \cdot \frac{2\zeta_0}{1 + \zeta_0} = \frac{e^{-\gamma}(1 - \varrho^2)}{1 - \varrho^2 \cdot e^{-2\gamma}} \tag{20}$$

$$A_R = \frac{v(0) - i(0)}{v(0) + i(0)} = -\varrho \frac{1 - e^{-2\gamma}}{1 - \varrho^2 \cdot e^{-2\gamma}} \tag{21}$$

Eqns. 20 and 21 are very general. For many cases of practical interest, however, they can be considerably simplified, as will be shown in a future publication[8].

(4) IMPLEMENTATION OF THE MODEL

The implementation of a model of a general transmission line, the electrical parameters of which should be variable and distributed over the whole physical length of the line, is rather difficult. In order to build a model with purely resistive parameters, sheets of resistive material, semiconductive plastics or thin- or thick-film technics may be used; the problem therefore becomes somewhat easier. Even for

this case, however, it appears that a model with lumped parameters is preferable; this means a model in which the electrical parameters, normally distributed over the length of the line, are concentrated in the form of resistors etc. A model of this type, however, can only approximate the real case with distributed values.

The most common representation of a transmission line by a lumped parameter network is a so-called T or Π ladder configuration. A T-equivalent is shown in Fig. 4. The branch impedances are here of course again pure resistances. In first order approximation they may be chosen equal to the series and shunt impedance of the line. The error committed in this way in the characteristic impedance and the propagation constant of the model as compared to the line is approximately[9]:

$$\zeta_0 = \sqrt{(\zeta_1 - \zeta_2)} = \zeta_{0T}\left(1 - \frac{\gamma_0^2}{8}\right)$$

$$\gamma_0 = \sqrt{\frac{\zeta_1}{\zeta_2}} = \gamma_{0T}\left(1 + \frac{\gamma_0^2}{24}\right) \qquad (22)$$

The index T refers to the corresponding characteristic value of the T-approximation. A Π-network using the same values results in errors of approximately the same size but of opposite sign.

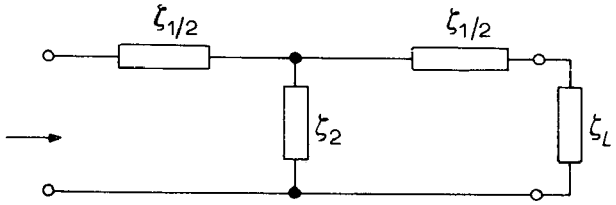


Fig. 4. Lumped T-approximation to a transmission line. $\zeta = R = 2S + K$; $\zeta_2 = 1/G = 1/K$; $\zeta_L = 1$.

For larger values of γ_0 the error becomes rather significant. To reduce it, corrections may be applied to the values of ζ_1 and ζ_2, which are illustrated in eqn. 23.

$$\zeta_1 = \zeta_0 \frac{e^{\gamma 0} - 1}{e^{\gamma 0} + 1}$$

$$\zeta_2 = \frac{\zeta_0^2 - \frac{\zeta_1^2}{4}}{\zeta_1} \qquad (23)$$

For the purposes envisaged in this application these corrections do not result in a convenient procedure of implementing the model; a better way of reducing the deviations of the model from the continuous line is to build the model from individual sections each representing a relatively short length of the line. The number of sections n should be chosen so that, depending upon the permissible overall error in γ, the propagation constant of the individual sections γ_s is $\leqslant 0.5$. For the parameters of the individual section we then obtain the relations:

$$\zeta_{1s} = \frac{\zeta_1}{n} \qquad \gamma_s \simeq \frac{\gamma_0}{n}$$

$$\zeta_{2s} = \zeta_2 \cdot n \qquad \zeta_{0s} \simeq \zeta_0 \qquad (24)$$

The error in characteristic impedance of the model is then determined by the value of γ_s according to eqn. 22. The error in propagation constant is determined, however, by the sum of the errors of the individual sections. For this reason n has to be chosen larger than at first might appear from eqn. 22. The schematic diagram of such a multisection model is shown in Fig. 5.

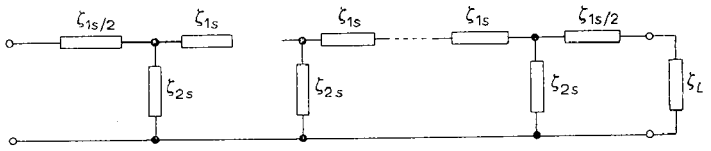


Fig. 5. A multisection transmission line model.

For our purposes the variable ΔK in the model should be represented both in the series and the shunt arms of the model according to Figs. 4 and 5 by independent variable resistances (see Fig. 6).

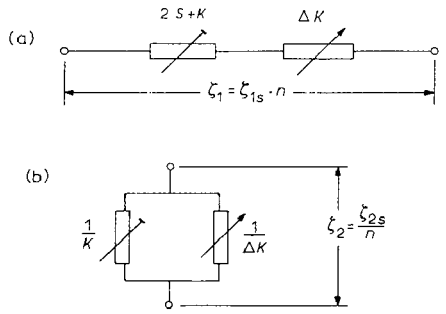


Fig. 6. The representation of the increment in absorption in the model.

ΔK is of course the increment in absorption due to the chromogen on the chromatogram. The elements marked with a —⊣ are to be set at the beginning of the measurement according to the optical parameters of the medium itself. The procedures for determining them will be described in a following paper[8].

Elements of the same type in all the sections should be mechanically coupled together so that a common control element may be used for adjustment.

Elements marked with an arrow —→ (representing ΔK) are to be adjusted during the measurement so that the values read on the model agree with the optical results obtained (see Section 6). The value of ΔK, corresponding to a given change in transmittance or reflectance, is read on a common dial. If required, automatic setting by a servo-follower system is feasible.

(5) MODELS WITH A SMALL NUMBER OF SECTIONS

The number of sections required in a model of this type to simulate the optical transmission of media with high optical density becomes very large. For example, when

simulating a medium with optical density 3.4 (Whatman No. 3 paper), γ becomes of the order of 6 to 8. To keep the error small, the value of γ_s per section has to be small also. Assuming a value of $\gamma = 0.25$ per section, then 25 to 30 sections are required. Though models of this size are technologically perfectly feasible, the large number of variable elements ganged together makes them cumbersome and costly. The difficulties increase if automatic and high-speed adjustment of the model is required. This would be the case, for example, if the model were to be incorporated into a scanning photodensitometer, in order to provide for a linear dependence of the output signal upon ΔK. In such a case digital modelling of eqns. 13 and 14 would seem to be the method of choice. On- or off-line digital modelling may be performed on almost any type of digital computer. On-line modelling, however, would require much computer time and in most cases this would be considered excessive. Digital modelling requires that the output data of the photodensitometer or reflectometer be available in digital form. Very few of the currently available instruments are so equipped and only a few laboratories possess the necessary accessories for digital conversion and storage of the analogue signal. This is one of the reasons why the experimental photodensitometer now being developed in our laboratories will be equipped with both a digital and an analogue output.

For media of medium and low optical density, perhaps up to a γ value of 3.2 (that is approx. 1.4 in decimal units) a multisection transmission line model of the type described seems to be quite adequate. Assuming eight sections with a transfer constant $\gamma_s \simeq 0.4$ per section, the overall error in γ is below 5%. The error in ζ_0 is about 2%; this means, that the error in ϱ may be neglected.

It should be noted that the optical density of the medium is not exclusively determined by $e^{-\gamma}$, but contains also a term dependent upon the reflection coefficient ϱ. In a medium with high scattering power such as paper, $1 - \varrho^2$ becomes small and this term may then contribute up to about one optical density unit to the density of the medium. This means that for media of this type the model described may be adequate up to an optical density value of approximately 2.5 decimal units. For reflectance measurements the value of ϱ alone is all important if γ exceeds the value of approximately 2.5 (see eqn. 21). Because of this we may conclude that a model with about eight sections is adequate for simulating the reflectance of a medium with any optical density, since ϱ is represented with satisfactory accuracy.

For high-speed on-line analog simulation of the effect of changes in absorbance of the medium, electronic techniques for adjusting ΔK and $1/\Delta K$ have to be employed. For a limited number of sections this approach need not be too costly, since relatively inexpensive switching modules in integrated design are now available. To adjust the transversal impedance $1/\Delta K$, one end of which is grounded, a simple counting circuit can be used. More difficulties are encountered with the longitudinal impedance ΔK, which is floating. A transistor operated reed relay arrangement probably represents the simplest solution. This should allow for speeds of adjustment of the order of milliseconds, and for most practical purposes this ought to be sufficient.

There are several special cases, however, quite frequently encountered in practice, where relatively simple special purpose circuits may be employed. An important application, which has already been mentioned, is the linearisation of the output of reflectometers and densitometers. If we are content with a limited range of applicability, these circuits are simpler and less expensive than digital instrumen-

tation. These aspects will be described in detail in another paper to be published soon.

(6) THE MEASUREMENT OF TRANSMITTANCE OR REFLECTANCE ON THE ELECTRICAL MODEL

To measure the transmittance or reflectance of a certain medium using the model, we have first to set the basic model parameters to the corresponding optical values S and K of the blank medium. How these are to be determined will be described in a later publication. The elements to be set in this way at the beginning of the measurement are marked in the figures by —|. The variable elements representing an increment in absorbance ΔK are marked by —→, and these are to be set in the zero position.

The actual measurement is done in accordance with the definitions set out in the right-hand part of eqns. 4 and 5. To simplify the procedure, $v(0) + i(0)$ is kept constant at a value E.

$$v(0) + i(0) \cdot 1 = E$$

$$A_T = \frac{j(1)}{j(0)} = \frac{v(1) + i(1)}{v(0) + i(0)} = \frac{2v(1)}{E}$$

$$A_R = \frac{r(0)}{j(0)} = \frac{v(0) - i(0)}{v(0) + i(0)} = \frac{v(0) - i(0)}{E} \tag{25}$$

To measure these values, the arrangement shown in Fig. 7 may be used. The transmission line model is here shown just as a black box M.

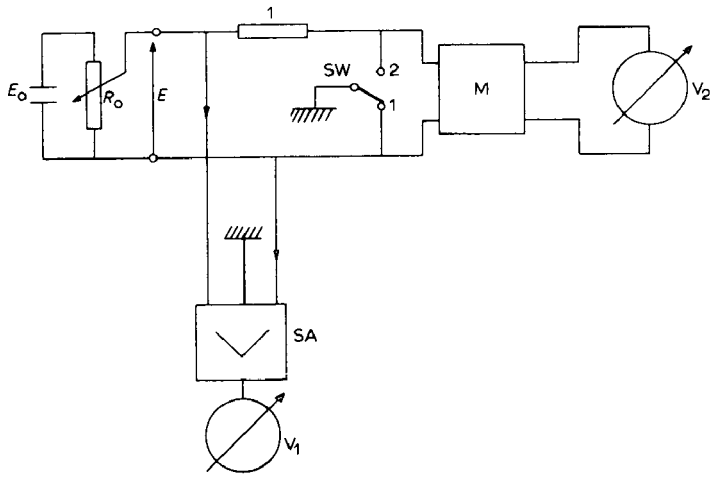


Fig. 7. Block diagram of the procedure for measuring A_T and A_R. SA = summing amplifier; M = transmission line model; SW = transmission–reflection measurement switch.

The circuit operates in the following way: first the variable parameters of the model have to be adjusted to the required values. Then with switch SW in position 1 the potentiometer R_0 is adjusted to give the reading 1 unit at meter V_1. This sets in

eqn. 25 $E = 1$; meter V_2 immediately reads the value of $A_T/2$. For measuring A_R switch SW is now brought into position 2. Meter V_1 then indicates the value of A_R under investigation. The gain of the summing amplifier SA also of course has to be considered in the calibration of the instruments; it is here assumed to be unity.

An actual fact of much more importance is the inverse procedure. Here the elements of the model marked with —⊣ (see Fig. 6) are set to represent the basic optical parameters of the medium. The elements modelling ΔK (marked —→) (see Fig. 6) are then varied, until the change in transmittance or reflectance measured on the medium is reproduced on the model. The corresponding value of ΔK is then read on the calibrated dial for these elements.

(7) CONCLUSIONS AND EXTENSIONS

It is hoped that the modelling approach described in this paper will represent a helpful and convenient procedure for solving, in terms of incremental absorbance, the transfer equations of a class of optical media for which the KUBELKA AND MUNK theory is valid. By replacing the resistances of the model by complex valued impedances, wavelength dependences of the optical parameters may to a certain extent be considered. A further possible extension of the method could be two- and three-dimensional models using semiconductive surface and volume conductors. In this way it might be possible to consider a certain degree of anisotropy of the medium. In general the application of the highly developed theory of homogeneous and non-homogeneous electrical transmission lines may become a helpful tool for the treatment of more complex and involved optical situations.

ACKNOWLEDGEMENTS

We thank the Psychiatric Services Branch, Province of Saskatchewan (A.A.B.), the Medical Research Council of Canada (A.A.B.) and the Canadian National Research Council (V.P.) for their support of this work.

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CHROM. 4690

NON-LINEARITY EFFECTS IN OPTICAL SCANNING OF CHROMATOGRAMS

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SUMMARY

The present paper is concerned with the error due to a finite spatial or spectral width of the illuminating scanning window in photodensitometers and reflectometers. The optical transfer characteristic of the medium is approximated by an exponential dependence upon the increment in absorbance, which produces the largest error. The results obtained are therefore worst case estimates. It is shown, that the absolute error in this case is with good approximation proportional to the mean square deviation of the absorbance from its mean value taken over the window. The more important percentage errors increase with increasing relative non-uniformity, that is with increasing ratio of variance to mean value. The results obtained are illustrated in three fairly typical numerical examples; the last of these showing that contrary to general practice relatively wide spectral windows may be tolerated without excessive error.

INTRODUCTION

In the optical scanning of chromatograms it is common practice to use an illuminated window covering the full active width of the medium. The scanning action is produced by moving the medium with a relative speed v perpendicularly to the slit (see Fig. 1).

The light transmitted or reflected by the illuminated area of the medium is collected by an optical system and concentrated upon a photodetector. The output signal of the photodetector is proportional to $I_0\bar{A}$, where I_0 is the illuminating light flux and $\bar{A}$ the general transfer function (transmittance or reflectance) of the medium. The bar over the symbol signifies that the mean value over the illuminated area is to be considered.

When an area of the medium containing an absorbing substance with a local

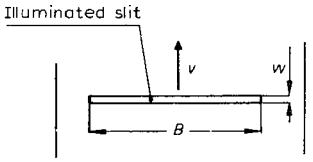


Fig. 1. Diagram of a slit scanning operation.

concentration $c(b)$ is reached, the absorbance increases by an amount $\Delta K(b)$ proportional to $c(b)$. We obtain therefore:

$$\Delta\bar{K} = \frac{1}{B}\int_B \Delta K(b)\,\mathrm{d}b = \frac{f}{B}\cdot Q \tag{1}$$

where Q is the amount of absorbing substance within the slit area and f is a proportionality constant, which is characteristic for the substance involved. ΔK causes a decrease ΔA of the transfer function A. The decrease in output of the photodetector is proportional to $\Delta\bar{A}$, that is to the mean value of ΔA over the slit. Unfortunately, however, $\Delta A(\Delta K)$ is in the general case a non-linear function of ΔK. As a result we obtain:

$$\frac{1}{B}\int_B [\Delta A(\Delta K(b)) - \Delta A(\Delta\bar{K})]\,\mathrm{d}b = \Delta\bar{A} - A(\Delta\bar{K}) = \varepsilon \neq 0$$

$$\Delta\bar{A} \neq A(\Delta\bar{K}) \tag{2}$$

There are only two cases where the unequality sign in eqn. 2 is not valid, *viz.* (1) if $\Delta K(b)$ is a constant, so that

$$\Delta K(b) - \Delta\bar{K} \equiv 0$$

and (2) if ΔA is a linear function of ΔK, that is if

$$\frac{\delta\Delta A(\Delta K)}{\delta\Delta K} = \text{const.} \tag{3}$$

If the unequality sign holds, the value of $\Delta\bar{A}$ obtained for a certain medium at a given Q is dependent upon the distribution $c(b)$ of investigated substance over the illuminated area. The purpose of all measurements of the type considered here is to determine $\Delta\bar{K}$ and from that Q as a function of the measured value $\Delta\bar{A}$. In the general case there is, however, no unique relationship between these two values. Since, in general, the distribution $c(b)$ is not known, it is common practice to neglect the non-linearity and to consider $\Delta\bar{A}(\Delta\bar{K})$ as equal to $A(\Delta\bar{K})$. Under this assumption a value for ΔK and from that a value for Q can be determined from the measured value $\Delta\bar{A}$ using a suitable calibration curve. This procedure introduces, however, an error ε_K the magnitude of which is determined by the left-hand side of eqn. 2. The purpose of this paper is to analyse the value of ε_K to be expected under some typical conditions.

Non-linearity errors due to a non-uniform distribution of investigated substance over the illuminated part of the medium may be reduced if the fixed slit illuminating optics are replaced by a flying spot scanning system. In this case, however, it becomes necessary to have an output signal, which is a linear function of concentration, if non-linearity errors in integrating the output of the individual spots over the whole zone

area are to be avoided. Flying spot scanning offers the advantage that in many cases it is possible to linearise the output signal either by analogue circuitry or by digital calculation, before summing is performed to determine the total amount of substance present. Flying spot scanning can therefore be made virtually independent of zone geometry; this is not the case when slit-shaped illumination is used, because part of the summing is performed in the photodetector itself with no possibility of subsequent linearisation.

In densitometry and reflectometry it has been common practice to employ a nearly monochromatic beam of light. Good monochromacity implies, however, a very narrow spectral width of the beam and therefore a considerable loss of light. If high sensitivities are required, the resulting decrease in illuminating intensity may be a severe limitation[1]. Using a beam with a relatively large spectral width may appreciably alleviate the situation and thus decrease the relative value of the electrical noise generated in the photodetector and the associated electronic circuitry. Since, however, the absorbance of a given substance is a function of wavelength, a non-linearity error entirely analogous to that defined in eqn. 2 arises.

$$\frac{1}{\Delta\lambda}\int_{\Delta\lambda} [\Delta A(\Delta K(\lambda)) - \Delta A(\Delta\overline{K(\lambda)})]\, d\lambda \neq 0$$

$$\Delta\overline{A(\lambda)} - A(\Delta\overline{K(\lambda)}) \not\equiv 0 \qquad (4)$$

$\Delta\lambda$ signifies here the spectral width of the scanning beam and $\Delta A(\lambda)$ the transmittance or reflectance as a function of wavelength. $\Delta\overline{K(\lambda)}$ is the mean value of the increment in absorbance $\Delta K(\lambda)$ over the band $\Delta\lambda$.

Eqns. 1 and 2 are virtually identical and can, therefore, be treated in a common way. The expressions derived below, which give an estimate of the magnitude of the non-linearity error incurred, apply therefore equally to both situations.

THE CASE OF BEER'S LAW

The optical behaviour of most chromatographic support media may be described with adequate accuracy by the KUBELKA AND MUNK equations. The controlling parameters are the coefficient of scattering S and the absorbance K. In general, it may be assumed that the chromogen does not affect the scattering, so that for the purpose envisaged here the optical transfer of the medium (that is either transmittance or reflectance) can be considered as a function of K only with the value of S as a constant parameter.

The dependence of the transfer function $A(K)$ upon the absorbance K can be shown[1] to vary from a linear law to the exponential dependence, known as Beer's Law describing the transmittance of a medium with negligible scattering. The non-linearity error ε_K is evidently dependent upon the curvature of the function $A(K)$. If the radius of curvature R is large, the error will be small and *vice versa*. Fig. 2 shows a graph of $R(K)$ for four extreme conditions of scattering. The curves shown there are calculated from the theoretical expressions for the transmittance $A_T(K)$ and reflectance $A_R(K)$ derived in ref. 2 on the basis of the KUBELKA AND MUNK theory. From the figure it may be seen that for practical values of K the strongest curvature occurs with the exponential dependence. With regard to non-linearity effects the exponential

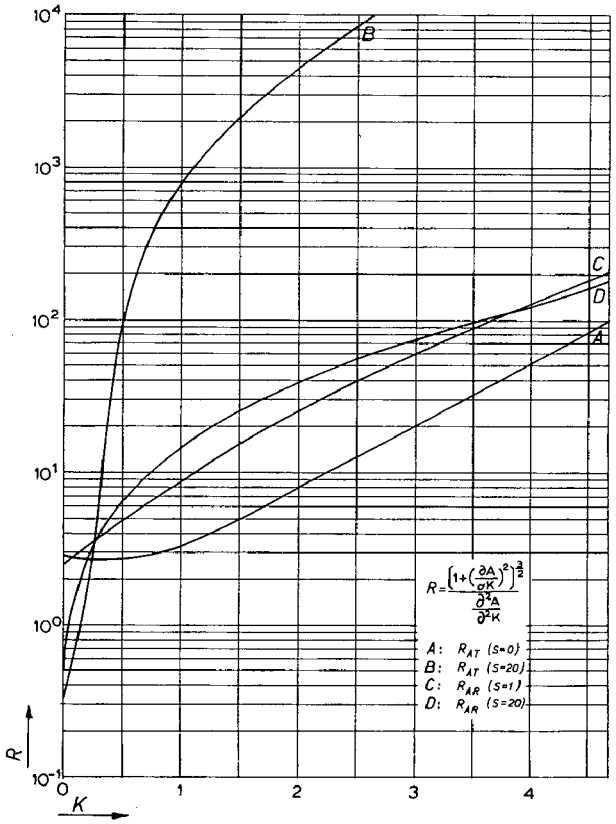


Fig. 2. The radius of curvature of transmittance and reflectance in dependence upon absorbance for extreme values of scattering. (A) R_{AT} ($S = 0$); (B) R_{AT} ($S = 20$); (C) R_{AR} ($S = 1$); (D) R_{AR} ($S = 20$).

dependence as postulated by Beer's Law represents the worst situation and produces the largest errors. A treatment based upon this law gives us therefore a worst case estimate.

The exponential relation representing Beer's Law can be expanded into a power series

$$e^{-K(x)} = 1 - K(x) + \frac{K(x)^2}{2!} - + \qquad (5)$$

In a qualitative way this may also be seen from Fig. 3 in ref. 3.

Breaking off the series after the nth term produces an error which can be shown to be less than the absolute value of the $(n + 1)$th term and to have the opposite sign as this term.

Let us now assume that in the region of concern the mean value of $K(x)$ is $\overline{K(x)}$, the bar again indicating the operation of averaging. The local value $K(x)$ will have a maximum absolute deviation of K_m from this value. Introducing $k(x)$ as a new variable, we obtain:

$$k(x) = K(x) - \overline{K(x)}$$

$$- K_m \leqslant k(x) \leqslant + K_m$$

Introducing this new variable, we may write:

$$A(K(x)) = e^{-K(x)} = e^{-[\overline{K(x)}+k(x)]} = e^{-\overline{K(x)}} \left[1 - \frac{k(x)}{1!} + \frac{k(x)^2}{2!} - +\right] \qquad (6)$$

Let the absorbance of the blank medium be K_0. The total absorbance of the medium, when an absorbing zone is scanned, is then of course the sum of K_0 and the increment $\Delta K(x)$ due to the absorbing substance. This gives us:

$$\overline{K(x)} = K_0 + \Delta\overline{K(x)} \qquad (7)$$

With a non-uniform distribution of $\Delta K(x)$ over the window space, as specified by the non-equality in eqn. 2, we obtain an output signal from the photodetector which is proportional to $\Delta\overline{A(K(x))}$. Since the distribution of $\Delta K(x)$ is in general not known, it is common to determine the required amount of investigated substance, Q, by inverting eqn. 6 and introducing the result into eqn. 1. This procedure entails, however, an error ε, equal to:

$$\varepsilon_K = -\left[\log \overline{A(K(x))} - \log A(\overline{K(x)})\right] = -\log \frac{\overline{A(K(x))}}{A(\overline{K(x)})}$$

$$= -\log \frac{\Delta\overline{A(\Delta K(x))}}{\Delta A(\overline{\Delta K(x)})} \qquad (8)$$

Introducing the expansion (5) we obtain:

$$\varepsilon_K = -\log \int_0^x \left[1 - \frac{k(x)}{1!} + \frac{k(x)^2}{2!} - +\right] \frac{dx}{X} \qquad (9)$$

X is here the maximum value of the variable x, specifying the width of the optical window. X may stand for any variable upon which the absorbance increment $\Delta K(x)$ may depend. In the cases examined here X may signify the width coordinate b of the sample or the spectral width λ.

The second term of the integral in eqn. 9 is by definition zero. If K_m is not too large, the expansion may be terminated after the second order term resulting in:

$$\varepsilon_K \simeq -\log \left[1 + \frac{\overline{k(x)^2}}{2}\right] \qquad (10)$$

The error in the term under the logarithm, committed in this way, is less than

$$\frac{1}{X} \int_0^x \frac{k(x)^3}{6} dx \Big|$$

(See remarks following eqn. 5.) Expression (10) can be further expanded, giving

$$\frac{k(x)^2}{2} = u$$

$$-\varepsilon_K = \log(1+u) \simeq u - \frac{u^2}{2} + \frac{u^3}{3} - \qquad (-1 < u \leqslant 1) \qquad (10a)$$

Terminating after the first term results in the important relation

$$-\varepsilon_K \simeq \frac{\overline{k(x)^2}}{2} \qquad \left(\frac{\overline{k(x)^2}}{2} \leqslant 1\right) \tag{11}$$

According to the statement concerning the magnitude and the sign of the error incurred, if an alternating uniformly converging series is terminated after a finite number of terms, which was discussed in relation to eqn. 5 the value of ε_K obtained in eqn. 11 is larger than the real value and therefore too unfavourable. If the term $\overline{k(x)^2}$ is kept smaller than unity the error committed by using only one term of the series in eqn. 10 is less than 1/8, which represents a maximum of approximately 12%.

The error ε_K as determined by eqn. 10 is expressed in natural units of absorbance. To obtain the error in optical density units, ε_K has to be divided by log 10 (*i.e.* 2.3).

The term $\overline{k(x)^2}$ in eqn. 10 is the mean square deviation of $\Delta K(x)$ from its mean value $\Delta\overline{K(x)}$. For practical applications the following relation, which can be proved easily, may be useful:

$$\overline{k(x)^2} = \frac{1}{X}\int_0^X \left[K(x) - \overline{K(x)}\right]^2 \mathrm{d}x = \frac{1}{X}\int_0^X \left[K(x)^2 - \overline{K(x)^2}\right] \mathrm{d}x$$

$$= \frac{1}{X}\int_0^x k(x)^2 \,\mathrm{d}x \tag{12}$$

Frequently it is not so much the absolute value ε_K of the error which is of importance but its relative value with respect to the total mean increase in absorbance $\Delta\overline{K(x)}$ over the absorbance value of the blank medium. Using eqns. 8 and 11 this relative error ε_{KR} turns out to be

$$\varepsilon_{KR}(\%) = 100\,\frac{\varepsilon_K}{\Delta\overline{K(x)}} \simeq -100\,\frac{\overline{k(x)^2}}{2\left[\log \Delta\overline{A(K(x))} - \frac{\overline{k^2(x)}}{2}\right]} \tag{13}$$

The conditions under which the right-hand approximation in eqn. 13 may be used are the same as those quoted in connection with eqns. 9 and 10a.

Once more it should be stressed that eqns. 11 and 13 are based upon Beer's Law and thus represent a worst case estimate; in most cases, and those with which practical photodensitometry or reflectometry are concerned, the curvature of A as a function of K is smaller than with Beer's Law and the error estimates obtained here are too unfavourable.

SOME NUMERICAL EXAMPLES

The implications resulting from eqns. 11 and 13 are probably best illustrated by considering some numerical examples, which approach the real situations frequently encountered in practice.

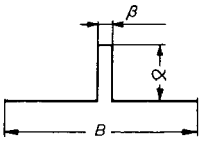


Fig. 3. Idealised absorbance profile of a narrow zone illuminated by a transversal slit.

As a first example we shall consider the extremely unfavourable case of a narrow zone, covering only a small part of the illuminated window. The idealised absorbance profile for this case is shown in Fig. 3.

If the zone is very narrow, that is if $\beta/B \ll 1$, we can put $k(b) \simeq K(b)$ and consequently

$$\overline{k^2(b)} \simeq \alpha^2 \cdot \frac{\beta}{B}$$

$$\Delta\overline{K(b)} = \frac{\alpha \cdot \beta}{B} \ll 1 \tag{14}$$

Introducing these relations into eqn. 13 we have:

$$\varepsilon_{KR}\,(\%) \simeq 100\,\frac{\alpha^2 \cdot \frac{\beta}{B}}{2\,\frac{\alpha \cdot \beta}{B}} = 100\,\frac{\alpha}{2}\,(\%) \tag{15}$$

As mentioned before, for the numerator in eqn. 15 to be valid without excessive error α should be $\lesssim 1$. As a test upon the accuracy of the approximations involved, let us consider the exact result for the case where $\alpha = 1$ and $\beta/B = 0.1$. The true value of $\Delta\bar{K}$ is evidently 0.1 and for $\Delta\bar{A}$ we obtain $0.1\ (1 - e^{-1}) \simeq 0.063$. From this we would obtain an apparent value of $\Delta\bar{K} = \log\,(1 - 0.063) \simeq 0.063$ resulting in a relative error ε_{KR} $(0.1 - 0.063)/0.1 = 37\%$. The approximate expression (15) gives for the same case an error of 50%, that is a value about 30% too unfavourable. Since, however, the conditions of this particular example are rather extreme, this accuracy may be considered adequate. For less extreme cases the accuracy in determining the error by eqn. 15 is much better.

From eqn. 15 it follows that in the case considered the integration error is rather severe; to keep it within tolerable limits α has to be very small, of the order of 0.2 (less in natural units). To obtain a reasonable accuracy when narrow zones with higher increments in absorbance are to be scanned, the conventional slit-shaped window has to be replaced by a flying spot system. As already shown (see refs. 4 and 5), a further advantage of flying-spot arrangements is the considerable improvement in optical signal-to-noise ratio. In order to make full use of the advantages of a flying spot system, however, linearisation of the output signal is essential. This can be done by digital processing or by analogue circuitry; the latter procedure will be extensively discussed in ref. 2.

If β/B becomes larger, the situation improves due to an increase in $\Delta\bar{K}$. Let us assume $\beta/B = \frac{1}{2}$, then

$$\Delta\overline{K(b)} = \frac{\alpha}{2}$$

$$\overline{k^2(x)} = \frac{a^2}{4}$$

$$\varepsilon_{KR}\,(\%) = 100\,\frac{a}{8} \tag{16}$$

and this results in a fourfold improvement against the previous case.

A more favourable situation, which is a good approximation similar to many practical cases, will now be discussed. Let us assume that the distribution of $K(x)$ over the slit is parabolic although this is by no means essential (see Fig. 4). Here we obtain:

$$aK_1 = K_2$$

$$K(b) = K_1\left[1 + a\left\{1 - \left(\frac{2b}{B}\right)^2\right\}\right]$$

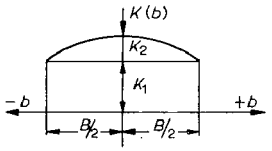


Fig. 4. Parabolic absorbance profile over the window aperture.

$$\overline{K(b)} = \frac{1}{B}\int_{-B/2}^{+B/2} K(b)\cdot \mathrm{d}b = K_1\left(1 + \frac{2a}{3}\right)$$

$$k^2(b) = \frac{1}{B}\int_{-B/2}^{+B/2}\left[K(b) - \overline{K(b)}\right]^2 \mathrm{d}b$$

$$= \frac{1}{B}\int_{-B/2}^{+B/2} K_1{}^2a^2\left[\frac{1}{3} - \left(\frac{2b}{B}\right)^2\right]^2 \mathrm{d}b = K_1{}^2a^2\left[\frac{1}{9} - \frac{1}{5}\right] \simeq 0.09\,a^2K_1{}^2 \tag{17}$$

Introducing this value into eqn. 13 we obtain:

$$\varepsilon_{KR}\,(\%) = 100\,\frac{0.09a^2K_1{}^2}{2K_1\left(1 + \frac{2a}{3}\right)} \simeq 4.5a^2K_1\,(\%) \qquad (a \ll 1) \tag{18}$$

If we assume $a = 0.3$ and $K_1 = 1$, we obtain an error $\varepsilon_{KR} \simeq 0.4\%$, a value which in most cases is negligible.

AN EXAMPLE FOR SPECTRAL NON-UNIFORMITY

Let us now consider a spectral window of finite width Λ (see Fig. 5). The absorbance of the chromogen will in general vary over this width. The real curve of

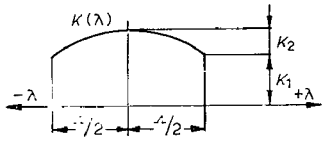


Fig. 5. Approximation of the spectral absorbance curve by a parabolic arc.

absorbance against spectral position within the window will again, as was done in the previous example, be approximated by a parabolic arc, though any other reasonable analytical expression (*e.g.* a $\cos^2$ or Gaussian curve) will lead to very similar results.

The total increment in absorbance is, of course, proportional to the concentration c of the chromogen in the scanned part of the zone. We can therefore apply the results obtained in the previous example, putting:

$$K_1 = \Delta K_{\max} \cdot \gamma$$

$$K_2 = aK_1$$

$$a = \frac{1 - \gamma}{\gamma}$$

This gives an error

$$\varepsilon_{KR}\,(\%) \simeq 4.5\,\frac{(1-\gamma)^2}{\gamma} \cdot \Delta K_{\max}\,(\%) \qquad (19)$$

Assuming a rather broad spectral window with $\gamma = 0.5$ and the peak absorbance increment $\Delta K_{\max} = 2.5$ the error still does not exceed approximately 5%. This illustrates that relatively broad spectral windows may be tolerated without any sizeable deterioration of measurement accuracy.

ACKNOWLEDGEMENT

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CHROM. 4726

APPROXIMATE SOLUTIONS TO THE KUBELKA AND MUNK EQUATIONS

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SUMMARY

The present paper discusses the simulation of the optical characteristics of plane parallel isotropic media under special conditions where the general solutions either degenerate or else may be considerably simplified. The first case is mainly that of a scattering medium with vanishing absorbance. The second one assumes high scattering and medium absorbance. In this latter case both transmittance and reflectance may be approximated by an $e^{-\text{const}\sqrt{K}}$ function. Other sections consider the determination of the basic constants of the solutions by optical measurements on the blank medium and the character of the optical noise in transmittance and reflectance measurements. Reflectance is shown to be less susceptible to optical noise than transmittance.

INTRODUCTION

In a recent paper[1] the authors discussed the electrical modelling of the optical behavior of plane parallel scattering media. The results derived were completely general and covered the whole range of media to which the KUBELKA AND MUNK theory[2] may be applied. There are, however, certain particular situations where the solutions obtained may be much simplified and which might, therefore, merit a more detailed consideration; several of these are considered in this paper.

It has been shown[1] that the transmittance A_T and reflectance A_R of a medium can be expressed in terms of the characteristic impedance ζ_0, the attenuation constant γ and the reflection coefficient ϱ of a 3-terminal electrical model circuit. The meaning of these terms was briefly described in ref. 1. They will not be repeated here. The relations found were:

$$A_T = \frac{e^{-\gamma}(1-\varrho^2)}{1-\varrho^2 \cdot e^{-2\gamma}} = \frac{2v(1)}{v(0)+i(0)} \tag{1}$$

$$A_R = -\varrho\frac{1-e^{-2\gamma}}{1-\varrho^2 \cdot e^{-2\gamma}} = \frac{v(0)-i(0)}{v(0)+i(0)} \tag{2}$$

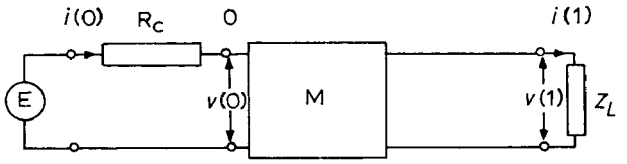


Fig. 1. General model diagram. M = Model; $R_c = 1$ = current measuring resistor; $Z_L = 1$ = load resistor; E = input generator with voltage E.

The symbols v and i on the right-hand side of eqns. 1 and 2 designate voltage and current, respectively, of the electrical model; the numbers in brackets refer to the terminal at which these values are observed, 0 marking the input and 1 the output (see Fig. 1).

The parameters ζ_0, γ, and ϱ are related to each other and to the coefficient of scattering S and of absorbance K of the medium by the following relationships:

$$\zeta_0 = \sqrt{\frac{2S + K}{K}}$$

$$\varrho = \frac{1 - \zeta_0}{1 + \zeta_0}$$

$$\gamma = \sqrt{\{K\,(2S + K)\}} = K \cdot \zeta_0 = K \frac{1 - \varrho}{1 + \varrho} \tag{3}$$

For ζ_0 and ϱ apply the limitations:

$$1 \leqslant \zeta_0 \leqslant \infty \tag{4}$$
$$-1 \leqslant \varrho \leqslant 0$$

THE DEGENERATE CASE WITH EITHER S OR $K \to 0$

A special situation arises if one of the variables S or K vanishes. In this case our model equations degenerate and cannot be applied immediately. The case of either S or K tending towards infinity is of no practical importance and need not be considered here.

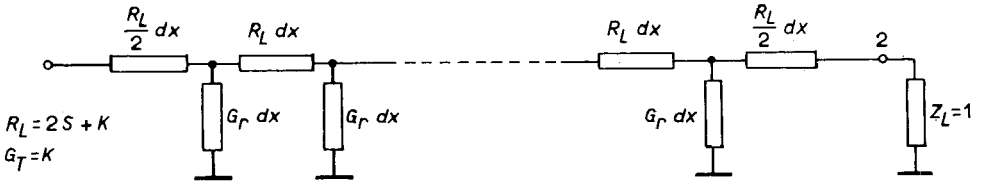


Fig. 2. Basic diagram of transmission line model.

If K tends to zero, the basic expressions (1) and (2) become meaningless. It is easy, however, to obtain the desired result by using the basic transmission line model illustrated in Fig. 2 (the derivation of this was discussed in ref. 1) and setting $K = 0$. What is obtained is a simple voltage divider network (see Fig. 3b).

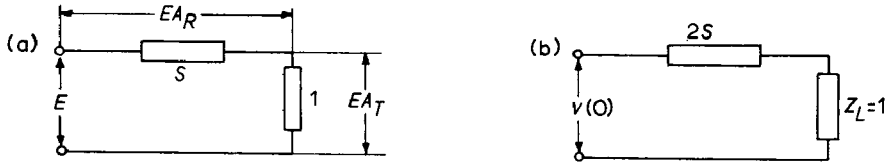


Fig. 3. (a) Equivalent circuit for transmission and reflection with $K = 0$. (b) Model of a degenerate case with $K = 0$.

Using eqns. 1 and 2 we find the transmittance and reflectance for this case from the model circuit in Fig. 3b which represents a degenerate line. Since scattering alone only changes the direction of flow of the radiant energy without producing any losses, the sum of transmittance and reflectance euals 1 (ref. 3).

$$A_T(0) = \frac{1}{1+S}$$

$$A_R(0) = 1 - A_T(0) = \frac{S}{1+S} \tag{5}$$

These particular relationships are modelled by the circuit shown in Fig. 3a.

The second limiting case with $S = 0$ can easily be determined from the basic equations, since in this case ζ_0 becomes 1 and $\varrho = 0$. We obtain an attenuating pad with attenuation $\gamma = K$ and characteristic impedance $\zeta_0 = 1$. This case corresponds of course to the ideal relations prescribed by Beer's Law. It can probably best be modeled by a bridged T-network (see Fig. 4). Reflection is zero, since the impedance

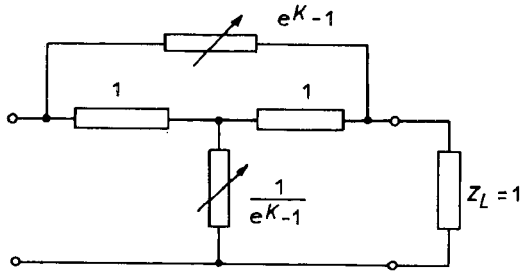


Fig. 4. Bridged T-model for case $S = 0$; $\zeta = 1$; $\gamma = \log e^K = K$.

of the model is constant (unity) and matched to the load at all values of K. Expressed in optical terms it is zero, because no backscatter occurs. The transmission A_T is obviously e^{-K}.

THE NEARLY DEGENERATE CASE WITH $0 < K \ll 1$

When a finite but very small amount of absorbance $K \ll 1$ is present on the medium, the equivalent transmission line ceases to be degenerate. The resulting decrement in transmittance ΔA_T or reflectance ΔA_K can of course then be found from the basic eqns. 1 and 2. The results may be read from the graphs of $A_T' = \partial A_T/\partial K$

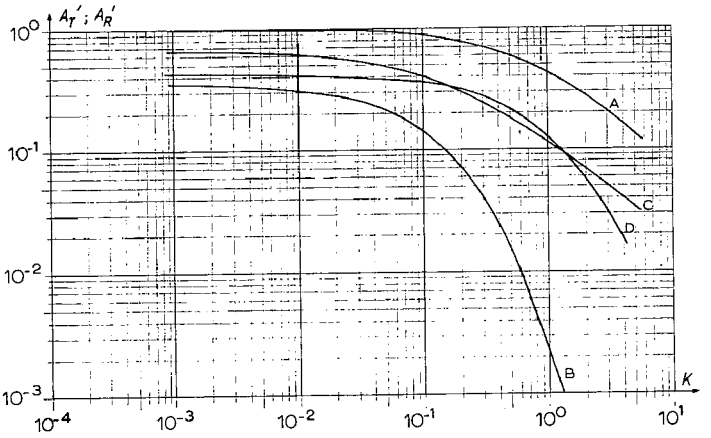


Fig. 5. Sensitivity curves for transmittance and reflectance at extreme values of S. (A) A_T' ($S = 0$); (B) A_T' ($S = 20$); (C) A_R' ($S = 1$); (D) A_R' ($S = 20$).

and $A_R' = \partial A_R/\partial K$, which are shown for two extreme values of S in Fig. 5. These curves were calculated from eqns. 1 and 2.

For small increments in absorbance, ΔK, both transmittance and reflectance can be approximated by a linear dependence,

$$\Delta A_T \simeq A_T' \cdot \Delta K$$
$$\Delta A_R \simeq A_R' \cdot \Delta K \qquad (6)$$

The useful signal obtained during actual measurement is proportional to ΔA_T or ΔA_R. A_T' and A_R' therefore characterise the absolute sensitivity of the method. From the graphs shown in Fig. 5 it may be seen that the largest value of the derivative of both transmittance and reflectance occurs at small values of K. With respect to S the highest sensitivity is obtained in transmittance measurements, if $S = 0$, that is if Beer's Law is valid. The smallest absolute change and thus the smallest value of useful signal for a given value of K is encountered when the medium is strongly scattering (*i.e.*, $S = 20$). Curves of $A_T' = f(K)$ for intermediate values of S will fill the space between these two extreme curves.

The sensitivity of reflectance measurements lies between the extreme values for transmittance. Quite surprisingly, however, the curves for different values of the coefficient of scattering intersect, so that the value of S giving the best sensitivity depends upon the absorbance K of the medium.

Instead of the absolute sensitivity it may sometimes be more convenient to consider the relative sensitivity $A'(K)/A(K)$ with $\Delta K = 0$. For small values of K the relative sensitivity increases with increasing scattering, whilst at larger values of K the opposite condition prevails.

THE REFLECTANCE OF MEDIA WITH MEDIUM TO LOW TRANSMITTANCE

A particular case, frequently encountered in technical application, is a medium with relatively strong scattering and a certain minimum of absorbance. This minimum

value of K is dependent upon the coefficient of scattering S and may be expressed as the requirement:

$$1 < \gamma = \sqrt{\{K\,(2S + K)\}} \simeq \sqrt{2SK} \tag{7}$$

Expression (2) may now be simplified to:

$$e^{-2\gamma} \ll 1$$
$$A_R \simeq \varrho \tag{8}$$

Most practical measurements using reflectance spectroscopy are based upon this approximation. Its accuracy increases with increasing values of γ and decreasing values of ϱ^2. Inspection of eqns. 3 reveals that the reflection factor ϱ is solely a function of the ratio K/S. The inverse expression $K/S = f(\varrho)$ is usually called the remission function*. It can be obtained by solving the equations defining ϱ for the variable $u = K/S$.

$$\frac{K}{S} = u$$

$$\zeta_0^2 = \left(\frac{1 - \varrho}{1 + \varrho}\right)^2 = \frac{2 + u}{u}$$

$$u = -\tfrac{1}{2}\left(\frac{1}{\sqrt{\varrho}} + \sqrt{\varrho}\right)^2 \tag{9}$$

A closer examination of eqn. 9 reveals that on a logarithmic scale for u the right-hand side is symmetrical to the axis $|\varrho| = 1$. A graph of $u = f(\varrho)$ is shown in Fig. 6. From this figure it is evident that in order to obtain good accuracy, the range of ϱ over which reflectance measurements are to be made, should be restricted to approximately $-1 \leqslant \varrho \lesssim -0.3$, or $K/S < 1$. At very small values of K/S it is necessary to be care-

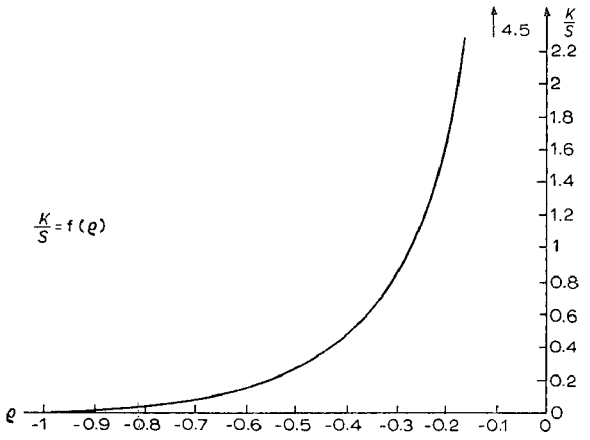


Fig. 6. The remission function $K/S = f(\varrho)$.

* Most frequently the remission function is defined in terms of $R = -\varrho$. See also ref. 4.

ful since γ may become too small for the approximation to be valid, in which case considerable errors will ensue.

In practice the approximate value for A_R may be used for all media with an optical density above about one unit. To illustrate this, the following argument may be used.

Optical density is defined as the decimal logarithm of A_T^{-1} or 2.3 times its natural logarithm. Inspection of eqn. 1, however, shows that the factor $(1-\varrho^2)/(1-\varrho^2 \cdot e^{-2\gamma})$ can only appreciably contribute to the optical density when $1-\varrho^2$ is very small. *E.g.*, for $\varrho = -0.9$ the smallest value of this factor is evidently 0.19, which is approximately 0.7 optical density units. For $\varrho = -0.9$ the value of K/S (read from Fig. 6) is, however, so small that it will only infrequently be encountered in actual practice. This means that a medium with an optical density D larger than 1 will nearly always have a γ value of at least $(D-0.7) \times 2.3$. For optical density values $D > 1$ we therefore nearly always find $e^{-2\gamma} \ll 1$.

AN APPROXIMATE EXPRESSION FOR THE REFLECTION FACTOR

It was pointed out above that for reasonable accuracy the value of ϱ should be below -0.35, or $u \lesssim 1$. It will now be shown that for this range, which is of considerable practical importance, the remission function $u = f(\varrho)$ can be closely approximated by an exponential function. In order to do this we write:

$$u = 2q^2$$

$$\varrho = \frac{1 - \zeta_0}{1 + \zeta_0} = \frac{q - \sqrt{(1 + q^2)}}{q + \sqrt{(1 + q^2)}}$$

$$\log \varrho = \log [q - \sqrt{(1 + q^2)}] - \log [q + \sqrt{(1 + q^2)}] \tag{10}$$

However

$$[q - \sqrt{(1 + q^2)}] [q + \sqrt{(1 + q^2)}] = -1$$

therefore

$$\log [q + \sqrt{(1 + q^2)}] = -\log | [q - \sqrt{(1 + q^2)}] |$$

$$\log | \varrho | = -2 \log [q + \sqrt{(1 + q^2)}] \tag{11}$$

The right-hand side of this relation can be expanded into a power series[5]:

$$\log [q + \sqrt{(q^2 + 1)}] = q - \frac{1}{2 \cdot 3} q^3 + \frac{1 \cdot 3}{2 \cdot 4 \cdot 5} q^5 - \frac{1 \cdot 3 \cdot 5}{2 \cdot 4 \cdot 6 \cdot 7} q^7 + - \tag{12}$$

The series expansion in eqn. 12 is valid for $q^2 < 1$. At the other end of the scale, that is for $q > 1$, another expansion may be applied.

$$\log [q + \sqrt{(q^2 + 1)}] = \log 2q + \frac{1}{2 \cdot 2} \cdot \frac{1}{q^2} - \frac{1 \cdot 3}{2 \cdot 4 \cdot 4} \cdot \frac{1}{q^4} +$$

$$+ \frac{1 \cdot 3 \cdot 5}{2 \cdot 4 \cdot 6 \cdot 6} \cdot \frac{1}{q^6} - + \tag{13}$$

There is a well known theorem about the error committed in terminating a uniformly convergent power series with terms of alternating sign. This theorem states that the error committed in this way is less in magnitude than the first omitted term. Applying this to series (12), we find that the first term in eqn. 12 is thus an adequate representation of the remission function provided the next term is reasonably smaller (*e.g.*, less than 10%). This leads to the approximation

$$\log \varrho \simeq 2q = \sqrt{\frac{2K}{S}}$$

If $\frac{q^3}{6q} = \frac{u}{12} \lesssim 10\%$

$u \lesssim 1.2$, that is $\varrho \lesssim -0.23$)

$$\varrho \simeq -e^{-\sqrt{\frac{2K}{S}}} \tag{14}$$

As already mentioned, the range for which this approximation is valid coincides closely with the range most suitable for reflectance spectroscopy.

For very large values of u, series (13) can be used as a basis for a simplified approximate expression. Allowing an error of similar magnitude, we obtain:

$$\log \varrho \simeq -2 \log 2q = -\log 2u$$

$$u = \frac{K}{S} = \frac{1}{2\varrho}$$

If $\frac{1}{4q^2 \cdot \log 2q} = \frac{1}{u \log 2u} \lesssim 10\%$

that is for $u \gtrsim 4.5$ (15)

In other words, for very large values of the ratio K/S, that is for media with little scattering and high absorbance, the remission function becomes inversely proportional to K.

THE TRANSMITTANCE OF MEDIA WITH STRONG SCATTERING AND INTERMEDIATE ABSORBANCE

The considerations in this section apply to the case where $\varrho^2 \ll 1$; a value of $|\varrho| \leq 0.4$ will in general be adequate. From Fig. 6 it may be seen that this corresponds to $K/S \geq 0.4$, and that beyond this value ϱ^2 changes only relatively slowly with K. Eqn. 1 thus reduces to:

$$A_T \simeq e^{-\gamma} \left(\frac{K}{S} \gtrsim 0.4\right) \tag{16}$$

In the range

$$1 \gtrsim \frac{K}{S} \gtrsim 0.4$$

we may make the further approximation

$$\gamma = \sqrt{\{(2S + K) \cdot K\}} \simeq \sqrt{2SK}$$

resulting in

$$A_T = e^{-\sqrt{2SK}} \tag{17}$$

Approximation (17) is appropriate principally for strong scattering and intermediate values of absorbance (*e.g.* paper). A comparison with expression (14) shows that in both cases the logarithm of the transfer function is proportional to $\sqrt{K}$. The range in which eqn. 14 may be applied includes that for expression (17), which is, however, somewhat more restricted towards smaller values of K/S.

DETERMINATION OF THE OPTICAL CONSTANTS OF THE MEDIUM

Before the transfer eqns. 1 and 2 can be solved for a particular application or before they can be simulated on a model arrangement, the optical constants of the blank (unstained) medium must first be determined by optical measurements. In general two independent measurements will be required to determine S and K. In order to eliminate statistical variations in the values of these parameters, however, it may frequently be desirable to perform two series of such measurements.

As described earlier, the reflectance of a layer (or multiple layers) of a medium with a sufficiently high optical density is equal to ϱ. Care has to be taken to exclude the surface component of the reflected light by using for example polarised light filters[4]. Using eqn. 9 or Fig. 6 the ratio K/S can be determined from the measured value of ϱ. The second measurement may be the transmittance of the same medium. Introducing the measured value of ϱ into expression (1), the corresponding value of $e^{-\gamma}$ (and from that of course γ) can easily be determined. Provided $e^{-2\gamma}$ is small enough, the simplified results are:

$$\gamma = \log \frac{A \varrho}{1 - \varrho^2} \tag{18}$$

$$K = \frac{\gamma}{\zeta_0} = \frac{1 + \varrho}{1 - \varrho} \cdot \log \frac{A_T}{1 - \varrho^2}$$

$$S = \frac{K}{2} (\zeta^2{}_0 - 1) = K \cdot \frac{2\varrho}{1 - \varrho^2} = \frac{2\varrho}{(1 - \varrho)^2} \cdot \log \frac{A_T}{1 - \varrho^2} \tag{19}$$

If the approximations (14) and (17) both hold, the relations above can be further simplified to yield:

$$\log \varrho \cdot \log A_T = 2K$$
$$\log A_T / \log \varrho = S \tag{20}$$

On occasions it may be preferable to measure just reflectance or else transmittance. In these cases a chromogen with known increments in absorbance is applied to the medium and from the values of the transfer for K and $K + \Delta K$ the values S and K may be calculated. The calculation is, of course, easier to perform if one of the approximations described in eqns. 8, 14 or 16 can be applied. If a general purpose

model such as the one described in ref. 1 is available, the calculation may be replaced by successive adjustment of the model parameters on a trial and error basis, until the values of A_R and A_T measured on the model agree with the optical values obtained from the medium. This procedure is very much facilitated if the approximate values of S and K are known beforehand.

Fluctuations in the basic parameters of the medium (variations in thickness, density, etc.) produce fluctuations in the transfer function, which for the purpose of measuring changes in absorbance are equivalent to the noise in a communication system[6].

As long as these fluctuations affect S and K equally, we can consider them as a change ΔX in the effective thickness of the medium. ζ_0 as a ratio value will in this case remain unchanged and so by the same token will ϱ. Only γ will be affected and that in proportion to ΔX:

$$\Delta\gamma = \gamma \cdot \Delta X \tag{21}$$

Turning back to eqns. 1 and 2 we find that A_R is principally a function of ϱ. Reflectance, therefore, does not change very much with variations in the optical thickness of the background medium. It is, however, sensitive to inhomogeneities in the medium, affecting K and S to a different degree and therefore producing fluctuations in ζ_0 and ϱ as well as in γ. In general, however, fluctuations of the first type seem to prevail, and as a consequence of this, reflectance measurements are relatively insensitive to optical noise, provided surface effects can be disregarded.

A_T, on the other hand, is influenced by fluctuations in the effective thickness of the medium.

$$\Delta A_T \simeq A_T(0) \cdot e^{-\Delta\gamma} = A_T(0) \cdot e^{-\gamma \cdot \Delta X} \tag{22}$$

From eqn. 22 we see that for a given variation ΔX the change in transmittance produced is proportional to the mean value of transmittance $A_T(0)$. In consequence of this, noise is produced which is basically multiplicative in nature. This is a fundamental difference from the predominantly additive noise in electrical systems. One important feature of multiplicative noise is that it can be largely suppressed by forming the ratio of two equally affected signals. Difference procedures, which would be the method of choice in additive systems, are less efficient in these cases.

CONCLUSION

Summarising the results obtained we arrive at the following conclusions:

As previously shown[1], a resistive transmission line is a convenient model for studying, in general, the optical properties of thin media that can be adequately described by the KUBELKA AND MUNK theory. For certain special cases, that is for some limited ranges of optical parameters, it can be shown that the general solutions may be very much simplified. Circuit arrangements to model these expressions will be described in a future publication.

Reflectance measurements are to be recommended mainly when the medium in question has high scattering power and low to medium absorption at high optical density. In this case such measurements are less susceptible to optical noise than is the case for transmission measurements.

Transmission measurements can be used in all cases provided the optical density is not too high and that the output signal is larger in amplitude than the optical equivalent of the electrical noise of the detector assembly. The optical noise in transmission measurements is essentially multiplicative and to reduce it, a double-beam arrangement with ratio forming at the output seems to be the best solution. The strictly exponential dependence of transmittance upon absorbance postulated by Beer's Law represents a limiting case, which is attained only if the coefficient of scattering S tends towards zero. In the practically important case of a medium with high scattering power, intermediate absorbance values and high optical density the law of dependence of both transmittance and reflectance can be approximated by an $e^{-\text{const}\sqrt{K}}$ function.

ACKNOWLEDGEMENTS

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ANALYTICAL STUDIES OF PYRETHRIN FORMULATIONS BY GAS CHROMATOGRAPHY*

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SUMMARY

Commercial formulations of mixtures of pyrethrins, piperonyl butoxide, and *n*-octyl bicycloheptene dicarboximide were quantitatively measured by gas chromatography. The three active ingredients of the formulations were measured simultaneously using gas chromatographic columns containing the support Chromosorb W coated with either 3% or 5% SE-30 silicone. The individual pesticide components present in the formulations, ranging in amounts of 0.05 to 50.0% of the mixtures, could be measured within a reasonable degree of accuracy.

INTRODUCTION

Pesticide chemists generally agree that the AOAC method[1] for the analysis of pyrethrin formulations is tedious, time-consuming, and lacks precision and accuracy; the latter is evident in the collaborative report by KELSEY[2]. The British Joint Committee of the Pharmaceutical Society and the Society for Analytical Chemistry on Methods of Assay for Crude Drugs made a comparative detailed study of the method with the PBK (Pyrethrum Board of Kenya) procedure[3] and concluded that the AOAC procedure was "inherently unsatisfactory", that it also measured "false materials", but they recommended its use as an interim measure.

The AOAC procedure measures the hydrolysis products of the pyrethrums, considered to be chrysanthemum monocarboxylic acids which are derived from pyrethrin I and cinerin I components of the pyrethrum mixture and are reported as pyrethrin I (ref. 4); the measured dicarboxylic acids are derived from the pyrethrin II and cinerin II components and are reported as pyrethrin II. Recent reports[5] indicate that jasmolin I would also be included in the pyrethrin I fraction and jasmolin

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II would be included in the pyrethrin II fraction. Pyrethrins are subject to degradation by air oxidation, exposure to light, and possibly by enzymatic activity[6], and the degraded components most probably yield acids on hydrolysis which would be measured and reported as true pyrethrins.

To facilitate process and formulation procedures, WACHS AND HANLEY[7] utilized the principle of the selective reaction of ethylenediamine with the cyclopentenolone esters of pyrethrums. The amine salts of the carboxylic acids are titrated with sodium methylate. The esters of cinerin II and pyrethrin II were not measured in this procedure and a molecular weight factor was used to calculate total pyrethrins, which assumed a 1:1 ratio of pyrethrins I and II. FURMANEC *et al.*[8] employed thin-layer chromatography for the separation of pyrethrins I and II, followed by treatment of the separated fractions with orthophosphoric acid and heat and the measurement of the reaction products by colorimetry at 550 mμ. In the latter procedure, decomposition problems could arise unless elaborate precautions were taken[9].

The most promising procedure for the screening of pyrethrum formulations, especially for the ones that contain small amounts (in the range of 0.05–0.50% pyrethrin concentrations) of the pesticide, includes the use of gas chromatography[9–16]. Further, if the synergist piperonyl butoxide (PBO) or *n*-octyl bicycloheptene dicarboximide (NOBD) are present in the pyrethrin formulation, all of the components can be measured simultaneously under the conditions reported in this study. It is conceded[5] that a pyrethrin primary standard or an accurately assayed pyrethrum extract must be available as a frame of reference for quantitation purposes. This report presents data on the analysis of pyrethrins and synergists by gas chromatography wherein the only variable parameter was the gas chromatographic column. The cleanup reagent Florisil was used with all samples to remove oil-based materials and other substances that would otherwise interfere with the gas chromatographic analysis of the NOBD component of the formulation.

MATERIALS AND METHODS

Materials

Gas chromatograph. A Varian Aerograph Series 1200, flame-ionization detector was employed. The column temperature was 190°, the injector and detector temperatures were 205°. Gas flow rates were nitrogen, 25 ml/min; hydrogen, 25 ml/min; air 200 ml/min. Three of the gas chromatographic columns used contained packings of 3%, 5%, and 7% SE-30 silicone, respectively, coated on a Chromosorb W support. A fourth column contained 5% SE-30 silicone coated on Gas-Chrom Q support. All supports were 60–80 mesh, acid-washed and treated with dimethyldichlorosilane. The gas chromatographic columns were $\frac{1}{8}$ in. I.D. by 5-ft. spiral borosilicate glass. A Leeds and Northrup Speedomax H recorder, 1 mV full scale, chart speed 0.5 in./min was used.

Chromatographic columns for Florisil cleanup. The columns consisted of 20 × 400 mm borosilicate glass with Ultramax stopcock and 300-ml reservoir.

Glass U-tubes for aerosol sampling. Two types of tubes were applied: (1) 1.5 mm I.D. × 7 mm O.D. capillary tubing with one end expanded to accept a 3-mm ($\frac{1}{8}$-in.) silicone 'O' ring. (2) 3-mm ($\frac{1}{8}$-in.) glass tubing fused inside 5 mm O.D. glass tubing

with an extension of about 12 mm of the $\frac{1}{8}$-in. tubing. A $\frac{1}{8}$-in. 'O' ring will seal the connection when the sample is withdrawn from the aerosol container.

Florisil. Florisil (Floridin Co., Pittsburgh, Pa.), 60–80 mesh, was heated for 16 h at 130° prior to use.

Reagents. Anhydrous sodium sulfate was heated for 16 h at 280° prior to use. Hexane, acetone, and carbon disulfide were redistilled in an all-glass system.

Pyrethrin and synergists standard solutions. Pyrethrin concentrate (20% assay) was supplied by Niagara Chemical Division, FMC Corp., 100 Niagara St., Middleport, N.Y. 14105; piperonyl butoxide, 100% (PBO) by Fairfield Chemical Division, FMC Corp., 441 Lexington Avenue, New York, N.Y.; and *n*-octyl bicycloheptene dicarboximide (NOBD) by McLaughlin Gormley King Co., 1715 Fifth St., S. E., Minneapolis, Minn.

Solutions of mixtures of the above compounds were prepared for gas chromatography standards as follows. The concentration of the compounds in carbon disulfide solution was 0.4 μg/μl for pyrethrin I, and 1.1 μg/μl each for PBO and NOBD; 2-μl or 3-μl aliquots were applied to the gas chromatographic column. A range setting of 10 and a variable attenuation setting of 16, 32, or 64 were used with the instrument. To quantitate the NOBD in the standard mixture, either a smaller aliquot, a change in attenuation setting, or an additional dilution of the standard solution was necessary to bring the NOBD curve within the linear range on the recorder chart (see Fig. 1).

The linearity range was 0.2 to 2.2 μg for pyrethrin I, 0.6 to 5.6 μg for PBO, and 0.3 to 1.7 μg for NOBD, with a minimum detectability of about 0.06 μg for each of the three components.

Preparation of samples

Aerosols. The cap and spray head are removed from the container. The remainder of the container and the appropriate U-tube are weighed. The U-tube is attached to the spray tube of the aerosol container. The aerosol container is shaken vigorously for at least 30 sec, the open end of the U-tube is plunged into 125 ml hexane contained in a separatory funnel and the U-tube is immediately depressed to remove 10–20 g of the sample from the container. The container and U-tube are again weighed to determine, by difference, the weight of the sample to be analyzed. If the sample is miscible with hexane, it is transferred to the Florisil column for cleanup. If the sample is a water–oil base emulsion, the pyrethrins are partitioned into a hexane layer and the hexane is concentrated to a small volume by evaporation on a steam bath with a stream of air. The residue is then transferred to the Florisil column with hexane.

Liquids (oil based). An appropriate amount of sample is weighed, diluted with 10 ml hexane, and transferred to the Florisil column for cleanup.

Dusts. Although dusts were not included in this study, if such materials are analyzed for the pyrethrins, the sample is extracted with either acetonitrile or chloroform (dust:solvent = 1:5) by shaking for 1 h on a mechanical shaker. The mixture is filtered, the solvent is removed by evaporation, and the residue is transferred to the Florisil column with hexane for cleanup.

Sample cleanup

The chromatographic column is packed with 5 g anhydrous sodium sulfate, followed by 20 g Florisil, and topped with 5 g anhydrous sodium sulfate. The column

TABLE I

ANALYTICAL DATA ON COMMERCIAL PYRETHRUM SAMPLES OBTAINED WITH VARIOUS GAS CHROMATOGRAPHIC COLUMNS[a]

Gas chromatographic column	*Sample*[b]								
	Py			*PBO*			*NOBD*		
	Range	*Average (%)*	*Label guarantee*	*Range*	*Average (%)*	*Label guarantee*	*Range*	*Average (%)*	*Label guarantee*
3% SE-30 on Chromosorb W									
Aerosol A[c]	0.18–0.21	0.19	0.20	0.42–0.56	0.50	0.50	0.43–0.57	0.51	0.50
Aerosol B	0.16–0.23	0.20	0.20	0.47–0.64	0.53	0.50	0.47–0.69	0.53	0.50
Aerosol C	0.19–0.22	0.20	0.20	0.46–0.53	0.49	0.50	0.46–0.66	0.54	0.50
Py 2.5	2.27–2.68	2.49	2.50	—	—	—	—	—	—
Py 53	4.80–5.69	5.11	5.00	49.0–56.1	52.4	53.1	—	—	—
65404	0.038–0.049	0.044	0.05	0.078–0.086	0.082	0.10	0.14–0.16	0.14	0.16
5% SE-30 on Chromosorb W									
Aerosol A	0.20–0.24	0.22	0.20	0.43–0.54	0.49	0.50	0.44–0.54	0.47	0.50
Aerosol B	0.20–0.24	0.21	0.20	0.48–0.59	0.51	0.50	0.45–0.58	0.51	0.50
Aerosol C	0.19–0.21	0.20	0.20	0.41–0.50	0.46	0.50	0.41–0.53	0.47	0.50
Py 2.5	2.30–2.58	2.42	2.50	—	—	—	—	—	—
Py 53	4.95–5.20	5.03	5.00	50.2–53.0	51.7	53.1	—	—	—
65404	0.041–0.050	0.045	0.05	0.075–0.085	0.079	0.10	0.13–0.15	0.14	0.16
7% SE-30 on Chromosorb W									
Aerosol A	0.17–0.25	0.20	0.20	0.38–0.53	0.45	0.50	0.48–0.60	0.55	0.50
Aerosol B	0.19–0.25	0.21	0.20	0.41–0.56	0.49	0.50	0.47–0.60	0.54	0.50
Aerosol C	0.16–0.21	0.19	0.20	0.46–0.64	0.54	0.50	0.41–0.50	0.45	0.50
Py 2.5	1.90–3.45	2.39	2.50	—	—	—	—	—	—
Py 53	3.85–4.65	4.33	5.00	43.5–54.0	48.8	53.1	—	—	—
65404	0.040–0.055	0.044	0.05	0.070–0.075	0.073	0.10	0.13–0.16	0.15	0.16
5% SE-30 on Gas-Chrom Q									
Aerosol A	0.17–0.22	0.19	0.20	0.45–0.58	0.50	0.50	0.46–0.53	0.49	0.50
Aerosol B	0.17–0.23	0.20	0.20	0.43–0.59	0.51	0.50	0.36–0.54	0.47	0.50
Aerosol C	0.17–0.21	0.18	0.20	0.44–0.62	0.51	0.50	0.34–0.52	0.46	0.50
Py 2.5	2.28–2.71	2.47	2.50	—	—	—	—	—	—
Py 53	4.55–5.40	4.70	5.00	50.0–54.2	51.7	53.1	—	—	—
65404	0.043–0.052	0.047	0.05	0.087–0.092	0.089	0.10	0.13–0.15	0.14	0.16

[a] Acid-washed, dimethyldichlorosilane-treated support, 60–80 mesh.

[b] Commercially available formulations which contained pyrethrins (Py), piperonyl butoxide (PBO) and *n*-octyl bicycloheptene dicarboximide (NOBD).

[c] Aerosols A, B, and C were samples of the same shipment of the type designed for household use. The remainder of the samples were oil-based formulations. The analytical range represents nine samplings of each sample for analysis, except No. 65404, which was four samplings.

TABLE II

OBSERVED VARIATIONS IN ANALYTICAL DATA FROM REPLICATE SAMPLINGS OF THE PYRETHRIN FORMULATIONS

Gas chromatographic column	*Sample component*[a]								
	Py			*PBO*			*NOBD*		
	Range	*Maximum variation (%)*	*Maximum variation from label guarantee (%)*	*Range*	*Maximum variation (%)*	*Maximum variation from label guarantee (%)*	*Range*	*Maximum variation (%)*	*Maximum variation from label guarantee (%)*
Aerosols A, B and C[b]									
3% SE-30 on Chromosorb W	0.16–0.23	0.07	35	0.42–0.64	0.22	44	0.43–0.69	0.26	52
5% SE-30 on Chromosorb W	0.19–0.24	0.05	25	0.41–0.59	0.18	36	0.41–0.58	0.17	34
7% SE-30 on Chromosorb W	0.17–0.25	0.09	45	0.38–0.64	0.26	52	0.41–0.60	0.19	38
5% SE-30 on Gas-Chrom Q	0.17–0.23	0.06	30	0.43–0.62	0.19	38	0.34–0.54	0.20	40
Py 2.5									
3% SE-30 on Chromosorb W	2.27–2.68	0.41	16.4	—	—	—	—	—	—
5% SE-30 on Chromosorb W	2.30–2.58	0.28	11.2	—	—	—	—	—	—
7% SE-30 on Chromosorb W	1.90–3.45	1.55	62.0	—	—	—	—	—	—
5% SE-30 on Gas-Chrom Q	2.28–2.71	0.43	17.2	—	—	—	—	—	—
Py 53									
3% SE-30 on Chromosorb W	4.80–5.69	0.89	17.8	49.0–56.1	7.1	13.4	—	—	—
5% SE-30 on Chromosorb W	4.95–5.20	0.25	5.0	50.2–53.0	2.8	5.3	—	—	—
7% SE-30 on Chromosorb W	3.85–4.65	0.80	16.0	43.5–54.0	10.5	19.8	—	—	—
5% SE-30 on Gas-Chrom Q	4.55–5.40	0.85	17.0	50.0–54.2	4.2	7.9	—	—	—
65404									
3% SE-30 on Chromosorb W	0.038–0.049	0.011	22.0	0.078–0.086	0.008	8.0	0.14–0.16	0.02	12.5
5% SE-30 on Chromosorb W	0.041–0.050	0.009	18.0	0.075–0.085	0.010	10.0	0.13–0.15	0.02	12.5
7% SE-30 on Chromosorb W	0.040–0.055	0.015	30.0	0.070–0.075	0.005	5.0	0.13–0.16	0.03	18.8
5% SE-30 on Gas-Chrom Q	0.043–0.052	0.009	18.0	0.087–0.092	0.005	5.0	0.13–0.15	0.02	12.5

[a] Py = pyrethrins; PBO = piperonyl butoxide; NOBD = *n*-octyl bicycloheptene dicarboximide.
[b] Aerosols, twenty-seven samplings; Py 2.5 and Py 53, nine samplings each; 65404, four samplings.

is prewashed with 100 ml hexane, leaving enough of the solvent in the column to cover the packed contents of the column. The sample residue is transferred to the column with 5–10 ml hexane, the column is then washed with 75 ml hexane; the hexane eluate is discarded. The pyrethrins and the synergistic compounds are then eluted from the column with 125 ml acetone. The acetone eluate is evaporated nearly to dryness by means of a stream of air and a warm steam bath. The residue is diluted to about 10 ml with carbon disulfide and passed through a small column of anhydrous sodium sulfate. The sodium sulfate column is washed with a small amount of carbon disulfide and the combined eluates are made to a definite volume for gas chromatographic analysis.

RESULTS AND DISCUSSION

The data shown in Tables I and II on the pyrethrins (Py) were obtained from the pyrethrin I fraction of the gas chromatographic curve. The calculated amount of

TABLE III

ANALYSIS OF PYRETHRIN CONTENT OF AEROSOL FORMULATIONS[a]

Sample	*Gas chromatographic column*			
	3% SE-30 on Chromosorb W (%)	*5% SE-30 on Chromosorb W (%)*	*7% SE-30 on Chromosorb W (%)*	*5% SE-30 on Gas-Chrom Q (%)*
	0.19	0.20	0.20	0.19
	0.19	0.21	0.17	0.18
	0.21	0.20	0.21	0.17
	0.19	0.20	0.19	0.17
A	0.19	0.20	0.25	0.18
	0.20	0.24	0.21	0.19
	0.21	0.24	0.21	0.19
	0.18	0.24	0.19	0.21
	0.19	0.22	0.19	0.22
	0.20	0.21	0.25	0.21
	0.16	0.18	0.19	0.18
	0.18	0.23	0.20	0.20
	0.21	0.21	0.21	0.19
B	0.23	0.21	0.22	0.20
	0.22	0.23	0.21	0.21
	0.22	0.24	0.20	0.23
	0.19	0.21	0.19	0.20
	0.20	0.20	0.19	0.17
	0.20	0.20	0.21	0.17
	0.20	0.19	0.19	0.18
	0.19	0.19	0.16	0.17
	0.19	0.21	0.17	0.17
C	0.19	0.21	0.21	0.17
	0.22	0.19	0.21	0.18
	0.19	0.20	0.21	0.21
	0.20	0.20	0.19	0.20
	0.19	0.20	0.20	0.21

[a] Pyrethrin I values, determined by gas chromatography, were multiplied by a factor of 2. Nine analyses were made on each of three aerosol formulations (all three aerosols of identical label; pyrethrin content 0.20%), on four gas chromatographic columns as described above.

pyrethrin I was multiplied by a factor of 2, since it is known that the two major components present in pyrethrums are pyrethrin I and pyrethrin II and normally occur in approximately equal amounts[6,14]. With one possible exception, the data in the tables and Fig. 1 indicate that a gas chromatographic column containing a 5% SE-30 silicone on Chromosorb W (AW-DMCS), 60–80 mesh, was preferable for the separation and quantitation of pyrethrin I, NOBD, and PBO. The exception was the samples packed in the aerosol containers, where statistical analysis (Tables III and IV) indicated that the 3% SE-30 silicone column was the preferred one.

TABLE IV

STATISTICAL DATA ON PYRETHRIN AEROSOL FORMULATION ANALYSIS[a]

Gas chromatographic column	*Mean value*	*Standard deviation*	*Coefficient of variation*
3% SE-30 on Chromosorb W	0.197	0.014	7.51
5% SE-30 on Chromosorb W	0.209	0.016	8.08
7% SE-30 on Chromosorb W	0.201	0.020	9.94
5% SE-30 on Gas-Chrom Q	0.190	0.017	9.28

[a] Based on nine samplings from each of three aerosols, or a total of 27 samplings.

ANALYSIS OF VARIANCE[b]

Source of variation	*Degrees of freedom*	*Sum of squares*	*Mean square*
Gas chromatographic column	3	50.2	16.73
Aerosol sample	2	23.7	11.85
Column × sample	6	209.9	34.94[c]
Determination within sample within column	96	85.1	0.89
Total	107	368.9	

[b] Calculations based on % values (see Table III) × 100 for simplicity.
[c] Significant level at 1%.

Under the experimental conditions specified in this report, the pyrethrin I component of the pyrethrum fraction (Fig. 1, curves A and B, peak 2) of the formulation was the only predominant peak of the pyrethrum fraction in the curve recorded by the gas chromatograph; the other pyrethrum components did not interfere with the simultaneous recording of the NOBD and PBO components of the mixture. The identity of the pyrethrin I fraction, collected from the gas chromatographic column, was confirmed by IR spectroscopy (see Fig. 2); the spectrum was in excellent agreement with the one reported by ELLIOTT[17].

The manipulative errors that may accumulate during the period of sampling, cleanup, and final concentration, prior to the sample reaching the gas chromatographic analysis stage must be considered in any comparative analytical techniques. One must assume that such errors will be minimal in the hands of an experienced analyst and, therefore, any major differences in the comparative data will be related to the gas chromatographic column performance. Visual evidence of this factor is apparent

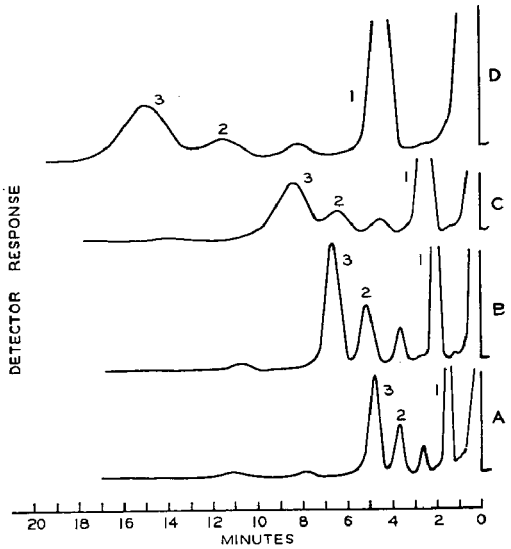


Fig. 1. Gas chromatographic curves of pyrethrin–synergist formulations. Curves A, B, and D from gas chromatographic columns of 3%, 5%, and 7% SE-30 silicone on Chromosorb W, respectively; curve C from a column of 5% SE-30 silicone on Gas-Chrom Q. Peaks 1, 2, and 3 are NOBD, pyrethrin I, and PBO, respectively.

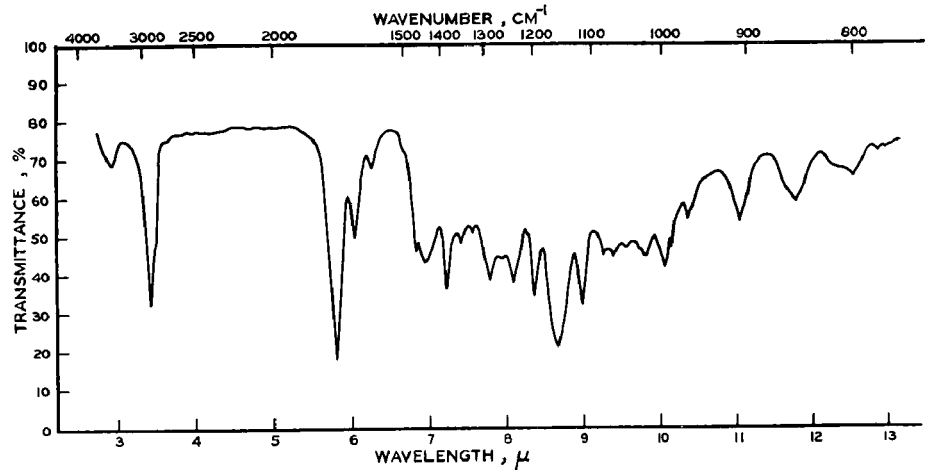


Fig. 2. IR spectrum of the pyrethrin I fraction obtained from the gas chromatographic column.

in curves C and D of Fig. 1; arithmetic recovery data (Table V) does not readily show this distinction. The proper sampling of aerosols is always difficult, as illustrated in Tables I, II, and VI.

The data indicate that the label guarantee of a commercial formulation which contains pyrethrins and synergists can be verified by analysis of the mixture by gas chromatography. If it becomes necessary to adhere to the conditions for the "permissive guarantee variations" or a "passed" judgment decision on the label guarantee

TABLE V

RECOVERY OF PYRETHRIN FORMULATION COMPONENTS FROM FLORISIL COLUMNS

Gas chromatographic column		*Pyrethrin I (%)*	*Piperonyl butoxide (%)*
3% SE-30 on Chromosorb W		87	90
		88	92
		94	94
	Average	90	92
5% SE-30 on Chromosorb W		90	95
		89	95
		96	95
	Average	92	95
7% SE-30 on Chromosorb W		91	93
		89	90
		84	86
	Average	88	90
5% SE-30 on Gas-Chrom Q		94	95
		92	93
		90	95
	Average	92	94

TABLE VI

EFFECT OF SAMPLING TECHNIQUE ON ANALYTICAL VALUES OF PYRETHRIN FORMULATIONS FROM AEROSOL CONTAINERS[a]

Sampling date	*Sample weight (g)*	*Sampling procedure*[b]		*Pyrethrins (%)*	*Piperonyl butoxide (%)*
May 21st	13.1	No shaking		0.19	0.47
	15.7			0.14	0.37
			Average	0.17	0.42
May 23rd	16.6	No shaking		0.23	0.58
	11.7			0.18	0.49
	17.3			0.27	0.73
	18.6			0.22	0.58
			Average	0.23	0.60
May 28th	18.0	Shaken		0.22	0.51
	18.0			0.20	0.51
	13.5			0.23	0.56
	15.5			0.21	0.47
	16.0			0.21	0.50
	27.0			0.17	0.45
			Average	0.21	0.50
Label guarantee				0.20	0.50

[a] The aerosol samples were a mosquito and fly spray containing an emulsion of water and oil base and isobutane propellant.

[b] No shaking means: the sample was taken with no prior mixing.
Shaken means: the aerosol can was shaken vigorously for 30 sec prior to each sampling.

of a commercial sample, prescribed by the Association of American Pesticide Control Officials (AAPCO)[18] as follows:

Pesticide active ingredient guarantee (%)	*Allowable deviation below guarantee*
<1.00	15% of guarantee
1.00– 19.99	0.1 plus 5% of guarantee
20.00– 49.99	0.5 plus 3% of guarantee
50.00–100.00	1.0 plus 2% of guarantee

It is possible that aerosol formulations with individual active ingredients of less than 1.00% could be analyzed within their prescribed limitations. It may also be possible to retain this degree of precision with oil-based formulations within the label guarantee range of 1 to 49.99%. However, when the active ingredient is $\geqslant 50\%$, such as piperonyl butoxide, it may be somewhat difficult to adhere to the AAPCO analytical restrictions.

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CHROM. 4724

ESSENTIAL OILS AND THEIR CONSTITUENTS

III. SOME NEW TRACE CONSTITUENTS IN THE ESSENTIAL OIL OF *SALVIA LAVANDULAEFOLIA*, VAHL.

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SUMMARY

The essential oil of *Salvia lavandulaefolia*, Vahl. was investigated by a combination of the usual techniques of fractional distillation, column chromatography, capillary and preparative gas chromatography. With the aid of a home-made pressure programmer, capillary chromatographic analysis was carried out more effectively. In the analysis, a number of hitherto unreported trace constituents were isolated and identified.

INTRODUCTION

The oil of *Salvia lavandulaefolia*, Vahl. (Spanish sage) is obtained from the distillation of a wild growing plant found in the Spanish provinces of Almeria, Granada, Jaën and Murcia, Granada being the main area producing the oil[1]. According to ARCTANDER[2], the annual production of the oil varies from 3 to 10 metric tons but accurate information is scanty as the oil is subjected to adulteration especially in conjunction with the oil of *Rosmarinus officinalis* L. (rosemary) and *Lavandula latifolia* Vill. (spike lavender).

During the last war, when the oil of Dalmatian sage (*Salvia officinalis*) became scarce, a number of companies in the food industry used the oil of Spanish sage together with other essential oils as a replacement for Dalmatian sage oil. There is still some usage (although very small) of Spanish sage oil in the food industry at this time.

The composition of the essential oil of *Salvia lavandulaefolia*, Vahl. has been the subject of a few investigations. Some of the compounds reported are as follows: α-thujene[6], α-pinene[3–6], tricyclene[5], camphene[6], β-pinene[4–6], myrcene[5,6], α-phellandrene[4–6], limonene[3–5], δ-3-carene[4], terpinolene[5,6], p-cymene[5,6], γ-terpinene[6], cineol[3–6], α-thujone[6], β-thujone[6], α-pinene epoxide[5], linalool[3–6], linalyl acetate[3–5], linalyl isobutyrate[3], camphor[3–6], isoborneol[4–6], isobornyl acetate[4,5], borneol[4–6], bornyl acetate[4–6], α-terpineol[4–6], α-terpinyl acetate[4–6], β-terpineol[5], carvotan acetone[5], carvone[4,6], caryophyllene[4–6], farnesene[5], bisabolene[6], humulene[5], viridiflorol[5] and geraniol[6].

It is the purpose of this paper to describe the detection and identification of a number of previously unreported trace constituents which we have found present in the oil of *Salvia lavandulaefolia*, Vahl.

EXPERIMENTAL

The Spanish sage oil used in this study was stated to be authentic and was obtained from G. Lueders, Toronto.

In the analysis, all of the compounds were characterized by carefully comparing their IR spectra with standard IR spectra which were obtained from reliable commercial sources or from previously published spectra.

Fractionation of the oil

Spanish sage oil (430 g) was fractionally distilled under vacuum through a twelve theoretical plate column. During the distillation, the pot temperature was maintained at 130–150° and the reflux ratio was held at 3:1 with an average throughput of 4 ml/min. Some problem was encountered with the camphor crystallizing in the condensor and trap, but this was alleviated by the careful application of external heat. The distillation was controlled by altering the vacuum from 11.5 to 0.1 cm of Hg and varying the pot temperature within the limits previously mentioned.

Twenty-six fractions were collected, their amount being composition dependent. The residue (45.8 g) was further distilled through a Podbielniak Spinning Band column and fractions 27–30 were collected. The final residue (29.7 g) was by this time a dark, viscous, polymeric mass.

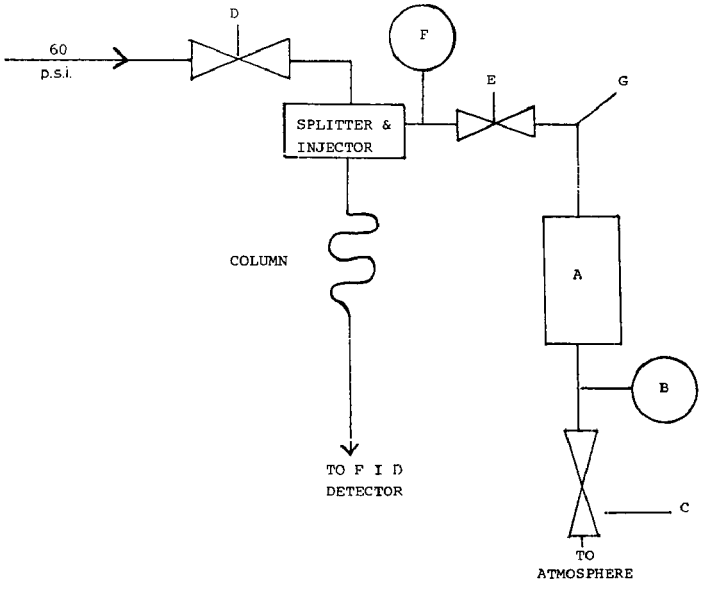


Fig. 1. A diagrammatic representation of the pressure programmer. A = Reservoir; B = pressure gauge; C = exhaust valve; D = carrier gas control valve; E = split control valve; F = pressure gauge; G = "normal" split outlet.

Chromatography

Because of the complexity of the distillation fractions, certain of them were subjected to alumina and silver nitrate alumina column chromatography[7]. The subfractions from each column chromatographic separation were examined gas chromatographically on Carbowax 20M capillary columns so that the amount collected in each fraction was composition dependent. As time was a limiting factor in monitoring each column chromatographic separation, a pressure programmer was designed so that the GLC runs could be carried out in less time. The pressure programming system used is shown diagrammatically in Fig. 1.

Pressure programmer

A Varian-Aerograph Model 1200 gas chromatograph which has been adapted for capillary work was used in this study. The inlet carrier gas was regulated by the

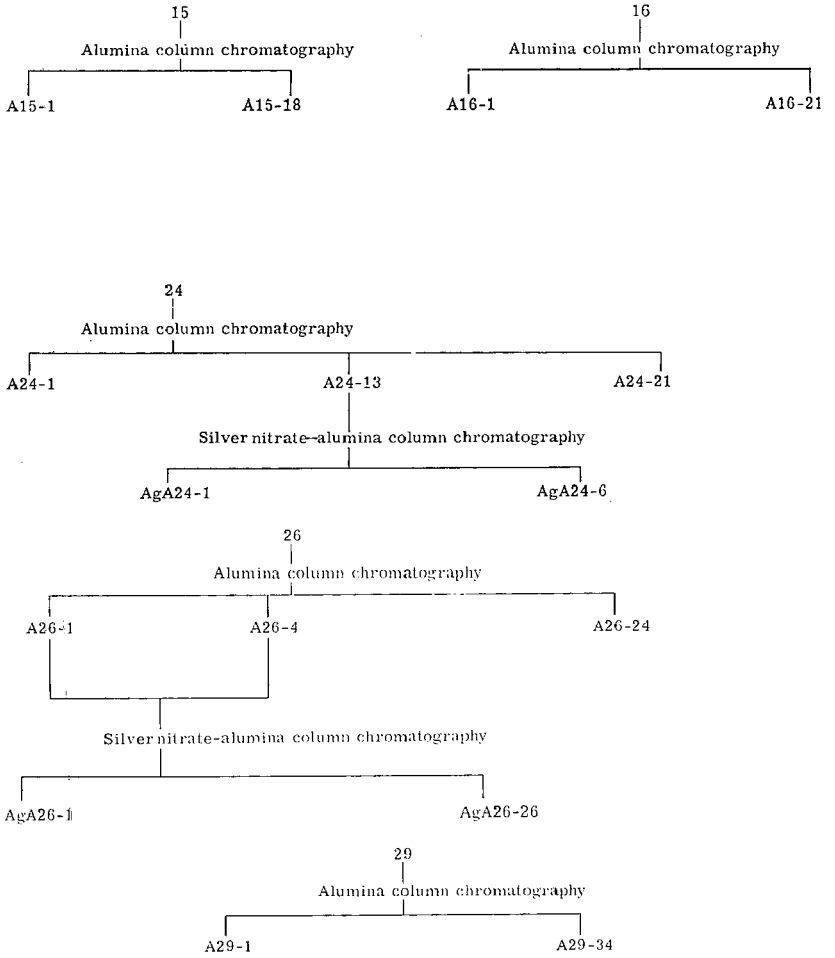


Fig. 2. Flow charts for subfractionation of distillation fractions on both alumina and silver nitrate–alumina columns.

needle valve D on the chromatograph and the flow of gas through the column was adjusted by the inlet splitter. The reservoir (A) (13 ft. × ¾ in. O.D. copper tube) equipped with a pressure gauge (B) and an exhaust valve (C) was connected to the split gas exhaust port (G) which is normally vented to the atmosphere. Initially, a column flow of 1.5 ml/min and a split flow of 90 ml/min was set. Upon injection of sample into the gas chromatograph, the exhaust valve (C) was closed. This caused an increase in reservoir pressure, which could be followed on the pressure gauge (B), and in turn caused the column inlet pressure to increase with time. At the end of a chromatographic run the exhaust valve (C) was opened and the reservoir was allowed to reach atmospheric pressure.

RESULTS

In the following compilation of analytical results, the figures in parentheses given after each compound designate the fraction or subfraction origin of that compound which can be followed on the flow charts (see Fig. 2).

TABLE I

COMPOSITION OF THE VOLATILE OIL OF *Salvia lavandulaefolia*, VAHL.

Peak No.	*Compound*	*Percentage composition*
1	α-Thujene	0.2
2	α-Pinene	8.6
3	Camphene	7.0
4	β-Pinene	4.5
5	Sabinene	1.3
6	Myrcene	3.2
7	Limonene	4.5
8	1,8-Cineol and *cis*-ocimene	23.2
9	*trans*-Ocimene	0.4
10	γ-Terpinene	tr
11	*p*-Cymene	1.9
12	Terpinolene	tr
13	*cis-allo*-Ocimene	0.1
15	α-*p*-Dimethylstyrene	0.3
16	*trans*-Sabinene hydrate	0.5
17	α-Cubebene	tr
18	Copaene	tr
19	Camphor	20.1
20	Linalool and α-gurjunene	3.6
21	Linalyl acetate and *cis*-α-bergamotene	
22	Bornyl acetate and *trans*-α-bergamotene	1.6
23	Terpinen-4-ol	1.0
24	Caryophyllene and aromadendrene	
25	δ-Terpineol and *allo*-aromadendrene	0.1
26	Sabinyl acetate	3.6
27	Sabinol, α-humulene and borneol	0.3
28	α-Terpineol	tr
29	α-Terpinyl acetate	11.2
32	δ-Cadinene	0.4
33	Aromatic curcumene	0.4
34	Nerol	0.1
35	Geraniol	0.4
Unidentified constituents		1.1

Monoterpene hydrocarbons (12)

α-Thujene (1), α-pinene (1), camphene (1), β-pinene (1), sabinene (1), myrcene (1), limonene (A15-3), γ-terpinene (A15-3), terpinolene (A15-3), *cis*-ocimene (A15-13), *trans*-ocimene (A15-13) and *cis-allo*-ocimene (A15-14).

Aromatic hydrocarbons (2)

p-Cymene (A15-13) and α-*p*-dimethylstyrene (A15-16).

Oxygenated terpenes (12)

1,8-Cineol (1), linalool (A15-18), *cis*-sabinene hydrate (A16-21), linalyl acetate (A24-11), bornyl acetate (A24-11), α-terpinyl acetate (A24-11), camphor (A24-16), terpinen-4-ol (A24-20), δ-terpineol (A24-20), geraniol (A24-21), nerol (A24-21) and α-terpineol (AgA24-5).

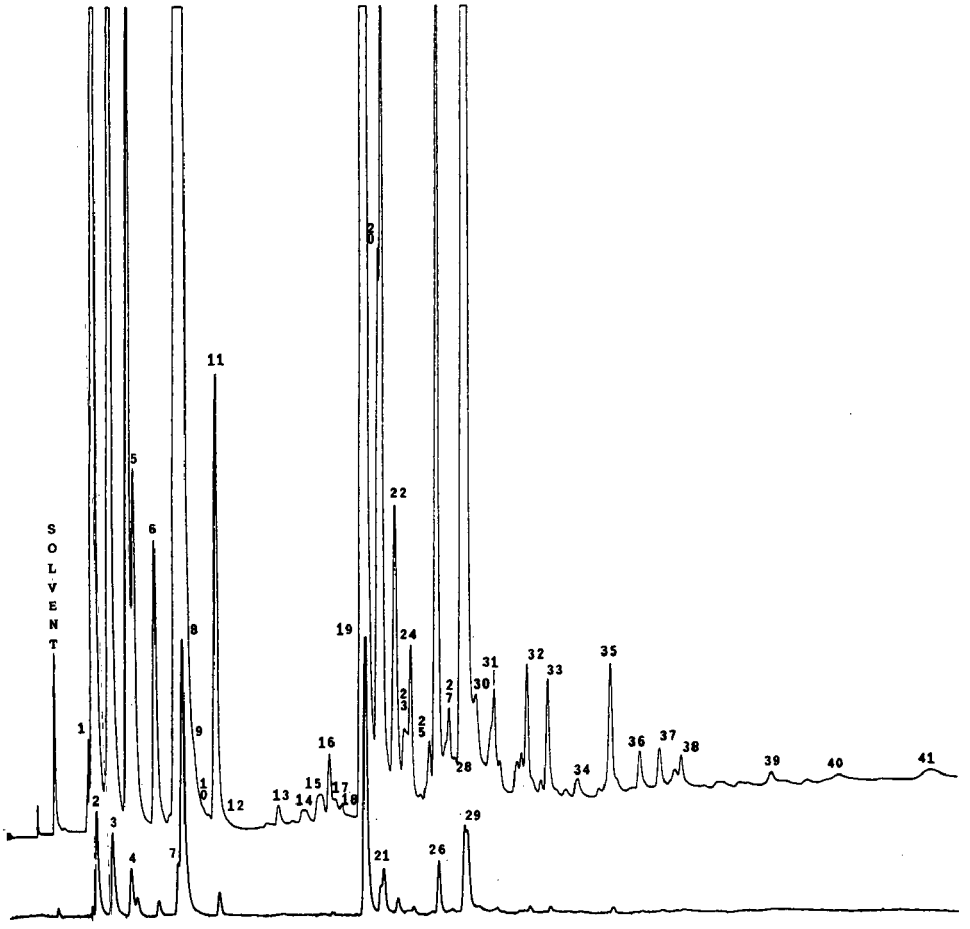


Fig. 3. A temperature and pressure programmed capillary GLC analysis of the volatile oil of *Salvia lavandulaefolia*, Vahl. using a 100 ft. × 0.01 in. I.D. stainless steel capillary column coated with F.F.A.P.

Sesquiterpene hydrocarbons (11)

α-Cubebene (A24-2), copaene (A24-2), α-gurjunene (A24-2), caryophyllene (A24-5), α-humulene (A24-5), *cis*-α-bergamotene (AgA26-18), *trans*-α-bergamotene (AgA26-18), aromadendrene (AgA26-20), *allo*-aromadendrene (AgA26-20), δ-cadinene (A29-2) and aromatic curcumene (A29-15).

A list of the above compounds along with their area percentages calculated from disc integration measurements, obtained using a flame ionization detector, is given in Table I. Also, the position of these same compounds on a chromatogram of the original oil can be seen in Fig. 3.

DISCUSSION

With the aid of the previously described home-made pressure programmer, which required no calibration once optimum conditions were established, the analysis was undertaken more effectively. Occasionally, the use of a dual-channel recorder was used for the same capillary GLC run. This procedure enabled both the major and minor peaks which were present in the oil to be monitored without having to continually attenuate (an example of this can be seen in Fig. 3). This technique was found to be invaluable when the isolated compounds were positioned on a chromatogram of the original oil. The conditions used for both preparative and capillary GLC are given in Table II. In the analysis reported, 22 of the 35 previously reported constituents were identified in addition to a further 18 constituents previously unreported.

TABLE II

CONDITIONS FOR GAS CHROMATOGRAPHY

Information	*Capillary*	*Preparative*
Instrument	Varian-Aerograph Model 1200	Varian-Aerograph Model 700
Columns	100 ft. × 0.01 in. stainless steel coil	20 ft. × 1/4 in. aluminum coil
Stationary phases	(1) SF-96 (2) Carbowax 20M (3) F.F.A.P.	(1) Carbowax 20M (2) Apiezon L
Flow rate	1–3.0 ml N_2/min	60 ml H_2/min
Split ratio	100:1	—
Pressure program	16 → 60 p.s.i.	—
Column temperature	70–180°	80–200°
Temperature program rate	mostly 4°/min	ballistically
Injector temperature	220°	250°
Detector temperature	220°	250°
Detector	Flame	Thermal conductivity
Recorder	Mosley	Leeds & Northrup
Chart speed	0.25 in./min	6 in./h
Sample size	0.2 μl	1–100 μl solutions

ACKNOWLEDGEMENTS

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CHROM. 4758

DETERMINATION OF THE STOKES RADIUS OF NATIVE AND SUCCINYLATED GLUTAMATE DEHYDROGENASE ON SEPHAROSE 4B

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SUMMARY

The Stokes radius of native and of succinylated beef liver glutamate dehydrogenase was measured by gel filtration on Sepharose 4B. Methods for the determination of the Stokes radius of a protein on a gel were compared. The succinylated enzyme was found to lose all glutamate dehydrogenase activity and has a very elongated conformation.

INTRODUCTION

It was observed by OLSON AND ANFINSEN[1] that beef liver glutamate dehydrogenase (GDH) associates reversibly at high enzyme concentrations. As reported in literature, the molecular weight of the dilute enzyme varies between 2.5 and 4.0×10^5 (refs. 2–5). Recently EISENBERG AND TOMKINS[6] and DESSEN AND PANTALONI[7] found 313,000 and 310,000, respectively, for the molecular weight of the protomers. The reported molecular weight of the oligomer varies between 1×10^6 and 2.2×10^6 (refs. 1, 2, 6, 8–10) and recent investigations point to a linear indefinite type of subunit association for this enzyme[7,11,12].

The size of the oligomer cannot be measured on Sephadex since it is completely excluded from those gels. Recently, agaropectin-free agarose was prepared in bead form by HJERTÉN[13]. This product is now available from Pharmacia under the trade name Sepharose and it can be used for the gel filtration of large macromolecules. Under these conditions, the Stokes radius of these molecules can be calculated from the elution volume, V_e, of the macromolecule and from the column constants using the basic equation of ACKERS[14]. The elution volume, V_e, is a function of the effective hydrodynamic volume of the molecule and a chemical modification of a protein may change the effective volume of the molecule by altering its shape.

In the present work, succinylated glutamate dehydrogenase was prepared and

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the Stokes radii of the native and the succinylated enzymes were determined and compared in order to find out if succinylation of the enzyme had caused a noticeable change in the shape of the molecule.

MATERIALS AND METHODS

Beef liver glutamate dehydrogenase (GDH) or L-glutamate NAD (P)-oxidoreductase (deaminating) EC 1.4.1.3, bovine thyroglobulin and catalase were obtained from Sigma Chemicals. Sepharose 4B was purchased from Pharmacia Canada Ltd.

Succinylation of glutamate dehydrogenase

The enzyme was succinylated according to the method of HABEEB[15]. The enzyme (110 mg, 10 mg/ml) was dialyzed for 20 h at 2° against 2 l of 0.05 *M* potassium phosphate at pH 7.6. The volume of the solution after dialysis was 25 ml. Three milliliters of dioxane containing 164 μmoles of succinic anhydride were added dropwise with stirring over a period of 30 min. This quantity of reagent represents a 24 times excess over the number of succinylable amino groups[5,16]. The pH was maintained at 7.8 with 1 *N* NaOH. The enzyme solution was then concentrated to a volume of 15 ml by the addition of dry Bio-Gel P-4 (ref. 17).

Column preparation

Sepharose 4B was obtained as a suspension which was diluted by the addition of 5 volumes of 0.05 *M* phosphate buffer at pH 7.6. The suspension was left at 10° for a few hours and then poured into a Pharmacia column (2.5 × 45 cm) up to a height of 40 cm. The column was equipped with an upflow adapter and washed with two volumes of eluent. The bed volume, V_t, was determined at the end of the experiment by filling the column to the same height with water[18].

Column standardization

The void volume, V_0, of the Sepharose 4B column was measured with 2 ml of a suspension of lyophilized *E. coli* cells (1 mg/ml, *E. coli* strain 9001 NCTC, Batch No. 6, Seravac Laboratories Ltd., Great Britain). The volume of eluent was measured directly in a graduate cylinder at the outlet of a LKB Uvicord monitor set at 254 mμ. Bovine thyroglobulin (10 mg, 1 ml), bovine liver catalase (40 mg, 2 ml), glutamate dehydrogenase (20 mg, 2 ml) and succinylated glutamate dehydrogenase (8.3 mg, 2 ml) were applied separately and in duplicate on top of the column. The column was run under a pressure of 25 cm of water giving a flow rate of 2.8 ml/cm^2/h.

Ultracentrifugation

Native and succinylated glutamate dehydrogenase were dialyzed for 24 h at room temperature against 4 l of 0.15 *M* phosphate buffer at pH 8.0. The concentration of enzyme was measured at 279 mμ (ref. 1). The sedimentation velocity was measured at 20° and at a concentration of 4.14 mg/ml in a Beckman ultracentrifuge Model E at 59,780 r.p.m.

Electrophoresis

Cellulose acetate strips (1 in. × 6$\frac{3}{4}$ in. Sepraphore III, Gellman Co.) were used.

The electrophoresis was run at a potential difference of 100 V for 2 h in cold 0.05 *M* phosphate buffer at pH 7.6. The strips were stained with Ponceau S (0.01% in 5% trichloroacetic acid), washed with acetic acid and ethanol and cleared with 10% acetic acid in methanol[19].

Ackers' equation[14]

This equation was used to calculate the Stokes radii.

$$K_D = \frac{V_e - V_o}{V_i} = \left(1 - \frac{a}{r}\right)^2 \left[1 - 2.104\left(\frac{a}{r}\right) + 2.09\left(\frac{a}{r}\right)^3 - 0.95\left(\frac{a}{r}\right)^5\right]$$

In this equation, K_D is the distribution coefficient of the protein on the gel, V_e, the elution volume, V_0, the void volume of the column, V_i, the effective imbibed volume of the gel, a, the Stokes radius of the molecule, and, r, the effective pore radius of the gel.

RESULTS

Succinylated glutamate dehydrogenase

Preliminary reports on the succinylation of glutamate dehydrogenase have been presented by FRIEDEN[20]. Succinylation renders the enzyme very soluble in 0.05 *M* Tris–HCl at pH 8 and the succinylated enzyme may be left at room temperature for at least one week without any increase in the turbidity of the solution. The extinction coefficient $E^{1\%}_{279\,m\mu}$ was found to be 9.48, which is close to the value found by OLSON AND ANFINSEN[1] for the native enzyme. This indicates that the tyrosine residues were not modified by succinylation in these conditions. A similar observation was reported for the acetylation of this enzyme[5] and for the succinylation of globular proteins[21]. The modified enzyme is inactive at concentrations 1.7 and 2.4 times the concentration normally used to determine the glutamate dehydrogenase activity. COLMAN AND FRIEDEN[5] have shown that an excess of acetic anhydride inactivates the enzyme.

Sedimentation velocities

Table I shows the sedimentation velocity of native and succinylated glutamate dehydrogenase. A single and symmetrical peak was obtained for both of these proteins at a concentration of 4.14 mg/ml. OLSON AND ANFINSEN[1] found a value $S_{20,w}$ of 25 *S* for the native enzyme at a concentration of 4 mg/ml. The sedimentation velocity of the succinylated enzyme was not standardized by the method of SCHACHMAN[22] since the partial specific volume of the modified enzyme is not known.

TABLE I

SEDIMENTATION VELOCITY OF NATIVE AND SUCCINYLATED GLUTAMATE DEHYDROGENASE

Protein	*Sedimentation velocity (S)*	*Standardized sedimentation velocity (S)*
Native glutamate dehydrogenase	22.2	24.8
Succinylated glutamate dehydrogenase	21.0	—

TABLE II

GEL FILTRATION OF PROTEINS ON SEPHAROSE 4B

Protein	V_e *(cc)*	V_e/V_o
Thyroglobulin	137	2.34
	137	2.34
Catalase	160	2.74
	158	2.71
Native GDH	130	2.22
	131	2.24
Succinylated GDH	71.3	1.22
	61.4	1.05

Electrophoresis

Native glutamate dehydrogenase gave a single band at 5.3 cm from the origin in the direction of the anode. Under the same conditions, the succinylated enzyme gave a single band which stayed at the origin.

Gel filtration

Two pure proteins of known Stokes radius were run on Sepharose 4B in addition to the native and succinylated enzyme. The results of the gel filtration are given in Table II. ACKERS' equation was used to calculate the effective pore radius, r, of the gel using K_D and the Stokes radius of thyroglobulin or catalase. The Stokes radius of catalase was calculated using ACKERS' equation and the r value obtained with thyroglobulin; the Stokes radius of thyroglobulin was then calculated in the same way using the r value obtained with catalase. The calculated Stokes radii of these two proteins are given in Table III. These are within $\pm 8\%$ of the values in literature. This gives a measure of the accuracy of ACKERS' equation when applied to filtration on Sepharose 4B in these conditions. Using the average value of r calculated previously, the Stokes radii of the native and succinylated enzyme were calculated (Table IV).

The logarithms of the Stokes radius of catalase and of thyroglobulin were plotted against their reduced elution volume V_e/V_0 (Fig. 1). From that curve, the

TABLE III

EFFECTIVE PORE RADIUS OF SEPHAROSE 4B[a]

Protein	K_D	a/r	r *(mμ)*	a *(mμ)*	
				Calculated value	*Accepted value*
Catalase	0.722	0.0750	69.8	4.82	5.23 (ref. 25)
Thyroglobulin	0.564	0.1262	64.2	8.81	8.10 (ref. 26)

[a] $K_D = (V_e - V_0)/V_i$, where V_e is the elution volume of the molecule, V_0, the void volume of the column and, V_i, the effective imbibed volume of the gel, a, the Stokes radius of the protein and r, the effective pore radius of the gel.

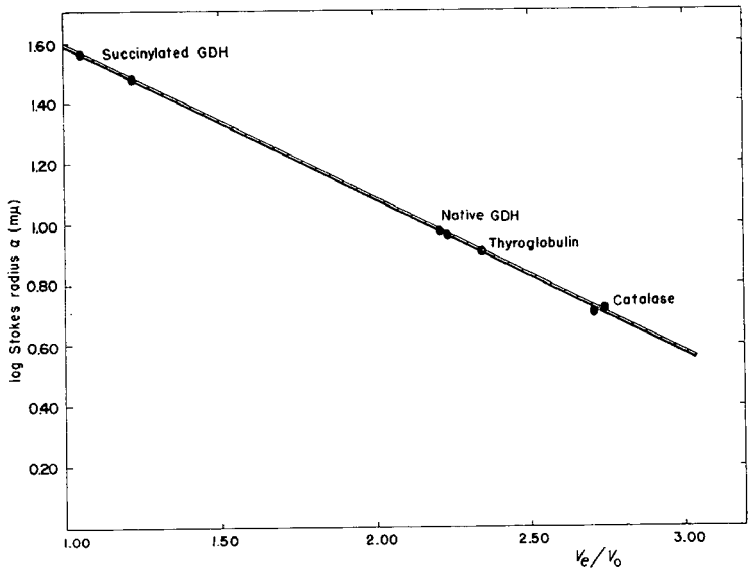


Fig. 1. Determination of the Stokes radius of native and succinylated glutamate dehydrogenase on Sepharose 4B.

Stokes radii of native and succinylated glutamate dehydrogenase were found to be 9.37 and 33.1 mμ, respectively. These values are comparable to the calculated values using ACKERS' equation (Table IV).

DISCUSSION

At a concentration greater than 0.8 mg/ml, glutamate dehydrogenase exists as an oligomer[1,4,6]. At this concentration, an average value of 9.5 mμ was obtained for the Stokes radius of the native enzyme on Sepharose 4B. Using the Stokes–Einstein equation[23], $D = kT/6\pi\eta a$ when D is the diffusion coefficient, k, the Boltzmann constant, T, the absolute temperature, η, the viscosity coefficient and a, the Stokes radius of the protein, the diffusion coefficient of the oligomer was found to be 2.15×10^{-7} cm²/sec. OLSON AND ANFINSEN[1] have reported a value of 2.38×10^{-7} cm²/sec for the diffusion coefficient of glutamate dehydrogenase at a concentration of 4 mg/ml. Gel filtration on Sepharose 4B provides a rapid method to determine the Stokes radius of large molecules. The ACKERS' equation or the use of a standard curve (log Stokes radius against V_e/V_0) gave comparable results.

TABLE IV

STOKES RADIUS OF NATIVE AND SUCCINYLATED GDH

Protein	K_D	a/r	a (mμ)
Native GDH	0.514	0.1445	9.7 ± 0.5
	0.521	0.1419	9.5 ± 0.5
Succinylated GDH	0.058	0.4961	33.3 ± 5.6

The observations made on the succinylated enzyme indicate that the succinylated molecule is considerably elongated. We observed also that succinylated glutamate dehydrogenase did not move on electrophoresis. The electrophoretic mobility of a molecule being proportional to its charge and inversely proportional to the square of its radius and to its degree of asymmetry[24], a large increase in the Stokes radius of the succinylated enzyme may explain its electrophoretic behavior.

The fast migration of the succinylated enzyme on Sepharose 4B is not due to an increase in molecular weight by agglomeration or polymerization since a decrease in the sedimentation velocity was observed (Table I). An increase in the Stokes radius of a molecule accompanied by a decrease in the sedimentation velocity indicates a decrease in the diffusion coefficient due to an increase in the frictional coefficient produced by an increase in the degree of asymmetry of the molecule. Succinylation of this enzyme does not seem to alter its state of aggregation, but causes a large increase in the Stokes radius of this molecule indicating an important change in shape.

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CHROM. 4742

SPEKTRALFLUOROMETRISCHE *IN SITU*-ANALYSE POLYCYCLISCHER AROMATEN NACH TRENNUNG AUF ACETYLIERTEN CELLULOSESCHICHTEN

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(Eingegangen am 19. März 1970)

SUMMARY

Spectrofluorometric in situ analysis of polycyclic aromatic compounds after separation on acetylated cellulose layers. I. Qualitative and quantitative evaluation

The evaluation of polycyclic aromatic compounds, separated by thin-layer chromatography on acetylated cellulose, is described. *In situ* measurements of the fluorescence, excitation and emission spectra on thin-layer plates by means of a spectrofluorometer allow the identification as well as the determination of most of these substances. The limit of detection depends on the intensity of fluorescence of the aromatic compounds, and was estimated to be 0.1 to 0.001 μg on average. The error in the reproducibility in spotting the sample on the thin-layer plate is in the order of 10%. Standard curves show a fairly good linearity up to 0.2 μg of the substance. The results obtained from the standard curves, corrected by an external standard, are independent of the adjustment of the instrument.

EINLEITUNG

Die Bestimmung polycyclischer Aromaten gewinnt ein immer grösseres Interesse, denn viele dieser Substanzen sind krebserregend. Ihre Identifizierung wird häufig mit Hilfe der Fluoreszenzspektroskopie durchgeführt, da die meisten Polycyclen eine charakteristische Fluoreszenz zeigen. Die Nachweisempfindlichkeit liegt bei 10^{-8}–10^{-9} g/ml, daher ist ein sicherer Nachweis von mindestens 0.1 μg dieser Stoffe in der Probe möglich. Voraussetzung für die Anwendung der Fluoreszenzspektroskopie ist allerdings, dass nicht mehrere polycyclische Aromaten in der Messlösung vorliegen, da sie sich in ihrer Fluoreszenz durch Löschung, Überlagerung usw. stark gegenseitig beeinflussen. Die daher notwendige Trennung einer Vielzahl von polycyclischen Aromaten, wie sie z.B. im Rauch vorkommen, erfolgt mit Hilfe der kombinierten Anwendung verschiedener chromatographischer Trennsysteme. Der durch Ausschütteln mit Lösungsmitteln gereinigte Extrakt lässt sich säulenchromatographisch

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an aktivem Aluminiumoxid[1-3] oder dünnschichtchromatographisch auf Silicagelplatten[4] in mehrere Gruppen auftrennen. Da die einzelnen Gruppen in der Regel mehrere Aromaten enthalten, ist ihre simultane fluorometrische Auswertung nur in wenigen Fällen möglich. Zur weiteren Trennung eignet sich acetylierte Cellulose in Form von Papier[3] oder Dünnschicht[5]. Auf diesem Material werden die Aromaten gut getrennt. Sie zeigen ausserdem eine wesentlich intensivere und differenziertere Fluoreszenz als auf Silicagel oder Aluminiumoxid. Es war daher zu erwarten, dass eine direkte qualitative und quantitative Auswertung solcher Dünnschichtchromatogramme *in situ* möglich ist.

Zur sicheren Identifizierung der Dünnschichtflecken ist die Aufnahme der Fluoreszenz-Anregungs- und Emissionsspektren erforderlich, wie sie SAWICKI und Mitarbeiter[6] bei gaschromatographisch vorgetrennten und auf Dünnschichtplatten kondensierten Substanzen vornahmen. BORNEFF UND FISCHER[3] konnten bereits 1961 einige Aromaten durch direkte Aufnahme ihrer Emissionsspektren von acetyliertem Papier identifizieren. Die quantitative Auswertung fluoreszierender Dünnschichtflecken wurde schon bei mehreren Substanzgruppen unter Anwendung verschiedener Dünnschichtauswertegeräte[7-10] mit Erfolg durchgeführt.

APPARATUR

Zur Ausführung der fluorometrischen Analysen stand ein Aminco-Bowman-Spectrophotofluorometer (American Instrument Co., Silver Springs, Md., U.S.A.) zur Verfügung[11,12]. Ein aufsetzbares Dünnschichtauswertgerät erlaubt die Aufnahme der Spektren direkt von einer Dünnschichtplatte.

Zur Erzeugung eines kontinuierlichen Lichtes (ab 200 nm) dient eine Xenon-Hochdruck-Lampe. Die Anregungs- und Emissions-(Gitter)-Monochromatoren (200–800 nm) sind horizontal im 90°-Winkel zueinander angeordnet. Zur linearen Veränderung der Wellenlänge dienen zwei mit den Gittern gekoppelte Synchronmotoren. Bei Benützung des Dünnschichtauswertgerätes wird mit Hilfe eines an Stelle des Küvettenhalters angebrachten Spiegel- und Linsensystems der zur Fluoreszenzerzeugung dienende Lichtstrahl im Winkel von 90° nach oben geleitet. Die zur Auswertung vorgesehene Dünnschichtplatte liegt mit der Schicht nach unten auf einem horizontal angebrachten Kreuztisch. Trifft das durch einen engen Spalt (0.5 × 5 mm) austretende Anregungslicht auf einen Substanzfleck, so durchdringt das erzeugte Fluoreszenzlicht Adsorbensschicht und Glasplatte und wird durch eine Glasfaseroptik zum Emissionsmonochromator geleitet. Mit Hilfe eines Elektromotors kann der Kreuztisch mit konstanter Geschwindigkeit bewegt werden, wodurch die quantitative Auswertung der Dünnschichtflecken ermöglicht wird. Der Kreuztisch und die optischen Systeme befinden sich in einem lichtdicht verschliessbaren Kasten.

Da die Auflösung der Fluoreszenz-Anregungs- und Emissionsspektren von der Spaltbreite entlang des Anregungs- und Emissionslichtweges abhängig ist, sind besonders angeordnete diskontinuierliche Spaltsysteme (von 0.5–5 mm) eingebaut. Als Photomultiplier wenden wir den IP-28 mit einer maximalen Empfindlichkeit bei 350 nm (Messbereich von 230 bis 600 nm) an. Der verstärkte Messstrom wird an einem Galvanometer angezeigt und von einem Schreiber ("Servogor" der Fa. Metrawatt, Nürnberg) registriert. Der Anschluss eines XY-Schreibers oder Oszillographen ist möglich.

VORBEREITUNG DER PROBE

Zur Isolierung und Reinigung der in verschiedenen Lebensmitteln, Wasser, Luftstaub, Rauchkondensat usw. vorkommenden polycyclischen Aromaten sind verschiedene Methoden beschrieben worden[1–6,13]. Als besonders geeignet erwies sich ein von GRIMMER UND HILDEBRANDT[1,2] ausgearbeitetes und auf verschiedene Lebensmittel angewandtes Verfahren. Die Aufarbeitung der von uns untersuchten Proben (Räucherkammerruss, -teer, Rauchkondensat, geräucherte Fleischwaren usw.) erfolgt nach dieser Methode, die wie folgt durchgeführt wird:

(a) Extraktion der zerkleinerten Proben mit Methanol und Cyclohexan,

(b) Flüssig–flüssig-Verteilung zwischen Cyclohexan und N,N-Dimethylformamid (90% + 10% Wasser) und Rückführung der Aromaten in Cyclohexan,

(c) Reinigung durch Säulenchromatographie an Silicagel.

Die Vortrennung der polycyclischen Aromaten in sechs Fraktionen führen wir bei Vorhandensein grösserer Mengen (>0.5 μg je Komponente) über einer Aluminiumoxidsäule nach dem von GRIMMER UND HILDEBRANDT[1] beschriebenen Verfahren aus; bei geringeren Mengen verwenden wir Silicagel-Platten, wie sie von FRITZ[4] empfohlen wurden. Beide Methoden ergeben eine ausreichende Vortrennung des Gemisches zu der nachfolgenden Feintrennung.

FEINTRENNUNG DER POLYCYCLISCHEN AROMATEN

Zur weiteren Auftrennung der einzelnen Fraktionen benutzen wir eine von KÖHLER UND EICHHOFF[5] empfohlene Mischdünnschicht aus Aluminiumoxid und acetylierter Cellulose zur zweidimensionalen Chromatographie. Obwohl wir die Feintrennung der Aromaten nur eindimensional vornehmen, hat sich die Anwendung der Mischdünnschicht im Hinblick auf die Trennschärfe als vorteilhaft erwiesen.

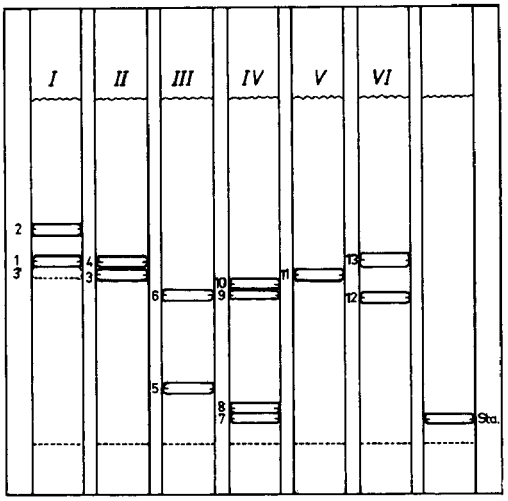


Fig. 1. Schematische Darstellung der Trennung einzelner aus Al_2O_3-Säule gewonnene Fraktionen (I–VI) eines Testgemisches auf Al_2O_3-acetylierten Cellulose-Mischplatten (1–13, siehe Tabelle I; Sta = Standard, 0.1 μg 3,4-Benzpyren).

Zur Herstellung der Mischplatten dient eine Mischung aus acetylierter Cellulose (MN 300 G/Ac 40% der Fa. Macherey, Nagel und Co.) und Aluminiumoxid (zur DC, neutral, Woelm) im Gewichtsverhältnis 2:1. Von diesem Gemisch werden 24 g in 30 ml Wasser und 30 ml Methanol mit einem Ultra-Turrax homogenisiert (60 sec) und zur Beschichtung von fünf Glasplatten (20 × 20 cm) mit einer Schichtdicke von 0.25 mm verwendet. Die vorerst an der Luft getrockneten Platten werden im Umlufttrockenschrank 1½ h bei 60° nachgetrocknet. Mit Hilfe eines Lineals und eines 3 mm breiten Spatels teilt man die Beschichtung in 2 cm breite Streifen auf (Fig. 1). Aliquote Anteile der eingeengten Fraktionen aus der Aluminiumoxidsäule[1] oder aus der Silicagel-Platte[4] und 0.1 μg 3,4-Benzpyren (Standard) werden in 2 cm Breite strichförmig auf die Startlinien aufgetragen.

Fig. 1 zeigt die Verteilung und Trennung des von GRIMMER UND HILDEBRANDT[1] vorgeschlagenen Testgemisches (bestehend aus dreizehn der am häufigsten vorkommenden polycyclischen Aromaten) auf der von uns benutzten Dünnschichtplatte, nach der vorangehenden Auftrennung in sechs Fraktionen (I–VI) auf einer Al_2O_3-Säule.

Ein gleichmässiges Auftragen ist hinsichtlich der Trennschärfe und Genauigkeit der Auswertung sehr wichtig. Als Laufmittel dient eine Mischung aus Methanol–Toluol–Essigsäureäthylester–Isobutylalkohol–Wasser (60:5:10:5:15). Die Höhe des ersten Laufes beträgt *ca.* 18 cm. Eine zwei- oder dreimalige Entwicklung der Platte nach Zwischentrocknen verbessert die Trennung wesentlich.

Die einzelnen Substanzzonen werden unter der UV-Lampe (lang- und kurzwellig) an beiden Enden mit kurzen Strichen markiert (Fig. 1).

AUFNAHME DER FLUORESZENZ-ANREGUNGS- UND EMISSIONSSPEKTREN

Die Dünnschichtplatte wird mit der Schicht nach unten auf den Kreuztisch des Dünnschichtauswertgerätes gelegt. Der durch einen 0.5 mm breiten und 5 mm langen Spalt begrenzte Anregungslichtstrahl soll die Substanzbande in der Längsrichtung und in ihrer Mitte treffen. Die Glasfaseroptik soll wegen der erreichbaren höheren

TABELLE I

ANREGUNGS- UND EMISSIONSWELLENLÄNGEN POLYCYCLISCHER AROMATEN ZUR AUFNAHME IHRER ORTSKURVEN

Nr.	*Polycyclische Aromaten*	*Anregung bei (nm)*	*Emission bei (nm)*
1	Phenanthren	295	410
2	Anthracen	378	430
3	Pyren	338	415
4	Fluoranthen	360	465
5	Chrysen	322	408
6	1,2-Benzanthracen	354	412
7	3,4-Benzpyren	388	430
8	3,4-Benzfluoranthen	355	452
9	Perylen	414	446
10	1,2-Benzpyren	332	408
11	1,12-Benzperylen	388	430
12	1,2:5,6-Dibenzanthracen	351	420
13	Coronen	308	452

Empfindlichkeit in 90° (Stellung für Messung der Transmission) und nicht in der vom Hersteller angegebenen 45°-Stellung zur Platte eingesetzt werden. Die Einstellung der Wellenlänge an den Anregungs- und Emissionsmonochromatoren ist von der zu messenden Substanz abhängig (Tabelle I). Durch wechselndes Verstellen der Wellenlänge wird bei unbekannten Substanzen die maximale Fluoreszenzintensität ermittelt.

Vor der Aufnahme der Anregungsspektren müssen die Spalten im Strahlengang des Anregungslichtes möglichst eng (0.5 mm) eingestellt werden, um eine ausreichende Auflösung der Spektren zu erreichen. Die Spaltsysteme des Emissionsmonochromators sollen möglichst weit geöffnet sein, um gut messbare Fluoreszenzintensitäten zu erhalten. Die Anregungsspektren werden durch kontinuierliche Veränderung der Wellenlänge (ab 200 nm) des Anregungslichtes bei der für eine maximale Fluoreszenz-

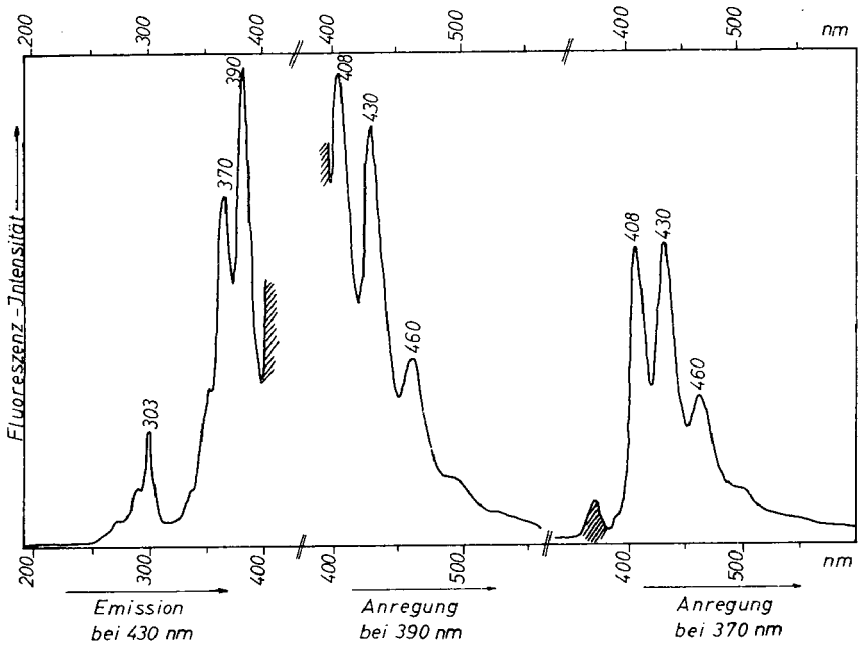


Fig. 2. Anregungs- und Emissions-Spektrum von 3,4-Benzpyren an acetylierten Cellulose-Mischplatten (Konz. 0.01 μg/Bande).

ausbeute notwendigen Einstellung des Emissionsmonochromators aufgenommen (Fig. 2). An Hand des Anregungsspektrums bestimmt man nun den zur Erzeugung der maximalen Fluoreszensintensität notwendigen Wellenlängenbereich genauer. Dieser wird dann am Anregungsmonochromator eingestellt.

Vor der Aufnahme des Emissionsspektrums wird durch Vergrösserung des Austrittsspaltes am Anregungsmonochromator (auf 3–5 mm) für ein intensiveres Anregungslicht und durch Einstellung sehr enger Eintritts- und Austrittsspalte am Emissionsmonochromator für eine gute Auflösung des Spektrums gesorgt. Falls nicht zu starkes Streulicht auftritt, stellt man die Anfangswellenlänge am Emissionsmonochromator niedriger als die des Anregungsmonochromators ein, andernfalls sollte die

Wellenlänge so niedrig eingestellt werden, wie es das Streulicht zulässt (Fig. 2). Durch kontinuierliche Veränderung der Wellenlängeneinstellung des Emissionsmonochromators bei konstanter Anregungswellenlänge und gleichzeitiger Registrierung der Fluoreszenzintensitätsänderungen erhält man das Emissionsspektrum der Substanz (Fig. 2).

AUFNAHME DER FLUORESZENZ-ORTSKURVEN

Das strichförmige Auftragen der einzelnen Fraktionen bewirkt, dass nach der Entwicklung der Platte die einzelnen Substanzen bandenförmig getrennt vorliegen. Das Auswertungsprinzip ist das gleiche wie bei der Elektrophorese[7]. Die Dünnschichtplatte wird mit konstanter Geschwindigkeit durch den Anregungslichtstrahl des Auswertgerätes gefahren. Hierbei wird ein aliquoter Anteil der Substanzbande von dem 0.5 mm breiten und 5 mm langen Anregungslichtstrahl kontinuierlich erfasst. Bei laufender Registrierung der Fluoreszenzintensitätsänderung mit einem Linearschreiber erhält man die entsprechenden Ortskurven (Fig. 3).

Bei der quantitativen Auswertung stellt man an dem Monochromator die zur Erzeugung der maximalen Fluoreszenzintensität erforderlichen stoffspezifischen Anregungs- und Emissionswellenlängen ein. Falls zu starkes Streulicht störend wirkt, wählt man eine höher liegende Emissions- oder eine niedrigere Anregungsbande (Tabelle I). Da praktisch jeder der zu untersuchenden polycyclischen Aromaten eine andere ideale Anregungs- und Emissionswellenlänge besitzt, wird eine Trennungsreihe so oft und bei so vielen Monochromatoreneinstellungen durchfahren, wie Substanzen quantitativ zu bestimmen sind.

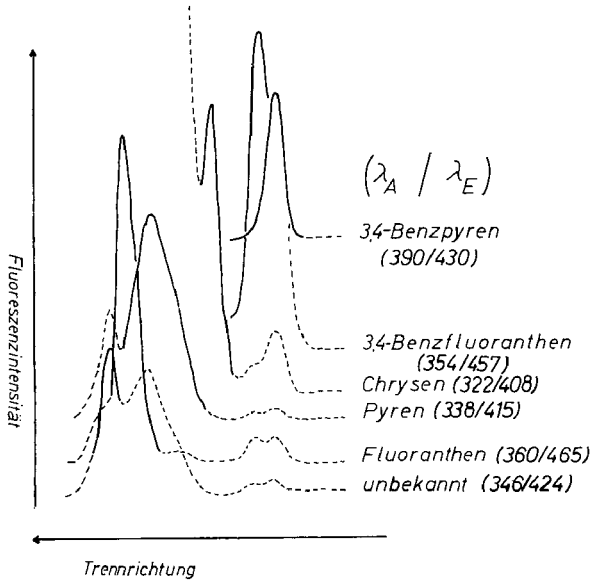


Fig. 3. Registrierung der Fluoreszenzortskurven einer Trennreihe (*ca.* 0.01 bis 0.3 μg je Komponente) bei substanzspezifisher Anregungs- und Emissionswellenlängen (λ_A = Anregungs-; λ_E = Emissionswellenlänge).

Wie Fig. 3 zeigt, gelingt auf diese Weise eine fast störungsfreie und sehr empfindliche Aufnahme der Ortskurven auch bei nicht vollständig getrennten Substanzbanden, wie es z.B. bei 3,4-Benzpyren und 3,4-Benzfluoranthen oder Pyren und Fluoranthen der Fall sein kann. Ist die Konzentration entlang den 2 cm langen Banden nicht ganz gleichmässig verteilt, so empfiehlt es sich, die Messung der einzelnen Substanzbanden an drei Stellen der Zone zu wiederholen und bei der Auswertung den Mittelwert zu bilden.

Bei der Aufnahme der Ortskurven müssen alle Messbedingungen konstant gehalten werden, da nur so vergleichbare Messgrössen zu erwarten sind. Man hält daher Spaltsystem, Photomultiplier, Empfindlichkeits- und Schreibereinstellung konstant, so dass nur die elektronische Abschwächung des maximalen Verstärkungsgrades in konstanten Proportionen (z.B. 1:3.0; 1:10; 1:30 usw.) mit Hilfe des Meter-Multipliers stattfindet. Bei der Aufnahme der Ortskurven wenden wir folgendes Spaltsystem an: 5; 0.5; 0.5-Platte-0.5; 0.5 mm. Bei dieser engen Spalteinstellung ist die Registrierung von 0.01 μg 3,4-Benzpyren, verteilt auf einer 2 cm langen Bande, mit einem 70-80%-igem Skalenausschlag bei maximaler Verstärkereinstellung (Meter-Multiplier: 0.001; Sensitivity: 20) möglich. Bei konstantem Kreuztischvorschub von 7.5 cm/min und Papiervorschub von 12 cm/min erhält man gut auswertbare Ortskurven.

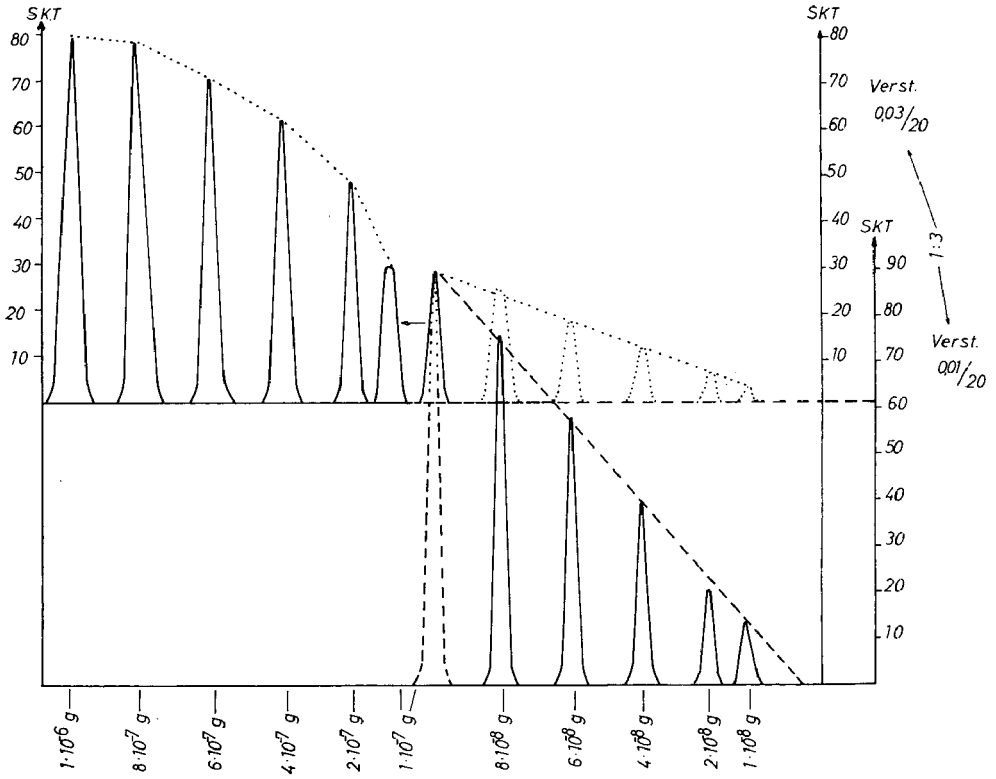


Fig. 4. Proportionalität zwischen Mengen von 3,4-Benzpyren und ihre Fluoreszenzortskurven (bei 10^{-7} g/Bande wurde die Verstärkung verdreifacht).

AUSWERTUNG DER ORTSKURVEN

Zur quantitativen Auswertung der Ortskurven ist die Kenntnis der Beziehungen zwischen Substanzmenge und Grösse der Ortskurven unerlässlich. Fig. 4 zeigt die Ortskurven unterschiedlicher Mengen an 3,4-Benzpyren. Eine lineare Beziehung zwischen Höhe der Ortskurven und Substanzmenge ist nur im Bereich von 0.01 bis 0.1 μg/Bande zu beobachten. Ab 0.2 μg wird der Höhenunterschied immer kleiner, die Breite der Ortskurven nimmt dagegen zu. Da sich die Breite der einzelnen Banden—und somit der Ortskurven—auch von Platte zu Platte verändert, wobei Durchmesser der Auftraglinie, Laufstrecke, Zahl der Entwicklungen usw. eine Rolle spielen, muss die Breite der aufgezeichneten Ortskurven zur Auswertung herangezogen werden.

Wir fanden, dass durch Multiplikation der gemessenen Fluoreszenzintensitätsmaxima (in Skalenteilen) und der korrigierten Fussbreite der Ortskurven B (in cm; Fig. 5) ein zur quantitativen Berechnung geeigneter Flächenwert (FlW) entsteht. Um die durch unterschiedliche Dicke der Schicht und der Glasplatte und durch unvermeid-

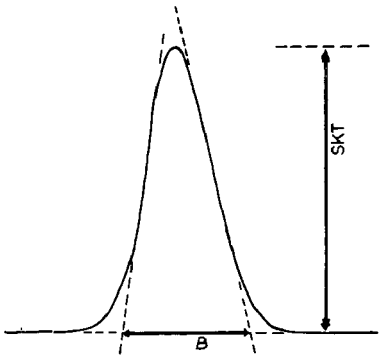


Fig. 5. Auswertung der Fluoreszenzortskurven nach Höhe (in SKT = Skalenteilen) und korrigierter Fussbreite (B, in cm).

liche apparative Veränderungen (z.B. bei der Xenonlampe, Photomultiplier usw.) bedingten Abweichungen zu kompensieren, müssen die Flächenwerte mit einem aus dem auf derselben Platte mitchromatographierten Standard (0.1 μg 3,4-Benzpyren) berechneten Faktor (F) multipliziert werden.

$$F = \frac{100}{\text{FlW des Standards}}$$

Bei 15 Messungen schwankte dieser Faktor z.B. zwischen 0.96 und 1.31 (Mittel 1.08).

Bei den von uns gewählten Messbedingungen ist zur Registrierung der Fluoreszenzintensität des Standards eine Abschwächung der maximalen Verstärkung von 1 zu 10 erforderlich. Zur Berechnung der Gesamtfluoreszenzintensität (GFI = Intensität bei maximaler Verstärkung) für 0.1 μg 3,4-Benzpyren muss der auf 100 korrigierte Flächenwert mit 10 multipliziert werden. Somit weist er eine GFI von 1000 auf (Fig. 6).

Die Berechnung der Gesamtfluoreszenzintensität aus den Ortskurven unbekannter Mengen erfolgt wie beim Standard:

$$\text{GFI} = \text{SKT} \times B \times F \times \text{Abschwächung}.$$

Zur Ermittlung der Substanzmengen, welche auf der Dünnschicht vorliegen, werden die Eichkurven verwendet (Fig. 6).

Zur Aufstellung der Eichkurven haben wir die einzelnen Substanzen in verschiedenen Mengen nach der beschriebenen Methode mehrfach chromatographisch ausgewertet und die Ergebnisse in ein doppeltlogarithmisches System eingetragen. Je nach Fluoreszenzintensität der einzelnen polycyclischen Aromaten verlaufen die Kurven zwar in unterschiedlichen Messbereichen, aber alle mit einer Neigung von *ca.* 45°, wenn ihre Menge unterhalb der 0.1 μg/Bande liegt. Bei grösseren Mengen flachen die Kurven ab, wie es nach Angaben von DANCKWORTT UND EISENBRAND[14] bei Messungen in Lösungen zu erwarten ist. Eine quantitative Auswertung ist bei

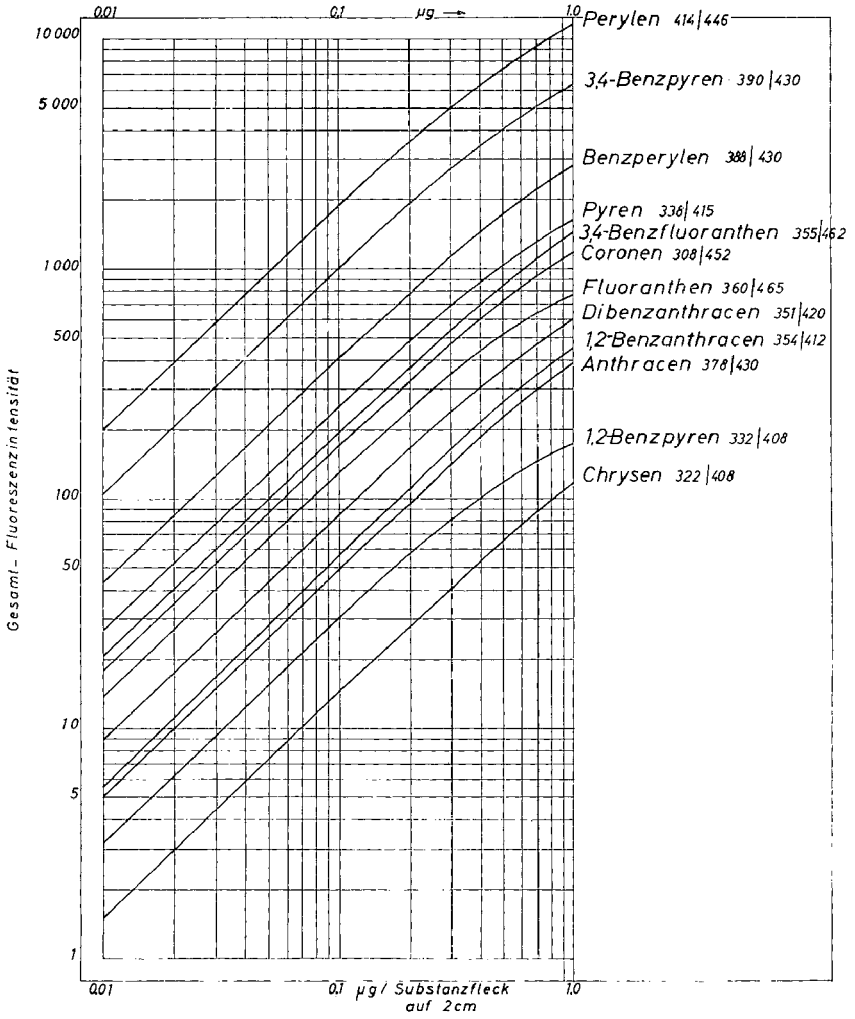


Fig. 6. Eichkurven verschiedener polycyclischer Aromaten im doppeltlogarithmischen System zur Ermittlung ihrer Menge auf den Al_2O_3-acetylierten Cellulose-Mischplatten, bei 2 cm Breite strichförmiger Auftragung und den angegebenen Anregungs- und Emissionswellenlängen.

Mengen über 2–3 μg nicht mehr möglich. Falls Substanzmengen unterhalb von 0.01 μg/Bande vorliegen sollten, erfolgt ihre Berechnung aus der in diesem Mengenbereich linearen Beziehung zwischen Gesamtfluoreszenzintensität und Menge. Bei Substanzmengen über 1 μg ist die Trennung mit kleineren Mengen zu wiederholen oder nach Extraktion des Fleckes durch UV- oder Fluoreszenz-Spektroskopie quantitativ zu ermitteln[1,4].

GENAUIGKEIT DER METHODE

Die Nachweisempfindlichkeit einzelner polycyclischer Aromaten ist durch ihre Fluoreszenzintensität begrenzt. Aromaten, die auf acetylierter Celluloseschicht sehr intensiv fluoreszieren, wie z.B. Perylen, 3,4-Benzpyren, 1,12-Benzperylen, Pyren usw., sind noch in ng-Mengen sehr gut nachzuweisen. Crysen, 1,2-Benzpyren, Anthracen, die nur schwach fluoreszieren, sind erst in Mengen über 0.1 μg, Phenanthren erst ab 1 μg zu erfassen.

Diese sehr unterschiedliche Nachweisempfindlichkeit beeinflusst die Genauigkeit der quantitativen Auswertung *in situ* sehr wesentlich. Die Reproduzierbarkeit der Messung und Auswertung bei der Chromatographie bekannter Mengen wies eine $\pm$ 10%ige Abweichung auf. Dies ist auf die Schwierigkeit der Auftragung quantitativer Mengen von Testgemischen mit Hilfe der von uns benutzten Mikropipetten zurückzuführen.

Mit der hier beschriebenen Methode konnten 75–85% der eingesetzten polycyclischen Aromaten (0.1 μg je Komponente) wiedergefunden werden. GRIMMER UND HILDEBRANDT[1] geben eine 95–100%ige Wiederfindung bei Anwendung der simultanen UV-spektroskopischen Methode an. Die eingesetzten Substanzmengen betrugen allerdings im Gegensatz zu unseren Versuchen mehrere μg je Komponente. Die von uns gefundenen höheren Verluste sind weitgehend mit der Schwierigkeit der quantitativen Übertragung der einzelnen Fraktionen auf die Dünnschicht zu erklären. Bei Anwendung eines automatischen Auftragegerätes dürften sowohl die Reproduzierbarkeits- als auch die Wiederfindungsfehler wesentlich gemindert werden.

ZUSAMMENFASSUNG

Die Auswertung der auf Dünnschicht (acetylierten Cellulose) getrennten polycyclischen Aromaten mit Hilfe eines Spektralphotofluorometers *in situ* erlaubt die Aufnahme der Fluoreszenz-Anregungs- und Emissionsspektren und somit ihre sichere Identifizierung, sowie die simultane quantitative Bestimmung einer Grosszahl von Substanzen. Die Nachweisbarkeitsgrenze liegt je nach Fluoreszenzintensität der Aromaten zwischen 0.1 und 0.001 μg. Die Reproduzierbarkeit bei bandenförmiger Auftragung weist 10% Fehler auf. Die angegebenen Eichkurven verlaufen bis *ca.* 0.2 μg Substanz/Bande weitgehend linear und können bei Benutzung eines externen Standards unabhängig von apparativen Veränderungen verwendet werden.

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CHROM. 4751

IDENTIFICATION DES ACIDES ORGANIQUES ET ÉVALUATION DE LEURS TENEURS INDIVIDUELLES DANS LES JUS DE RAISIN ET LES VINS PAR CHROMATOGRAPHIE ET PHOTODENSITOMÉTRIE

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AVEC LA COLLABORATION TECHNIQUE DE N. HEREDIA

(Reçu le 19 Décembre 1969; manuscrit modifié reçu le 25 Mars 1970)

SUMMARY

Identification of organic acids and determination of their individual amounts in grape-juices and wines by chromatography and photodensitometry

Tartaric, malic, lactic, succinic, citric and citromalic acids are identified by separation with one-dimensional thin-layer chromatography on cellulose. The individual amounts are determined by photodensitometry of the spots.

The method is applicable to the free acids and their salts present in wines, grape-juices or vegetable extracts.

INTRODUCTION

Parmi les nombreuses méthodes de séparation des acides organiques sur papier ou sur couche mince, certaines permettent la séparation d'un nombre important d'acides grâce à l'emploi de deux solvants migrant selon deux dimensions du support (CARLES *et al.*[1]). D'autres, n'utilisent qu'une seule migration: RASMUSSEN[2], BRUN ET GRAU[3], BACHUR[4], KUNKEE[5]; selon cette dernière technique certains auteurs tels que COOKE[6], LINDNER ET JURICS[7], ont cherché la possibilité d'évaluation quantitative par photodensitométrie. Dans cette voie nous avons obtenu une méthode convenant aux acides contenus dans les jus de raisin, les vins et les extraits végétaux.

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SÉPARATION CHROMATOGRAPHIQUE

Mode operatoire

Préparation des couches. Les plaques (20 × 20 cm) sont préparées avec de la poudre de cellulose (Macherey, Nagel et Co. MN 300) et stockées dans une enceinte exempte de vapeur d'acide.

Dépôt des solutions. Les solutions à analyser sont déposées en bandes fines de 20 mm de longueur disposées à 2 cm du bord inférieur de la plaque.

Les vins rouges sont débarrassés préalablement des anthocyanes qui se superposent avec le spot de l'acide tartrique. Ils sont traités avec du charbon végétal à raison de 250 mg/10 ml ou avec une résine échangeuse de cations (type Dowex 50).

Humidification et développement. L'enceinte devant servir au développement est saturée de vapeur d'eau au moyen de deux béchers de 25 ml dans lesquels on verse une vingtaine de millilitres d'eau à 70°. Dix minutes après, on introduit deux plaques dans la cuve, la couche cellulosique tournée vers les béchers (vers l'intérieur). On referme et après deux heures d'humidification, on verse le solvant de développement constitué par la phase supérieure du mélange butanol–acide formique–eau (4:2:5), préparé depuis un mois.

Révélation. La révélation a fait l'objet de nombreux essais. Le mélange glucide-aniline a été préféré aux autres révélateurs (bleu de bromophénol, pyridine, 2′,7′-dichlorofluoresceine); parmi les glucides, le *d*-arabinose donne les meilleurs résultats: fond uniforme et très clair, taches très contrastées. La pulvérisation du révélateur après développement s'est montrée supérieure aux autres types d'application (mélange à la suspension de cellulose avant étalement, mélange au solvant de développement, imbibition par les vapeurs).

On utilise le mélange suivant:

20 ml d'une solution aqueuse de *d*-arabinose (2 g dans 20 ml); 22 ml d'une solution alcoolique d'aniline (2 ml d'aniline récemment distillée dans 20 ml d'ethanol); 58 ml de *n*-butanol débarrassé de ses impuretés acides par agitation 2 h sur la soude (30 g de soude par litre de butanol) puis distillation.

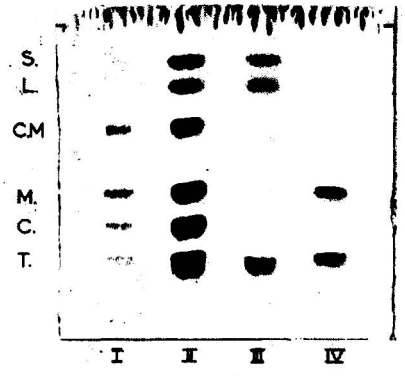


Fig. 1. T = Acide tartrique; C = Acide citrique; M = Acide malique; C.M. = Acide citramalique; L = Acide lactique; S = Acide succinique. I = Solution-témoin (10 méquiv. de chaque acide par litre); II = Solution-témoin (50 méquiv. de chaque acide par litre); III = Vin rouge de carignan 1967; IV = Jus de raisin rouge 1968.

Ce mélange doit être conservé à basse température et à l'abri de la lumière. Il est pulvérisé sur les plaques posées horizontalement à raison de 10–12 ml sur chacune. Les plaques sont mises dans une étuve à 90° bien ventilée et obscure, pendant 6 min puis elles sont placées dans une enceinte, à l'abri des vapeurs d'acide.

Le lendemain, la révélation est d'une netteté suffisante.

Résultats

La Fig. 1 reproduit un chromatogramme de quatre échantillons. On notera l'efficacité de la séparation et la netteté du contour des taches. On observe les échantillons suivants:

Échantillon No. 1: 20 μl d'une solution aqueuse d'acides organiques à 10 méquiv./l chacun; échantillon No. 2: 20 μl d'une solution aqueuse d'acides organiques à 50 méquiv./l chacun; échantillon No. 3: 20 μl d'un vin rouge de carignan. Macération de sept jours (chromatographie après décoloration); échantillon No. 4: 20 μl d'un jus de raisin rouge sulfité puis désulfité. (Chromatographie après dilution dans 3 volumes d'eau).

Les R_F des différents acides sont recueillis dans la Tableau I.

TABLEAU I

VALEURS DES R_F DES PRINCIPAUX ACIDES CONTENUS DANS LES VINS, LES JUS DE RAISIN ET LES EXTRAITS VÉGÉTAUX

Acide	R_F
Tartrique	0.13
Citrique	0.23
Malique	0.34
Citramalique	0.55
Lactique	0.69
Succinique	0.79

Discussion

L'étalement important des différents R_F assure une bonne efficacité à la séparation. Celle-ci est encore améliorée par le fait que l'acide tartrique, généralement le plus important, est celui qui migre le moins. On peut souligner d'autre part la netteté des taches de R_F élevé (acides citramalique, lactique et succinique) difficile à obtenir avec d'autres méthodes.

Un acide organique donné et ses différents sels sont isolés au sein d'une même tache chromatographique. Nous reviendrons sur cette intéressante particularité dans la partie quantitative.

Sur les chromatogrammes réalisés à partir d'une solution d'acide lactique, celui-ci donne naissance à deux taches, une principale de $R_F = 0.69$ due à l'acide lui-même et une plus réduite de $R_F = 0.86$ due vraisemblablement à un lactide (résultat de l'estérification réciproque des fonctions alcool et acide de deux molécules). Pour éviter cet inconvénient on peut procéder à partir d'une solution de lactate de sodium.

Par ailleurs, il y a une tendance inexpliquée de l'acide tartrique à donner deux taches contigües, lorsque les doses de départ sont élevées (1 microéquivalent).

Nous avons envisagé le devenir de quelques acides exogènes (antiseptiques

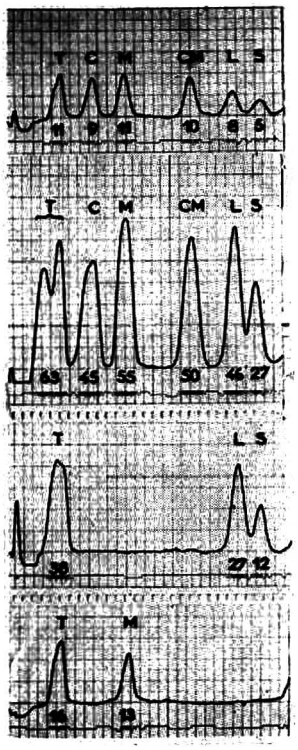


Fig. 2. I = Solution-témoin (10 méquiv. de chaque acide par litre); II = Solution témoin (50 méquiv. de chaque acide par litre); III = Vin rouge de carignan 1967; IV = Jus de raisin rouge 1968. Le chiffre en dessous de chaque pic correspond au nombre de "tops".

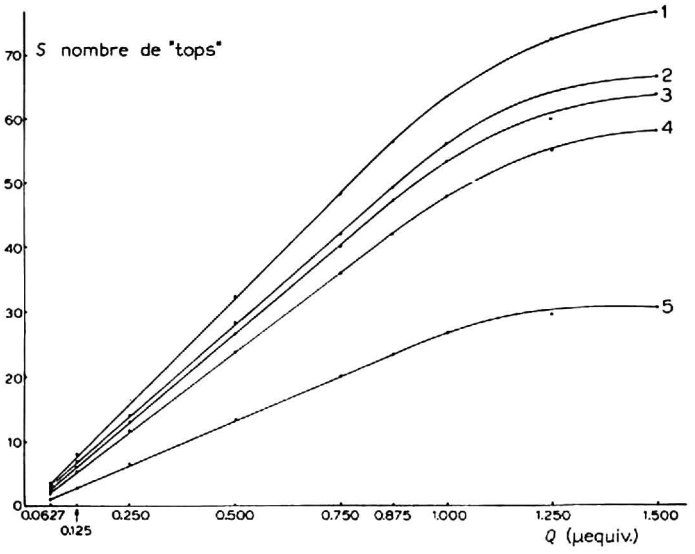


Fig. 3. Relation entre la surface d'un pic et la quantité d'acide déposée. (1) = acide tartrique; (2) = acide malique; (3) = acide citrique; (4) = acide lactique; (5) = acide succinique.

TABLEAU II

VALEURS DES R_F DE QUELQUES ACIDES GÉNÉRALEMENT PRÉSENTS À L'ÉTAT DE TRACES

Acide	R_F	*Acide*	R_F
Benzoïque	0.03	Galacturonique	0.02
Cyanhydrique	0.04	Gluconique	0.44
Salicylique	0.00	Glutamique	0.04
Sorbique	0.00	Glucuronique	0.13
Sulfureux	0.03	Malonique	0.61
α-Cétoglutarique	0.55	Oxalique	0.06
Chlorhydrique	0.05	Phosphorique	0.06
Dimethylglycerique	0.64	Shikimique	0.20
		Sulfurique	0.00

autorisés ou frauduleux) ou naturels qui, généralement présents à l'état de traces, peuvent prendre exceptionnellement une certaine importance. Les R_F de ces acides sont recueillis dans le Tableau II.

La plupart de ces acides ne migrent pas ou migrent très peu et de ce fait ne peuvent interférer. Ceux qui migrent sont généralement présents dans les vins à l'état de traces non révélables. Il convient toutefois d'être prudent avec les vins issus de vendanges botrytisées ou eudémisées car la teneur en acide glucuronique peut ne plus être négligeable et la tache brune correspondante chevauche alors celle de l'acide tartrique.

ÉVALUATION PHOTODENSITOMETRIQUE

Mode opératoire

Les mesures sont effectuées à l'aide du Photomètre intégrateur enregistreur Vernon* type T.R.D. 5, (un photodensitomètre Joyce-Loebl, type Chromoscan** a également été utilisé). Ces appareils sont employés en transparence. La longueur du faisceau lumineux est supérieure de quelques fractions de millimètres à celle des taches.

Afin d'augmenter le contraste, un filtre Wratten (47 B) est placé entre la plaque chromatographique et la cellule photoélectrique.

La surface de chaque pic est intégrée en unités arbitraires, au moyen de "tops" successifs inscrits au bas de la feuille d'enregistrement (Fig. 2).

La Fig. 3 montre que la surface d'un pic (intégrale de la densité optique des différents points de la tache correspondante), est proportionnelle à la quantité d'acide déposée jusqu'à un microéquivalent; au delà de cette dose, la relation n'est plus linéaire.

Pour un même dépôt c'est le composé obtenu par la révélation de l'acide tartrique qui a le coefficient d'extinction moléculaire le plus important, celui obtenu par la révélation de l'acide succinique étant le plus faible.

Sur une même plaque de 20 × 20 cm, on dépose cinq taches selon une bande de deux centimètres de longueur:

* Fabriqué par les Établissements Vernon, 5, rue Ed. Temblay 94, Vitry sur Seine.
** Joyce Loebl, France, 84, rue de Verdun, 75, Suresnes.

Solution étalon: 0.2 microéquivalent de chaque acide; solution étalon: 1.0 microéquivalent de chaque acide; trois échantillons à analyser.

Les doses de 0.2 et 1.0 microéquivalent sont à l'intérieur des limites où l'évaluation photodensitométrique reste linéaire. Le recours à deux mélanges synthétiques de concentrations voisines des extrêmes est rendu obligatoire par la variabilité de la révélation.

Résultats et discussion

Précision des résultats. Les causes d'erreurs en photodensitométrie ont été envisagées dans une étude générale de SHELLARD[8].

Les évaluations réalisées à partir de chromatographie sur papier ou sur couche mince peuvent être entachées d'une erreur dont la principale est due à l'impossibilité du transfert sur le support de la totalité du volume choisi.

Les recherches de FAIRBAIRN ET RELPH[9] montrent que l'ascension du liquide sur la paroi extérieure de la pipette, et la capillarité à l'intérieur, conduisent à des erreurs sur le volume déposé. Ces erreurs sont estimées plus importantes que celles dues à toutes les autres opérations réunies. Les auteurs concluent à la nécessité d'effectuer les dépôts au moyen d'un "dispositif à éjection forcée de gouttes". Dans le présent travail, cette technique n'a pas été employée.

Parmi les autres causes d'erreurs, le mode d'application et la variation du volume de révélateur utilisé ne paraissent pas intervenir, le volume choisi étant en large excès. Par contre, le chauffage fait parfois apparaître une hétérogénéité du fond près des bords latéraux. En conséquence, il est préférable de ne pas effectuer de dépôt à moins de 3 cm de ceux-ci.

TABLEAU III

PRÉCISION DES RÉSULTATS DES DÉTERMINATIONS DES TENEURS INDIVIDUELLES EN ACIDE TARTRIQUE ET MALIQUE D'UN JUS DE RAISIN (méquiv./l)

Dosages	*Acide tartrique*	*Acide malique*
1	69.6	23.6
2	67.8	22.5
3	65.7	21.8
4	66.3	22.2
5	68.4	22.7
6	67.8	22.1
7	69.3	23.4
8	67.5	22.6
9	68.1	22.8
10	69.0	22.5
11	67.5	23.2
12	64.8	21.4
Moyenne arithmétique	67.65	22.56
Écart type d'une mesure	1.442	0.642
Erreur d'une mesure ($P = 0.05$)	3.17	1.41
Erreur relative d'une mesure	± 4.70%	± 6.26%
Écart type de la moyenne (erreur-type)	0.416	0.185
Erreur de la moyenne ($P = 0.05$)	0.915	0.407
Erreur relative de la moyenne	± 1.35%	± 1.80%

TABLEAU IV

DÉTERMINATION PHOTODENSITOMÉTRIQUE APRÈS ADDITION D'UNE QUANTITÉ CONNUE D'ACIDES TARTRIQUE ET SUCCINIQUE À UN VIN BLANC

Acide	*Avant addition*	*Après addition de 10 méquiv./l*
Tartrique	31	41
Succinique	9	18

A partir d'un même échantillon nous avons fait douze déterminations complètes des teneurs en acide tartrique et malique. Les résultats obtenus et l'étude de leur variabilité figurent dans le Tableau III.

Une étude analogue sur un vin montre que les résultats concernant les teneurs en acide succinique et lactique peuvent être évaluées avec une précision de l'ordre de celle qui est obtenue pour l'acide malique.

L'addition d'une quantité connue (10 méquiv./l) d'acides tartrique et succinique à un vin blanc se retrouve lors de la détermination photodensitométrique ainsi que le confirme le Tableau IV.

Évolution des taches en fonction du temps. Comme cela a été signalé dans la première partie, après pulvérisation et chauffage, il convient d'attendre le lendemain pour procéder aux déterminations photodensitométriques. En effet, aussitôt après le chauffage, les taches sont de très faible intensité et rougeâtres. Après quinze à vingt heures d'attente, l'intensité a fortement augmenté et la teinte a viré au brun rougeâtre. Ce délai nous paraît être suffisant.

En fait, même après une vingtaine d'heures d'attente il y a encore une évolution de la teinte vers le brun et également une légère augmentation du contraste.

L'évolution des taches témoins étant la même que celle des taches correspondant aux vins et aux jus de raisins, les résultats sont sensiblement les mêmes si on fait la détermination photométrique le surlendemain au lieu du lendemain.

Ce fait confirmé par les résultats mentionnés dans le Tableau V, accroit la commodité d'utilisation de la méthode.

TABLEAU V

RÉSULTATS DES DÉTERMINATIONS PHOTODENSITOMÉTRIQUES EFFECTUÉES 24 h OU 40 h APRÈS LA RÉVÉLATION (méquiv./l)

Détermination	*Acide tartrique*		*Acide malique*		*Acide lactique*		*Acide succinique*	
	24 h	*40 h*	*24 h*	*40 h*	*24 h*	*40 h*	*24 h*	*40 h*
Vin de carignan rouge								
No. 1	24.0	25.0			22.0	22.0	20	21.0
No. 2	22	21.6			22.0	21.7	19.5	19.2
No. 5	23.6	23.5			21.0	21.0	21.5	21.4
No. 7	25.0	24.0			23.0	22.5	23.0	22.7
Jus de raisin rouge								
No. 1	44.0	46.0	45.2	44.0				
No. 5	50.0	48.0	48.0	48.4				

TABLEAU VI

SURFACE DES PICS (NOMBRE DE "TOPS") OBTENUS À PARTIR DE DÉPÔTS DE 0.8 μéquiv. D'ACIDE TARTRIQUE LIBRE ET DE TARTRATES ACIDE ET NEUTRE DE POTASSIUM)

Acide tartrique	*Tartrate acide de potassium*	*Tartrate neutre de potassium*
46	46	44

Influence éventuelle de la salification. Les manipulations précédentes ont été réalisées avec des acides libres. La méthode étant destinée aux moûts et aux vins, il était important de savoir si ceux-ci devaient être préalablement décationisés ou non.

Nous avons pu observer que la chromatographie d'un acide libre et de un ou plusieurs de ses sels, donne une tache unique. De plus l'addition d'acide sulfurique à un échantillon ne modifie pas les résultats quantitatifs obtenus. Enfin, le Tableau VI montre que la surface des pics est la même pour un même nombre d'équivalents, que l'acide soit libre, partiellement ou totalement salifié.

CONCLUSION

La méthode d'isolement et de révélation établie, conduit à une bonne séparation des acides organiques libres et salifiés, des jus de raisin et des vins. Les acides tartrique, citrique, malique, citramalique, lactique et succinique et leurs sels donnent des taches nettes, bien isolées. Il en est de même pour d'autres acides moins importants en oenologie, tel que les acides gluconique, diméthylglycérique, shikimique.

Sur la base de ces résultats, il apparaît que la méthode photodensitométrique permet de déterminer rapidement les teneurs individuelles en acides organiques libres ou salifiés des jus de raisin et des vins. La précision des résultats est suffisante pour convenir non seulement aux laboratoires d'analyses courantes mais aussi aux laboratoires de recherches. On peut envisager d'étendre le champ d'application de cette méthode à d'autres domaines que l'oenologie, en biochimie végétale notamment.

RÉSUMÉ

Les acides tartrique, malique, lactique, succinique, citrique et citramalique sont identifiés après séparation par chromatographie monodimensionnelle sur couche mince de cellulose. Les teneurs individuelles sont évaluées par photodensitométrie des taches.

La méthode est applicable aux acides libres ou salifiés contenus dans les vins, les jus de raisins ou les extraits végétaux.

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CHROM. 4739

SENSITIVITY OF PIG LIVER ESTERASE IN DETECTING TWELVE CARBAMATE PESTICIDES ON THIN-LAYER CHROMATOGRAMS

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SUMMARY

A comparison of pig and beef liver extracts for the detection of twelve carbamates on thin-layer plates is discussed. The esterase in frozen extracts of pig liver was found sensitive to inhibition by the carbamates studied at the nanogram to picogram levels. Bromine or ultraviolet light was shown to destroy the inhibitory property of the pesticides. Microslides were useful for rapid detection of the carbamates. Silica Gel G and G-HR were superior among the different types of gel layers studied. The hR_F values of the standards in different solvent systems were given. The advantages of using the freeze-dried extracts were discussed.

INTRODUCTION

The thin-layer chromatographic–enzyme inhibition (TLC–EI) technique developed in our laboratory has been used to detect carbamate and organophosphorus pesticides[1,2]. WINTERLIN *et al.*[3] demonstrated by TLC that bee brain cholinesterase was generally more sensitive for detection of these pesticides. Bee brain cholinesterase, in particular, was more sensitive to inhibition by carbaryl (Sevin) than human plasma cholinesterase while the opposite held for aldicarb (Temik). Likewise, we observed that pig liver esterase was more sensitive in detecting carbaryl than beef, sheep, monkey, or chicken liver esterase[4]. Therefore, pig liver esterase was further studied for TLC detection of twelve carbamate pesticides. In addition, the effects of UV light or bromine on these compounds were evaluated. The use of microscope slides for rapid detection of the carbamates was also studied.

MATERIALS AND METHODS

Enzyme solution preparation

The pig and beef liver esterases were extracted following the procedure of MENDOZA *et al.*[4]. The 2000 × *g* extracts were either frozen directly or freeze-dried. Before use, the frozen extracts were diluted eight times with 0.05 *M* Tris–HCl buffer

at pH 8.3. The freeze-dried extracts were reconstituted with the same buffer solution to approximate the amounts of solids present in the spray solution made of frozen extracts. Solids obtained after freeze-drying the pig and beef extracts were 5.8 and 5.6 g/100 ml, respectively.

Preparation of TLC plates

Coating materials were applied approximately 450 μ thick on TLC plates by means of a Desaga applicator (obtainable from Desaga, Heidelberg, G.F.R.). The list below includes the sources of the materials and the amounts used to make slurries.

Aluminum Oxide DS-5, Camag (Arthur H. Thomas Co., Philadelphia, Pa.) 75 g/60 ml of distilled water.

Kieselgel D-5 (silica gel), Camag (Arthur H. Thomas Co.) 50 g/100 ml of distilled water.

MN-Kieselgel G-HR (silica gel), (Macherey, Nagel and Co., Düren, G.F.R.) 50 g/100 ml of distilled water.

Silica Gel H (E. Merck AG, Darmstadt, G.F.R.) 50 g/100 ml of distilled water.

Silica Gel G (E. Merck AG) 50 g/100 ml of distilled water.

SilicAR TLC-7 (Mallinckrodt Chemical Works, St. Louis, Mo.) 50 g/100 ml of distilled water.

Polyamide II (E. Merck AG) 50 g/50 ml of distilled water and 50 ml of acetone.

Some TLC plates with agar-agar were prepared according to the procedure of MAINI[5]. Agar-agar, 0.4 g, was dissolved in 120 ml of boiling distilled water and 50 g of MN-Kieselgel G-HR was added while the solution was still hot. The mixture was shaken vigorously for approximately 1 min before applying it on the preheated plates at 450 μ thickness.

All plates were heated at 110° in an oven for 1 h before use.

Preparation of TLC micro-plates

TLC micro-plates were coated with MN Kieselgel G-HR with or without agar-agar by using the apparatus developed by LOVELADY[6]. The space between the shoe and the micro-plates was adjusted to 450 μ before use. A thin plexiglass slide was fitted snugly in the loader closing the space between the shoe guide and the main body to prevent the slurry from flowing out freely. Before moving the loader across the micro-plates, the plexiglass slide was pulled up to let the slurry out. The slurry was made of 5 g of silica gel in 10 ml of distilled water. The coated micro-plates were incubated 1 h at 110°.

Solvent systems and development of the plates

The standards were applied 2.5 cm from the edge of the gel and were resolved in the solvent systems listed on Table I. The enzyme extracts were sprayed on the plates. As soon as the plates were dry, they were sprayed with the 5-bromoindoxyl acetate solution[7]. Areas of inhibition appeared as white spots on a blue background.

The carbamates spotted on micro-plates were resolved in a 200-ml beaker with 10 ml of solvent and a petri dish cover.

Carbamate standards

Common or proprietary names, chemical nomenclatures, and manufacturers

TABLE I

SOLVENT SYSTEMS USED TO RESOLVE THE TWELVE CARBAMATES AND THE ROOM TEMPERATURE DURING THE TEST

Solvent systems	*Composition*	*Temp. (°C)*
1	20% acetone in cyclohexane	25
2	20% acetone + 10% benzene in hexane	28
3	20% acetone in pentane	28
3[a]	20% acetone in pentane	25
4	30% acetone in hexane	27
4[a]	30% acetone in hexane	25
5	20% acetone + 10% benzene in pentane	28
6	20% acetone in hexane saturated with methanol	28
7	20% acetone in hexane	25
8	20% acetone in 1 part of hexane: 1 part of cyclohexane	26
9	30% acetone in cyclohexane	25
9[a]	30% acetone in cyclohexane	25
10	30% acetone in heptane	25
10[a]	30% acetone in heptane	25
11	20% acetone in 1 part of pentane: 1 part of hexane	25
12	20% acetone in 1 part of hexane: 1 part of heptane	25
17	cyclohexane	28
18	ethanol	28
22	10% ethanol + 10% benzene + 20% acetone in heptane	28
23	chloroform	25
24	20% methanol in benzene	25

[a] Solvent systems were used on plates made with agar-agar.

are listed below. The purity determined by the suppliers is expressed in percent. Carbofuran 3-OH analogue and Mobam did not have purity assay; however, each gave only one spot under the TLC–EI conditions used.

Aldicarb = 2-methyl-2-(methylthio) propionaldehyde O-(methylcarbamoyl)-oxime (Union Carbide, Olefins Division, New York, N.Y.), 99.7%.

Banol = 2-chloro-4,5-xylenol methylcarbamate (Tuco Products Division, Upjohn, Kalamazoo, Mich.), 100%.

Baygon = *o*-isopropoxyphenyl methylcarbamate (Chemagro Corporation, Kansas City, Mo.), 99.2%.

Carbaryl = 1-naphthyl methylcarbamate (Union Carbide), 99.7%.

Carbofuran = 2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate Niagara Chemical Division, (FMC Corporation, Middleport, N.Y.), 99.2%.

Carbofuran 3-OH analogue = 2,3-dihydro-2,2-dimethyl-3-hydroxy-7-benzolfuranyl methylcarbamate (FMC Corporation).

Matacil = 4-dimethylamino-*m*-tolyl methylcarbamate (Chemagro Corporation), 99.4%.

Mesurol = 4-(methylthio)-3,5-xylyl methylcarbamate (Chemagro Corporation), 100%.

Mobam = 4-benzothienyl methylcarbamate (Mobil Chemical Company, Industrial Chemical Division, Richmond, Va.).

Ortho 5353 = *m*-(1-ethylpropyl) phenyl methylcarbamate and *m*-(1-methylbutyl) phenylmethylcarbamate mixture is approximately 1:4 proportions (Chevron Chemical Company, Ortho Division, Richmond, Calif.), 99.2%.

TABLE II

DETECTION LIMITS IN NANOGRAMS OF TWELVE CARBAMATE PESTICIDES ON MN KIESELGEL G-HR LAYERS BY TLC–EI PROCEDURE USING PIG AND STEER LIVER EXTRACTS

(+) = TLC spots corresponding to the areas where enzymes were inhibited lasted 5 min; (++) = lasted more than 5 min but less than 30 min; (+++) 30 min or more. (±) = spots lasted 1–2 min, and (−) = no enzyme inhibition detected. TLC plates were coated with 450 μ thick layer of Kieselgel G-HR. The solvent system was 20% acetone in cyclohexane.

Compounds	*Liver extracts*			
	Pig		*Steer*	
	Frozen	*Freeze-dried*	*Frozen*	*Freeze-dried*
Aldicarb	50 (±)	100 (±)	100 (±)	100 (±)
	30 (−)	50 (−)	50 (−)	50 (−)
Banol	0.5 (+)	5 (±)	5 (±)	10 (±)
	0.1 (−)	1 (−)	1 (−)	5 (−)
Baygon	10 (±)	50 (±)	100 (±)	100 (±)
	5 (−)	10 (−)	50 (−)	50 (−)
Carbaryl	0.1 (+)	0.5 (±)	0.5 (±)	5 (+)
	0.05 (−)	0.1 (−)	0.1 (−)	1 (−)
Carbofuran	1 (+)	50 (+)	100 (±)	100 (+)
	0.5 (−)	10 (−)	50 (−)	50 (−)
Carbofuran 3-OH	10 (±)	100 (±)	100 (±)	400 (±)
	5 (−)	50 (−)	50 (−)	300 (−)
Matacil	10 (±)	50 (±)	50 (±)	50 (+)
	5 (−)	10 (−)	10 (−)	10 (−)
Mesurol	0.1 (±)	10 (±)	10 (±)	10 (±)
	0.05 (−)	5 (−)	5 (−)	5 (−)
Mobam	0.5 (+)	1 (±)	5 (++)	5 (+)
	0.1 (−)	0.5 (−)	1 (−)	1 (−)
Ortho 5353	1 (+)	5 (±)	5 (+)	5 (+)
	0.1 (−)	0.5 (−)	1 (−)	1 (−)
Tranid	100 (±)	500 (±)	300 (++)	100 (±)
	50 (−)	400 (−)	200 (−)	50 (−)
Zectran	50 (+++)	50 (++)	50 (±)	50 (±)
	10 (−)	10 (−)	10 (−)	10 (−)

Tranid = 5-chloro-6-oxo-2-norbornanecarbonitrile O-(methylcarbamoyl)oxime (Union Carbide), 99.9%.

Zectran = 4-dimethylamino-3,5-xylyl methylcarbamate (Dow Chemical Company, The Agricultural Products Department, Midland, Mich.), 99%.

All the standards were dissolved in methanol and kept in a refrigerator.

RESULTS AND DISCUSSION

Sensitivity tests

Table II shows the detection limits in nanograms (ng) of different carbamate pesticides on MN Kieselgel G-HR layer by TLC–EI procedure using pig and steer liver extracts. In general, the pig liver extracts and frozen extracts were more sensitive than the steer liver extracts and freeze-dried extracts, respectively, in detecting the pesticides tested. Carbaryl was detected at 0.1 ng by the frozen extract of pig livers and at 0.5 ng by the freeze-dried extract of pig livers or frozen extract of steer livers. With the frozen extract of pig livers, Banol and Mobam were both detectable at the

TABLE III

DETECTION LIMITS IN NANOGRAMS OF TWELVE CARBAMATE PESTICIDES ON SILICA GEL G AND ALUMINUM OXIDE DS-5 LAYERS USING FROZEN PIG LIVER EXTRACTS

(+) = TLC spots corresponding to the area where enzymes were inhibited lasted 5 min, (++) = lasted more than 5 min but less than 30 min, (+++) = 30 min or more.

Compounds	*Types of layer*	
	Silica Gel G	*Aluminum Oxide DS-5*
Aldicarb	30 (±)	10 (±)
	10 (−)	5 (−)
Banol	0.5 (++)	0.5 (++)
	0.1 (−)	0.1 (−)
Baygon	10 (±)	10 (+)
	5 (−)	5 (−)
Carbaryl	0.1 (+)	0.1 (±)
	0.05 (−)	0.05 (−)
Carbofuran	1 (±)	1 (±)
	0.50 (−)	0.5 (−)
Carbofuran 3-OH	10 (+)	5 (+)
	5 (−)	1 (−)
Matacil	5 (++)	1 (±)
	1 (−)	0.5 (−)
Mesurol	0.1 (±)	0.1 (±)
	0.05 (−)	0.05 (−)
Mobam	0.1 (+)	0.5 (+++)
	0.05 (−)	0.1 (−)
Ortho 5353	1 (±)	1 (±)
	0.5 (−)	0.5 (−)
Tranid	50 (+)	5 (++)
	10 (−)	1 (−)
Zectran	0.5 (±)	5 (+++)
	0.1 (−)	1 (−)

0.5-ng level and carbofuran and Ortho 5353 at the 1-ng level. The carbofuran 3-OH analogue was less sensitive than carbofuran. Tranid, the least inhibitory among the pesticides tested, was detected at 100–500 ng levels.

The sensitivity of detection was found to be affected by the types of gel layers used, Table III. Silica Gel G was much more sensitive than MN Kieselgel G-HR (*cf.* Table II) in detecting aldicarb, Matacil, Tranid and Zectran. The detectability of Banol, Baygon, carbaryl, carbofuran, carbofuran 3-OH analogue, and Ortho 5353 on Silica Gel G was comparable to that on MN Kieselgel G-HR. The aluminum oxide TLC plates did not give discrete spots corresponding to the carbamates tested. The spots, though larger than those on Silica Gel G or G-HR, were diffused. Nevertheless, the detectable levels of aldicarb, carbofuran 3-OH analogue, and Tranid, were lower on this layer than on Silica Gel G.

The TLC spots, corresponding to the areas where the enzymes were inhibited, were clearly defined on Silica Gel G and MN Kieselgel G-HR layers. The development of the background and the spots were consistently rapid and intense on Silica Gel G. Therefore, considering the low limit of detection, Silica Gel G is as useful as MN Kieselgel G-HR in the TLC–EI procedure for these carbamates.

Silica Gel D-5 was satisfactory for use in carbamate detection but less superior

than Silica Gel G and G-HR. It did not give a uniform background as did the Silica Gel G and G-HR.

The use of Aluminum Oxide DS-5 layer may be limited for the detection of Aldicarb, Carbofuran 3-OH analogue, and Tranid.

Polyamide 11 did not form a uniform suspension in water unless acetone was added. The carbamates spotted on the polyamide TLC plates streaked. The aqueous spray droplets were not readily adsorbed on the layer, thus the blue background obtained was uneven.

Silica TLC-7 and Silica Gel H layers gave poor TLC background which obliterated spots where the pesticides were located.

TABLE IV

EFFECT OF BROMINE AND UV ON TWELVE CARBAMATE INSECTICIDES RESOLVED ON MN KIESELGEL G-HR AND SPRAYED WITH THE PIG LIVER EXTRACTS

Compounds	*Levels evaluated (ng)*	*Br_2*	*UV*
Aldicarb	1000	slight decrease	decrease
Banol	10	decrease	decrease
Baygon	100	decrease	decrease
Carbaryl	1	increase	decrease
Carbofuran	10	decrease	decrease
Carbofuran 3-OH	10	decrease	increase
Matacil	1000	slight increase	decrease
Mesurol	10	slight decrease	decrease
Mobam	10	no change	no change
Ortho 5353	100	no change	no change
Tranid	100	no change	no change
Zectran	100	decrease	decrease

Effect of bromide and UV on twelve carbamate pesticides

Table IV shows that bromine and UV generally reduced the ability of the compounds to inhibit the pig liver esterase. However, carbaryl exposed to bromine vapor and carbofuran 3-OH analogue exposed to UV showed an appreciable increase in their ability to inhibit the enzymes used. Also, a slight increase in enzyme inhibition was observed with Matacil exposed to bromine. The decrease in enzyme inhibition due to 10 ng of Banol that was exposed to bromine contradicted the observation of WALES *et al.*[2]. Both bromine and UV had negligible effect on Mobam, Ortho 5353, and Tranid.

Solvent system on different TLC plates

Table V shows the hR_F values of the eleven carbamates resolved with different solvent systems on MN Kieselgel G-HR. The best separation was obtained with solvent system 5; only 4 pairs of carbamates had overlapping hR_F values (in parenthesis): (a) Matacil (70)–Mobam (69); (b) Baygon (77)–carbaryl (77); (c) carbofuran (80)–Mesurol (80); (d) Banol (89)–Ortho 5353 (89). Carbamates with the same or a difference of two hR_F values were considered overlapping. With the other solvent systems, three to six carbamate hR_F values overlapped at one area.

The only advantage obtained with MN Kieselgel G-HR and agar-agar com-

TABLE V

hR_F VALUES OF ELEVEN CARBAMATES RESOLVED WITH DIFFERENT SOLVENT SYSTEMS ON MN KIESELGEL G–HR

Compounds	*1*	*2*	*3*	*3*[a]	*4*	*4*[a]	*5*	*6*	*7*	*8*	*9*	*9*[a]	*10*	*10*[a]	*11*	*12*	*17*	*18*	*22*	*23*
Aldicarb (500)[b]	20	33	66	60	45	50	62	23	21	27	33	35	25	32	29	30	0	75	33	11
Banol (50)	33	48	77	72	65	57	89	40	35	39	46	47	39	40	40	32	3	79	45	33
Baygon (500)	27	40	76	63	50	53	77	28	30	32	35	33	38	35	27	25	1	76	35	11
Carbaryl (5)	24	38	75	63	43	57	77	28	29	28	35	27	25	37	32	24	1	75	35	25
Carbofuran (500)	26	39	71	61	49	43	80	29	20	27	37	38	31	33	31	24	2	70	33	8
Carbofuran 3-OH (500)	6	13	23	22	32	35	28	7	7	7	33	20	15	19	7	5	0	55	39	0
Matacil (500)	27	37	75	67	54	55	70	28	28	33	39	40	30	37	33	25	2	70	33	7
	P 0[c]	P 0	P 0	P52	P31	P47	P 0	P15		P20		P27	P 0		P 0	P 0		P 0		
	P 6	P 9	P48		P39		P10						P10			P 3				
		P24			P67		P27						P18			P12				
							P53						P25			P20				
Mesurol (50)	32	48	77	72	64	61	80	40	35	39	45	51	39	43	37	31	0	77	37	30
													P 7					P 0		
Mobam (5)	22	37	71	61	50	52	69	26	25	28	35	37	27	33	32	21	3	76	33	23
Ortho 5353 (50)	37	53	80	77	68	65	89	44	39	41	51	53	40	47	40	33	5	78	43	33
										P35										
Zectran (500)	35	48	79	77	66	61	86	50	37	40	46	47	40	47	39	33	5	77	43	27
		P26	P 0	P53	P25	P47	P 0	P17		P 7		P27	P 0		P10	P 6	Po			
			P57		P40		P 5			P20			P 7		P20	P13				
							P25			P30			P23							
							P53						P27							

[a] The TLC plates were prepared with agar-agar.
[b] Figures in parenthesis indicate the pesticide amounts (in ng) spotted.
[c] P = inhibitor detected in the standard solution.

bination was the improvement of resistance to mechanical breakage[5]. The carbamate hR_F values on these plates were comparable to those on TLC plates without agar-agar.

It is interesting to note that the hR_F values of some pesticides increased while those for the others decreased or remained the same in another system. The position of carbofuran 3-OH relative to other carbamates shifted most often (*cf.* solvent system 22 *versus* solvent systems 1, 2, 5, 6, 7, 8, 11, or 12; *cf.* 3 *versus* 4; *cf.* 5 *versus* 9).

The carbamates on MN Kieselgel G-HR migrated to the solvent front when developed with 30% chloroform and 10% ethanol in petroleum ether or with acetonitrile; the exceptions were carbofuran 3-OH analogue and Tranid, with hR_F values of approximately 90. No migration of the carbamates was observed with pentane, hexane, heptane, benzene, or petroleum ether. These solvents may be important in certain TLC clean-up procedures for carbamates.

With ethanol, solvent system 18, the hR_F values for the carbamates were approximately 90 on Silica Gel H and SilicAR TLC-7 and approximately 80 for MN Kieselgel G-HR. As previously observed, carbofuran and Tranid migrated slower than the other carbamates studied.

Two-dimensional TLC in one solvent system was carried out to determine whether additional spots for Matacil, Mesurol, Ortho 5353, and Zectran standards were in the standard solution or breakdown products during chromatography (see Table V). For Matacil, Mesurol, and Ortho 5353, additional spots were obtained after the first resolution only indicating that the standards had some impurities. Zectran gave several spots after the first and one spot, besides that of Zectran, after the second resolution. Therefore, Zectran standard had impurities and was breaking down during chromatography.

The hR_F values of twelve carbamates on Silica Gel G, D-5, H, and SilicAR

TABLE VI

hR_F VALUES OF TWELVE CARBAMATES ON DIFFERENT TYPES OF GEL LAYERS AND RESOLVED WITH SOLVENT SYSTEM 5 OR 7 AT 25°

Compounds	*Types of layer*									
	Silica Gel G		*Silica Gel D–5*		*Silica Gel H*		*SilicAR TLC–7*		*Aluminum Oxide DS–5*	
	5	*7*	*5*	*7*	*5*	*7*	*5*	*7*	*5*	*7*
Aldicarb	43	14	38	8	43	17	—[b]	—	60	20
Banol	64	26	55	18	63	28	—	27	73	42
Baygon	57	22	42	14	49	22	47	23	63	20
Carbaryl	46	18	33	12	51	22	52	26	57	20
Carbofuran	45	18	33	12	51	21	53	26	57	20
Carbofuran 3-OH	20	5	12	2	10	8	20	13	3	0
Matacil	45	18	33	12	51	21	47	21	57	22
Mesurol	54	22	47	18	60	27	62	33	63	27
Mobam	45	14	33	9	51	20	47	26	52	16
Ortho 5353	65	25	61	19	68	32	60	35	73	30
Tranid	12	3	7	1	16	4	15	5	3	0
Zectran	64	24	61	19	71	30	60	39	73 P o[a]	30

[a] P = inhibitor detected in the standard solution.
[b] — = area of the spot, not definite.

TABLE VII

MEAN hR_F VALUES OF TWELVE CARBAMATES RESOLVED ON SILICA GEL G WITH 20% METHANOL IN BENZENE SYSTEM IN GLASS TANKS WITH (A) AND WITHOUT (B) FILTER PAPER LINING

Each mean was based on four observations.

Compounds	*A*	*B*
Aldicarb	52.0 ± 1.4	68.0 ± 5.2
Banol	61.8 ± 1.1	76.0 ± 3.4
Baygon	55.5 ± 1.0	68.2 ± 3.7
Carbaryl	54.8 ± 0.6	65.0 ± 2.9
Carbofuran	56.5 ± 0.6	68.0 ± 2.3
Carbofuran 3-OH	39.8 ± 3.4	54.5 ± 2.7
Matacil	53.8 ± 0.8	65.0 ± 2.8
Mesurol	60.2 ± 1.0	74.2 ± 1.4
Mobam	53.0 ± 0.0	67.5 ± 2.8
Ortho 5353	62.2 ± 1.1	78.8 ± 2.4
Tranid	39.2 ± 3.2	53.5 ± 1.0
Zectran	62.0 ± 1.2	78.2 ± 2.4

TABLE VIII

MEAN hR_F VALUES OF TWELVE CARBAMATES ON MICROSLIDES (75 × 25 mm) COATED WITH MN KIESELGEL G-HR WITH OR WITHOUT AGAR-AGAR

Compounds	*Solvent*[a] *system*	*Number observed*	*Mean hR_F*	
			Without agar-agar	*With agar-agar*
Aldicarb	7	4	24.9 ± 0.6	25.5 ± 1.0
	9	2	47.5	42.5
Banol	7	4	34.8 ± 1.4	34.3 ± 0.7[b]
	9	3	46.7 ± 0.9	47.3 ± 0.7
Baygon	7	3	32.0 ± 1.0	30.3 ± 1.2
	9	3	43.7 ± 1.7	44.7 ± 0.9
Carbaryl	7	4	24.9 ± 1.4	28.5 ± 1.3
	9	3	48.0 ± 1.0	45.0 ± 0.0
Carbofuran	7	4	25.8 ± 1.6	29.5 ± 1.5
	9	5	44.4 ± 1.4	43.6 ± 1.0
Carbofuran 3-OH	7	4	5.5 ± 0.3	6.2 ± 1.2
	9	4	17.0 ± 0.7	17.5 ± 0.5
Matacil	7	3	25.7 ± 0.7	24.7 ± 0.9
	9	3	41.3 ± 0.3	40.7 ± 0.3
Mesurol	7	4	33.0 ± 0.9	32.0 ± 0.0[b]
	9	3	46.7 ± 0.3	47.7 ± 0.3
Mobam	7	3	23.0 ± 1.0	22.3 ± 0.9
	9	3	44.0 ± 1.0	42.7 ± 0.3
Ortho 5353	7	4	37.5 ± 2.0	38.8 ± 1.3
	9	3	50.0 ± 1.0	49.3 ± 0.3
Tranid	7	4	0.0	0.0
	9	3	14.7 ± 0.3	14.3 ± 0.7
Zectran	7	3	34.7 ± 0.3	35.0[c]
	9	3	48.7 ± 1.8	51.0 ± 0.0

[a] 7 = 20% acetone in hexane, 9 = 30% acetone in cyclohexane.
[b] Based on three observations only.
[c] A mean of two observations, both 35.

TLC-7, and Aluminum Oxide DS-5 are shown in Table VI. Solvent systems 5 and 7 were used. The carbamates had lower hR_F values on these gels than on MN Kieselgel G-HR when solvent systems 5 and 7 were used. The exception was Banol, which had an hR_F value of 42 on Aluminum Oxide DS-5 and only 35 on MN Kieselgel G-HR with solvent system 7 (*cf.* Table V). With the same solvent system 7, carbofuran had hR_F values of 21, 20 and 20 on Silica Gel H, Aluminum Oxide DS-5 and MN Kieselgel G-HR, respectively; carbofuran 3-OH had hR_F values of 8 on Silica Gel H and 7 on G-HR (*cf.* Table V). Solvent system 24 gave variable hR_F values for the carbamates on Silica Gel G, Table VII. The hR_F values in the same solvent system decreased when the tanks were lined with filter papers. Moreover, the standard deviations of the means also decreased, with the exception of those for carbofuran 3-OH analogue and Tranid.

The migration rates of the twelve carbamates on microslides coated with MN Kieselgel G-HR with or without agar-agar were comparable and consistent in either solvent systems 7 or 9, Table VIII. However, the migration rates were much increased when solvent system 9 was used. As previously stated, agar-agar improved the mechanical resistance of the gel layer.

CONCLUSIONS

The results showed that pig liver esterase was more sensitive in detecting carbamates than was beef liver esterase. Although the esterase lost some sensitivity to inhibition when the extracts were freeze-dried, the amounts detected were still at the ng levels. Pig liver esterase was particularly sensitive to inhibition by carbaryl.

In comparison with the data of WINTERLIN *et al.*[3], the three types of esterases are given in the order of increasing sensitivity for (a) aldicarb: human plasma cholinesterase (H) > bee head cholinesterase (B) > pig liver esterase (P); (b) carbaryl: B > P > H; (c) carbofuran: B > P = H. Comparative study of some published methods using enzymes is in progress.

The results also showed that bromine or UV generally decreased the inhibitory property of the carbamates. The exception was carbaryl whose inhibitory property was markedly enhanced after exposure to bromine. The effect of UV may be comparable to the effect of sunlight on the carbamates.

The sensitivity of the TLC–EI detection of the pesticides studied was affected by the types of gel layers. Nanograms to picograms of standards were consistently detected on Silica Gel G and G-HR; these gels readily produced uniform blue backgrounds. For rapid detection of the carbamates, microslides could be used.

The freeze-dried liver extract has properties that are desirable for storage, transport, and large scale preparation of the extracts. They are as follows: (1) The storage is limited to approximately 6 g of powder for every 100 ml of extracts freeze-dried instead of the usual 10 to 12 test tubes (13 × 100 mm). (2) The freeze-dried extracts can readily be reconstituted with water or the buffer solution. (3) The exact amount of the freeze-dried enzyme needed for spraying can easily be weighed. Thawing out the whole tube of the frozen enzyme, when only a small amount of spray is needed, is wasteful. (4) Like the frozen extracts, the freeze-dried extracts can be used even after a year of storage at below 0°.

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CHROM. 4730

NACHWEIS VON FREIEN IONEN IN MARKIERTEN ÖLEN

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SUMMARY

Detection of free ions in labelled oils

For the detection of free radionuclide ions in labelled mineral and vegetable oils and some of their components thin-layer chromatography, paper chromatography and paper electrophoresis were used. According to the results obtained thin-layer chromatography was particularly suitable for the examination of ^{51}Cr-, ^{59}Fe- and ^{65}Zn-labelled oils, whereas for ^{82}Br-labelled compounds paper electrophoresis was the method of choice.

EINFÜHRUNG

In vorangegangenen Arbeiten wurde die Herstellung von öllöslichen ^{51}Cr, ^{54}Mn, ^{59}Fe, ^{65}Zn, ^{82}Br und ^{131}J beschrieben[1–5], die eine Reihe von Anwendungen wie Ermittlung von Strömungsgeschwindigkeiten, Bestimmung von Mischzeiten etc. in nichtwässerigen Systemen ermöglichen. Die markierten Öle dürfen die genannten Radionuklide jedoch nur in organisch gebundener Form und nicht als Ionen enthalten, denn beispielsweise würde freies Radiobrom oder- jod and Metalloberflächen adsorbiert werden und fehlerhafte Resultate verursachen.

In der vorliegenden Arbeit wurde daher versucht möglichst einfache Nachweisverfahren für Radionuklide in Ionenform in markierten organischen Verbindungen aufzufinden. Als Methoden wurden Dünnschichtchromatographie, Papierchromatographie und Elektrophorese in die engere Wahl gezogen, da sie bereits für ähnliche Problemstellungen verwendet worden waren[1–8].

EXPERIMENTELLES

Materialien und Geräte

Es wurden die folgenden Materialien verwendet: ^{51}Cr in 0.1 *N* HCl, 35 μCi/ml, Träger 1 mg Cr/ml; ^{54}Mn in 0.1 *N* HCl, 10 μCi/ml, Träger 10 μg Mn/ml; ^{59}Fe in 0.1 *N* HCl, 1 μCi/ml, Träger 1 mg Fe/ml; ^{65}Zn in 0.1 *N* HCl, 3 μCi/ml, Träger 2 mg Zn/ml;

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^{82}Br als 0.1 *N* NH_4Br-Lösung, 100 μCi/ml. Markierte Mineral- und Pflanzenöle, Öl- und Naphthensäure. Kaliumferrocyanid, 2%ige wässerige Lösung; 0.1% Auritricarbonsäure in 1%iger wässeriger Ammoniumacetatlösung; 1% Diphenylcarbacidlösung in Äthanol; Veronalpufferlösung pH 8.6. Petroläther, Diäthyläther, Tetrachlorkohlenstoff, Benzol, Essigsäureäthylester und Äthanol. HCl, NaOH, NH_3 usw. Kieselgel zur Analyse (Merck, Darmstadt).

Die nachstehenden Geräte wurden angewandt: Glasplatten (20 × 20 cm); Papierstreifen (2 × 20 cm) Whatman Nr. 3MM, Schleicher & Schüll 2043b; Elektrophoreseapparatur; 3 × 3 in. NaJ(Tl) Kristall "Harshaw" mit "Intertechnique"; 400 Kanal Impulshöhenanalysator mit Schreiber und Drucker; Dünnschicht-Scaner "Berthold" LB 2721 mit Servogor-Kompensationsschreiber Type RE 511.

Dünnschichtchromatographische Untersuchungen

Nach Literaturangaben sind für die Trennung von Mono-, Di- und Triglyceriden Mischungen von Petroläther mit 10–15% Diäthyläther geeignet[1–4]. Dieselbe Laufmittelmischung kann auch zur Trennung von organischen Substanzen, die freie Fettsäuren enthalten, verwendet werden. Allerdings ist ein Zusatz von 1–2% Eisessig zu empfehlen um Streifen und Schwanzbildungen durch die Fettsäuren zu verhindern[1,2,5,6]. Pflanzenöle, die Epoxy- und Hydroxyverbindungen enthalten sind polarer als die meisten tierischen Fette und lassen sich ebenfalls mit Gemischen von Petroläther auftrennen[7]. Die zuletzt genannten Fliessmittel schienen daher sehr allgemein verwendbar und für unsere Zwecke geeignet.

Zur Ausführung wurden Glasplatten mit Kieselgel (Merck) als Adsorbens beschichtet und 24 Std. stehen lassen. Vor den Versuchen wurden die Platten 1 Std. bei

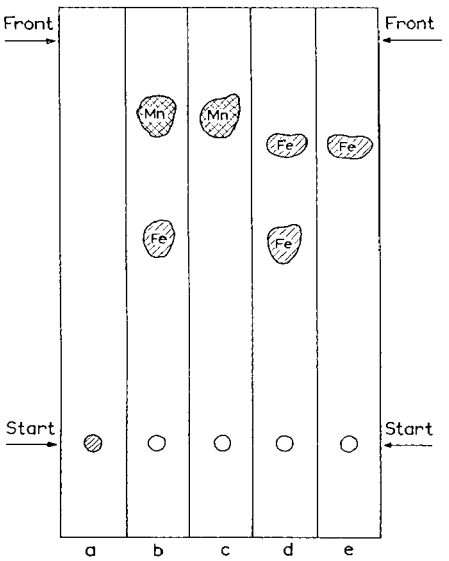


Fig. 1. Nachweis von freien Ionen in ^{54}Mn- und ^{59}Fe-markierter Naphthensäure und Spindelöl. a = Wässerige ^{54}Mn- und ^{59}Fe-Lösung; b = ^{54}Mn– und ^{59}Fe–Naphthensäure, R_F-Werte 0.92 und 0.75; c = ^{59}Fe–Naphthensäure, R_F-Wert 0.75; d = ^{54}Mn–Naphthensäure, R_F-Wert 0.92; e = ^{59}Fe–Spindelöl, R_F-Wert 0.83.

100° getrocknet. Die fertigen Gelschichten wiesen eine Dicke von 1 mm auf. Nach Einritzen in 2 cm breite Streifen wurden auf jedem, verschiedene Proben in Mengen von 1–2 μl auf die am unteren Ende befindliche Startlinie aufgetropft. Die beladenen Gele wurden anschliessend in eine Mischung von Petroläther–Diäthyläther–Eisessig (80:20:1) gestellt und das Fliessmittel in einem geschlossenen Gefäss 1 Std. lang einwirken lassen. Nach Beendigung des Versuches und Verdunsten des Lösungsmittels, wurden die Eisenverbindungen mit einer frisch zubereiteten 2%igen wässerigen Kaliumferrocyanid Lösung sichtbar gemacht. Mangan konnte dagegen durch Besprühen der Platten mit einer 1%igen Lösung von Diphenylcarbacid in Äthanol lokalisiert werden.

Fig. 1 zeigt die bei der Auftrennung von markierten Naphthenaten bzw. markierten Spindelöl erhaltenen Ergebnisse. Als sehr wesentlicher Befund ergab sich, dass die für die Markierung verwendeten ionenhältigen Radionuklidlösungen unter den angegebenen Bedingungen am Startfleck verblieben. Lagen die Radionuklide jedoch in organischer Bindung vor, wanderten sie mit dem Lösungsmittelgemisch und es zeigten sich in den erhaltenen R_F-Werten deutliche Unterschiede für ^{59}Fe und ^{54}Mn.

Unter den gleichen Bedingungen wurde auch mit ^{59}Fe und ^{54}Mn markierter Ölsäure, Rüböl und Triolein chromatographiert und entwickelt. Wie Fig. 2 beweist ergaben sich auch hier dieselben Laufdifferenzen. Fe^{3+} und Mn^{2+} Ionen wanderten

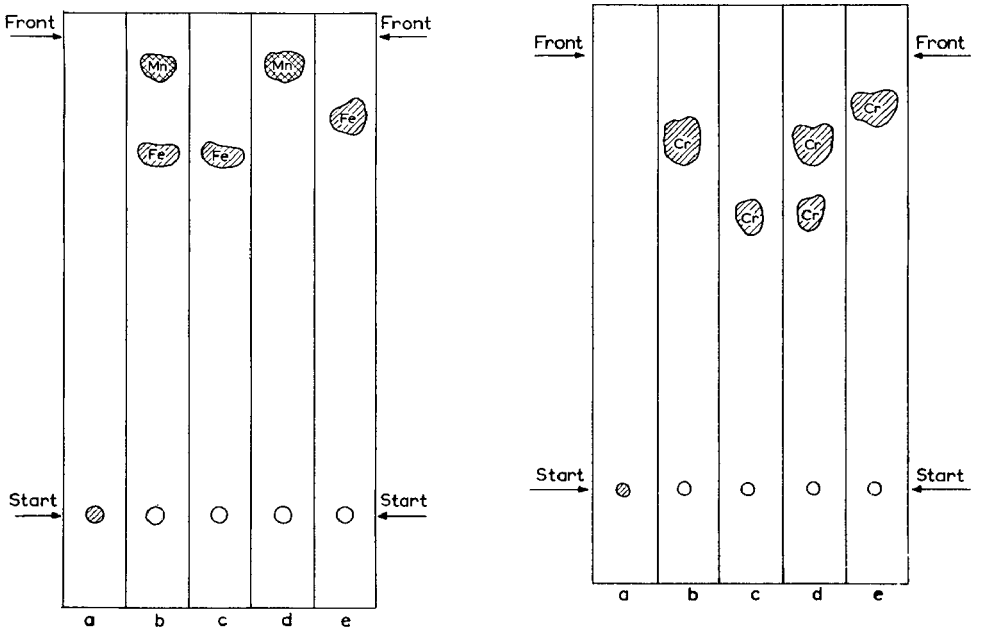


Fig. 2. Nachweis von freien Ionen in ^{54}Mn- und ^{59}Fe-markierter Ölsäure, Rüböl und Triolein. a = Wässerige ^{54}Mn- und ^{59}Fe-Lösung; b = ^{54}Mn- und ^{59}Fe-Ölsäure, R_F-Werte 0.81 und 0.42; c = ^{54}Mn-Ölsäure, R_F-Wert 0.80; d = ^{59}Fe-Rüböl, R_F-Werte 0.74 und 0.46; e = ^{54}Mn- und ^{59}Fe-Triolein, R_F-Werte 0.91 und 0.74.

Fig. 3. Nachweis von freien Ionen in ^{51}Cr-markiertem Triolein, Ölsäure, Rüböl und Naphthensäure. a = Wasserige ^{51}Cr-Lösung; b = ^{51}Cr–Triolein, R_F-Wert 0.80; c = ^{51}Cr–Ölsäure, R_F-Wert 0.63; d = ^{51}Cr–Rüböl, R_F-Werte 0.80 und 0.63; e = ^{51}Cr–Naphthensäure. R_F-Wert 0.88.

TABELLE I

TRENNUNG VON MARKIERTEN ÖLEN BZW. ÖLKOMPONENTEN DURCH PAPIERCHROMATOGRAPHIE

Papiersorte: Whatman Nr. 3MM. Laufzeit: 50 min. Fliessmittel: (I) Petroläther–Diäthyläther–Eisessigsäure (80:20:1); (II) Petroläther–Diäthyläther–Eisessigsäure (70:30:2); (III) Chloroform–Benzol (2:1); (IV) Diäthyläther–Petroläther–Essigsäureäthylester (1:1:1); (V) Benzol–Äthanol abs. (9:1); (VI) Tetrachlorkohlenstoff–Benzol–Äthanol abs. (16:8:1).

Markierte Substanz	R_F-*Werte*																	
	I			*II*			*III*			*IV*			*V*			*VI*		
	^{59}Fe	^{51}Cr	^{65}Zn	^{59}Fe	^{51}Cr	^{65}Zn	^{59}Fe	^{51}Cr	^{65}Zn	^{59}Fe	^{51}Cr	^{65}Zn	^{59}Fe	^{51}Cr	^{65}Zn	^{59}Fe	^{51}Cr	^{65}Zn
Naphthensäure	—	0.82	—	—	0.86	—	0.89	0.96	0.93	0.89	0.91	0.92	0.91	0.92	0.92	0.94	0.88	0.90
Ölsäure	—	0.78	—	—	0.87	—	0.85	0.88	0.83	0.93	0.87	—	0.92	0.92	0.89	0.83	0.79	0.83
Rüböl	—	0.58	—	—	0.73	—	—	0.64	—	—	0.88	—	0.82	—	—	0.81	—	—
Triolein	—	—	—	—	—	—	—	—		—	0.85	—	—	—	—	—	—	—

nicht, dagegen wurden für organisch gebundenes ^{59}Fe und ^{54}Mn deutliche Differenzen in den Wanderungsstrecken erhalten.

Dasselbe Laufmittel wurde auch zur Untersuchung von ^{51}Cr-markierten Triolein, Ölsäure, Rüböl und Naphthensäure benützt. Gleichzeitig wurde auch die für die Markierung verwendete Chrom(III)chlorid-Lösung mit in die Versuchsreihe aufgenommen. Es wurde ebenfalls eine Wanderung des organisch gebundenen ^{51}Cr beobachtet, nachdem Chrom duch Besprühen mit 0.1% von Auritricarbonsäurelösung und nach Entwicklung mit einer 25%igen Ammoniaklösung sichtbar gemacht worden war (Fig. 3). Wie im Falle von ^{59}Fe-markierten Rüböl (Fig. 2) konnten auch hier zwei Flecke von ^{51}Cr festgestellt werden. Diese Tatsache deutet auf die gleichzeitige Markierung von zwei in Rüböl vorhandene Substanzen hin. Gegenüber den mit ^{59}Fe und ^{54}Mn markierten Ölen, wanderten solche die ^{51}Cr enthielten langsamer, und gute Trennungen wurden erst nach 80 Min. Laufzeit erhalten.

Papierchromatographische Untersuchungen

Da Öle und Ölsäure bereits durch papierchromatographische Verfahren[8–11] analysiert wurden, haben wir auch diese Methode geprüft. Die Auffindung eines geeigneten Laufmittels war jedoch schwierig, da sich nur wenige Arbeiten mit einer ähnlichen Problemstellung beschäftigt hatten und daher wenig Hinweise vorhanden waren. So verwendeten RUSSIN *et al.*[12] eine Mischung von Butanol–Dioxan–Ammoniak, 2 *N* (4:1:1.5) zum Nachweis von freien ^{131}J in jodierten Ölen, während ANGHILERI[13] für qualitative Bestimmung von $Na^{131}J$ in markierter Ölsäure und Triolein, mit Kupferhydroxyd imprägniertes Whatman-Papier empfehlen. Das Jodid verblieb auf der Startstelle zurück während deutliche Unterschiede zwischen den schnell laufenden

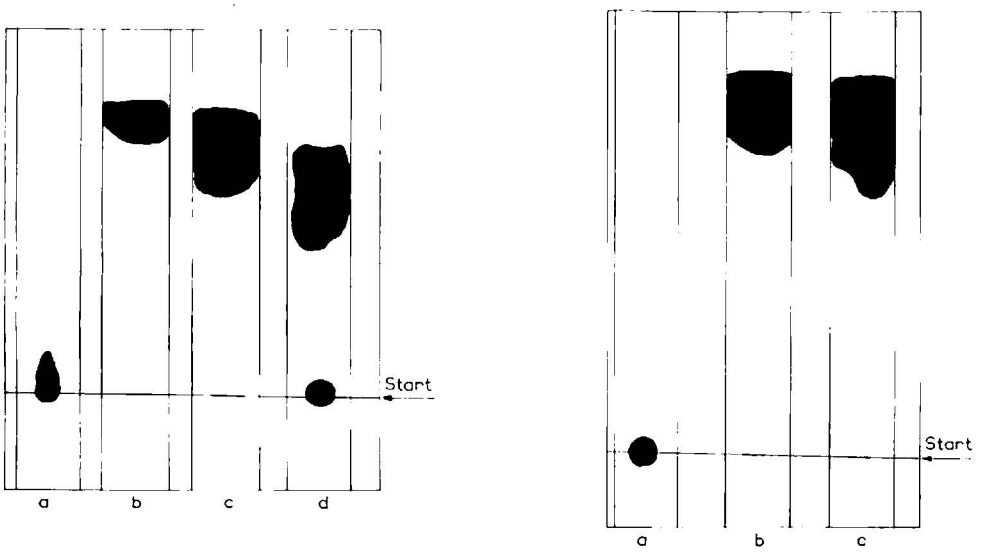


Fig. 4. Nachweis von freien Ionen in ^{59}Fe-markierter Naphthensäure, Ölsäure und Rüböl. a – Wässerige ^{59}Fe-Lösung; b – ^{59}Fe-Naphthensäure; c – ^{59}Fe–Ölsäure; d – ^{59}Fe-Rüböl.

Fig. 5. Nachweis von freien Ionen in ^{65}Zn-markierter Naphthensäure und Ölsäure. a – Wässerige ^{65}Zn-Lösung; b – ^{65}Zn-Naphthensäure; c – ^{65}Zn–Ölsäure.

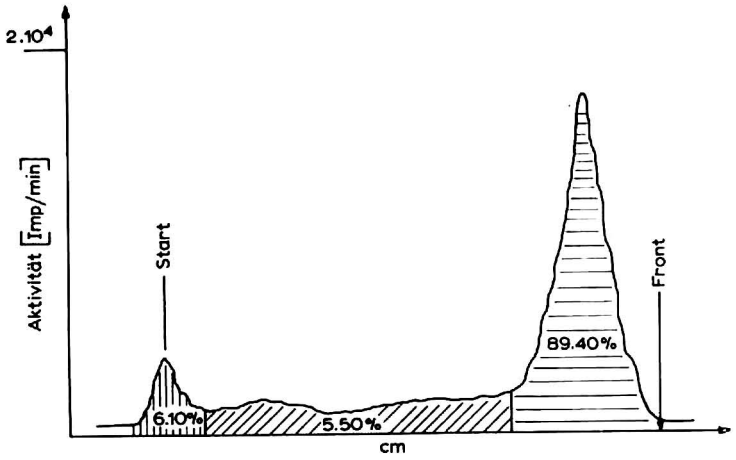


Fig. 6. Scanning diagramm einer mit ^{65}Zn markierten Naphthensäure.

Triolein und der langsam wandernden Ölsäure zu beobachten waren. Wir verwendeten dagegen das sich schon bei der Dünnschichtchromatographie bewährte Gemisch von Petroläther, Diäthyläther und Eisessigsäure. Die Papierstreifen wurden nach Aufbringung von ^{51}Cr-, ^{59}Fe- bzw. ^{65}Zn-markierten Ölen aufsteigend 50 Min. lang chromatographiert.

Als weitere Lösungsmittelgemische wurden noch Chloroform–Benzol (2:1), Benzol–Äthanol (9:1), Diäthyläther–Petroläther–Essigsäureäthylester (1:1:1), sowie Tetrachlorkohlenstoff–Benzol–Äthanol (16:8:1) verwendet. Die Ergebnisse sind in Tabelle I zusammengefasst. Bei allen Mischungen traten deutliche Schleierbildungen auf (Fig. 4 und 5).

Zur quantitativen Erfassung der Verteilung wurden die Streifen daher millimeterweise abgetastet. Ein Scanning-Diagramm ist in Fig. 6 zu sehen und zeigt, dass sich am Start 6.1%, im Mittelteil 5.5% und im eigentlichen Peak 89.4% der Aktivität befanden. Die Ergebnisse weiterer Versuche sind ebenfalls in Tabelle II zusammengestellt. Es wird noch versucht die Zusammensetzung und Bindung der am Start und im Mittelfeld verbliebenen Aktivität aufzuklären.

TABELLE II

AKTIVITÄTSVERTEILUNG IN DEN PAPIERSTREIFEN NACH CHROMATOGRAPHIE

Markierte Substanz	*Aktivität (%) am*		
	Start	*Zwischenteil*	*Front*
^{65}Zn–Naphthensäure	6.10	5.50	89.40
^{59}Fe–Naphthensäure	1.87	5.12	93.01
^{51}Cr–Naphthensäure	3.45	8.20	82.10
^{65}Zn–Ölsäure	3.05	4.10	92.85
^{59}Fe–Ölsäure	5.50	10.30	84.20
^{51}Cr–Ölsäure	1.20	4.65	94.15
^{59}Fe–Rüböl	9.40	8.50	82.10

Elektrophoretische Untersuchungen

Wie bereits Versuche mit jodierter Ölsäure oder Ölen gezeigt hatten[11,12,14] ist es mit Hilfe der Papierelektrophorese möglich den umgekehrten Effekt zu erzielen, d.h. die freien Ionen wandern zu lassen, während organisch gebundene Radionuklide am Startpunkt verbleiben. Wegen der bestehenden Analogie zu ^{131}J-hältigen Ölen haben wir daher die von uns mit ^{82}Br-markierten Öle ebenfalls mit dieser Methode untersucht.

Es wurden 2 cm breite und 30 cm lange Whatmann Nr. 3MM Papierstreifen verwendet. Die Aufbringung der Proben erfolgte aber hier im Gegensatz zu der Papierchromatographie nicht am Ende sondern in der Mitte. Nach dem Anfeuchten der Streifen mit Veronalpufferlösung wurde eine Spannung von 180 V angelegt. Die Stromstärke betrug durchschnittlich 1.6 mA und die Versuchsdauer 90 Min.

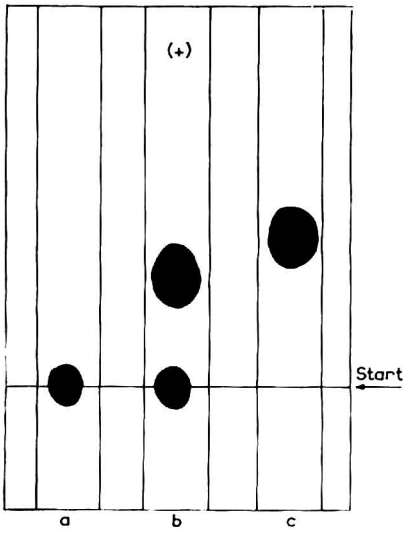


Fig. 7. Nachweis von freien Ionen in ^{82}Br-markiertem Olivenöl. a = ^{82}Br–Olivenöl allein; b = $NH_4{}^{82}$Br-Lösung und ^{82}Br-Olivenöl; c = $NH_4{}^{82}$Br-Lösung.

Nach Trocknen der Papierstreifen wurde von jedem eine Autoradiographie angefertigt. Fig. 7 zeigt das Ergebnis einer derartigen Untersuchung von ^{82}Br-markiertem Olivenöl. Auf die Startlinie des Streifens "a" wurde nur reines markiertes Olivenöl, auf Streifen "c" $NH_4{}^{82}$Br-Lösung und auf Streifen "b" ein Gemisch der beiden aufgebracht. Das Autoradiogramm lässt daher den Schluss zu, dass das markierte Olivenöl keine freien ^{82}Br-Ionen enthielt.

DISKUSSION

Mit Hilfe der Dünnschichtchromatographie unter Verwendung einer Mischung von Petroläther, Diäthyläther und Eisessig konnten freie Ionen deutlich von ^{51}Cr-, ^{54}Mn-, ^{59}Fe-, bzw. ^{65}Zn-markierten Pflanzen- oder Mineralölen unterschieden werden, da die wässerigen Radionuklidlösungen in diesem Milieu nicht wanderten.

Die Dünnschichtchromatographie erlaubte in einigen Fällen noch weitere Aus-

sagen. So deutete die Auffindung von zwei ^{59}Fe-Flecken mit verschiedenen R_F-Werten bei Rüböl darauf hin, dass zwei verschiedene Verbindungen mit ^{59}Fe markiert wurden. Ein Vergleich der Autoradiographien der Platten von markierten Rüböl und Triolein ergab auf Grund der Übereinstimmung der Wanderungswege, dass das im Rüböl vorhandene Triolein ebenfalls mit ^{59}Fe markiert wurde. Genau dieselbe Übereinstimmung konnte auch bei ^{51}Cr-markiertem Rüböl bzw. Ölsäure und Triolein gefunden werden.

Bei Doppelmarkierungen können die einzelnen Radionuklide ebenfalls durch Dünnschichtchromatographie aufgetrennt werden. So wurden beispielsweise bei ^{54}Mn- und ^{59}Fe-hältiger Öl- oder Naphthensäure für beide Radionuklidverbindungen verschiedene R_F-Werte erhalten.

Obwohl die Dünnschichtchromatographie sehr gute Ergebnisse zeigte, wurde versucht die teuren Gelplatten durch die wesentlich billigeren Papierstreifen zu ersetzen. Bei Verwendung der gleichen Laufmittelmischung trat jedoch starke Schleier- und Schwanzbildung auf (Fig. 4 und 5). Diese konnte auch nicht durch die Wahl anderer Lösungsmittel (Tabelle II) unterdrückt werden. Die Autoradiographien der Papierchromatogramme zeigten jedoch einwandfrei, dass die markierten Verbindungen mit der Lösungsmittelfront wanderten.

Mit ^{82}Br-markierten Mineral- und Pflanzenölen wurden auf Grund der von uns bei ^{131}J-markierten Öle gemachten guten Erfahrungen mit Hilfe der Papierelektrophorese auf freie Radionuklid-Ionen untersucht. Die Autoradiographien—als Beispiel sei Fig. 7 genannt—bewiesen, dass organisch gebundenes ^{82}Br am Startfleck verblieb, während freie Ionen im elektrischen Feld längere Strecken wanderten und so deutlich identifiziert werden konnten.

DANK

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ZUSAMMENFASSUNG

Zum Nachweis von freien Radionukliden in markierten Mineral- und Pflanzenölen sowie einigen ihrer Komponenten wurde Dünnschichtchromatographie, Papierchromatographie und Papierelektrophorese herangezogen. Nach den bisher vorliegenden Ergebnissen war für die mit ^{51}Cr, ^{59}Fe, ^{54}Mn und ^{65}Zn markierten Öle die Dünnschichtchromatographie als Untersuchungsmethode besonders geeignet, während mit ^{82}Br markierte, organische Verbindungen mit Vorteil durch Papierelektrophorese analysiert werden konnten.

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Notes

CHROM. 4772

A simple method for the calculation of the minimum number of theoretical plates required for a chromatographic separation

In earlier publications[1–4] the planning of chromatographic separations of inorganic and organic substances on ion-exchange columns has been reported. Using complex forming agents in the eluent, the most convenient pH and concentration of the solution were calculated by means of equations, which formulated the relation between the distribution coefficient of the substance and the pH of the eluent at a given concentration.

Now an attempt has been made to provide a simple formula for calculating the minimum value of the number of theoretical plates necessary to obtain a clear separation of two components of similar behaviour.

The following symbols are used:

a = void fraction of the column;
B, A = substances to be separated;
$c_{max,B}$ = the maximum concentration of component B in the effluent;
D_B = volume distribution coefficient of substance B;
m_B = total amount of substance B (mmole);
N = number of theoretical plates;
v_B = the base line of the triangle in the chromatogram (ml) (see Fig. 1);
$v_{max,B}$ = total eluent volume (ml) (see Fig. 1);
X = total volume of the column (ml).

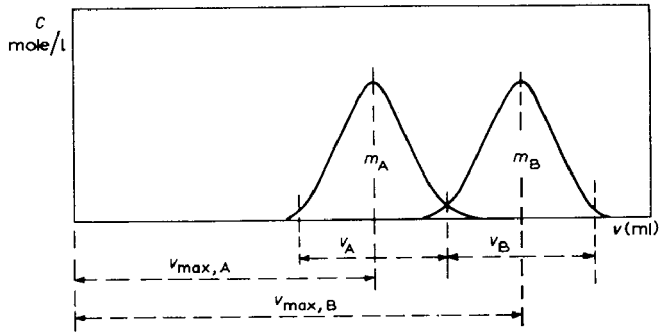


Fig. 1. Elution diagram of two components of similar behaviour.

Let us consider first an ideal chromatogram of two components (see Fig. 1). To obtain a clear separation of the two substances, the distance between the two peaks must be greater than the sum of the two half base lines. Thus,

$$\left(\frac{v_{max,B}}{v_{max,A}} - 1\right) v_{max,A} \geq \tfrac{1}{2} v_A + \tfrac{1}{2} v_B \qquad (1)$$

Using the fundamental equation deduced by GLUECKAUF[5]

$$c_{max} = \frac{m}{v_{max}} \sqrt{\frac{N}{2\pi}} \tag{2}$$

considering that

$$m_B \approx \tfrac{1}{2}\, v_B \cdot c_{max,B} \tag{3a}$$
$$m_A \approx \tfrac{1}{2}\, v_A \cdot c_{max,A} \tag{3b}$$

we obtain for v_B

$$v_B \approx 2 \cdot v_{max,B} \sqrt{\frac{2\pi}{N}} \tag{4}$$

and a very similar equation for v_A.

Since v_{max} can be expressed in terms of the distribution coefficient and column data

$$v_{max} = X\,(D + a) \tag{5}$$

eqn. 1 can be transformed:

$$\frac{v_{max,B}}{v_{max,A}} - 1 \geq \frac{\tfrac{1}{2}\,(v_A + v_B)}{v_{max,A}}$$

$$\frac{D_B + a}{D_A + a} - 1 \geq \frac{(D_A + a) + (D_B + a)}{(D_A + a)} \sqrt{\frac{2\pi}{N}}$$

$$N \geq 2\pi \left[\frac{\dfrac{D_B + a}{D_A + a} + 1}{\dfrac{D_B + a}{D_A + a} - 1} \right]^2 \tag{6}$$

If the distribution coefficients of the two substances to be separated are known, the number of theoretical plates necessary can be calculated. Eqn. 6 differs fundamentally from equations of similar form recommended in the literature[6,7] for calculation, since the ratio of the $(D + a)$ values corresponding to the ratio of the real eluent volumes (v_{max}) and not the ratio of the distribution coefficients (*i.e.* separation factor) corresponding to the ratio of net eluent volumes is used.

Since in liquid chromatographic separations the first substance is always eluted with an eluent volume comparable with the dead volume of the column, in calculations the latter may not be neglected.

Another advantage of eqn. 6 is that the ratio and not the difference of the terms is used, the latter being more common[8]; the ratio is more characteristic for the expression of the effectiveness of separations.

The values obtained at various $(D_B + a)/(D_A + a)$ ratios were calculated and compared with the data of the diagram of GLUECKAUF[5], which is based on rigorous mathematical treatment of the overlapping ratio of the two curves. The values calculated by eqn. 6 correspond to separations with an impurity of less than 0.3%, if the ratio of the amount of the two components is 1:1. Since the real form of the curves always differs from the ideal one, and therefore the calculations are in any way

only approximative, eqn. 6 may be proposed for informatory calculations in the planning of liquid chromatographic separations.

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CHROM. 4782

Imbrication patterns

WALRAVEN, LADON and KEULEMANS have recently described[1–3] imbrication plots or roofing tile patterns as an aid in the tentative identification of organic compounds. Isomers of homologous compounds were shown to form staggered parallel plots depending on the carbon number when examined on two stationary phases. It is shown that the series of parallel linear relationships representing isomeric compounds that are produced when logarithmic expressions of retention on two stationary phases are plotted according to carbon number are simply a particular extension of established homologous plots. The overlapping plots are parallel only when the structural parameters of the isomers are non-interacting or where interactions are present these are independent of the structures of the variable portions of the isomers. An example of the plots is shown in Fig. 1a, using data of methyl esters of monocarboxylic acids[4].

Linear relationships of homologous compounds on two stationary phases are well known. JAMES[5] with aliphatic amines plotted relative retention on a polar and a non-polar stationary phase to produce linear plots radiating from the origin for each homologous series. With hydrocarbons a series of parallel lines were produced by JAMES AND MARTIN[6] using the logarithm of retention obtained on two phases. Concurrently primary, secondary and tertiary alkanols, alkanones and *n*-alkanes were shown to produce parallel linear plots using two polar phases[7]. Similar results have been achieved with fatty esters by the use of a "new" and an "aged" polyester stationary phase[8]. Differences in behaviour of these esters have also been observed by the use of a single polar column using two temperatures[9] while plots of isomeric alkanes C_5–C_9 have been reported using squalane at 30° and 70° (ref. 10).

This work shows the mathematical basis of parallel plots of isomeric homologous series on two stationary phases and of the parallel (imbrication) plots linking the isomeric compounds of the same carbon number.

Discussion

Fig. 1 shows a typical example of an imbrication pattern with parallel plots of someric esters of the same carbon number. The dotted lines on the figure show parallel plots of the methyl esters of (a) *n*-carboxylic acids and (b) 2,2-dimethylcarboxylic acids. The other points shown similarly produce linear plots of the methyl esters of the 2-, 3- and 4-methylcarboxylic acids and of the 3-methyl-3-ethyl- and 4-methyl-4-propylcarboxylic acids.

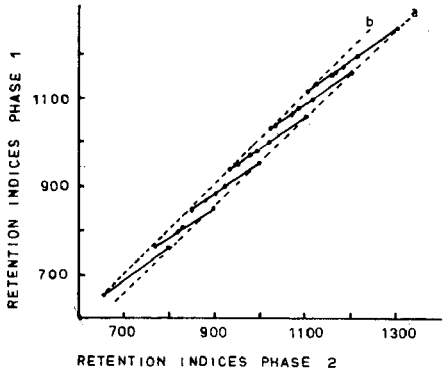


Fig. 1. Retention plots of the methyl esters of homologous monocarboxylic acids on a polar and a non-polar stationary phase. Solid lines show parallel plots of esters of the same chain length and dotted lines of esters of (a) *n*-carboxylic acids and (b) 2,2-dimethylcarboxylic acids.

It is shown mathematically that homologous series which produce parallel plots of slope m_1 with any logarithmic representation of retention and carbon number on one stationary phase and similar parallel plots of slope m_2 on a second phase will produce further parallel plots of slope m_3 when retention values on both phases form the co-ordinates.

If the separation factors for several homologous series are constant on two phases with values x_1 and x_2 then multiple retention representation will again produce parallel plots.

Simple retention plots (*i.e.* log retention *versus* carbon number) of a homologous series on two stationary phases (1) and (2) may be shown as

$$\text{Log } r_1 = m_1 C + a_1 \quad (1)$$

$$\text{Log } r_2 = m_2 C + a_2 \quad (2)$$

$$\text{Log } r_1 - \log r_2 = (a_1 - a_2) + C(m_1 - m_2) \quad (3)$$

From eqn. 2 follows:

$$C = \frac{1}{m_2} \log r_2 - \frac{a_2}{m_2}$$

Eqn. 3 now becomes:

$$\text{Log } r_1 = \log r_2 + (a_1 - a_2) + (m_1 - m_2)\frac{1}{m_2}\left(\log r_2 - \frac{a_2}{m_2}\right) \quad (4)$$

$$\text{Log } r_1 = a_1 - a_2\frac{m_1}{m_2} + \log r_2\left(1 + \frac{m_1 - m_2}{m_2}\right) \quad (5)$$

$$\text{Log } r_1 = \frac{a_1 m_2 - a_2 m_1}{m_2} + \frac{m_1}{m_2}(\log r_2) \quad (6)$$

As eqn. 6 is of the form for a linear plot the multiple retention plots of the homologous series are parallel with slope m_1/m_2.

It has been shown with the α-alkyl acrylic esters[11] that a network of plots is produced with a linear relationship existing between isomers of the same total carbon number when a variable structural parameter and the logarithm of retention form the co-ordinates. While one series of slopes has been calculated the second series may be similarly obtained. This situation is analogous to that described by eqns. 1 and 2 although a normal slope linking the isomers does not occur and the linear parallel nature of the imbrication pattern may be shown by obtaining slopes on pairs of points:

$$\text{Log } r_{11} = a_{11} + m_1 C \ldots \log r_{21} = a_{21} + m_2 C$$
$$\text{Log } r_{12} = a_{12} + m_1 C \ldots \log r_{22} = a_{22} + m_2 C$$
$$\text{Log } r_{13} = a_{13} + m_1 C \ldots \log r_{23} = a_{23} + m_2 C$$

The slope of a line between two isomer points is obtained from the equation above.

$$\frac{\text{Log } r_{11} - \log r_{12}}{\text{Log } r_{21} - \log r_{22}} = \frac{a_{11} - a_{12}}{a_{21} - a_{22}}$$

The slope is independent of the carbon number, constant for the various sets of points and two series of parallel lines exist.

While WALRAVEN *et al.*[2,3] have reported parallel plots for isomers of carboxylic esters, hydrocarbons and phenyl and cycloalkanes, many series of isomers do not show this linearity. The linearity exists where the structural parameters of the isomers are non-interacting or, if interactions occur, these are independent of the structurally variable portion of the isomers. In such series retention is simply an additive effective or a summation of contributions due to component parts of the molecule and ac-

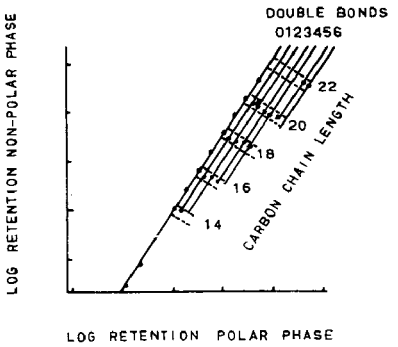


Fig. 2. Retention plots of saturated and unsaturated fatty esters on a polar and a non-polar stationary phase[12].

cordingly retention of near homologues may be calculated with considerable precision.

Imbrication plots were in effect proposed by JAMES[12] as shown in Fig. 2 for determining the number of double bonds in unsaturated fatty esters. Linear plots were not achieved as the positions of unsaturation are not equivalent; however, the grouping of the points into zones was of some value.

Recognition of the end carbon chain relationship[13] allows a network plot of unsaturated fatty esters[14] but the relationships are restricted to isomers where unsaturation is isolated from the chain extremities. This limitation applies to the variety of mathematical procedures that have been developed as aids in the tentative identification of unsaturated fatty esters[15–17]. Retention data available for complete series of fatty compounds have been listed[14] and the inequality of retention contributions due to the position of unsaturation or chain substituents is widely apparent. In such cases linear imbrication plots are not obtained.

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Alkali metal fluorides as column packing for gas–solid chromatography

Several publications have recently appeared on the use of simple inorganic alkali metal salts as solid adsorbents in gas–solid chromatography (GSC). One of these, a comprehensive report on the application of alkali metal chlorides and nitrates in GSC[1], encompassed the chromatography of a rather wide range of aliphatic and aromatic compounds, including some with polar hydroxyl and amine substituents. The authors concluded that "physical adsorption", not chemisorption, predominated in the solute–adsorbent interaction. Little stress was placed on the analytical applications of these separations because of the relatively small elution times exhibited by most of the solutes.

In contrast, the use of alkali metal fluorides as a solid adsorbent has not heretofore received attention. This is somewhat surprising, as the formation of adducts between alkali metal fluorides and non-metallic oxides and oxyfluorides has been known for several years. Examples illustrative of such adduct formation are the reactions of SO_2 (ref. 2), $F_2C{=}O$ (ref. 3) $(CF_3)_2C{=}O$ (ref. 3) and SOF_4 (ref. 4) with either potassium or cesium fluorides.

$KF + SO_2 \rightleftarrows Cs^+[SO_2F]^-$

$CsF + F_2C{=}O \rightleftarrows Cs^+[OCF_3]^-$

$CsF + (CF_3)_2C{=}O \rightleftarrows Cs^+[OCF(CF_3)_2]^-$

$CsF + F_4SO \rightleftarrows Cs^+[OSF_5]^-$

For a specific nonmetallic oxide or oxyfluoride the relative ratio of dissociation, *in vacuo*, at any specified temperature, is inversely proportional to the size of the alkali metal cation[3,5].

REDWOOD AND WILLIS have taken this as direct corroboration of the formation of an oxyfluoride anion in the adduct as opposed to the coordination complex, $[Cs \leftarrow OCF_2]^+F^-$. For a series of totally fluorinated alkali metal alkoxides ($M^+[OCF_3]^-$, $M^+[OCF_2CF_3]^-$ and $M^+[OCF(CF_3)_2]^-$), these authors have also shown that the extent of dissociation (*i.e.* $M^+[\overset{/}{\underset{\backslash}{OCF}}]^- \rightarrow MF + O{=}\overset{/}{\underset{\backslash}{C}}$), at a specified temperature increases with increasing bulk of the nonmetallic oxyfluoride[6]. All the adducts studied by REDWOOD AND WILLIS are characterized by a rapid and reversible equilibrium with the dissociation products.

Considering these properties of alkali metal fluorides and their adducts, it seemed likely that the metal fluorides could have utility in GSC separation of gaseous mixtures containing certain metal oxides and oxyfluorides. In order to test this utility, the present investigation was conducted. The study was, however, limited to those salts (KF, RbF, CsF) which readily form adducts with F_2CO and similar compounds.

Experimental

The gas chromatographic experiments were performed using a Model 5000 Barber-Colman gas chromatograph fitted with a linear six-port manual gas sampling valve.

Pure CaF_2 (Harshaw Chemical Company) was ground to 60–80 mesh and ignited at 700° to remove all traces of organic impurities. High purity (99.9%) CsF (Ozark-

Mahoning Co.) was screened in a dry box and the 120–200 mesh fraction retained.

Sufficient CsF was added to the CaF_2 to make a 10% by weight mixture of CsF in CaF_2*. The mixture was then heated for 18 h at 250° and transferred under dry conditions to a 12-ft. × ¼-in. O.D. copper tube. After the column was attached to the chromatograph it was further conditioned by heating at 250° for 18 h while purging with helium (22 cc/min) was effected.

An equimolar mixture of $F_2C{=}O$, CF_3CFO, and $(CF_3)_2C{=}O$ was prepared and combined with $CF_2{=}CF_2$ in the approximate volume ratio of 1:1:1:20. A representative chromatogram of this sample mixture obtained with a 1000 μl vapor sample and a delayed temperature program (30° for 5 min followed by 25°/min program up to 250°) is shown in Fig. 1. All four components in the sample mixture are clearly separated.

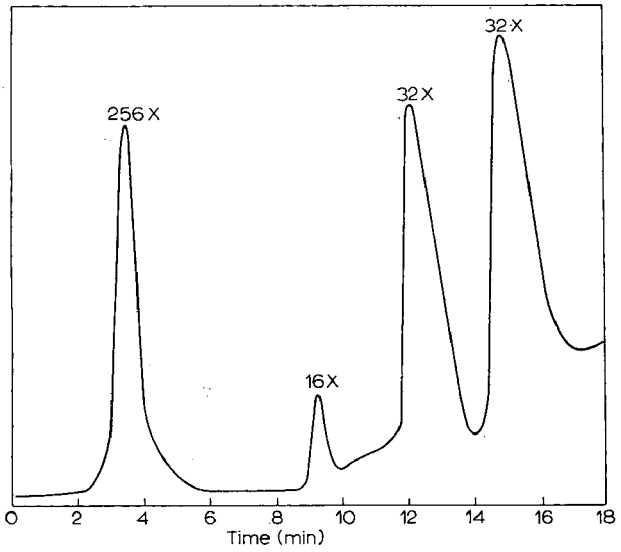


Fig. 1. Separation of $F_2C{=}O$, CF_3CFO and $(CF_3)_2C{=}O$. Conditions: 12 ft. × ¼ in. O.D. 10% CsF–90% CaF_2 column; He 20 ml/min; isothermal 30° for 5 min, programmed 25°/min to 250°. Elution order: $CF_2{=}CF_2$ 3.4 min; $(CF_3)_2C{=}O$ 9.2 min; CF_3CFO 12.0 min; and $F_2C{=}O$ 14.8 min.

Results and discussion

Tetrafluoroethylene is not chemisorbed and elutes rapidly. The order of elution, Fig. 1, for the three oxyfluoro-compounds is the inverse of the order of their boiling points ($F_2C{=}O$, −83°; CF_3CFO, −59°; $(CF_3)_2C{=}O$, −28°). However, their relative retention does parallel the decomposition rates determined by REDWOOD AND WILLIS[6]. For these selected compounds a reversible chemisorption process is therefore the predominant factor in achieving a chromatographic separation.

Separations other than those described can be visualized. Alkali metal fluorides discriminate, via adduct formation, between many nonmetallic oxyfluorides and their hydrolysis products. For example, CsF has been used in the laboratory scale sepa-

* The CaF_2 here serves only as a support for the alkali metal fluorides. Unlike the latter, CaF_2 and other Group II fluorides show no tendency to form complexes with the oxides and oxyfluorides under consideration[7].

rations[7] of SF_4 from SOF_2, COF_2 from CO_2, and SOF_4 from SO_2F_2. In all cases the alkali metal salt forms an adduct with the more highly fluorinated parent compound and not with its hydrolysis product, thus permitting a simple purification of the parent oxyfluoride.

Use of an alkali metal fluoride column in GSC is limited by two common impurities, H_2O and HF. Because of the deliquescent nature of the metal fluorides care must be taken to minimize the adsorption of atmospheric water vapor during the preparation and packing of the column. Adsorption of moisture by the fluoride results in a marked decrease in the surface area and consequently in the number of active acceptor sites.

Hydrogen fluoride also readily interacts with the alkali metal fluorides to form the very stable bifluorides, MHF_2.

Regeneration of an HF contaminated column has not been generally successful. As some HF cannot generally be avoided in the majority of samples containing nonmetallic oxyfluorides, the only recourse is a periodic column replacement.

Although, as we have noted, there are some difficulties involved in the use of alkali metal fluoride columns in GSC, the novel separation which can be achieved warrants their consideration in any attempt to separate nonmetallic oxyfluoride derivatives.

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Nickel complexes as adsorbents in gas–solid chromatography

The first attempt at modifying stationary phases of gas chromatographic columns with an inorganic electrolyte was by BRADFORD[1] and consisted of silver nitrate dispersed in glycol. Since this time, a number of papers concerned with using inorganic salts and salt-modified stationary phases has appeared[2–5]. PHILLIPS *et al.*[6] reported on the use of N-dodecyl salicylaldimines of Ni, Pd, Pt, and Cu and the methyl-*n*-octyl glyoximes of Ni, Pd, and Pt as stationary phases. They found that retardation

factors for paraffins and olefins were indicative of interactions between the metal complexes and aromatic hydrocarbons. The interaction between primary and secondary amines with the salicylaldimine complexes increased in the order Pt, Pd, Ni, while the order of interaction is reversed in the glyoxime complexes. *n*-Nonyl-*β*-diketone complexes of Be, Al, Ni, and Zn were used as liquid phases by CARTONI *et al.*[7]. Relative retention volumes of various compounds on metal complex columns *versus* the relative retention volume on a squalane column yielded straight lines passing through the origin. The values of alcohols on the metal complex columns increased from tertiary to primary alcohols. PECSOK AND VARY[8] employed transition metal–phthalocyanine complexes in silicon oil as the stationary phase, and attributed variations in retention volume to interactions between the measured compound and the metal ion. More recently, ILIE[9] reported on the use of transition metals with 1,10-phenanthroline and 2,2'-bipyridine as stationary phases. It was noted both by PECSOK AND VARY[8] and ILIE[9] that the interaction of oxygen-containing compounds on these complexes decreased in the order Fe > Ni > Co > Zn > Cu.

No one has compared the environment surrounding any one transition metal ion. The glyoximes and salicylaldimines offer an ideal case as the substituted groups on either ligand can be easily varied.

In this study we have prepared several sets of complexes utilizing nickel as the transition metal ion, and show the dependence of retention volume of various organic liquids upon the environment surrounding it.

Experimental

Materials. Columns using bis(dimethylglyoximato)nickel(II), bis(1,2-cyclohexanedionedioximato)nickel(II), bis(salicylaldimino)nickel(II), and nickel perchlorate as stationary phases were prepared, and are referred to as Ni-DMG, Ni-CHD, Ni-SAL, and $Ni(ClO_4)_2$, respectively. After the columns were prepared the per cent composition was determined by dissolving the complex and found to be of the following composition: Ni-DMG, 4.5%, 8.2%, and 26.8%; Ni-CHD, 12.7% and 27.7%; Ni-SAL, 3.8%, 12.6%, and 25.7%. The nickel perchlorate column was 26.0%. A column using only the solid support was used as a reference. The solid support, used as received, was Gas-Chrom P, 100–120 mesh, acid/base washed, and was obtained from Applied Science Laboratories.

All other chemicals were of reagent grade quality and were used as received. The chemicals used for investigation gave only one peak when chromatographed and were assumed pure enough for use.

Apparatus. A Perkin-Elmer Model 154 gas chromatograph fractometer modified with a thermostatically controlled oven (± 2.0°) and a thermister detector was used with a Leeds and Northrup Speedomax H recorder for retention volume measurements. All measurements were made isothermally at 50°, 85°, and 125°. Thermogravimetric data were obtained on a Dupont 950 thermogravimetric analyzer using a nitrogen atmosphere and a heating rate of 20°/min.

Copper tubing, 6 ft. × 1.4 in. O.D., was filled with the dried packing material. Uniform distribution was achieved by vibration of the column.

Results and discussion

Thermogravimetric analysis on the solid packing materials showed absolute

TABLE I

NET RETENTION VOLUME (ml) ON NICKEL COLUMNS AT 50°[a]

Compound	*26.8% Ni-DMG*	*27.7% Ni-CHD*	*25.7% Ni-SAL*	*26.0% $Ni(ClO_4)_2$*	*Gas-Chrom P*
Hydrocarbons					
1-Pentene	6.7	8.1	2.0	0.6	0.3
n-Pentane	7.7	7.0	1.9	0.4	—
Allyl chloride	7.5	10.7	2.6	1.4	0.8
n-Hexane	13.4	17.7	4.1	1.2	0.7
Cyclopentane	9.5	13.2	3.2	0.8	0.5
Allyl bromide	13.7	26.0	6.2	2.7	1.9
Cyclohexane	23.2	33.5	6.9	1.4	0.8
Cyclohexene	27.2	40.6	8.4	2.7	1.9
2-Bromobutane	28.6	45.1	10.1	4.3	2.7
n-Heptane	32.1	45.7	9.4	3.0	1.7
n-Bromobutane	37.8	64.7	14.6	6.0	3.9
Benzene	24.0	40.9	9.0	2.9	2.4
Toluene	60.2	106	22.8	9.3	4.3
n-Butylbenzene	N.P.	N.P.	279	129	55.8
Chlorobenzene	115.8	239	51.5	13.2	7.2
m- and *p*-xylene	140	262	58.2	25.2	11.1
o-Xylene	169	325	72.0	30.0	13.2
Bromobenzene	239	538	120	28.9	13.7
Mesitylene	334	683	132	68.4	26.5
p-Cymene	499	N.P.	215	105	40.3
Benzyl chloride	477	N.P.	259	119	443
Aldehydes					
Propionaldehyde	5.4	9.0	3.4	4.7	11.1
Isobutyraldehyde	8.9	12.6	3.9	3.7	6.0
Butyraldehyde	12.4	19.2	5.6	6.8	7.5
n-Valeraldehyde	20.9	49.6	17.0	21.0	17.8
n-Heptaldehyde	167	293	91.1	N.P.	101
2-Ethylhexanal	254	464	113	N.P.	199
Benzaldehyde	321	N.P.	256	N.P.	55.1
Salicylaldehyde	N.P.	N.P.	N.P.	—	—
Esters					
Methyl acetate	7.1	9.4	3.2	5.4	5.3
Cyclohexanone	161	383	116	80.4	102
2,6-Dimethyl-4-heptanone	312	525	127	79.8	174
2-Octanone	375	712	211	84.7	196
Ethers					
Ethyl ether	5.4	6.4	1.1	1.7	2.4
Tetrahydrofuran	17.4	24.5	9.0	7.2	9.6
Dioxane	32.1	57.0	17.6	7.8	22.6
Di-*n*-butyl ether	144	224	48.2	18.5	88.9
Anisole	211	454	111	—	33.2
Alcohols					
Methanol	4.8	16.6	27.1	23.9	3.6
Ethanol	6.2	18.3	22.2	29.9	14.5
tert.-Butanol	11.8	14.9	5.8	N.P.	31.8
Isopropanol	7.5	16.2	11.6	70.5	18.1
n-Propanol	14.9	42.5	39.6	61.8	37.8
Isobutanol	25.0	58.0	39.3	70.5	73.0
n-Butanol	37.4	102	93.9	N.P.	84.9
2-Pentanol	43.7	96.3	49.8	N.P.	—
Isopentanol	69.0	179	N.P.	N.P.	—
n-Pentanol	95.5	280	N.P.	N.P.	203
n-Hexanol	234	567	N.P.	N.P.	207

TABLE I *(continued)*

Amines					
tert.-Butylamine	16.2	13.4	5.2	9.9	14.9
Diethylamine	17.4	N.P.	0.7	N.P.	8.2
n-Butylamine	51.8	87.3	N.P.	N.P.	42.1
Triethylamine	28.2	44.8	14.9	N.P.	47.5
Pyridine	90.2	254	N.P.	N.P.	63.0
Aniline	N.P.	N.P.	N.P.	N.P.	74.2
N,N-Dimethylaniline	N.P.	N.P.	488	103	128
Acids					
Acetic	N.P.	531	N.P.	N.P.	N.P.

[a] In the case of slight peak asymmetry retention times were measured to peak maxima. N.P. = no peak.

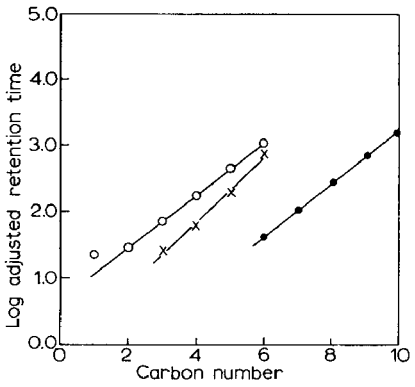
thermal stability of all supports to 220° in a nitrogen atmosphere. Significant decomposition of the nickel complexes occurred only above 250°. At 150°, a temperature exceeding that of any of the chromatographic studies, column packings showed a weight loss of less than 0.1% upon an extended heating period of 100 h.

Net retention volumes for seventy-four compounds on five of the columns at 50° are presented in Table I. The members within each class of compounds have been arranged in order according to boiling point. With few exceptions, it is noted that retention volumes increase in this same order.

Retention volumes for a compound, measured at the same temperature, were observed to pass through a minimum as the per cent nickel complex on the solid support increased. The retention volumes for a given compound on the same column decreased as the temperature was increased, but the relative order of elution of a class of compounds remained unchanged. In Fig. 1, linear plots for homologous series of alcohols, aldehydes, aliphatic hydrocarbons, and aromatic hydrocarbons are presented. In one case, the first member in the series shows anomalous behavior in being retained in the column for an abnormally long time. This is attributed to the relatively high dipole moment of the small molecule, and its relative ease of polarizability.

Pronounced tailing was observed for compounds on the column packed with uncoated Gas-Chrom P. Peaks obtained on the nickel perchlorate column were less well defined than peaks obtained for the same compounds on the columns made using the nickel complexes as stationary phases. Only aliphatic and aromatic hydrocarbons were eluted from this column at 50°.

For the columns containing nickel complexes as the solid phase peak symmetry was improved by increasing the per cent complex on the solid support (Fig. 2). Comparing retention volumes obtained at the same temperature on columns made from the same complex, varying only in per cent loading, it was noticed that the retention volumes passed through a minimum and increased as the per cent stationary phase increased. This may be explained by attributing the retention volumes on the 4.5% Ni-DMG and the 3.8% Ni-SAL columns to a solid support incompletely covered by the complex and the adsorbates being influenced by the solid support. On the 8.2% Ni-DMG and 12.6% Ni-SAL columns, there may be a thin layer of complex that neutralized the active sites on the solid support, allowing a minimum of interaction with an adsorbate. On columns with higher per cent complex coatings, there may be



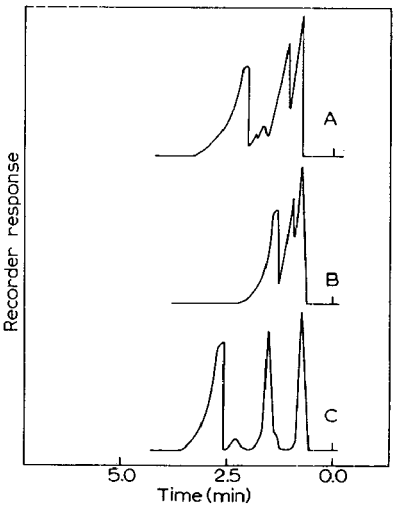
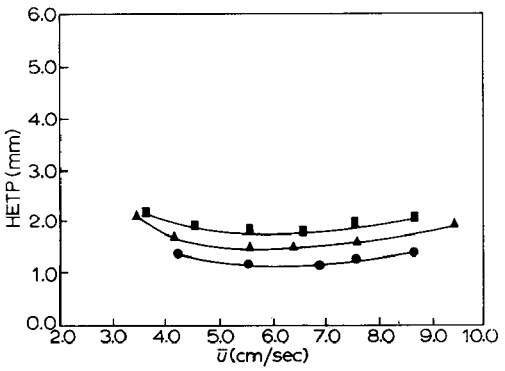


Fig. 1. Logarithm of adjusted retention time for homologous series *versus* carbon number on the 26.8% Ni-DMG column at 50°. ○, Alcohols; ×, aldehydes; ●, aromatics.

Fig. 2. Separation of pentane, hexane, and heptane on Ni-DMG columns. (A) 4.5%; (B) 8.2%; (C) 26.8%.

an excess of complex that completely eliminates active sites on the solid support and allows a maximum of interaction of the adsorbent with the adsorbate.

Satisfactory separation of C_3–C_7 aldehydes was observed using the 26.8% Ni-DMG column; however, a mixture of alcohols was incompletely resolved. Acetic acid was eluted from this column as a symmetrical peak but was not eluted at all from the Ni-SAL column.



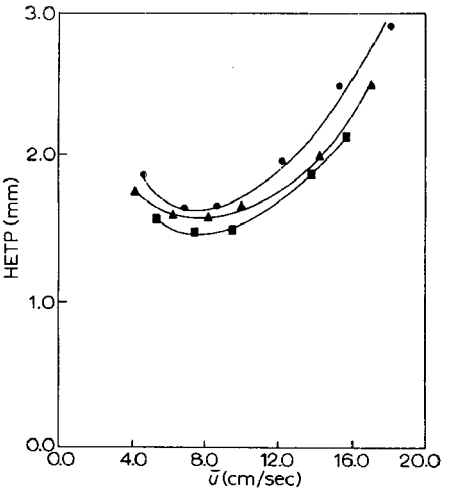


Fig. 3. Comparison of HETP curves for *n*-hexane at 50° (●), 85° (▲), and 125° (■) on the 26.8% Ni-DMG column.

Fig. 4. Comparison of HETP curves for *n*-hexane at 50° (●), 85° (▲), and 125° (■) on 3.8% Ni-SAL column.

The elution of amines from columns constructed of Ni-CHD and Ni-SAL resulted in asymmetrical peaks. Symmetry of these peaks was improved considerably by preconditioning the columns with three to five injections before data were collected. Amine peaks with the best symmetry were obtained using the 26.8% Ni-DMG column.

The number of theoretical plates obtained on the columns varied from 450 to 1600, and a minimum HETP value of 0.98 mm was observed on the 27.7% Ni-CHD column at 50°. For the Ni-DMG columns, plate height increased as column temperatures increased (Fig. 3), but for Ni-SAL a decrease was noted (Fig. 4). Typical Van Deemter data were obtained on the Ni-CHD column. Optimum flow rates were found to be between 6.0 and 8.5 cm/sec.

Benzene and *n*-hexane were used to study the effect of increasing sample size on retention volume (Fig. 5). In all cases, the sample size varied from 0.05 to 5.0 μl. For

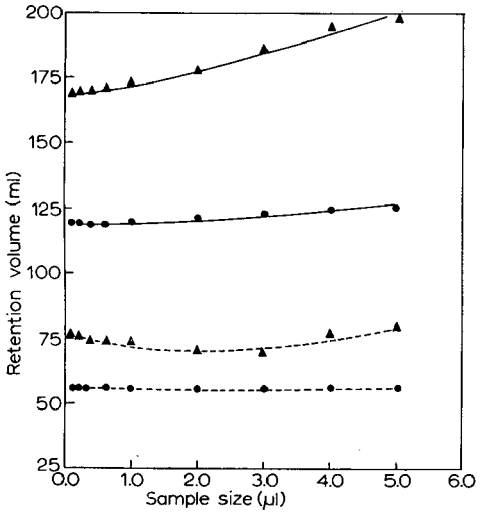


Fig. 5. Retention volume *versus* sample size (μl) at 50° on 26.8% Ni-DMG (▲—▲, benzene; ●—●, *n*-hexane) and 25.7% Ni-SAL (▲— — —▲, benzene, ●— — —●, *n*-hexane).

the ten columns studied, the average change of retention volume over the range of sample size examined was only 2–4%. The decrease in retention volume indicates a heterogeneous surface and strongly suggests that adsorption isotherms are concave toward the pressure axis.

Heats of adsorption for a few of the compounds studied on the complexes used as stationary phases were calculated in the usual manner[10] and are presented in Table II. Results are shown for the most symmetrical peaks with retention times being measured at the peak maximum. Comparing the data in Table II for the three columns of interest, 26.8% Ni-DMG, 27.7% Ni-CHD, and 25.7% Ni-SAL, shows that ΔH values are not as large on the Ni-DMG column as the Ni-CHD column, and almost identical to those obtained on the Ni-SAL column. The order of decreasing heats of adsorption, and an indication of increased interaction between adsorbents and adsorbates is Ni-DMG = Ni-SAL < Ni-CHD. This is also the order of increased tailing of compounds on the metal complex columns. The heats of adsorption may be used,

TABLE II

HEATS OF ADSORPTION (kcal/mole)

Columns: (A) 26.8% Ni-DMG;
(B) 27.7% Ni-CHD;
(C) 25.7% Ni-SAL

Compound	*Column*		
	A	*B*	*C*
n-Pentane	−5.7	−5.8	−5.5
n-Hexane	−5.8	−6.3	−5.1
n-Heptane	−7.0	−7.0	−7.2
Diethyl ether	−5.7	−6.0	−3.5
Dibutyl ether	−8.5	−8.9	−8.6
Dioxane	−6.5	−7.1	−6.6
THF	−6.1	−6.3	−6.1
Benzene	−6.3	−6.5	−5.0
p-Xylene	−8.1	−7.5	−6.2
Mesitylene	−9.1	−8.3	−5.9
Bromobenzene	−8.5	−8.9	−6.1

therefore, to explain partially the difference in the amounts of tailing on the three sets of columns studied.

In conclusion, we can say that it is possible to make a chromatographic packing of a slightly soluble complex on a solid support. This type of column has shown selective retention for various classes of organic compounds and can be used for separations of mixtures. Of the metal complexes used as stationary phases, tailing increased in the order: Ni-DMG < Ni-SAL < Ni-CHD. Tailing of the liquids decreased as per cent stationary phase increased, and was found to follow the order: aliphatic hydrocarbons < ethers < esters < ketones < aldehydes < alcohols = amines.

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CHROM. 4748

Investigation into the composition of lavender and lavandin oil

Some new components

The oils of lavender, lavandin and spike lavender, belonging to the plant family of the *Labiatae*, show characteristic differences with regard to their chemical composition. These differences are shown clearly by gas chromatographic analysis of the major components *viz.* camphor, 1,8-cineol, linalol and linalyl acetate.

In genuine French lavender oil we found less than 1% 1,8-cineol and camphor and about equal amounts of linalol (31%) and linalyl acetate (38%). Samples of genuine French lavandin oil contained 6% cineol, 7% camphor, 35% linalol and 19% linalyl acetate, whereas samples of genuine Spanish spike lavender oil showed a greater amount of cineol (25%) and camphor (14%) and a negligible percentage of linalyl acetate (0.3%). The percentage of linalol turned out to be 47%. Comparable investigations have been carried out by NAVES[1], HOLNESS[2], KOLSEK AND MATICIC[3], STELTENKAMP AND CASAZZA[4] and ILLE[5].

The relative retention times of the main constituents on diethylene glycol succinate (DEGS), Carbowax 20M, Carbowax monostearate and Castorwax are listed in Table I. Castorwax appeared to be selective for the separation of linalol, camphor and linalyl acetate. This is in agreement with the results, obtained by KOLSEK[3]. On a packed column with Carbowax 20M complete separation of the three compounds could only be obtained by programming the temperature slowly. Camphor elutes from this phase and also from Carbowax monostearate *before* linalol and linalyl acetate, while from DEGS it occurs *after* the latter compounds.

TABLE I

RELATIVE RETENTION TIMES OF SOME MAJOR COMPONENTS OF LAVENDER, LAVANDIN AND SPIKE LAVENDER OIL

Analytical conditions:

[a] Column 3 m, 1/8 in. O.D., loaded with 10% (w/w) diethylene glycol succinate on acid-washed Embacel support. Temperature: programmed from 75–200°, 20 min at a rate of 1.1°/min, followed by 2°/min.

[b] Column 3 m, 1/4 in. O.D., loaded with 10% (w/w) Carbowax monostearate on acid-washed Embacel support. Temperature: programmed from 75–200°, at a rate of 3°/min.

[c] Column 2 m, 1/8 in. O.D., loaded with 10% (w/w) Castorwax on acid-washed Embacel support. Temperature: isothermal 120°.

[d] Column 2 m, 1/8 in. O.D., loaded with 10% (w/w) Carbowax 20M on acid-washed Embacel support. Temperature: programmed from 70–200°, 12 min at a rate of 0.9°/min, followed by 1.1°/min.

	Relative retention times (camphor = 1.00)			
Component	*DEGS*[a]	*CMS*[b]	*Castorwax*[c]	*Carbowax 20M*[d]
1,8-Cineol	0.30	0.28	0.35	0.31
Camphor	1.00	1.00	1.00	1.00
Linalol	0.92	1.05	0.78	1.08
Linalyl acetate	0.95	1.35	1.15	1.13

TABLE II

LIST OF NEW CONSTITUENTS FOUND IN LAVENDER AND LAVANDIN OIL

Lavender oil		*Lavandin oil*	
Constituent	*Method of identification*[a]	*Constituent*	*Method of identification*
β-Phellandrene	RRT	β-Phellandrene	RRT
Terpinolene	RRT, IR	Menthone	RRT, IR
α-Thujene	RRT	*p*-Methyl acetophenone	RRT, IR
α-Phellandrene	RRT		
Perillyl alcohol	RRT		
Perillaaldehyde	RRT		
n-Hexanal	RRT		
n-Heptanal	RRT		
Phellandral	RRT		

[a] RRT = relative retention time; IR = infrared spectrum.

Further investigation into the composition of lavender and lavandin oils led to the identification of some constituents not previously reported to be present in these oils. They are listed in Table II.

Trace amounts of β-phellandrene have been identified by gas chromatography on different liquid phases[6]. The hydrocarbon fraction was isolated by percolation of the oil over silica gel with pentane. In addition, the pentane eluate of lavender oil showed the presence of α-thujene, α-phellandrene and terpinolene. Of these, terpinolene has also been found in lavandin oil[4], its presence being confirmed by us. In the carbonyl fraction of lavandin oil, isolated by treating the oil with Girard-P reagent in the presence of methanol and acetic acid, menthone and *p*-methyl acetophenone were identified by IR spectroscopy. The latter has hitherto only been found in a few essential oils. It also appeared to be a constituent of spike lavender oil[7,8]. Up to now we have not detected it in lavender oil. The carbonyl fraction of lavender oil, isolated by means of sodium bisulfite contained perilla-aldehyde, *n*-hexanal, *n*-heptanal and

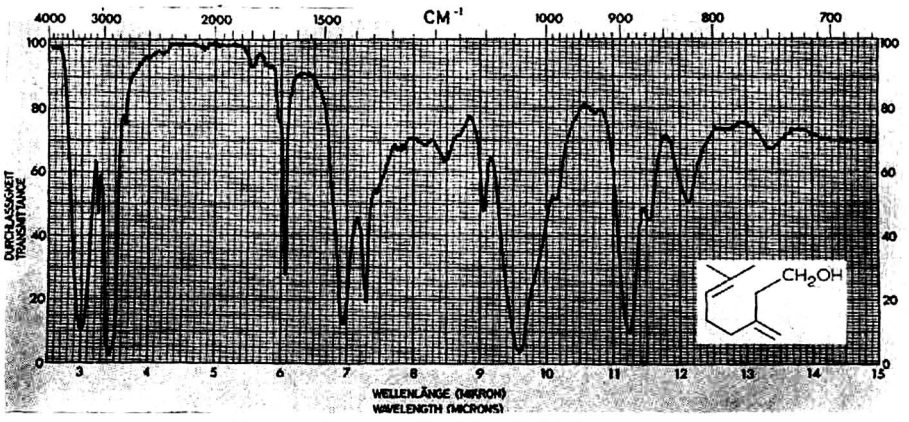


Fig. 1. IR spectrum of isogeraniol. Pure liquid, cell thickness 0.023 mm.

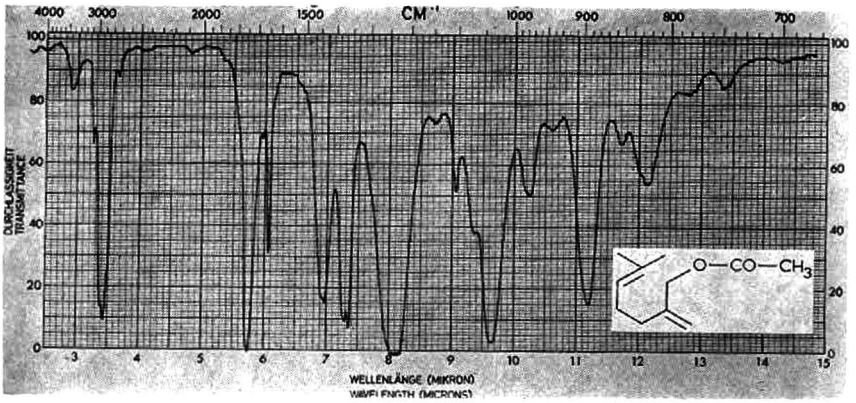


Fig. 2. IR spectrum of isogeranyl acetate. Pure liquid, cell thickness 0.021 mm.

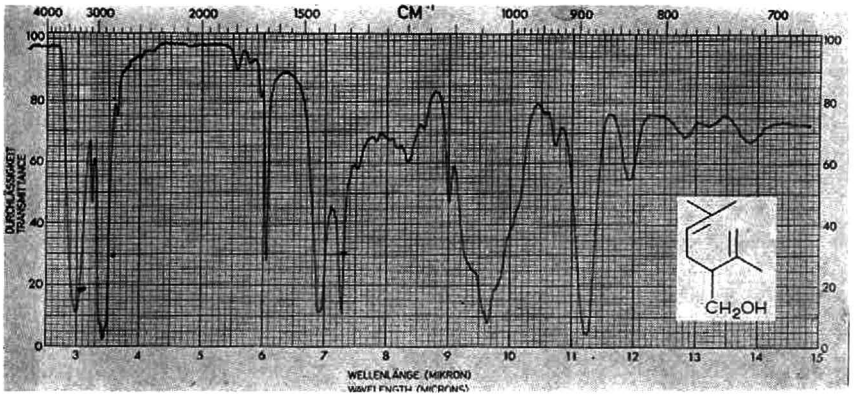


Fig. 3. IR spectrum of lavandulol. Pure liquid, cell thickness 0.021 mm.

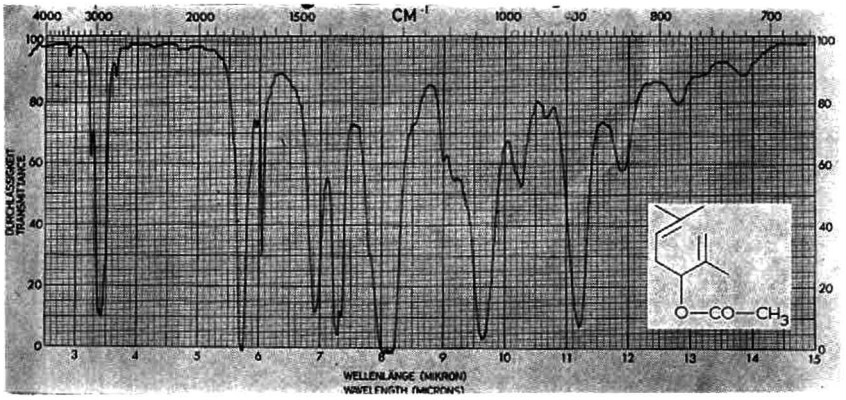


Fig. 4. IR spectrum of lavandulyl acetate. Pure liquid, cell thickness 0.021 mm.

phellandral as new constituents. They were identified by gas chromatography on packed and capillary columns. We also detected a trace of perillyl alcohol in the same fraction. Authentic samples were available of all the components identified. The methods of identification are also shown in Table II.

OGNYANOV and co-workers[9,10] have reported isogeranyl acetate as a new component in lavender oil. The identification was based upon the similarity of the IR spectrum of the reduced ester (*i.e.* isogeraniol) with that of published spectra[11,12]. However, careful comparison of the IR spectra (published by the Bulgarian investigators[9]) with those of authentic and pure samples of isogeraniol and isogeranyl acetate (Figs. 1 and 2) revealed that they were not identical. The spectra of OGNYANOV and co-workers appeared to be those of lavandulol and lavandulyl acetate. (Figs. 3 and 4). In spite of the close resemblance between the spectra of isogeraniol and lavandulol, they can be easily distinguished by:

(a) the shape of the C–OH stretching band at 9.6 μ (1042 cm^{-1});

(b) the positions and pattern of the bands in the region of 7.4–9.1 μ (1351–1099 cm^{-1});

(c) the position of the CH out of plane deformation band of the tri-substituted ethylenic bond, which in isogeraniol is positioned at 12.13 μ (824 cm^{-1}), in lavandulol at 11.92 μ (839 cm^{-1}) and in the spectrum of OGNYANOV at 11.9 μ (840 cm^{-1});

(d) the position of the bands in the 12.7–14 μ (787–714 cm^{-1}) region.

The IR spectra of the acetates show characteristic differences with respect to the position of the out of plane deformation band of the tri-substituted double bond, which occurs at 12.14 μ (824 cm^{-1}) in isogeranyl acetate, at 11.90 μ (840 cm^{-1}) in lavandulyl acetate and at 11.88 μ (842 cm^{-1}) in the spectrum of OGNYANOV. Conformity of the bands in the region of 12.5–14 μ (800–714 cm^{-1}) in the spectra of lavandulyl acetate and the spectrum under discussion is easily established. In our sample of lavender oil we found 5.0% of lavandulyl acetate, but only 0.4% in lavandin oil. The identification was also confirmed by NMR studies.

Another compound of importance in the oils of the genus lavandula appeared to be terpinen-4-ol. OGNYANOV and coworkers[9] found 12% of this alcohol in Bulgarian lavender oil, while NAVES AND TULLEN[13] found 1.5–3.5% in French lavender oil. SFIRAS AND VANDERSTREEK[14] detected the alcohol in lavandin oil, but its presence in this oil has not since been confirmed.

Our investigations also led to the identification of terpinen-4-ol by IR techniques. The highest percentage (5.6%) was found in French lavender oil; lavandin oil contained 2.4% of this alcohol and Spanish spike lavender oil 0.8%.

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CHROM. 4771

Gas chromatographic determination of some maleimides produced by the oxidation of heme and chlorophyll *a*

Our studies of chlorophyll biosynthesis (*e.g.*, see ref. 1), which involve the feeding of specifically labeled ^{14}C compounds to plants and the determination of the ^{14}C incorporation into the various portions of the chlorophyll molecule, have been hindered because of the lack of a method for the determination of microgram quantities of maleimides produced upon chromic acid oxidation of milligram quantities (usually available for these studies) of chlorophyll*. Only very recently have we established that the technique of chromate oxidation can be used with less than 10 mg quantities of porphyrin[2].

MORLEY AND HOLT[3] have shown that gas phase chromatography is an excellent method for non-destructively purifying milligram quantities of maleimides. Based on their report, a study was made of the feasibility of gas phase chromatography for the quantitative estimation of microgram quantities of maleimides. This report describes a gas chromatographic procedure found to be suitable for the determination of some maleimides, derived from the oxidation of heme and chlorophyll, in concentrations as low as 0.050 $\mu g/\mu l$ to within 3% experimental error.

Methods and materials

Preparation of maleimides. Maleimide, succinimide, citraconimide, methylethylmaleimide, hematinic acid methyl ester, and dihydrohematinic acid methyl ester were used for the sensitivity and reproducibility tests described herein. These compounds were prepared and characterized during an earlier study[2].

Gas phase chromatography. Maleimides were separated and estimated by gas phase chromatography on a 10 ft. $\times$ $\frac{1}{8}$ in. stainless-steel column packed with 20%

* The maleimides obtainable from the chromic acid oxidation of chlorophyll *a*, which are demonstrated in Fig. 1, include methylvinylmaleimide (ring I) which has been isolated only recently[4], methylethylmaleimide (ring II), methylmaleimide (ring III—citraconimide) when the oxidation is performed after degradation of pheophorbide *a* to pyrroporphyrin, and dihydrohematinic acid (ring IV).

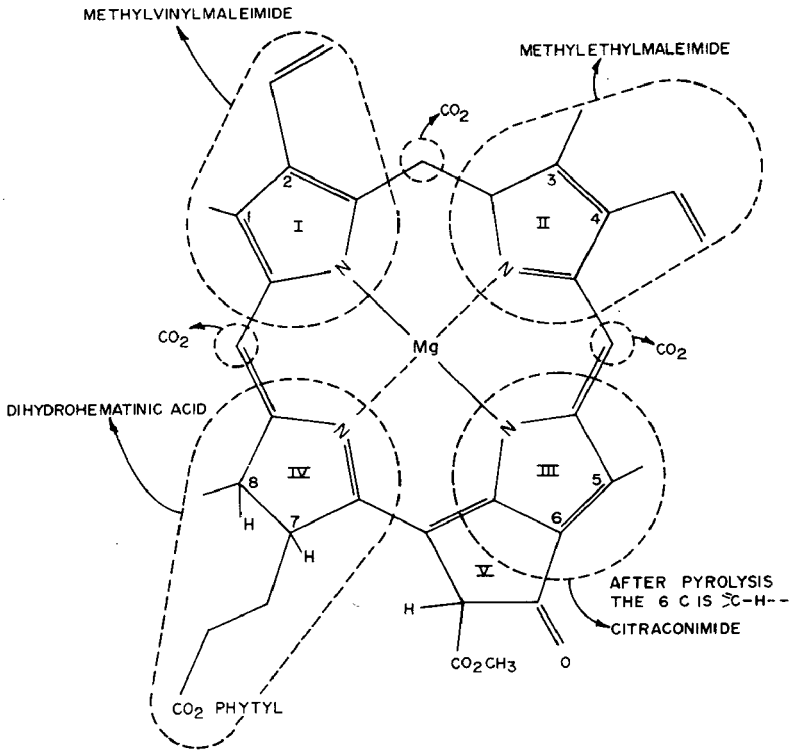


Fig. 1. Chromic acid oxidation products of chlorophyll *a*.

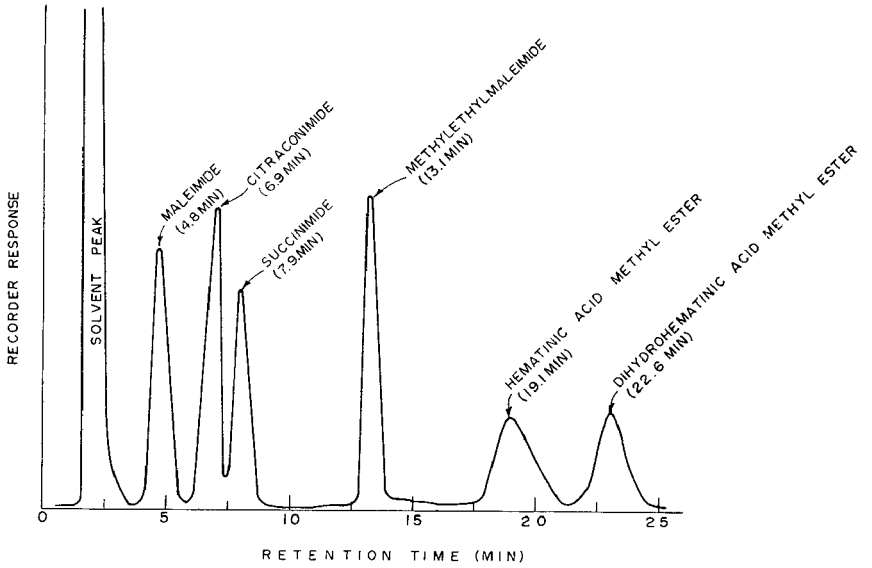


Fig. 2. A typical gas chromatographic separation of a mixture of maleimides (concentrations unequal).

Apiezon M on a support of 100–120 mesh dimethyldichlorosilane-treated and acid-washed Chromosorb W. The chromatograph used was an Aerograph Hi-Fy Model 600-D (Varian Aerograph, Walnut Creek, Calif.) equipped with a hydrogen flame detector (the hydrogen was generated by an Aerograph, Model 650, hydrogen generator). The Brown recorder (Minneapolis-Honeywell Regulator, Co., Brown Instrument Division, Philadelphia, Pa.) was equipped with a disc integrator (Disc Instruments, Inc., Santa Anita, Calif.). Prepurified nitrogen was used as the carrier gas. Injections of solutions of maleimides in reagent grade acetone into the chromatograph were made with a Hamilton (Hamilton Co., Whittier, Calif.) 10-μl syringe (701N-W/g). The chromatographic conditions used were as follows: nitrogen flow rate, 30 ml/min; hydrogen flow rate, 30 ml/min; chromatograph oven temperature (injector port and chromatographic column), 200°.

Results and discussion

A typical chromatogram demonstrating the separation of maleimides achieved with this system is presented in Fig. 2. The retention time obtained for each of the maleimides did not vary from those values observed when a mixture of the maleimides was run. The retention times, however, did change appreciably with small changes in temperature (*e.g.*, 10°) and in flow rate (*e.g.*, 5 ml/min).

TABLE I

MALEIMIDE SOLUTIONS DETERMINED BY GAS CHROMATOGRAPHY

Imide	*Lowest concentration achieved (μg/μl)*	% *error*[a]
Maleimide	1.00	± 3
Succinimide	0.50	± 3
Citraconimide	0.50	± 2
Methylethylmaleimide	0.050	± 2
Hematinic acid methyl ester	0.050	± 3
Dihydrohematinic acid methyl ester	0.050	± 3

[a] These values represent deviations (determined for ten 1-μl injections of each concentration indicated) of integrator counts at the concentration shown.

The data in Table I represent the sensitivity limits, and error at the limits shown, for this method of maleimide determination on the instrument used. When considerably higher concentrations of maleimide were used (about 100 times those indicated in Table I) errors of less than 2% were routinely observed.

The reliability of this method was tested when an independent worker prepared acetone solutions of the various maleimides. These unknowns were then given to a second worker to perform the analysis. In all cases the maleimide was identified and then quantitatively estimated to within 3% error when the various "unknown" maleimide solutions were run by the analyst. These error limits were well within the range desired of this procedure to fill our needs of maleimide determination.

Finally, the sensitivity of the entire procedure, for our previously stated use, was tested. A sample of approximately 5 mg pure chlorophyll *a* was oxidized with

chromic acid[2]. The residue, redissolved in ether, was treated with diazomethane[5] to esterify the dihydrohematinic acid produced during the oxidation. After evaporation of the ethereal diazomethane solution, the residue was redissolved in 0.5 ml acetone and run on the gas chromatograph. The methylethylmaleimide and dihydrohematinic acid methyl ester fractions were collected and then redissolved in 0.5 ml acetone. Their concentrations were found to be 0.21 and 0.15 $\mu g/\mu l$, respectively. This result indicates that the method is sensitive enough for our work with the [^{14}C]chlorophylls.

It should be noted that the limit of maleimide detection was at least an order of magnitude lower in maleimide concentration than reported herein, but the error in quantitative determination at those levels was very high (*i.e.*, 10–15%). The concentration limit for maleimide detection was about 1 ng/μl.

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CHROM. 4775

Deactivation of the gas chromatographic support for the separation of aliphatic amines

The main difficulty encountered in the gas–liquid chromatographic separation of the aliphatic amines is caused by the adsorption of the latter compounds to the quasi-inert chromatographic support[1]. The adsorption effect, when fairly weak, leads to the formation of asymmetric peaks, making impossible the proper quantitative evaluation of the separated compounds. In a less favorable case when the adsorption is very strong, no elution of the chromatographed compounds occurs. Probably the most disadvantageous situation may be found in the analysis of mixtures containing minor concentrations of strongly adsorbed compounds. It may happen that such compounds will be retained in the column, thus escaping identification[2,3].

Various methods have been proposed to suppress or diminish adsorption of the compounds to be chromatographed on the inert support. However, it is impossible to review them all in this note—only a short account on that subject is given here. Thus, generally speaking, these methods are concerned either with the deactivation of the support by alkali treatment[4,5] or with the application of high-boiling organic bases[2,6,7] (including detergent powders[8,9]) as liquid phases. The application of poly(tetrafluoroethylene) molding powder as the inert support[10] or the saturation of the carrier gas with an amine[11] have also been proposed.

In the present note a very simple and convenient method of deactivation of the inert support prior to its proper impregnation with a liquid phase suitable for resolving the primary and secondary aliphatic amines is described. This method consists in treating the inert support (such as Chromosorb P or W) with sodium metanilate(3-aminobenzene-1-sulphonate). Metanilic acid, unlike the other aminobenzenesulphonic acids, is thermally and chemically stable (it is very resistant, for instance, to desul-

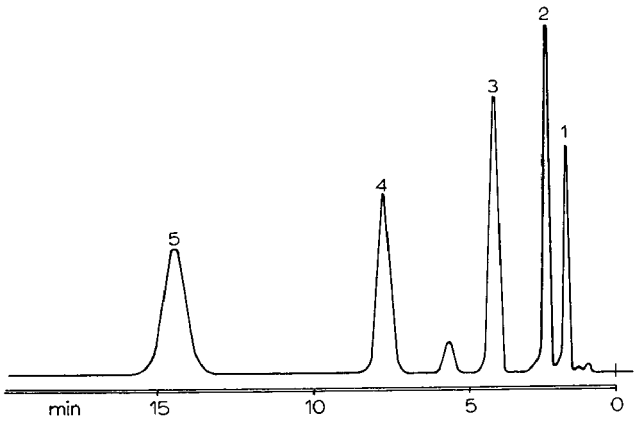


Fig. 1. The resolution of C_2–C_4 aliphatic secondary amines. 1 = diethylamine; 2 = diisopropylamine; 3 = di-*n*-propylamine; 4 = diisobutylamine; 5 = di-*n*-butylamine.! Apparatus: Perkin-Elmer gas chromatograph, Model 452, equipped with a hot-wire detector, stainless-steel column (200 × 0.4 cm) filled with Apiezon L (10%) on deactivated Chromosorb W, 60/80 mesh; temperature, 104°; carrier gas hydrogen (40 c.c./min); sample size, 1 μl.

phonation[12]). It was considered that sodium metanilate would block the active sites of the support and consequently suppress the adsorption of the amino groups.

An example will illustrate the method: an appropriate amount of the inert support was treated with 5% (calculated on the support) sodium metanilate in methanol (the latter should totally cover the support), the solvent consequently evaporated to dryness. The support so pretreated was in turn impregnated with Apiezon L or any other liquid phase, the only limitation being that the solvent used for impregnation should not dissolve sodium metanilate. With such a pretreated support, the following column fillings were prepared: Apiezon L (10%), Carbowax 5000 (10%) and Reoplex 400 (10%), all on Chromosorb P and W. All these fillings were investigated for resolution of aliphatic primary and secondary amines at temperatures up to 160°. The column containing Apiezon L was used even at 180° for several weeks without any deterioration of its efficiency. A typical separation of low-boiling aliphatic secondary amines on Apiezon L is shown in Fig. 1, with the experimental details given in the legend. Quite satisfactory results as to the shape of peaks were obtained on columns containing Carbowax 5000 and Reoplex 400. However, the resolution of the analysed amines on the polar liquid phases, especially on Reoplex 400, was less interesting due to the superimposition of some peaks.

The analyses of pyridine bases (picolines and some lutidines) were also effected on the Apiezon L and Reoplex 400 columns with satisfactory results.

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CHROM. 4747

An eluent programmer for column chromatography

Reproducible eluent additions are desirable for column chromatography procedures particularly when several samples are being compared. A versatile device is described which can reproducibly add eluents to a column as a series of stepwise additions or as a combination of stepwise and gradient additions. A chosen pattern can be easily modified or changed completely. The apparatus can be built with a minimum of available shop facilities and its cost is largely a function of the sophistication desired.

The versatility of this unit is due to the programmer which is used to open and close a series of relays. The programmer is basically a rotating drum with removable cams. These cams mechanically open or close a series of contacts according to a preset pattern. To prevent electrical overloading, each contact activates a relay which in turn activates a particular function of the unit for variable periods of time.

Liquid flow system

Flow from each reservoir to a manifold is controlled by an electrically operated solenoid valve (Model EDV-1-22, Fluorocarbon, Pfeifer and Co., Fort Washington, Pa.*). All connections are made with Chromatronix fittings and connecting fluid-lines are 0.16 I.D. teflon standard wall tubing. The manifold is machined from plexiglas to mate with these fittings. A bubble trap with approximately 2 ml capacity is placed after the manifold to prevent air bubbles from reaching the column. Gradients can be formed by connecting two reservoirs by the usual methods. Magnetic stirrers are positioned under the mixing reservoirs and are controlled by the electrical system.

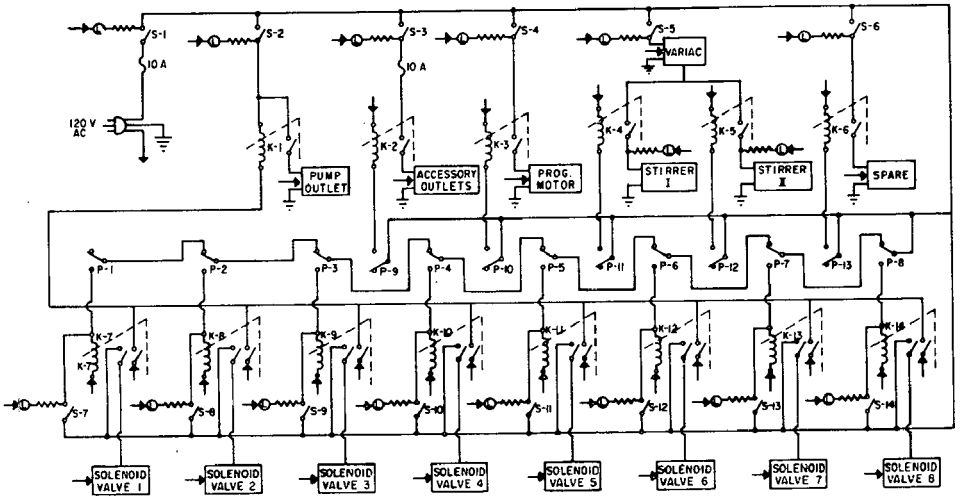


Fig. 1. Electrical schematic of eluent programmer. All resistors are 10 W, 3000 Ω. S-1 to S-14 are SPDT pushbutton switches with pilot lights. P-1 to P-13 are programmer contacts. K-1 to K-6 are Potter–Blum 5AGSPDT relays and K-7 to K-14 are Potter–Blum 11AGDPDT relays.

* Mention of commercial products does not imply their endorsement by the Department over similar products not mentioned.

Electrical system

The programmer used in this unit is a Sealectroswitch, Model No. 92-2066-203 (Sealectro Corp., Narbeth, Pa.). A one revolution per day motor is used to rotate the drum. There are 60 positions for a cam under each contact and at this speed each cam closes a contact for 24 min. Programmers can be purchased with a variety of motor speeds so that shorter time segments can be used.

Relays, activated by the programmer, are used to control each liquid flow valve, the magnetic stirrers, auxiliary outlets, pump and programmer motor. By

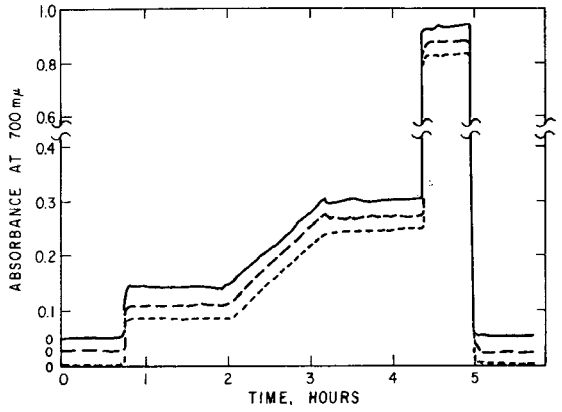


Fig. 2. Absorption of the copper sulfate solutions indicated in Fig. 3 demonstrating the reproducibility of the eluent profiles. Two of the profiles are offset from the origin to separate the runs. Flow rate was 2.4 ml/min.

controlling the latter recycling is prevented. The auxiliary outlets are used to shut down accessory equipment such as a fraction cutter, UV monitor, etc., at the end of the run.

The electrical schematic is shown in Fig. 1. There are several features of the unit which should be pointed out. Each valve may be controlled either manually or by programmed operation. The condition of each valve under either type of operation

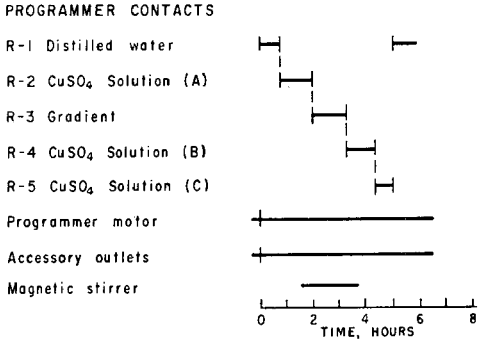


Fig. 3. Cam pattern used to program the eluent profile seen in Fig. 2. Each line indicates the period that a particular contact is closed. R-1 to R-5 are 500 ml-reservoirs. Solutions A, B and C are copper sulfate solutions with absorbances at 700 mμ of 0.1, 0.25 and 0.8, respectively; copper sulfate solutions of 1.0 and 0.1 absorbance were used to form the gradient.

is indicated by a pilot light. The pump is connected in series with the valves as a safety feature to prevent the pump from operating unless at least one valve is open. In addition the programmer is wired so that there is a simultaneous opening and closing of valves in any sequence.

Operation

To demonstrate the reproducibility and versatility of this unit a particular pattern of eluent addition, including a linear gradient, was decided upon and then matched by setting the programmer. The fluid lines and reservoirs were filled with appropriate copper sulfate solutions. Eluent absorbance at 700 mμ was monitored in a flow-through cell on a Gilford 2000 spectrophotometer. In order to make the gradient absorbance match the desired pattern the equation of BOCK AND LING[1] was used:

$$[C] = C_2 - (C_2 - C_1)\left(1 - \frac{\text{ml of eluent}}{\text{total volume of reservoirs}}\right)$$

where

$[C]$ = concentration of gradient
C_2 = concentration of solution in 2nd reservoir
C_1 = concentration of solution in mixing reservoir.

The results of three successive runs are shown in Fig. 2. The program used is shown in Fig. 3.

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CHROM. 4757

Sephadex G-15 column separation of [9,10-^{3}H]stearyl coenzyme A

[9,10-^{3}H]Stearyl coenzyme A, which was synthesized according to the procedure of YOUNG AND LYNEN[1], was isolated by column chromatography using Sephadex G-15 as the stationary phase. Previous methods, by which the acyl-CoA esters were separated by precipitation in acidic solution, were insufficient with respect to a small-scale preparation (10 μmoles), or in cases where only small amounts of the fatty acids had reacted with the coenzyme A. The isolated [9,10-^{3}H]stearyl-CoA was identified by common procedures.

Experimental procedure

An amount of 2.85 mg (10 μmoles) [9,10-^{3}H]stearic acid from Amersham/Searle (spec. act. 2,500 mCi/mmole) was converted into the mixed anhydride (9.5 μmoles), which was then reacted with 17 mg (11 μmoles) coenzyme A from Sigma (in the reduced form) to yield the [9,10-^{3}H]stearyl-CoA[1]. The mixture was then acidified to pH 5 with 1% $HClO_4$. After removal of the tetrahydrofuran from the reaction mixture under reduced pressure and extraction of the unreacted stearic acid with light petroleum ether, the solution (pH 7.5) was applied to a 42 × 1.2 cm column, packed with Sephadex G-15 from Pharmacia. The column chromatographic separation was operated at 4°. The eluting solvent was water containing 0.5% mercaptoethanol to prevent oxidation of the product. Fractions of 2 ml were collected; the elution profile (Fig. 1) was obtained by UV absorption (at 260 mμ, using a Gilford 240 spectrophotometer) and by radioactivity measurements (cocktail: 5 g PPO per 1000 ml dioxane). The fractions 1 to 5 were collected, the solvent removed by freeze drying, and the com-

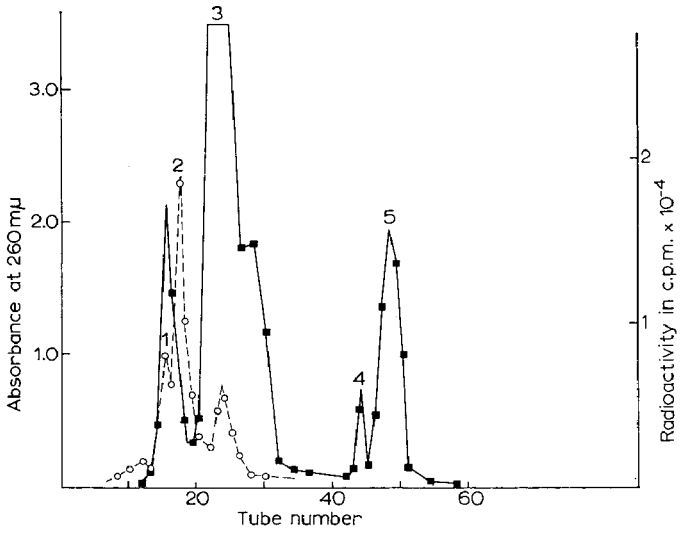


Fig. 1. Separation of the [9,10-^{3}H]stearylCoA on a Sephadex G-15 column. The dimensions of the column were 42 × 1.2 cm. The eluent was water containing 0.5% mercaptoethanol. Fractions of 2 ml were collected. — — — —, Radioactivity curve; ———, UV absorption at 260 mμ. Peaks 1–5 represent fractions 1–5.

pounds were then chromatographed on Whatman No. 3 paper using water–pyridine–isopropanol (1:2:2) as the solvent system[2]. The R_F values were as follows: fract. 1, 0.07; fract. 2, 0.12; fract. 3, 0.31; fract. 4, 0.80; fract. 5, 0.88. The [9,10-^{3}H]stearyl-CoA (fract. 1 and 2) was dissolved in water (pH 7.5) and assayed by absorption at 260 mμ (adenine ring) and by the hydroxamate formation[2]. The yield was 1.6 μmoles (17%).

The occurrence of two peaks (fract. 1 and 2) for the stearyl-CoA, as has also been described for the synthesis of acetyl-CoA[3], cannot be explained at present. The small radioactivity peak under the free coenzyme A peak (fract. 3) may be explained as a result of an incomplete separation of the stearyl-CoA ester from the unreacted CoA. The presence of the two low-molecular weight compounds (fract. 4 and 5) is probably the result of partial alkaline hydrolysis of some of the ester linkages of the coenzyme A molecule[4].

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CHROM. 4768

A note on the use of Florisil adsorbent for the separation of polychlorobiphenyls from chlorinated pesticides*

The analytical procedures for various types of materials for chlorinated pesticide residues by gas chromatography usually require some form of clean-up prior to the application of the sample to the gas chromatograph. The adsorbent Florisil[1] is commonly used in the clean-up step, and based on BURKE AND MALONE'S report in 1966[2], it was hopefully assumed that a modified product, Florisil PR (pesticide residue grade), had become available with consistent and reliable adsorbent properties. However, MILLS' work[3] in 1968 indicated that soluble sulfates, reported as Na_2SO_4, varied from 0.15 to 2.44% in twelve batches of the Florisil PR grade, and that this variation would affect the adsorptive characteristics of the Florisil to the detriment of pesticide recoveries from sample materials. It has been recommended to either pretest the adsorbent with a lauric acid procedure[3] or with a known group of chlorinated pesticides[4].

A new problem, which may now exist in pesticide residue analysis, is the possible presence of polychlorobiphenyls (PCB's) in the sample material[5-8]. The chromatographic behavior of the PCB's is so similar to that of some of the chlorinated pesticides that if they are not separated from the pesticides prior to analysis by gas chromatography, the end result will be a misinterpretation of the analytical data.

REYNOLDS[5] recently reported a method which included the use of Florisil, presumably the regular grade and not the PR grade, which separated the PCB's from some of the chlorinated pesticides including DDT. With this procedure, the PCB's were eluted from the column with hexane, and DDT and other pesticides were eluted

TABLE I

PER CENT RECOVERIES OF TECHNICAL GRADE DDT FROM FLORISIL COLUMNS USING THE REYNOLDS PROCEDURE[5]

Adsorbent	*Solvent*	*Eluting agents*[a]	
		200 ml hexane	*250 ml 20% diethyl ether in hexane*
Florisil PR grade	Petroleum ether[b]	97	0
	Hexane	97	0
Florisil regular grade	Petroleum ether	90	7
	Hexane	57	43

[a] The column was first eluted with 200 ml hexane, followed immediately by elution of the same column with 250 ml 20% diethyl ether–hexane solution.

[b] The DDT (10 μg) was placed on the column with either 50 ml of petroleum ether or 50 ml of hexane.

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TABLE II

PER CENT RECOVERIES OF *p,p'*-DDT[a] FROM FLORISIL COLUMNS USING THE REYNOLDS PROCEDURE[5]

Adsorbent	*Eluting agents*				
	Hexane				*250 ml 20% diethyl ether in hexane*
	1st fraction (50 ml)	*2nd fraction (50 ml)*	*3rd fraction (50 ml)*	*4th fraction (50 ml)*	
Florisil PR grade	0	35	51	5	0
Florisil regular grade	0	0	0	23	70

[a] The *p,p'*-DDT (10 μg) was placed on the column with a minimum amount (2 ml) of hexane.

TABLE III

PER CENT RECOVERIES OF CHLORINATED PESTICIDES FROM FLORISIL COLUMNS USING THE REYNOLDS PROCEDURE[5]

Adsorbent	*Eluting agent*	*Lindane*	*Heptachlor*	*Aldrin*	*Heptachlor epoxide*	*DDE*	*DDD*	*p,p'-DDT*	*Dieldrin*
Florisil PR grade[a]	200 ml hexane	89	91	91	0	87	83	88	0
Florisil regular grade		7	94	95	0	93	0	100	0
Florisil PR grade	250 ml 20% diethyl ether in hexane	6	0	0	96	0	0	0	97
Florisil regular grade		92	0	0	67	0	94	0	94

[a] The columns were packed with PR and regular grades of Florisil which had been desiccated 24 h after treatment for 16 h at 130°. All other columns described in Tables I–III were packed with hot Florisil immediately after its removal from a 130° oven.

TABLE IV

PER CENT RECOVERIES OF CHLORINATED PESTICIDES FROM FLORISIL COLUMNS USING THE FDA PROCEDURE[4]

Adsorbent	*Eluting agents*							
	200 ml 6% diethyl ether in petroleum ether						*200 ml 15% diethyl ether in petroleum ether*	
	Lindane	*Heptachlor*	*Aldrin*	*Heptachlor epoxide*	*DDE*	*DDD*	*p,p'-DDT*	*Dieldrin*
Florisil PR grade	89	96	99	100	125[a]	98	100	39
Florisil regular grade	91	88	96	93	92	93	93	95

[a] DDE and dieldrin superimposed on gas chromatograph chart under the conditions used; therefore the recovery estimate is based on DDE calculation.

with a subsequent mixture of diethyl ether–hexane. Using both the regular grade and PR grade of Florisil, we were unable to repeat the work of REYNOLDS.

As noted in Tables I–III, all or part of the DDT was eluted from Florisil columns of either grade with the hexane solution, depending upon the conditions used. Table IV shows that our supply of Florisil regular grade performed satisfactorily as prescribed by the official procedure of the Food and Drug Administration (FDA)[4]. However, our supply of Florisil PR grade did not differentiate wholly the dieldrin fraction.

It is apparent that the procedure recommended by REYNOLDS[5] for the separation of PCB's from pesticides will require a preliminary examination of the pesticide elution characteristics of the Florisil, regular or PR grade, prior to the use of the adsorbent for this purpose.

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CHROM. 4767

A chromogenic agent for detecting dimethylurea herbicides on thin-layer chromatographic plates

The methods for the detection of dimethylurea herbicides on thin-layer chromatographic plates are numerous[1–4]. In addition to these existing methods we found that spraying the chromatogram with aqueous potassium permanganate and then exposing the plate to UV irradiation is a general and reproducible method for the detection of dimethylurea herbicides. Test herbicides were spotted on 250-μ Silica Gel G plates and were developed with benzene–acetone (2:1) or benzene–methyl ethyl ketone (2:1). The herbicides were detected by spraying the plates with saturated aqueous potassium permanganate and exposing the sprayed plates to a mercury vapor arc lamp (Hanovia No. 30620).

TABLE I

TLC OF HERBICIDES ON SILICA GEL G PLATES

Herbicides	*Amounts detected (μg)*	*R_F values*	
		Benzene–acetone	*Benzene–methyl ethyl ketone*
Fenuron	1–2	0.52	0.29
Monuron	1–2	0.58	0.30
Fluormeturon	1–2	0.52	0.33
Diuron	2–4	0.67	0.36
Chloroxuron[a]	1	0.49	0.26
p-Bromofenuron	1–2	0.53	0.29

[a] This spot is detectable without UV irradiation.

Table I gives the amounts in which the herbicides were detectable as a yellow spot on a purple background.

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CHROM. 4743

Thin-layer chromatography of some plant phenolics

In the course of studies on the role of phenolics in plant metabolism it became necessary to separate and identify a number of phenolic compounds. Although there have been many reports in the literature on thin-layer chromatography (TLC) used for this purpose[2–5] much of the information available for the compounds of interest has been obtained using paper chromatography[6–8]. In order to obtain the advantages of speed and sensitivity available with TLC we felt it necessary to determine the R_F values of various standards by this technique.

TLC was carried out on 20 × 20 cm MN-polygram CEL 300 cellulose sheets obtained through Brinkmann Instruments, Inc. The solvents used were 2% formic acid (A), 20% potassium chloride (B), isopropyl alcohol–ammonium hydroxide–water, 8:1:1 (C), and 10% acetic acid (D). The solvents were prepared fresh immediately before use. The plates were examined under short-wave UV light after developing and the properties of the compounds were noted. The plates were then sprayed with freshly prepared diazotized *p*-nitroaniline or diazotized sulfanilic acid[1] and the colors which developed were noted within 20 min.

The characteristics of a number of phenolics, coumarins and flavonoids in this system are reported in Table I. Detection of the compounds could be made at the 0.2–1.0 μg range.

TABLE I

CHARACTERISTICS OF SOME PHENOLICS, FLAVONOIDS AND COUMARINS

Compound	R_F				*Detection*		
	A	*B*	*C*	*D*	*UV*	*DSA*	*DNA*
Phenolics							
Acids							
m-Anisic	0.66	0.67	0.49	0.65	F[a]	lt. tan	—
p-Anisic	0.56	0.62	0.49	0.53	Q[b]	tan	tan
Caffeic	0.11	0.16	0.23	0.36	bl. F	tan	grey
Chlorogenic	0.08	0.45	0.49	0.68	bl. F	grn-tan	tan
trans-Cinnamic	0.64	0.57	0.44	0.54	Q	—	—
o-Coumaric	0.34	0.39	0.29	0.51	F	orange	violet
m-Coumaric	0.43	0.26	0.33	0.52	F	orange	violet
p-Coumaric	0.34	0.33	0.28	0.47	Q	red	blue
3,4-Dihydroxyhydrocinnamic	0.18	0.64	0.70	0.76	Q	brown	violet
3,4-Dimethoxybenzoic	0.53	0.32	0.50	0.66	F	tan	violet
Ferulic	0.28	0.21	0.19	0.43	bl. F	purple	blue
Gallic	0.09	0.33	0.31	0.46	F	grn-brwn	tan
Gentisic	0.29	0.37	0.32	0.54	bl. F	yel-brwn	tan
o-Hydroxybenzoic (salicylic)	0.70	0.57	0.44	0.62	bl. F	yellow	red
m-Hydroxybenzoic	0.37	0.63	0.58	0.70	F	yellow	rose
p-Hydroxybenzoic	0.27	0.59	0.52	0.70	Q	yellow	red
3-Hydroxy-4-methoxycinnamic	0.26	0.14	0.21	0.39	F	orange	purple
Isochlorogenic	0.05	0.04	0.10	0.26	F	grn-tan	yel-tan
p-Methoxycinnamic	0.63	0.09	0.14	0.32	Q	—	—
5-Methoxysalicylic	0.07	0.44	0.41	0.60	bl. F	brown	—

TABLE I *(continued)*

Protocatechuic	0.05	0.51	0.44	0.60	bl. F	tan	grey
Pyrocatechuic	0.19	0.48	0.44	0.63	bl. F	tan	violet
α-Resorcylic	0.21	0.48	0.45	0.59	Q	red-orange	yellow
β-Resorcylic	0.30	0.40	0.39	0.54	bl. F	yel-brwn	yellow
γ-Resorcylic	0.71	0.39	0.66	0.81	F	brown	orange-tan
Sinapic	0.18	0.14	0.23	0.38	bl. F	violet	purple
Syringic	0.10	0.46	0.43	0.63	F	red	blue
Vanillic	0.18	0.52	0.44	0.63	F	orange	violet
Aldehydes							
2,4-Dihydroxybenzaldehyde	0.37	0.43	0.54	0.65	F	brown	brown
2,5-Dihydroxybenzaldehyde	0.38	0.52	0.62	0.71	bl. F	grn-brwn	green
3,4-Dimethoxybenzaldehyde	0.94	0.45	0.66	0.78	y. F	—	—
4,6-Dimethoxysalicylaldehyde	0.90	0.08	0.18	0.38	F	yellow	—
m-Hydroxybenzaldehyde	0.79	0.57	0.69	0.78	Q	yellow	—
p-Hydroxybenzaldehyde	0.64	0.56	0.68	0.76	Q	yellow	tan
Protocatechualdehyde	0.36	0.50	0.60	0.68	Q	grn-grey	tan
Salicylaldehyde	0.63	0.52	0.63	0.71	F	orange	—
Syringaldehyde	0.46	0.48	0.62	0.73	Q	pink	bl-grey
Vanillin	0.62	0.53	0.63	0.73	Q	orange	violet
o-Vanillin	0.61	0.49	0.61	0.74	F	yellow	pink
Alcohols and phenols							
Catechol	0.42	0.65	0.69	0.77	Q	brown	brwn-viol
Coniferyl alcohol	0.89	0.38	0.56	0.66	Q	violet	bl-grey
Eugenol	0.98	0.09	0.27	0.32	—	red	blue
Guaiacol	0.95	0.69	0.71	0.79	—	orange	violet
o-Hydroxybenzyl alcohol	0.92	0.71	0.83	0.85	Q	gold	rose
Isoeugenol	0.94	0.06	0.15	0.21	Q	peach	violet
Isovanillyl alcohol	0.83	0.67	0.79	0.83	Q	orange	purple
Phloroglucinol	0.16	0.42	0.53	0.63	—	yel-brwn	brown
Flavonoids							
Apigenin	0.17	0.00	0.00	0.00	Q	gold	yellow
Catechin	0.08	0.20	0.31	0.58	Q	yel-grn	brown
Chrysin	0.64	0.00	0.00	0.04	Q	yellow	orange
Fisetin	0.05	0.00	0.00	0.02	y. F	orange	oran-brwn
Fustin	0.09	0.24	0.33	0.50	F	tan	tan
Hesperetin	0.32	0.02	0.04	0.16	Q	orange	violet
Kaempferol	0.02	0.00	0.00	0.02	or. F	yellow	yellow
Morin	0.02	0.00	0.02	0.13	or. F	orange	yellow
Myricetin	0.00	0.00	0.00	0.00	y. F	grn-brwn	grn-brwn
Naringenin	0.48	0.03	0.04	0.19	—	gold	yel-brwn
Phloretin	0.48	0.00	0.03	0.05	Q	orange	yellow
Quercetin	0.03	0.00	0.00	0.01	gr. F	grey-grn	brown
Rutin	0.04	0.15	0.29	0.49	Q	tan	yel-grn
Coumarins							
Coumarin	0.46	0.43	0.56	0.72	Q	orange	violet
5,7-Dihydroxy-4-methylcoumarin	0.45	0.06	0.10	0.34	F	orange	orange
5,7-Dimethoxycoumarin	0.97	0.06	0.14	0.25	bl. F	yellow	red-brwn
Esculetin	0.16	0.21	0.28	0.46	bl. F	yel-brwn	yellow
Esculin	0.45	0.54	0.61	0.75	F	yellow	—
4-Hydroxycoumarin	0.72	0.41	0.50	0.57	F	yellow	violet
4-Methyldaphnetin	0.21	0.19	0.29	0.47	F	yellow	yellow
4-Methylumbelliferone	0.62	0.21	0.34	0.64	bl. F	tan	tan
Scopoletin	0.47	0.17	0.32	0.49	bl. F	violet	yellow
Umbelliferone	0.60	0.32	0.43	0.61	bl. F	yel-brwn	yel-brwn

[a] F indicates fluorescence.
[b] Q indicates quenching.

The system used proved satisfactory in most respects. Two-dimensional chromatography using the following solvent combinations could be used to separate mixtures of phenolics: A–C, B–C, D–C, and D–B. Solvent C proved to be unsatisfactory for use as the first solvent in a two-dimensional system.

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CHROM. 4776

A spray reagent for methylated phenolic compounds on thin-layer plates

Phosphomolybdic acid is a nonspecific reagent which gives blue spots with phenolic compounds and their methyl ethers[1] and also with lipids[2] on thin-layer plates. In order to develop a specific spray reagent for some classes of methylated phenols, use has been made of the characterist iccolour reaction of certain phenolic methyl ethers when these are dissolved in conc. HNO_3 (ref. 3). Examination of conc. HNO_3 as a spray reagent with various types of phenolic methyl ethers has shown its suitability as a sensitive spray reagent on thin-layer chromatograms of Silica Gel G (E. Merck). The results are detailed in Table I.

TABLE I

COLOUR DETECTION OF PHENOLIC METHYL ETHERS
The sensitivity in each case was 5 μg.

Sample No.	*Name of the compound*	*Colour produced*	*Time taken for colour development*
1	Phloroglucinol trimethyl ether	Deep blue	Immediately
2	Resorcinol dimethyl ether	Green	Immediately
3	Tetra-O-methyl-ellagic acid	Light blue	Immediately
4	Vanillin	Deep yellow	Immediately
5	Ferulic acid	Deep yellow	2 min
6	Vanillic acid	Yellow	5 min

Fully methylated ethers of pyrogallol, catechol and gallic acid produce no specific colour even when present in higher concentration ($> 10\ \mu g$); only faint dirty yellow spots are produced (due to nitric acid). This spray reagent does not give any colour with nonmethylated phenolic compounds.

The study of fully and partially methylated derivatives of phenolic compounds is of great importance in the determination of the structure of flavonoid compounds and condensed tannins. From the results, it appears that those compounds with a free methine (=CH–) group between two methoxyl groups or having an activating group such as carbonyl or else unsaturation in their structures produce characteristic colours, whereas methyl ethers of vicinal trihydroxy phenolic compounds do not produce characteristic colours. Due to a lack of authentic samples, we have not tested for reactions with methylated derivatives of flavonoids (esp. flavanones), but flavonoids containing a carbonyl group are likely to give characteristic colour reactions.

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CHROM. 4745

The separation and detection of methionine and its α-keto and α-hydroxy analogues on thin-layer chromatograms

The study of the biological interconversion of methionine, 2-keto-4-methylthiobutyric acid (methionine keto analogue, MKA), and 2-hydroxy-4-methylthiobutyric acid (methionine hydroxy analogue, MHA) required a method for their chromatographic separation and qualitative detection. The method described herein fulfills the need for such a system.

Standard solutions of methionine, MKA, MHA, and a mixture of the compounds were spotted on thin-layer chromatograms (silica gel; Eastman Chromagram Sheet No. 6061) and dried for 10 min in an oven at 85°. The chromatogram was developed, ascendingly, using 1-butanol–acetic acid (glacial)–water (170:25:5) until the solvent front had migrated 15 cm past the origin (about 2 h) after which the chromatogram was air dried. Methionine was detected by spraying with a 1% acetone solution of ninhydrin followed by heating at 85°. After circling the methionine spot, MKA and MHA were detected by a modification of the method of McCarthy and Sullivan[1]. The chromatogram was sprayed with a 1% aqueous solution of glycine immediately followed by spraying with a 14.3 *N* NaOH solution. After 30 sec the excess fluid was removed by patting with filter paper followed by spraying with a 10% aqueous solution of sodium nitroprusside. The MKA immediately appeared as a deep purple spot. The chromatogram was again patted dry and the location of the MKA was noted. The chromatogram was then heated for 5 min at 85° during which the MKA spot disappeared. Spraying with an acid mixture (12 *N* HCl–85% H_3PO_4, 9:1) resulted in the MHA appearing as a light violet-red spot and after about 5 min the MKA area reappeared as a light blue area, with all spots fading within 30 min. To establish that the MKA spot was actually the keto analogue, a separate chromatogram was sprayed with 0.4% 2,4-dinitrophenylhydrazine (in 2 *N* HCl) and 10% NaOH according to the method of Newcombe and Reid[2]. The yellow spot that developed coincided exactly with the deep purple and light blue spots noted as belonging to the MKA in the former method.

The 2-keto-4-methylthiobutyric acid was a gift of Dr. Minoru Nakano, Department of Biochemistry, School of Medicine, Gunma University, Maebashi, Gunma,

TABLE I

THE R_F VALUES AND MINIMUM DETECTABLE QUANTITIES (MDQ) OF METHIONINE, 2-KETO-4-METHYLTHIOBUTYRIC ACID, AND 2-HYDROXY-4-METHYLTHIOBUTYRIC ACID ON SILICA GEL THIN-LAYER CHROMATOGRAMS

Developing solvent: 1-butanol–glacial acetic acid–water (170:25:5). Detection methods are given in the text.

Compound	R_F	*MDQ (μmoles)*
Methionine	0.35	0.001–0.01
2-Keto-4-methylthiobutyric acid	0.68	0.1 (purple)
		0.05 –0.1 (l. blue)
2-Hydroxy-4-methylthiobutyric acid	0.86	0.1 –0.5

Japan. The 2-hydroxy-4-methylthiobutyric acid is a commercial product of the Monsanto Company, St. Louis, Mo. 63166, U.S.A. and was donated through the courtesy of Mr. N. L. REDING.

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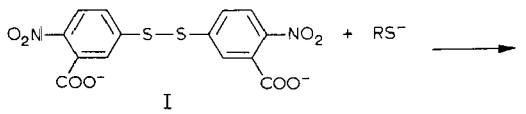
J. Chromatog., 50 (1970) 150–151

CHROM. 4738

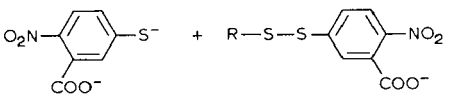
Chromatographic detection of thiols, disulfides, and thioesters with 5,5′-dithiobis(2-nitrobenzoic acid)

Few reagents are available for the detection of sulfhydryl containing compounds on chromatograms. Sodium nitroprusside[1], platinic iodide[2], or various quinones[3], which are not specific for sulfhydryl, have been used. Recently GRASSETTI AND MURRAY[4] reported the use of 2,2′-dithiobis(5-nitropyridine) as a selective reagent for the detection of thiols.

We have used alcohol–buffer solutions of 5,5′-dithiobis(2-nitrobenzoic acid)[5] (I, DTNB, ELLMAN reagent) as a convenient and sensitive spray reagent for the visualization of thiols on chromatograms as yellow spots*. The reagent reacts specifically with sulfhydryl groups by a disulfide exchange reaction to give the yellow thioanion (II) of 2-nitro-5-mercaptobenzoate[5].



I



II

We have extended this method to the chromatographic detection of disulfides and thioesters, which are both easily converted to their thiol derivatives: the former by reduction with sodium borohydride and the latter by alkaline hydrolysis.

* GRASSETTI AND MURRAY[4] reported that DTNB "does not appear to be suited as a spray reagent in organic media" for the chromatographic detection of thiols.

Experimental

Reagents

(a) DTNB reagent: 0.1% solution of 5,5'-dithiobis(2-nitrobenzoic acid) (Calbiochem, Los Angeles, Calif., U.S.A.) in a 1:1 mixture of ethanol and 0.45 *M* tris-(hydroxymethyl)aminomethane hydrochloride buffer at pH 8.2.

(b) Borohydride reagent: freshly prepared 0.4% solution of sodium borohydride (Metal Hydrides, Inc., Beverly, Mass., U.S.A.) in 95% ethanol.

(c) Acid solution: a mixture of glacial acetic acid, 6 *N* hydrogen chloride and acetone (8:2:90).

(d) Hydrolysis reagent: 0.2 *N* sodium hydroxide solution in 50% aqueous ethanol.

(e) pH indicator: 0.1 g phenol red (phenolsulfonephthalein) in 28.2 ml 0.01 *N* NaOH, diluted with water to 250 ml.

Procedures

All reagents were applied by aerosol spray (Nutritional Biochemicals, Cleveland, Ohio, U.S.A.) in a ventilated hood. Approximately 10^{-6} mole of each compound to be tested was spotted on filter paper or on thin-layer plates [silica, aluminum oxide, cellulose, polyamide (Eastman Kodak, Rochester, N.Y., U.S.A.)]. Development of a yellow color within a few seconds after spraying the DTNB reagent was called a "positive result". The color persisted for at least 15 h.

Thiols. The DTNB reagent was applied.

Disulfides. The borohydride reagent (one volume) was applied to the chromatogram. 15–20 min later the borohydride was destroyed by spraying with the acid solution (three volumes)*. The chromatogram was air dried for 1 h. pH indicator (Phenol Red) was spotted in several positions along the edges of the chromatogram which was then exposed to an ammonia atmosphere in a glass cylinder containing a dish with concentrated ammonia until the indicator spots turned red. After air drying for 4–5 min the DTNB reagent was applied.

Thioesters. The chromatogram was sprayed with the hydrolysis reagent followed 2 to 3 min later by the DTNB reagent. A yellow background gradually developed on standing.

Results and discussion

Compounds (thiols) which gave yellow spots on chromatograms within a few seconds after spraying with a solution of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB reagent) are listed in Table I. Cysteine was easily detected at 2×10^{-9} mole. 2-Mercaptouracil and 6-mercaptopurine gave negative results, probably due to the double bond character of the thiol function.

The specificity of DTNB for sulfhydryl was further corroborated by negative results obtained with S-protected cysteine derivatives, thioethers, disulfides, and thioesters (Table I). None of the common amino acids gave a positive test except cysteine. The high sensitivity and specificity of this procedure equals that obtained

* Complete decomposition of the reducing agent is essential. Residual sodium borohydride would interfere with the thiol test due to reduction of DTNB. Other reducing agents such as sodium bisulfite and sodium thiosulfate interfere similarly.

TABLE I

RESULTS OF DTNB SPRAY ON CHROMATOGRAMS

+, Appearance of yellow color within a few seconds. —, No color within 15 h.

Compound	*DTNB reagent alone*	*After reduction*	*After alkaline hydrolysis*
Cysteine	+		
N-*tert.*-Butyloxycarbonylcysteine methyl ester	+		
2-Mercaptopyridine	+		
Dithiothreitol	+		
Glutathione (reduced)	+		
Coenzyme A	+		
S-blocked cysteine derivatives[a]	—		
Methionine	—	—	
Thiourea	—	—	
Biotin	—	—	
Cystine	—	+	
Glutathione (oxidized)	—	+	
Vasopressin	—	+	
Neocarzinostatin	—	+	
Lysozyme	—	+	
Acetyl coenzyme A	—		+
N-Acetylhomocysteine thiolactone	[b]		+
Ethyl thioacetate	—		+[b]
n-Propyl thiopropionate	—		+
n-Butyl thioacetate	—		+
N-Benzyloxycarbonyl-S-acetylthiothreonine methyl ester	—		+
Butyryl thiocholine iodide	—		+

[a] Blocking groups used were: methyl, benzyl, diphenylmethyl, triphenylmethyl, benzylthiomethyl, benzyloxycarbonyl, tetrahydropyranyl, and ethylcarbamoyl.

[b] See discussion.

with 2,2′-dithiobis(5-nitropyridine) which recently has been recommended[4] for the chromatographic detection of thiols.

Disulfides were detected by DTNB reagent after reduction to thiols (Table I). An efficient reducing agent for this purpose was sodium borohydride in 95% ethanol. This test should be particularly useful for the detection of disulfide-containing amino acids and peptides on two-dimensional chromatograms. A peptide map of a peptic–chymotryptic hydrolysate of lysozyme gave seven yellow spots of disulfide-containing peptides*. The proteins lysozyme and neocarzinostatin gave positive results for disulfide without denaturation prior to application on filter paper.

The DTNB reagent was also used for the detection of thioesters on chromatograms after alkaline hydrolysis by spraying with aqueous ethanolic sodium hydroxide (Table I). N-Acetylhomocysteine thiolactone gave a positive result after 5 to 10 min with the DTNB reagent alone, probably due to hydrolysis at pH 8.2.

Highly volatile thiols generated on disulfide reduction or thioester hydrolysis may escape detection. However, by applying the reagents within 1 min, it was possible to detect ethyl mercaptan obtained from ethyl thioacetate.

* From the amino acid composition of the spots the positions of all four disulfide bonds of lysozyme could be independently assigned. This rapid determination of the positions of disulfide bridges in proteins will be reported elsewhere[6,7].

In conclusion, a rapid and sensitive technique has been developed for the identification and differentiation of thiols, disulfides, and thioesters on thin-layer and paper chromatograms. Thiols are detected as yellow spots after spraying chromatograms with an alcohol-buffer solution of the readily available 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman reagent, DTNB). Disulfides can be detected after reduction to thiols with sodium borohydride and application of the DTNB spray reagent. Thioesters are hydrolyzed by alkali and the resulting thiols are treated with DTNB.

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CHROM. 4744

Chromatography of sulphur: separation from hydrocarbons*

In an investigation of components of sewage sludges, we have found elemental sulphur in hydrocarbon fractions eluted from liquid adsorption columns. Free sulphur is commonly found in hydrocarbon sources such as petroleum and lake sediments, and is to be expected in any organic matter that has undergone successive reducing and oxidising conditions. However, there is little available information on the chromatographic behaviour of sulphur and its separation from hydrocarbons with which it is found.

Sulphur has been separated from polycyclic aromatic components of coal tar pitch by chromatography on alumina[1] and from the compounds S_7NH, $S_6N_2H_2$, and S_4N_4 on columns of alumina or silica gel[2-4], but no retention of sulphur relative to the solvent was reported.

This report shows that elemental sulphur can be separated from very weakly adsorbed classes of hydrocarbons, but not from slightly more strongly adsorbed classes, by chromatography on the usual adsorbents. Most of the experiments were carried out using alumina but similar results were obtained with silica gel and Florisil.

Experimental

Alumina (Aluminum oxide, neutral, Brockmann activity, grade 1; J. T. Baker Chemical Co.) was activated at 170° overnight. Columns were prepared in 1 cm diameter tubes by the slurry technique using *n*-heptane (*n*-heptane, pure grade, Phillips Petroleum Company) that had been distilled before use. Packed column length was 20 cm.

The hydrocarbon (100 mg) to be chromatographed was dissolved in 3 ml of *n*-heptane that had been kept over powdered sulphur. The solution was added to the column and the components were eluted with *n*-heptane. The eluate was collected in fractions (*ca.* 2 ml) from which the solvent was removed by evaporation in a stream of nitrogen.

Visual inspection or determination of the IR absorption spectrum identified the residue.

Results

On columns of alumina under the described conditions, eicosane (*n*-$C_{20}H_{42}$) and squalane (2,6,10,15,19,23-hexamethyltetracosane) were eluted before sulphur and were completely separated from it. Tetradecahydrophenanthrene was eluted ahead of sulphur but the separation was not quite complete. 1-Phenyldodecane was eluted slightly ahead of sulphur but the resolution was far from complete. Biphenyl was eluted after sulphur and was completely separated from it.

For comparison purposes mixtures of the hydrocarbons were chromatographed in a similar manner. Under these conditions eicosane and squalane were not appreciably separated from each other, but they were eluted before tetradecahydrophenanthrene and were largely resolved from it. All three saturated hydrocarbons were eluted before 1-phenyldodecane and were completely resolved from it.

* Report No. OR 69-6.

Conclusions

Elemental sulphur will pass through liquid chromatographic procedures along with hydrocarbons, and can be expected to contaminate the polycyclic naphthenic and monocyclic aromatic fractions of hydrocarbons purified or fractionated by this technique.

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CHROM. 4773

Isolation of ammonia by use of zirconium phosphate cation exchanger

Most methods currently utilized for the separation of ammonia from interfering compounds are based on the principle of gaseous diffusion of ammonia from an alkaline medium and absorption by an acidic medium[1–3].

This communication describes a simple method for separating ammonia from interfering substances. Zirconium phosphate is utilized as an inorganic cation exchanger to adsorb the ammonium ion thus separating it from interfering compounds. Once eluted, the ammonium ion can be oxidized by hypochlorite and reacted with phenol to form a compound whose absorbance obeys Beer's law[4].

Reagents and chemicals

For the preparation of sodium phenolate 100 g NaOH were dissolved in water and 120 g phenol added; the final volume was brought to 500 ml. Commercial grade clorox was used as the source of sodium hypochlorite. Zirconium phosphate (50–100 mesh) was supplied by Bio-Rad Laboratories.

Other chemicals used were concentrated nitric acid and cesium chloride.

Column apparatus

The zirconium phosphate column (0.8 cm × 0.8 cm) is connected to two reservoirs by a 3-way stopcock (Fig. 1). Flow rates are controlled by a modification of the Mariotte principle[5]. The column is equipped with a removable cap to facilitate loading.

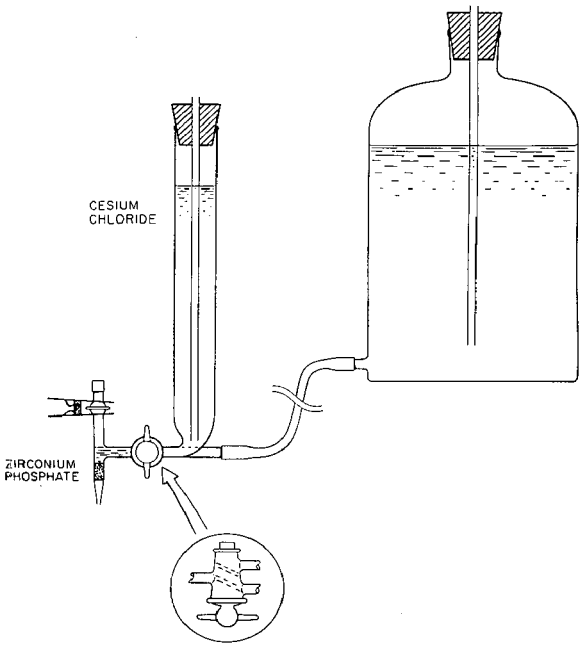


Fig. 1. Zirconium phosphate column for isolation of ammonia.

Method

This procedure contains two fundamental operations. First, ammonia is separated from indigenous interfering substances by column chromatography. Then it is eluted from the column and quantified.

A 1-ml aliquot of sample is adsorbed on the ion-exchange column. The column is rinsed with water (3 ml) at a flow rate of 7.5 ml min^{-1} cm^{-2} to remove contaminants. The adsorbed ammonia is displaced by flowing 1 ml of cesium chloride (1.24 *M*) at a

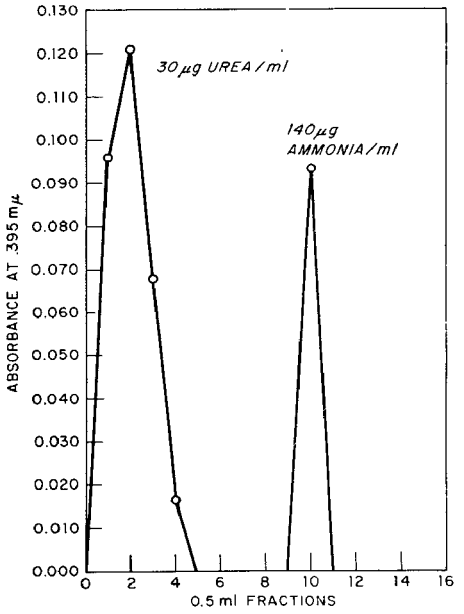


Fig. 2. Elution curve showing the separation of ammonia and urea on the zirconium phosphate column.

rate of 0.05 ml min^{-1} cm^{-2}. Subsequent to elution of ammonia the column is regenerated with concentrated nitric acid and the cesium recovered for re-use. After elution of the cesium, residual acid is removed by rinsing with water.

To 1 ml of the eluate from the zirconium phosphate column 0.7 ml phenol reagent and 0.4 ml sodium hypochlorite (5.25%) are added. After 5 min the absorbance is measured at a wavelength of 395 mμ or 625 mμ, depending on the concentration range.

Results and discussion

Ammonium ion can be eluted from zirconium phosphate either by increasing the acidity[6] or displacing it by the addition of a competing ion with a larger distribution

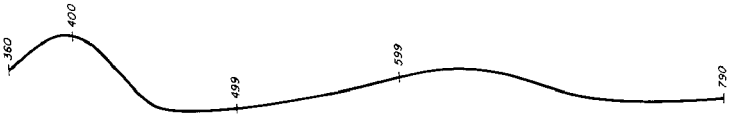


Fig. 3. Absorption spectrum of the phenol–indophenol complex.

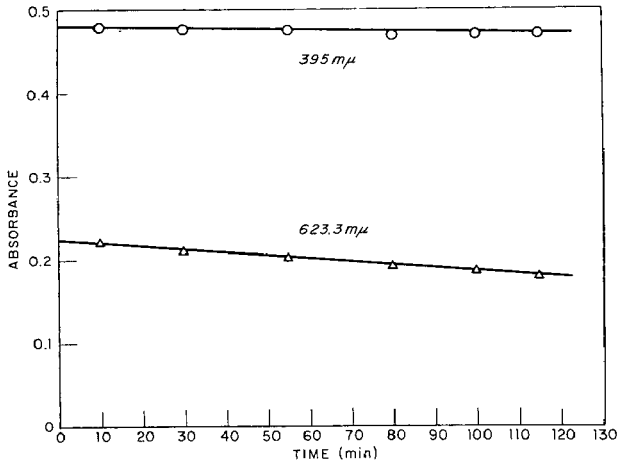


Fig. 4. Stability of the phenol–indophenol compound with time at 395 mμ and 623.3 mμ.

coefficient. The high distribution coefficient of the ammonium ion necessitated the utilization of concentrated nitric acid for its elution from the ion-exchange column. The concentrated acid, however, interfered with subsequent operations. Therefore, displacement of ammonia with cesium was chosen.

To evaluate the ability of this procedure to separate ammonia adequately from interfering biological substances two probable contaminants were tested. Fig. 2 indicates the ease by which ammonia can be isolated in the presence of urea. Similar separations were obtained in the presence of glutamine.

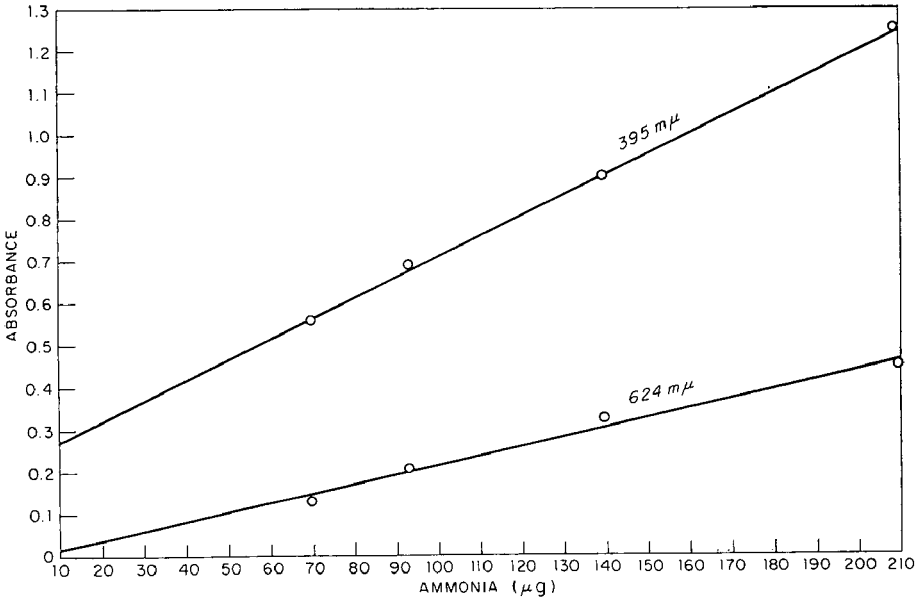


Fig. 5. Calibration curve relating the absorbance at 395 mμ and 623.3 mμ with concentration of ammonia.

An absorption spectrum (Fig. 3) of the phenol–indophenol complex was taken and from this spectrum it is apparent that measurement can be made at either of two wavelengths. Experience indicated that the stability of the compound with time was different at the two wavelengths (Fig. 4). Decrease in absorbance occurred at 625 mμ but the absorbance at 395 mμ remained stable. Since absorbance at 395 mμ was stable with time and gave maximum sensitivity, this became the wavelength of choice. Eqns. 1 and 2 are the equations of the line of best fit derived by linear regression from the calibration data (Fig. 5) taken at 624 and 395 mμ, respectively.

$$\mu g\ NH_4 = \frac{abs + 0.003}{0.00222} \quad (1)$$

$$\mu g\ NH_4 = \frac{abs - 0.225}{0.00488} \quad (2)$$

The zirconium phosphate column has possible applications to a wide range of chemical systems. Modifications of the chromatographic parameters would make possible measurement of ammonia in biological extracts and other preparations.

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Letters to the Editor

CHROM. 4759

In the note "A gas chromatographic study of the kinetics of the uncatalysed esterification of acetic acid by ethanol" (*J. Chromatog.*, 47 (1970) 265–268) the authors conclude from the linear time dependence of $1/(a-x)$ that the reaction is of second order and proceed to draw further conclusions from the rate constants thus found. Now, from the experimental procedure described, it follows that the reaction medium is an equimolecular mixture of ethanol and acetic acid without a solvent, in which at small conversions the *concentrations* of the reactants do not diminish even when their amounts decrease. Kinetic equations, however, refer to concentrations. The total conversion in the experiment given in detail is only 3.5%, and no appreciable decrease in reaction rate with conversion can be expected. Indeed, $(a-x)$ is equally linear with time which formally would indicate a zero-order reaction! No conclusions as to the kinetics of the reaction can be drawn from these experiments, and hence E appears to be the only numerical value of any significance.

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CHROM. 4765

In two recent papers[1,2] referring to two earlier papers of mine[3,4], Dr. GRAHAM suggests that it is not certain whether the ion-exchange mechanism was effected using my experimental technique, since the paper strips were impregnated with free amine so that amine thiocyanate could only be formed *in situ* during development with the aqueous solutions of NH_4SCN.

I would like to explain that the paper strips were, in fact, impregnated with the thiocyanate form of the amine, prepared by shaking 0.2 *M* benzene solutions of amine with aqueous solutions of NH_4SCN acidified with an equivalent amount of H_2SO_4. In view of the higher affinity of HSCN to amines, extraction of sulphate ions is negligible[5].

The misunderstanding is presumably due to the fact that in the preliminary communication[3] and in the English summary of the full text[4] it is stated that paper strips were impregnated with amine, full details of the technique being given in the Polish text[4].

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Book Review

CHROM. 4779

An Introduction to Gel Chromatography, by L. FISCHER, North-Holland Publishing Company, Amsterdam, London, 1969, 246 pages, price D*f* 25.—, $ 2/18/—.

This book is a paperback pocket edition of volume I, part II, of the series *Laboratory Techniques in Biochemistry and Molecular Biology*, edited by T. S. WORK AND E. WORK. The technique described is the one more commonly but less aptly known as "gel filtration". The variant known as "gel permeation chromatography", which uses more specialised equipment and is concerned with the fractionation of organic polymers in non-aqueous solvents, receives only brief mention.

The author first explains the parameters used to describe solute behaviour in gel chromatography, then describes the various types of gel and the equipment used in this technique. Experimental procedures for column and thin-layer gel chromatography are minutely explained. The description of preparative and analytical applications includes short accounts of the estimation of molecular weights and molecular weight distribution and of the measurement of complex formation between small and large molecules. The present uncertainty about the mechanism of gel chromatography is discussed in the final chapter. Useful items in the appendices include a table of manufacturers and suppliers of materials and equipment for gel chromatography and some analytical and detection methods for solutes in column effluents and thin gel layers.

The book succeeds in its aim to give sound practical advice to readers with no previous experience of gel chromatography, and it should enable them to use the technique satisfactorily. In a book at this level, however, more illustrated examples of the fractionation of mixtures of well-characterised substances would have been helpful, particularly if separations obtained with different gels had been compared. The author does not deal with the fractionation of major classes of substances in a systematic manner, and it is surprising to find no typical separations indexed under "peptides", "proteins", "oligosaccharides" or "polysaccharides". Anyone wishing to use gel chromatography for studying hydrogen exchange or interacting systems, or even for molecular size or molecular weight estimation, would need to find more information elsewhere.

The emphasis in this book is on the use of Pharmacia products, but this is probably as much a reflection of current practice as of the author's affiliation.

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Bibliography Section

EDITORIAL

In the last several years, man's attempt to learn more about the essentials of life has benefited from advances in separation procedures. A considerable increase in the number of papers published about the chromatographic and electrophoretic procedures for investigating proteins, enzymes and nucleic acids has been the result. The classification system used in the Bibliogrpahy in the past is now inadequate as it does not allow one rapidly to locate separation procedures for the substances mentioned above. To make the classification scheme more useful, some alterations have been made, mainly in the sections concerning column chromatography and electrophoretic techniques. Beginning with this issue of the *Journal of Chromatography*, this modification starts to appear in Sections 20 (proteins) and 21 (nucleic acids). Although it is valid for each section it will be especially useful in the sections on column chromatography and electrophoretic techniques. Currently there are no substantial problems encountered in formulating a classification scheme for enzymes and nucleic acids; however, an exhaustive classification for proteins is still difficult to present. Duplications always exist, especially when the division of proteins into those with and without enzymatic activity has to be respected for practical reasons. The result of our effort is as follows:

20. Proteins (including enzymes).
    - 20a. Proteins of plant origin including bacteria.
    - 20b. Plasma proteins.
    - 20c. Structural proteins and other nuclear proteins.
    - 20d. Protamines, histones and other nuclear proteins.
    - 20e. Chromoproteins.
    - 20f. Varia, with special reference to non-identified and tissue proteins.
    - 20g. Enzymes: oxidoreductases.
    - 20h. Enzymes: transferases.
    - 20i. Enzymes: hydrolases.
    - 20k. Enzymes: lyases.
    - 20l. Enzymes: isomerases.
    - 20m. Enzymes: ligases.
    - 20n. Complex mixtures of enzymes.
21. Purines, pyrimidines, nucleic acids and their constituents.
    - 21a. Purines, pyrimidines, nucleosides, nucleotides.
    - 21b. Nucleic acids: RNA
    - 21c. Nucleic acids: DNA.
    - 21d. Nucleoproteins.
    - 21e. Complex mixtures of nucleic acids.
    - 21f. Structural studies on nucleic acids.

Some further explanations should be supplied to facilitate the use of the above scheme, *viz.*

(1) All structural studies, including those dealing with macromolecular protein fractions, will be listed in Section 19b.

(2) Proteins containing a substantial proportion of non-protein material will be listed according to the nature of the non-protein material, *e.g.* glycoproteins, lipoproteins, and nucleoproteins. However, proteins with a trace amount of non-protein

material, which frequently difficult to distinguish from possible contamination, will be listed in Section 20. Cross-references will be supplied.

(3) In our classification, preference is generally given to the chemical nature of the substance listed. Thus, for example, enzymes containing a metal atom will be included in the section on metalloproteins (Section 20e).

(4) Proteins of bacterial and microbial origin, unless further specified as enzymes or nuclear proteins, will be listed in Section 20a.

(5) Proteins extracted from animal tissues, including hormones of proteinous nature (not peptides), will appear in Section 20f.

(6) The latter statement does not hold for plasma proteins, which are listed separately in Section 20b.

We hope that this new classification system will make the Bibliography more useful both in general and for those who seek separation procedures for a certain type of protein or enzyme.

Paper Chromatography

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See TLC section 15.

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CHROM. 4750

THERMODYNAMIC TREATMENT OF PARTITION EXPERIMENTS WITH SPECIAL REFERENCE TO MOLECULAR-SIEVE CHROMATOGRAPHY

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SUMMARY

Using thermodynamics an equation (eqn. 8) has been derived for the relationship between distribution coefficients and the parameters characterizing the solute (*e.g.* partial molal volume and molecular area) and the gel bed (*e.g.* pressure and interfacial tension). When active transport can be neglected the equation might also give a qualitative picture of the factors that determine the distribution of solutes between the internal space of a living cell and its surroundings. Introduction of a series of assumptions, which are all discussed, leads to simplified formulae (eqns. 14 and 15), which, in spite of their approximate nature, seem to be applicable in most experiments. The considerations herein also apply to regular partition experiments, including ALBERTSSON's aqueous two-phase systems, recalling that no pressure difference exists between two liquid phases, as between the interior of a gel and the surrounding medium.

INTRODUCTION

In molecular-sieve chromatography separation occurs on the basis of relative molecular size. It is therefore natural that a great deal of effort has been made to derive mathematical expressions for the relationship between chromatographic parameters (such as distribution coefficients and elution volumes) and parameters characterising the size of the molecules separated (for instance molecular weight and Stokes radius). Such mathematical treatments have been presented by PORATH[1], SQUIRE[2], LAURENT AND KILLANDER[3], and ACKERS[4] and are reviewed by the present author in ref. 5. The various formulae which have been deduced are different as they are based upon different physical models for the separation mechanism. In this paper thermodynamic considerations are presented and therefore no assumptions about the separation mechanism are required. A series of simplifications must however be introduced in order to arrive at practicably useful expressions. These expressions (eqns. 14 and 15) are therefore very approximate.

DERIVATION OF THE GENERAL FORMULA (EQN. 8)

We first consider, separately, the different terms which make up the final expression for the chemical potential g_i of a solute i.

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(1) *Activity*

The activity a_i of a solute i is by definition related to the chemical potential g_i by the well-known equation

$$g_i = \mu_i = \mu_i^0 + RT \cdot \ln a_i \tag{1}$$

where μ_i^0 refers to the standard state; μ_i is a function of temperature, pressure and the nature of the solvent. Putting $a_i = f_i \cdot c_i$ (f_i = the activity coefficient; c_i = the concentration in moles/l) we obtain

$$g_i = \mu_i = \mu_i^0 + RT \cdot \ln f_i \cdot c_i \tag{2}$$

(2) *Temperature and pressure*

With variations in temperature T and pressure p, g_i changes in accordance with the relation

$$\mathrm{d}g_i = -s_i \cdot \mathrm{d}T + v_i \cdot \mathrm{d}p \tag{3}$$

where s_i is partial molal entropy and v_i is partial molal volume.

(3) *Interfacial tension*

The chemical potential is also a function of the interfacial tension γ_i for the interface between the solute i and the surrounding medium. If A_i is the area of N molecules (N = Avogadro number) of the solute i we obtain

$$\mathrm{d}g_i = \gamma_i \cdot \mathrm{d}A_i \tag{4}$$

(4) *Electric potential*

Assume that each molecule of the solute i has a net electronic charge Z_i ($Z_i < 0$ for anions and > 0 for cations) and that the molecules are located where the electric potential is ψ. The chemical potential for this system is then described by the expression

$$g_i = F \cdot Z_i \cdot \psi \tag{5}$$

where F is the Faraday constant.

(5) *Adsorption*

In the cases where there are other types of interaction between the solute i and the bed material (*i.e.* the gel particles) than the above electrostatic interaction, we get the following contribution to the chemical potential

$$g_i = -E_i \tag{6}$$

where E_i (> 0 for adsorption) is the energy required to desorb N molecules of the solute i.

The total chemical potential is obtained by summation of eqns. 2–6 which gives

$$g_i = \mu_i^0 + RT \cdot \ln f_i \cdot c_i - \int_{T^0}^{T} s_i \mathrm{d}T + \int_{p^0}^{p} v_i \mathrm{d}p + A_i \cdot \gamma_i - A_i^0 \cdot \gamma_i^0 + \\ + F \cdot Z_i \cdot \psi - F \cdot Z_i^0 \cdot \psi^0 - (E_i - E_i^0) \tag{7}$$

where superscript ^{0} refers to the standard state.

For any solute i, distributed between two phases s and m, the chemical potential in phase s is equal at equilibrium to the chemical potential in phase m ($g_{i,s} = g_{i,m}$). Applying this law to a solute i, distributed between the stationary phase (s) and the mobile phase (m) of a chromatographic bed, we obtain the following relationship from eqn. 7 (to obtain a simpler formula we drop the subscripts i)

$$RT \cdot \ln K + \mu_s^0 - \mu_m^0 + v_s p_s - v_m p_m + A_s\gamma_s - A_m\gamma_m + F(Z_s\psi_s - Z_m\psi_m) -$$

$$- (E_s - E_m) + RT \cdot \ln \frac{f_s}{f_m} - (v_s p_s^0 - v_m p_m^0) - (A_s^0\gamma_s^0 - A_m^0\gamma_m^0) -$$

$$- F(Z_s^0\psi_s^0 - Z_m^0\psi_m^0) + (E_s^0 - E_m^0) = 0 \qquad (8)$$

where K is the distribution coefficient, defined by

$$K = \frac{c_s}{c_m} \qquad (9)$$

Selection of standard states

The standard states may be defined in different ways. The following choice is convenient for the present treatment.

In the standard state for the solute in the mobile phase the concentration of the solute (c_m^0) is such that $a_m^0 = f_m^0 \cdot c_m^0 = 1$ ($f_m = a_m/c_m \rightarrow 1$ when $c_m \rightarrow 0$). The pressure is then p_m^0 and the interfacial tension γ_m^0.

In the standard state the solute in the stationary phase is in equilibrium with the solute in the mobile phase, *i.e.*, $g_s^0 = g_m^0$ or according to eqn. 7 $\mu_s^0 = \mu_m^0$; the solute in the stationary phase has at this equilibrium an activity which we put equal to 1. The pressure is then $p_s^0 (p_s^0 \neq p_m^0)$ and the interfacial tension $\gamma_s^0 (\gamma_s^0 \neq \gamma_m^0)$.

APPROXIMATIONS OF THE GENERAL EQN. 8

Eqn. 8 was derived under the assumption that the partial molal volume v is independent of the pressure. This assumption involves no limitations in practice. For example, for proteins v is changed by only about 1 % when the pressure is changed by 1000 atm. (ref. 6). In many experiments the parameters v, A, and Z for a given solute can be expected to have the same values in the two phases. With the above standard states ($\mu_s^0 = \mu_m^0$) eqn. 8 then takes the form

$$RT \cdot \ln K + v[p_s - p_m - (p_s^0 - p_m^0)] + A[\gamma_s - \gamma_m - (\gamma_s^0 - \gamma_m^0)] +$$

$$+ FZ[\psi_s - \psi_m - (\psi_s^0 - \psi_m^0)] - [Es - E_m - (E_s^0 - E_m^0)] + RT \cdot \ln \frac{f_s}{f_m} = 0 \qquad (10)$$

As v, A, and Z are treated as constants in eqn. 10, this equation is only valid if the solute molecules do not alter their structure or configuration, including the "thickness" and the nature of the double layer and the degree of hydration when they are transferred from one phase to the other. Globular proteins probably fulfil this requirement better than chain molecules with a loose structure (flexible polymers). In molecular-sieve chromatography on very tight gels (for instance Sephadex G-10 and Bio-Gel P-2) one may sometimes expect that the concentrations of the ions in

the eluting medium (the buffer) are not the same in the two phases. In such a case $Z_s \neq Z_m$ (the net charge of the solute in the stationary phase differs from the net charge in the mobile phase) as the net charge Z is a function of the composition of the eluting medium. Under these conditions the fourth term in eqn. 10 should be replaced by $F(Z_s\psi_s - Z_m\psi_m) - F(Z_s^0\psi_s^0 - Z_m^0\psi_m^0)$. The net charge has, however, no influence on the K values as long as the gel matrix is neutral, *i.e.* when $\psi = 0$. It should be noted that the potential may differ from zero even if the gel polymer does not contain any charged groups, such as carboxylic groups, since certain ions in the eluting medium may have a greater tendency for interaction with the gel matrix than others. An example of this is shown by droplets of *n*-decanol which migrate in an electric field due to such a type of interaction[7].

We now intend to introduce a series of assumptions in order to simplify eqn. 10 (in several experiments some of these assumptions involve so large an approximation that they cannot be considered as justified):

(*a*) $\psi_s = \psi_m = \psi_s^0 = \psi_m^0 = 0$ *and* $E_s = E_m = E_s^0 = E_m^0 = 0$

In this case, the solute does not show any electrostatic or other interaction with the gel bed. The condition $E = 0$ is not fulfilled when low molecular weight aromatic compounds are chromatographed on tight gels. It should also be recalled that ψ may differ from zero even if the polymer is devoid of dissociable groups, as mentioned above.

(*b*) $v = \bar{v} \cdot M = constant \cdot M$

Here $\bar{v}$ is the partial specific volume and M is the molecular weight of the solute. The approximation that $\bar{v}$ is constant is only justified when groups of solutes having similar chemical structures are considered, so-called isochemical substances. It is well known, for example, that most proteins have a partial specific volume close to 0.74 $cm^3\ g^{-1}$ and carbohydrates close to 0.60. The greater the similarity between the repeating units of a polymer solute, the better is the approximation; it is probably quite satisfactory for members of a homologous series.

(*c*) $p_s - p_m = constant$ *for any given gel bed*

This approximation is equivalent to the assumption that the pressure difference is determined by the nature and the composition (the concentration) of the gel polymer, but not of the solutes.

(*d*) $\gamma_s - \gamma_m = constant$ *for isochemical solutes on any given gel bed*

An indirect indication that the approximations (c) and (d) — and (f) below — may be justified is the observation that the K values vary only slightly with alterations in solute and buffer concentrations[8,9]; see also experiment III in Fig. 1.

(*e*) *Molecular area*

The difficulty of defining the area of a molecule is obvious, because neither the shape, nor the degree of solvation can be determined exactly. In the mathematical treatment we must assume that there is a sharp boundary between the solute and the surrounding medium, although in reality there is a smooth transition (similar assumptions are made in the current theoretical treatment of electrophoretic migration

where the conception of a "slipping plane" is introduced). We will now consider separately non-coiled chain molecules, globular polymers, and flexible polymer chains.

(*e1*) $A = constant \cdot M$. The constant is assumed to have the same value for isochemical substances. This proportionality may be a satisfactory approximation when the solute molecule is in the form of a non-coiled chain. Low molecular weight substances in a homologous series belong to this class of substances.

(*e2*) $A = constant \cdot M^{2/3}$. Here again the constant is assumed to have the same value for isochemical substances. This approximation is valid for solutes which can be considered spherical and therefore approximate for globular proteins. However, the same relation might also be approximately valid for flexible polymer chains. Before producing evidence for this it should be remembered that flexible polymers, for instance dextran chains, are "non free-draining" molecules: TANFORD[10] states that the solvent within the interior of a flexible polymer (perhaps within the radius of gyration) is trapped and is therefore essentially indistinguishable, from a hydrodynamic point of view, from a solvent which might be inherently combined with the polymer chain.

Following the treatment of ONCLEY[11] we write the volume V of a solvated molecule as

$$V = \frac{M}{N}(\bar{v} + \delta \cdot v_s) \tag{11}$$

where δ is the number of grams of solvent per gram of dry macromolecular material and v_s is the specific volume of pure solvent. We now introduce the concept of an equivalent hydrodynamic sphere, which can be visualised as a solid sphere of radius a and with a *volume* equal to the volume of the solvated molecule. Accordingly

$$\frac{4}{3}\pi a^3 = \frac{M}{N}(\bar{v} + \delta \cdot v_s) \tag{12}$$

The *area* of the sphere will differ from the area of the macromolecule. The difference is, however, not large if the solute molecules are not too elongated, as numerical calculations show: if the macromolecules are cylindrical with radius r and height $h = r$, $2r$, and $4r$ the difference is 21, 14, and 20 %, respectively. We can therefore put $A = C^0 \cdot 4\pi a^2 \cdot N$, where C° has a value fairly close to unity (a is not necessarily equal to the Stokes radius). Using eqn. 12 we then obtain

$$A = C^0 \cdot (4\pi N)^{1/3} \cdot 3^{2/3} \cdot (\bar{v} + \delta \cdot v_s)^{2/3} \cdot M^{2/3} \tag{13}$$

The parameters C°, $\bar{v}$, and δ can be considered as constants for isochemical flexible polymer chains, *i.e.*, $A = \text{constant} \cdot M^{2/3}$, which is the same expression as that for globular proteins. The constant has, however, a considerably higher value for flexible polymers, because δ, the degree of solvation, is much larger for these than for globular proteins. One should observe that for $\delta = 0$ and $C^0 = 1$ eqn. 13 gives the area of N rigid spheres.

(*f*) $f_s/f_m = constant$

This assumption involves that the ratio between the activity coefficients in the

stationary and mobile phase has the same value for all isochemical solutes irrespective of their concentrations. This approximation might be valid for low solute concentrations.

Case (A)

When conditions (a), (b), (c), (d), (e1), and (f) are fulfilled, eqn. 10 can be simplified to

$$-\log K = C_1' \cdot M + C_0 \tag{14}$$

where C_1' is a sum of two terms, one similar to C_1 (eqn. 16) and the other similar to C_2 (eqn. 17).

Case (B)

When conditions (a), (b), (c), (d), (e2), and (f) are fulfilled, eqn. 10 can be simplified to

$$-\log K = C_1 \cdot M + C_2 \cdot M^{2/3} + C_0 \tag{15}$$

where

$$C_1 = \frac{\bar{v} \cdot [p_s - p_m - (p_s^0 - p_m^0)]}{2.3 \cdot RT} \tag{16}$$

$$C_2 = \frac{C^0 \cdot (4\pi N)^{1/3} \cdot 3^{2/3} \cdot \bar{v}^{2/3} (\bar{v} + \delta \cdot v_s)^{2/3} [\gamma_s - \gamma_m - (\gamma_s^0 - \gamma_m^0)]}{2.3 \cdot RT} \tag{17}$$

$$C_0 = \log \frac{f_s}{f_m} \tag{18}$$

We now make the assumption that in some experiments the constant C_2 in eqn. 15 is so small that the term $C_2 \cdot M^{2/3}$ is negligible. This equation then takes the form

$$-\log K = C_1 \cdot M + C_0 \tag{19}$$

In the cases when C_1 can be put = 0, for instance in ALBERTSSON's two-phase systems[12], eqn. 15 is reduced to

$$-\log K = C_2 \cdot M^{2/3} + C_0 \tag{20}$$

EXPERIMENTAL DETERMINATION OF THE CONSTANTS IN EQN. 14

The distribution coefficient K is calculated from the relationship

$$K = \frac{V_e - V_0}{V_i} \tag{21}$$

where

V_e = the elution volume of the solute of interest,
V_0 = the void volume, and
V_i = the volume of the solvent imbibed by the gel particles.

In order to determine the constants in eqn. 14 $-\log K$ is plotted against M.

From the resulting straight line (Fig. 1) C_1' is obtained as the slope and C_0 as the ordinate at the origin. For subsequent discussion we write C_1' as

$$C_1' = \frac{\mathrm{d}(-\log K)}{\mathrm{d}M} \tag{22}$$

EXPERIMENTAL DETERMINATION OF THE CONSTANTS IN EQN. 15

Alternative I

A smooth curve is drawn through the points obtained when $-\log K$ is plotted against M (see Fig. 3, the solid lines). Derivation of eqn. 15 gives

$$\frac{\mathrm{d}(-\log K)}{\mathrm{d}M} = C_1 + \frac{2C_2}{3} \cdot \frac{1}{\sqrt[3]{M}} \tag{23a}$$

The numerical value of $\frac{\mathrm{d}(-\log K)}{\mathrm{d}M}$ for a certain M value is obtained as the slope of the tangent to the curve for this M value. C_1 is then obtained as the ordinate at the

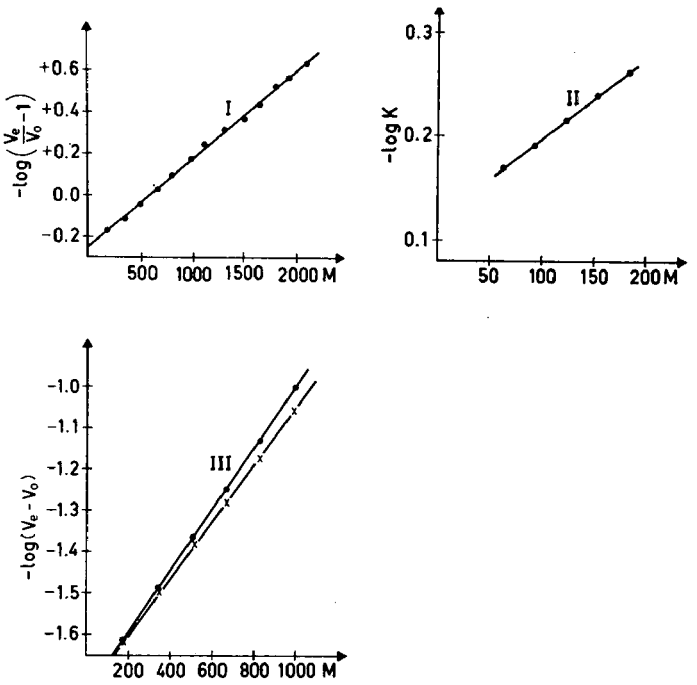


Fig. 1. Plots of $-\log K$ (K = the distribution coefficient) against the molecular weight M o *low* molecular weight *homologues* (see eqns. 14 and 19). As only V_e/V_0 values and $V_e - V_0$ values but not K values, were reported in the experiments I and III, we have, for these experiments, plotted the parameters $-\log [(V_e/V_0) - 1]$ and $-\log (V_e - V_0)$, which are related linearly to $-\log K$. I = oligosaccharides on Bio-Gel P-2 (ref. 29); II = polyhydric alcohols on a tightly cross-linked dextran gel (DVS 9; water regain 0.93 (ref. 26)); III = isomaltodextrins on Sephadex G-15 in distilled water (×—×) and in 0.1 M Tris–HCl, pH 7.0, +0.3 M NaCl (●—●) (ref. 31). The references apply to papers from which the parameters plotted have been taken. We have also obtained a similar linear relationship when plotting published data from partition chromatography experiments on paper.

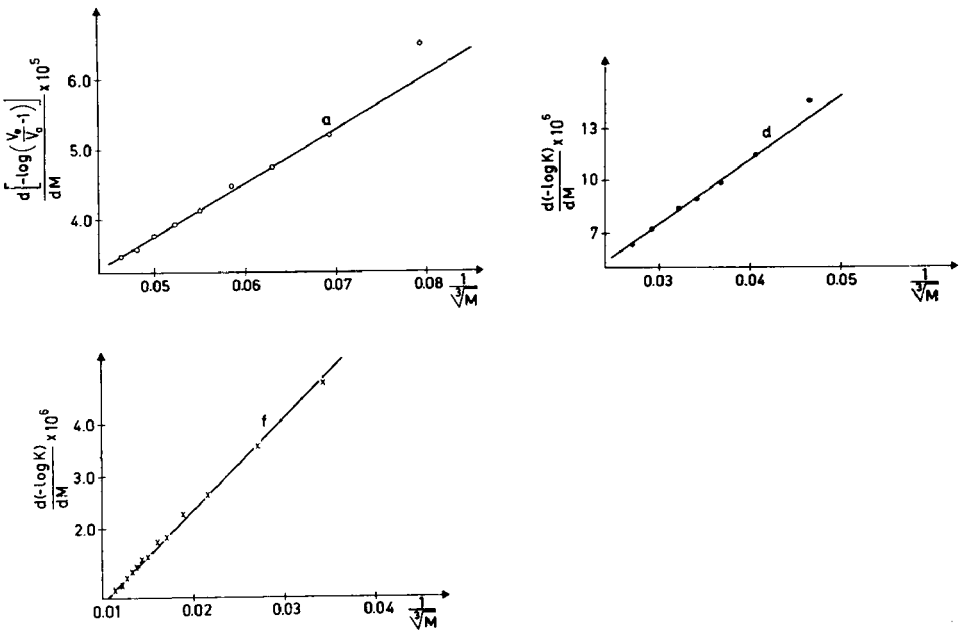


Fig. 2a. Plots of $\frac{d(-\log K)}{dM}$ against $1/\sqrt[3]{M}$ (K = the distribution coefficient; M = the molecular weight of *high* molecular weight *globular* or *flexible* polymers). (a) Polypeptides in a random coil conformation on a polyacrylamide gel, Bio-Gel P-150 (ref. 32). As only V_e/V_0 values, but not K values, were reported for the experiment (a), we have in this case plotted the parameter

$$\frac{d\left[-\log\left(\frac{V_e}{V_0}-1\right)\right]}{dM},$$

which is equal to $\frac{d(-\log K)}{dM}$. (d) Highly branched polysaccharides (ficoll fractions) on Sephadex G-200 (ref. 33). (f) Globular proteins on Sephadex G-200 (ref. 18). The linear relationship obtained is in accordance with eqn. 23a, which is the derived form of eqn. 15. These three experiments are found also in Figs. 3 and 4 with the same notations (a, d, f).

origin and C_2 as 3/2 times the slope of the straight line obtained when $\frac{d(-\log K)}{dM}$ is plotted against $1/\sqrt[3]{M}$ (see Fig. 2a); if a curved line is obtained for a certain molecular weight range, eqn. 15 is not valid in this range. The constant C_0 is equal to $-\log K$ for M equal to zero. If such an extrapolation cannot be performed accurately it might be better to make an estimation from the equation

$$3C_0 = -\log K_1 - \log K_2 - \log K_3 - C_1M_1 - C_1M_2 - C_1M_3 - \\ - C_2M_1^{2/3} - C_2M_2^{2/3} - C_2M_3^{2/3} \qquad (24)$$

where C_1 and C_2 have been determined graphically as described above; K_i (i = 1, 2, 3) corresponds to the molecular weight M_i.

Alternative II

The drawback of the above method of plotting is that it requires that the derivative $\frac{d(-\log K)}{dM}$ can be determined relatively accurately, *i.e.* the shape of the curve obtained when $-\log K$ is plotted against M must be accurately known. As this is

seldom the case we have plotted $(-\log K - C_0)/M$ for most experiments instead of $\frac{d(-\log K)}{dM}$ against $1/\sqrt[3]{M}$ where C_0 has previously been graphically determined after extrapolation, as described above. This plotting technique should also give a straight line which is evident from eqn. 15, written in the form

$$\frac{-\log K - C_0}{M} = C_1 + \frac{C_2}{\sqrt[3]{M}} \tag{23b}$$

Some examples are given in Fig. 2b. From these lines, C_1 and C_2 can be determined as the ordinate at the origin and the slope, respectively.

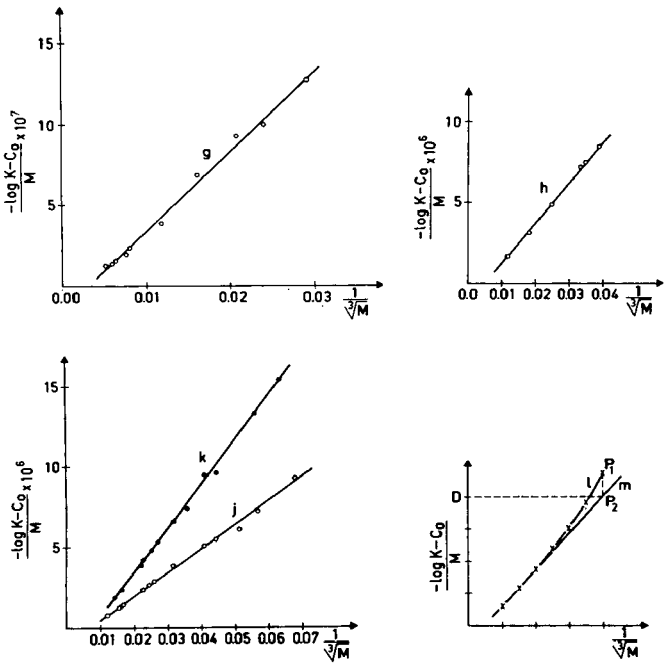


Fig. 2b. Plots of $(-\log K - C_0)/M$ against $1/\sqrt[3]{M}$ for *high* molecular weight *globular* or *flexible* polymers (see eqn. 23b). (g) Polysaccharides (dextrans) on an agarose gel, Sepharose 2 B (ref. 35). (j) Polysaccharides (dextrans) on an agarose gel, Sepharose 4 B (ref. 35). (k) Polysaccharides (dextrans) on an agarose gel, Sepharose 6 B (ref. 35). (h) Proteins on a polyacrylamide gel, T = 6.5%, C = 15% (ref. 36). (l, m) An outline of the procedure for the calculation of C_0 according to the formula $(-\log K - C_0)/M = D$. The experiments (h) and (g) are also referred to in Fig. 4.

If the value of C_0 is not accurately known a line will result, which is straight for high molecular weights and curved for low molecular weights (line l in Fig. 2b). This is due to the fact that small errors in C_0 may cause grave errors in $(-\log K - C_0)/M$ for low $-\log K$ values (low molecular weights) but not for high $-\log K$ values (high molecular weights). However, it is possible to calculate a more correct C_0 value from the expression $(-\log K - C_0)/M = D$, where D is the ordinate for a point P_2 on the extrapolated straight line m in Fig. 2b; the points P_1 and P_2 in this figure have the same abscissa. A new plot of $(-\log K - C_0)/M$ (with this new C_0 value) against $1/\sqrt[3]{M}$ is made. If this second plot does not yield a straight line,

another C_0 value is calculated from the new D value that this plot gives. This procedure is repeated until a C_0 value is obtained such that all the points scatter around a straight line.

VERIFICATION OF THE FORMULAE 14 AND 15

The experimental data were next investigated as to whether they fit the formulae derived, as this is a necessary condition for the correctness of the formulae (it is, however, not a sufficient condition).

(*a*) *Low molecular weight homologues (eqn.* 14)

Eqn. 14 requires that a plot of $-\log K$ against M results in a straight line. Fig. 1 shows that this relationship is obtained.

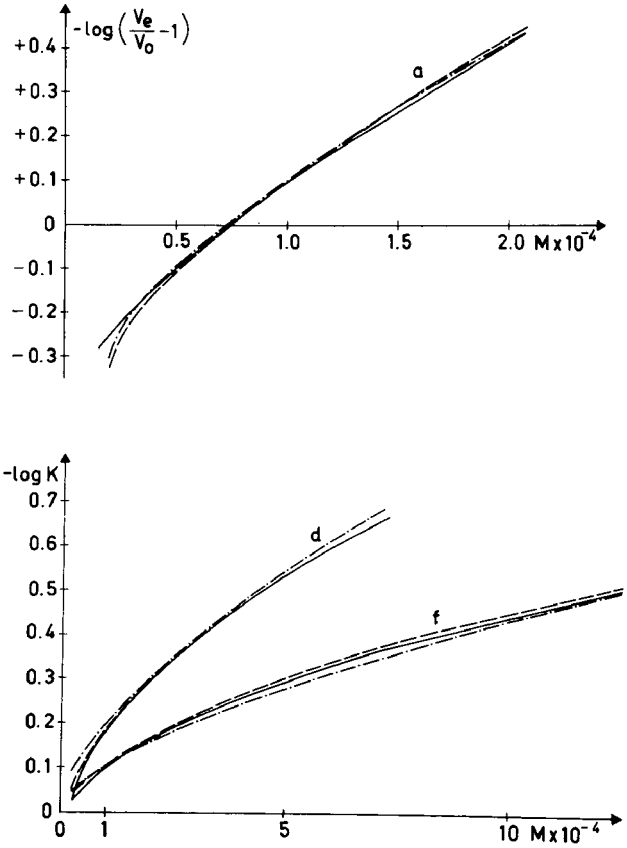


Fig. 3. Plots of $-\log K$ and $-\log [(V_e/V_0) - 1]$ against M for the experiments (a), (d), and (f), referred to in Fig. 2a (and Fig. 4). As only V_e/V_0 values, but not K values were reported for the experiment (a), we have, for this experiment, plotted the parameter $-\log [(V_e/V_0) - 1]$, which is related linearly to $-\log K$. (———) experimental curve; (– – – –) theoretical curve: $-\log K = C_1 \cdot M + C_2 \cdot M^{2/3} + C_0$ (eqn. 15). The constants C_1, C_2 and C_0 have been estimated as described under EXPERIMENTAL DETERMINATION OF THE CONSTANTS IN EQN. 15. (–·–·–) approximate curve: $-\log K = R \cdot M^{2/3} + S$ (eqn. 25). R and S have been determined as described under VERIFICATION OF THE FORMULAE 14 AND 15.

TABLE I

APPROXIMATE VALUES OF SOME CHROMATOGRAPHIC PARAMETERS FOR GELS WITH AN OPEN STRUCTURE, SUITABLE FOR THE SEPARATION OF HIGH MOLECULAR WEIGHT SUBSTANCES

The constants C_1, C_2, and C_0 refer to eqn. 15: $-\log K = C_1 \cdot M + C_2 \cdot M^{2/3} + C_0$. The values of $\Delta\Delta p = p_s - p_m - (p_s^0 - p_m^0)$, $\Delta\Delta\gamma = \gamma_s - \gamma_m - (\gamma_s^0 - \gamma_m^0)$, and f_s/f_m have been estimated from eqns. 16, 17, and 18, respectively. $\Delta\Delta\gamma$ is calculated under the assumption that $C^0 = 1$ and $\delta = 0$. This assumption might be justified for globular proteins (for ovalbumin $\delta = 0.18$ (ref. 38)). However, as the polysaccharides and polypeptides, referred to in this table, are more or less in a random coil conformation, their δ values are very high and unknown. We cannot therefore calculate $\Delta\Delta p$ and $\Delta\Delta\gamma$ for these polymers. The data used for the calculation of the different parameters are taken from the references given. All values listed in the table are so extremely approximate, that they only reflect the order of magnitude.

Solute	*Solvent*	*Gel type*	*Reference*	$C_1 \times 10^6$ ($moles^{-1}g$)	$C_2 \times 10^4$ ($moles^{-3/2}g^{3/2}$)	C_0	$\Delta\Delta p$ (*atm*)	$\Delta\Delta\gamma$ (*dyne* cm^{-1})	$\frac{f_s}{f_m}$
Polysaccharides									
Dextran fractions	0.3 % NaCl	Sepharose 2B	35	−0.3	+0.5	+0.053			1.1
Dextran fractions	0.3 % NaCl	Sepharose 4B	35	−1	+1	+0.032			1.1
Dextran fractions	0.3 % NaCl	Sepharose 6B	35	−3	+3	+0.021			1.1
Ficoll fractions	0.2 % NaCl	Sephadex G-200	33	−4	+6	−0.043			0.91
Proteins	0.05 *M* Tris–HCl, pH 7.5 + 0.1 *M* NaCl	Sephadex G-200	18	−1	+3	−0.007	−0.1	+0.1	0.98
Proteins	0.5% NaCl	Polyacrylamide, T = 6.5%, C = 15%	36	−2	+3	+0.049	−0.2	+0.1	1.1
Polypeptides (random coil conformation)	0.05 *M* Tris–HCl, pH 7.5 + 1 *M* $CaCl_2$	Bio-Gel P-150	32	−0.3	+12	–[a]			–

[a] The C_0 value (and the f_s/f_m value) could not be calculated for this experiment as only V_e and V_0 values, but not K values, have been reported.

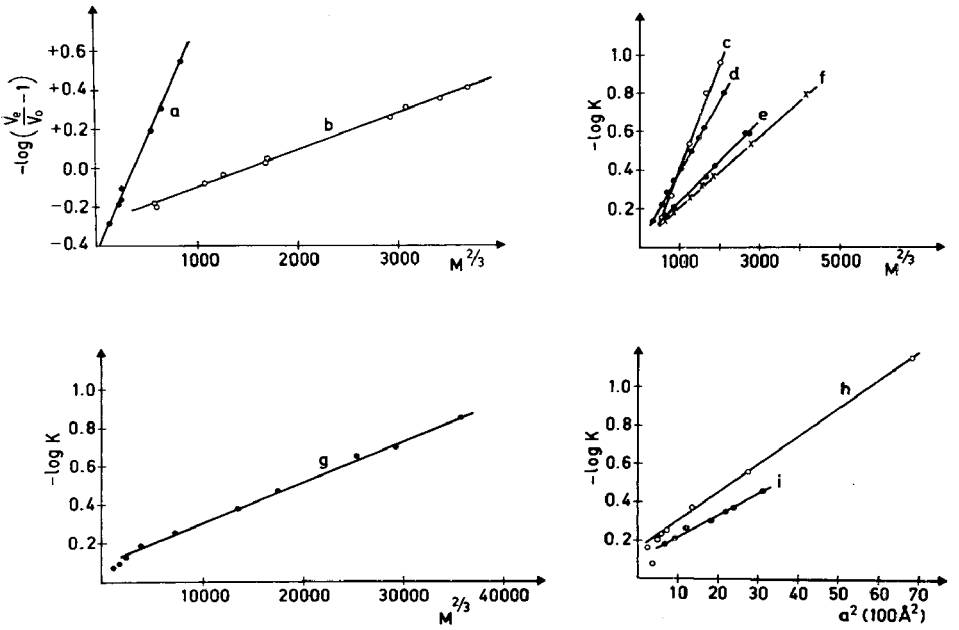


Fig. 4. Plots of $-\log K$ (K = the distribution coefficient) against $M^{2/3}$ (M = the molecular weight of *high* molecular weight *globular* or *flexible* polymers) or a^2 (a = Stokes radius) (see eqn. 25). As only V_e/V_0 values, but not K values, were reported for the experiments (a) and (b), we have plotted the parameter $-\log [(V_e/V_0) - 1]$ for these experiments, which is related linearly to $-\log K$. (a) Polypeptides on a polyacrylamide gel, Bio-Gel P-150 (ref. 32). (b) Proteins on Sephadex G-200 (ref. 19). (c) Proteins on Sephadex G-100 (ref. 34). (d) Polysaccharides on Sephadex G-200 (ref. 33). (e) Proteins on Sephadex G-200 (ref. 34). (f) Proteins on Sephadex G-200 (ref. 18). (g) Polysaccharides on an agarose gel, Sepharose 2B (ref. 35). (h) Proteins on a polyacrylamide gel, T = 6.5%, C = 15% (ref. 36). (i) Polysaccharides on a 6% agarose gel[37]. The references apply to papers from which the plotted parameters have been taken. As indicated in the diagrams, the points scatter around straight lines for the major part of the separation range. Such linear relationships are not to be expected in all experiments with proteins or polysaccharides, as eqn. 25 is only a formal approximation of the more generally applicable eqn. 15; thus, a straight line is not obtained when $-\log K$ is plotted against $M^{2/3}$ for experiments (j) and (k), referred to in Fig. 2b.

(b) High molecular weight globular proteins and flexible polysaccharides (eqn. 15)

A necessary condition for eqn. 15 to be valid is that a plot of $\frac{d(-\log K)}{dM}$ or $(-\log K - C_0)/M$ against $1/\sqrt[3]{M}$ gives a straight line (eqns. 23a and 23b). Using chromatographic data from the literature we have obtained such a relationship in most cases. Some examples are given in Figs. 2a and 2b.

The constants C_0, C_1, and C_2 in eqn. 15 were determined as described in the previous sections (in some experiments alternative I was used and in others alternative II). A graphic representation of eqn. 15 with these values of the constants is shown in Fig. 3 (the broken curves). These curves are in good agreement with the experimental curves (solid lines). Figs. 2a and 2b show that $C_1 \neq 0$; furthermore, C_1 is not sufficiently small compared with C_2 (Table I) that the term C_1M in eqn. 15 can be neglected in comparison with the term $C_2M^{2/3}$ for molecular weights over *ca.* 1000. Consequently, eqn. 20 which corresponds to eqn. 15 for $C_1 = 0$ is not applicable to molecular-sieve chromatography. This statement is at first a little surprising with

respect to the linear relationship often obtained in plots of $-\log K$ against $M^{2/3}$ (see Fig. 4). However, this empirical relationship

$$-\log K = R \cdot M^{2/3} + S \tag{25}$$

can be considered a formal approximation of eqn. 15, as it appears to be possible to find such values of R and S that $-\log K = C_1 M + C_2 M^{2/3} + C_0$ is virtually equal to $R \cdot M^{2/3} + S$ (the constants R and S have no evident physical significance). Examples of the feasibility of approximating eqn. 15 with eqn. 25 satisfactorily are given in Fig. 3 (the R and S values are those obtained graphically from the straight line obtained in plots of $-\log K$ against $M^{2/3}$). As eqn. 25 is applicable in several experiments it is not surprising that a plot of $-\log K$ against a^2 (a = Stokes radius) also often results in a straight line (see Fig. 4, experiments h and i).

DISCUSSION

A comparison between the present treatment and previous ones

The present theoretical treatment of molecular-sieve chromatography is a thermodynamic one and thus differs fundamentally from those of many other authors[1–4], in that no physical model is needed to explain the separations obtained. These previous hypotheses of molecular-sieve chromatography are based upon different physical models, which have a common factor in that the sieving properties of the gel columns are assumed to be due to steric exclusion and/or restricted diffusion. An inspection of eqn. 10 shows that thermodynamic considerations provide another explanation; namely that in ideal molecular-sieve chromatography ($\psi = E = 0$) the size-sieving properties of gels are due to: (a) differences in pressure between the gel phase and the mobile liquid phase, and (b) alterations in the interfacial tension of the solute when it moves from one phase to the other. In a recently published communication POLSON AND KATZ[13] advanced the hypothesis that chromatographic molecular sieving may partly be explained by osmotic effects. From this point of view a paper by EDMOND *et al.*[14] on the osmotic behaviour of dextran gels is of interest.

In most of the previous hypotheses it has been assumed that the solute concentrations are the *same* in the mobile phase and the part of the stationary phase that is available to the solute. In thermodynamic treatment the solute concentration in the stationary phase (the gel grains) is assumed to be different from the concentration in the mobile phase and it is not necessary to assume a non-uniform distribution of the solute in the gel grain. This fundamental dissimilarity is reflected in the definition of the distribution coefficients: in the present treatment the distribution coefficient K is equal to the ratio between the concentrations in the stationary and the mobile phase (eqn. 9), while in the previous treatments K is equal to the fraction of the inner volume V_i that is available to the solute. (LAURENT[37] has defined K in a somewhat different way.)

Although it has been almost generally accepted that solute molecules of both low and high molecular weight substances do penetrate the *whole* gel particle, experimental evidence is still lacking. From the thermodynamic point of view the penetration depth is not of interest: no assumptions about the dimensions of the stationary phase are required.

Many experiments have been reported in which solutes are not eluted according to molecular weight. This elution order is, however, not always to be expected — not even in the absence of interaction between solute and gel polymer — since it is the

partial molal volume v, the area A of the solute molecules and the interfacial tension γ between the solute molecules and the surrounding medium that primarily determine the distribution coefficients as eqn. 10 shows; it is only when certain conditions, mentioned under APPROXIMATIONS OF THE GENERAL EQN. 8, are fulfilled, that there is a positive correlation between elution volume and molecular weight (as for instance for globular proteins and for solutes of a homologous series: eqns. 15 and 14). In an extensive study of the behaviour of amino acids on Sephadex G-10, EAKER AND PORATH[15] observed that the distribution coefficients of these solutes were markedly changed with alteration in buffer composition. Several explanations were given by the authors. It is also probable that these changes in the K values may in part result from Donnan effects (see below) and alterations in the above parameters (v, A, γ): these effects are more pronounced in experiments with low molecular weight ions, such as amino acids, on tight gels.

Some authors have stated that the physical molecular sieving model used for the derivation of an equation is correct if the experimental data fit the equation. Such a verification is no proof, it is only a necessary, but not a sufficient condition, for the correctness of the model.

Estimation of the molecular size of a solute by the approximate eqns. 14 and 15

The transformation of the general eqn. 8 into eqns. 14, 15, 19, and 20 involves a series of approximations, mentioned above in section APPROXIMATIONS OF THE GENERAL EQN. 8. It should also be stressed that all the equations presented in this paper are based upon the assumption that both the mobile and stationary phase are homogeneous. This might be a simplification when the stationary phase is a granular gel: microheterogeneities in the gel may mean that the parameters p, γ, ψ, and E in eqn. 8 have different values in different regions of the gel grain. The approximate nature of eqns. 14 and 15 is thus obvious. The molecular weight estimated by these equations — or by any other of the previously published formulae[1-4] — must therefore be regarded as tentative, particularly when the shape of the molecule is unknown. An example of how large the errors may become is given by the case of globular proteins which, after denaturation by urea to a random coil conformation, migrate close to the void volume on a 6 % agarose gel (Bio-Gel A-5 m), while the same proteins in their native, globular state require almost double this volume for elution[16].

Without attempting a rigorous explanation of this "anomaly" we will call attention to the fact that the transition of a macromolecule from a globular to a random coil state involves an enormous increase in the area of the molecule (the amount of solvent in the macromolecule may exceed the amount of the dry macromolecular material by a factor of 100 (ref. 10), which means that δ in eqn. 13 — and consequently the area A — is much larger for denatured than for native proteins). As $\gamma_s - \gamma_m - (\gamma_s^0 - \gamma_m^0) > 0$ according to Table I an increase in A in eqn. 10 corresponds to a decrease in K provided that the other terms do not affect K in an opposite direction, *i.e.* a randomly coiled molecule will migrate with a lower K value than a globular molecule of the same molecular weight. This could also explain the high chromatographic migration rates of linear polyethylene glycol molecules as compared with globular proteins of the same molecular weight[17]. It is thus evident that the constants in the various proposed equations for the relation between K values and molecular size have different values for different kinds of solutes, depending on their nature (for

instance, whether they are proteins or carbohydrates) and shape (for instance, whether they are linear, globular, or randomly coiled). A molecular size calculated by these equations may consequently be entirely erroneous. The same uncertainty is, of course, associated with the use of empirical calibration diagrams. Different authors have proposed plotting different parameters in order to obtain a straight line in such diagrams[1–4, 18]. The present treatment and Figs. 1 and 4 indicate that there is a rather high probability that a straight line will be obtained if $-\log K$ (or $-\log[(V_e/V_0) - 1]$, or $-\log(V_e - V_0)$) is plotted against M for low molecular weight compounds and against $M^{2/3}$ for both globular and flexible macromolecules, provided the solutes of interest are isochemical substances, for instance homologues or polymers where the repeating units are similar. It is, however, irrelevant which plotting techniques are used, because in practice none of them will give a straight line in all cases (nor is it necessary that the calibration curve is a straight line).

It has been proposed[16] that the estimation of the molecular weight of a protein of an unknown shape should be performed in the presence of urea or guanidine hydrochloride in order to transform this protein and the reference proteins to the same conformation (random coil).

The dependence of solute concentration, temperature, ionic strength, and Donnan effects on the distribution coefficients

WINZOR AND NICHOL have found that the elution volumes or K values are somewhat dependent on the solute concentration[8]. This is explained by eqn. 8, as the partial molal volume, the pressure, the interfacial tension, and the activity coefficients vary with the concentration of the solute. Eqn. 8 also shows that the K values are somewhat temperature and ionic strength dependent, which has been experimentally verified in refs. 9 and 19 and in experiment III in Fig. 1. Molecular-sieve chromatography should therefore not be used, as has been proposed[9], for studying the effects of ionic strength and temperature on the shape of the solute molecules. NICHOL *et al.*[20] have reported that Donnan effects — although small — can be experimentally established in molecular-sieve chromatography of charged macromolecules, which means that the terms of the form $FZ\psi$ in eqn. 8 may sometimes play a certain role, even if they are negligible in most experiments. The factors Z and ψ are discussed under APPROXIMATIONS OF THE GENERAL EQN. 8. It is to be expected that the Donnan effects are more pronounced when small molecules are chromatographed on gels with low water regain (for instance Sephadex G-10 and Bio-Gel P-2).

The Brønsted formula

In 1962 HJERTÉN AND MOSBACH advanced different hypotheses to explain the molecular-sieving action of polyacrylamide gels[21]. One of the hypotheses was based upon the assumption that the solutes are distributed between the stationary and mobile phases in a gel bed according to the BRØNSTED formula[22]

$$K = e^{-\frac{\lambda}{kT}} \tag{26}$$

where:

K = the distribution coefficient,

λ = a parameter which depends on the molecular size of the solute,

k = the Boltzmann constant, and

T = the absolute temperature.

This formula is obtained directly from the assumption that the solute molecules partition between the two phases according to the Boltzmann distribution formula.

ALBERTSSON[12] has proved Brønsted's statement[22] that λ for macromolecules in a two-phase system is proportional to the area of the solute. For such solutes eqn. 26 can be written

$$-\log K = \text{constant} \times A \tag{27}$$

which is a special case of eqn. 10 when all terms, except the first and the third, can be neglected (the constant has the same value for "isochemical" substances). For a spherical molecule one thus obtains the relation

$$-\log K = \text{constant} \times M^{2/3} \tag{28}$$

which corresponds to eqn. 20 for $C_0 = 0$.

In many experiments we have found that a plot of $-\log K$ against $M^{2/3}$ gives a straight line (Fig. 4). In some cases it passes through the origin in accordance with eqn. 28; examples are found in Fig. 4, experiments d, e, and f. However, eqn. 28 also requires that a straight line through the origin is obtained when $\frac{(d - \log K)}{dM}$ is plotted against $1/\sqrt[3]{M}$. As in practice this line does not pass through the origin (which an extrapolation of the lines in Fig. 2a indicates), it is more correct to consider eqn. 28 a formal approximation of eqn. 15; the arguments are the same as those mentioned under VERIFICATION OF THE FORMULAE 14 AND 15 where it was proved that eqn. 25 is an approximation of eqn. 15. The reasons why the Brønsted formula is only approximately valid for chromatographic molecular sieving are as follows: (a) the interactions between the solute and the surrounding medium that give rise to a term containing activity coefficients (C_0 in eqn. 15) have been neglected; (b) the formula has been derived for conventional two phase systems where the pressure in the top phase is the same as in the bottom phase, while the "swelling pressure" in a gel particle causes a pressure difference between the interior and exterior of the gel particle[10], resulting in the term $C_1 \cdot M$ in eqn. 15.

According to Brønsted, λ for low molecular weight substances is proportional to the molecular weight M of the solute[22]. Eqn. 26 then takes the form

$$-\log K = \text{constant} \times M \tag{29}$$

This equation can be considered a special case of eqn. 19. The latter equation written in the form $-\log K = \text{constant} \times v + C_0$, where v is the partial molal volume, has been used in the treatment of the behaviour of neutral low molecular weight substances on ion exchange resins[23], and has been found to conform to experimental data for cellodextrins on Sephadex G-25 (ref. 24). (See also ref. 25.)

Homologous series

If is assumed that eqn. 14, or eqn. 19, is applicable for substances which are members of a homologous series. For the pairs n and $n + 1$ in such a series we get

$$-\log K_n = C_1' \cdot M_n + C_0$$

$$-\log K_{n+1} = C_1' \cdot M_{n+1} + C_0$$

or

$$-\Delta(\log K_n) = C_1' \cdot \Delta M \tag{30}$$

As this equation is valid for any n value one can conclude that the difference between the log K values for any two consecutive members of a homologous series is constant. MARSDEN[26] has arrived at the same conclusion using an assumption that MARTIN[27] employed in his theoretical studies on partition chromatography, namely that the free energy required to transport a molecule from one solvent to another is the sum of the free energies for transport of the individual constituent groups of the molecule. MARSDEN[26] also verified that $\Delta(\log K)$ values for two consecutive members of a series of polyols were approximately constant.

The above conclusion that the difference between the log K values for any two consecutive members of a homologous series is constant is evident also from eqn. 22 and is in agreement with MARTIN's statement[27] that "the addition of a group X changes the partition coefficient by a given factor depending on the nature of the group, and on the pair of phases employed, *but not on the rest of the molecules*". MARTIN considers this "prediction contrary to usual expectation. It is usually felt that the formation of a derivative of greatly increased molecular weight will "swamp" any differences that exist and will render separation more difficult. This, however, is not to be expected if such a derivative be chosen that the same pair of phases can be employed while still maintaining convenient values for the partition coefficients". However, MARTIN has not taken into account that the resolution of two solutes is a function not only of the ΔK values but also of the K values (see refs. 28 and 5), the resolution in general decreasing with a decrease in K values. In the case of molecular-sieve chromatography one may therefore expect the resolution to decrease when one goes up through a series of homologues, which is in agreement with what is found in practice (see for instance Fig. 3, ref. 29). This decrease in resolution with diminishing K values (*i.e.* with increasing values of the molecular weights M) is still more pronounced for solutes (such as many proteins) whose chromatographic behaviour is determined by eqn. 15 instead of eqn. 14. This is evident from eqn. 23a which shows that $\frac{d(-\log K)}{dM}$ is not constant (as in eqn. 22), but decreases with M ($C_2 > 0$ according to Table I).

These considerations may partly explain why the resolution of proteins in molecular sieving is far inferior to that of low molecular weight homologues.

Chromatographic parameters (Tables I and II)

In Table I we have listed the values of the constants C_1, C_2, and C_0 for high molecular weight substances on loose gels, estimated as described under EXPERIMENTAL DETERMINATION OF THE CONSTANTS IN EQN. 15; the corresponding values of $\Delta\Delta p = p_s - p_m - (p_s^0 - p_m^0)$, $\Delta\Delta\gamma = \gamma_s - \gamma_m - (\gamma_s^0 - \gamma_m^0)$, and f_s/f_m obtained from eqns. 16, 17, and 18 are also given.

The negative value of $p_s - p_m - (p_s^0 - p_m^0)$ means that the difference in pressure between the stationary and mobile phase is larger in the standard state than in the actual experiment; the pressure in the gel must always be larger than outside[10]. According to Table I $\Delta\Delta\gamma$ has a value of 0.1 dyne cm^{-1}. Direct measurements of the interfacial tension between two phases of aqueous polymers have given values of 0.003–0.1 dyne cm^{-1} (ref. 30). As expected the activity coefficient of a solute has about the same value in the stationary and in the mobile phase ($f_s/f_m \sim 1$).

In Table II chromatographic data from experiments with low molecular weight homologues on tight gels are collected. The constants C_1', C_1, and C_0 are those in

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TABLE II

APPROXIMATE VALUES OF SOME CHROMATOGRAPHIC PARAMETERS FOR TIGHT GELS, SUITABLE FOR THE SEPARATION OF LOW MOLECULAR WEIGHT SUBSTANCES (HOMOLOGUES)

The constants C_1', C_1, and C_0 are those in the formula $-\log K = C_1' \cdot M + C_0$ (eqn. 14) and $-\log K = C_1 \cdot M + C_0$ (eqn. 19). C_1' and C_1 have the same numerical value but a different physical meaning. The values of $\Delta\Delta p = p_s - p_m - (p_s^0 - p_m^0)$ refer to the alternative situation where eqn. 19 is applicable. The data used for the calculation of the different parameters are taken from the references given. All values listed in the table are so extremely approximate, that they only reflect the order of magnitude.

Solute	*Solvent*	*Gel type*	*Reference*	$C_1' \times 10^4$ $(= C_1 \times 10^4)$ $(moles^{-1}\ g)$	C_0	$\Delta\Delta p$ *(atm)*	$\frac{f_s}{f_m}$
Polyhydric alcohols	0.05 *M* Tris–HCl, pH 7.6	Dextran gel, DVS 9	26	4	0.14	(+30)	1.4
Oligosaccharides	Water	Bio-Gel P-2	29	4	—[a]	(+30)	—
Isomaltodextrins	Water	Sephadex G-15	31	6	—[a]	(+50)	—
Isomaltodextrins	0.1 *M* Tris–HCl, pH 7.0 + 0.3 *M* NaCl	Sephadex G-15	31	7	—[a]	(+50)	—

[a] C_0 values (and f_s/f_m values) could not be calculated for these experiments as only V_e and V_0 values but not K values are reported.

eqns. 14 and 19. Knowledge of the value of C_1' does not permit calculation of the values of $\Delta\Delta p$ and $\Delta\Delta\gamma$. The values of $\Delta\Delta p$ given in Table II do not therefore refer to eqn. 14 but to eqn. 19. As it is difficult to decide if the latter equation is applicable, the $\Delta\Delta p$ values have been put in parentheses. Fortunately eqns. 14 and 19 are formally very similar inasmuch as they predict that a straight line should be obtained when $-\log K$ is plotted against M — a relationship which we have obtained experimentally in all the cases we have studied. It should be noted, however, that eqn. 14 and eqn. 19 give a different physical interpretation of the slope of the straight line. The ratio between the activity coefficients f_s/f_m seems to differ more from unity for hard gels (Table II) than for loose gels (Table I). This finding is to be expected.

Verification of the formulae

From Figs. 1–3 it is evident that the experimental data conform to the formulae derived. Another condition for the validity of the derived formulae is that they give values of $\Delta\Delta\gamma$ and f_s/f_m which have the expected order of magnitude (see the above section *Chromatographic parameters*).

Applicability of the thermodynamic considerations in areas other than chromatography

The thermodynamic considerations presented in this paper have been centred around molecular-sieve chromatography but will — with due modifications — also apply to conventional two phase partition experiments, for instance those described by ALBERTSSON[12] from whose theoretical treatment of aqueous two-phase systems the author has obtained many ideas. In these systems one may put $p_s = p_m$ (ref. 10); accordingly the constant C_1 (eqn. 16) has the value zero, *i.e.* formulae similar to eqns. 14 and 20 are applicable (the constant C_1' in eqn. 14 consists in this case of only one term).

All the considerations throughout this paper are based upon the assumption of equilibrium between two phases and cannot therefore be used with any exactness for living systems. The treatment may, however, yield a qualitative picture of the solute distribution across a cell membrane when active transport can be neglected. Thus eqn. 10 shows that the concentration of a solute (positively charged) within the particles (for instance mitochondria or bacteria) can be extremely small if the electric potential and the pressure in the particle is sufficiently high and/or the interfacial tension γ for a solute is higher on the inside than the outside of the particle (see also ref. 26). Similar considerations might be applicable to fluids streaming in the semipermeable capillaries in living tissue. It may be mentioned that the pressure in tightly cross-linked resin particles used for ion-exchange chromatography has been estimated to several hundred atm[23].

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CHROM. 4790

AN IMPROVEMENT IN THE ELUTION TECHNIQUE FOR MEASUREMENT OF ADSORPTION ISOTHERMS BY GAS CHROMATOGRAPHY

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SUMMARY

The measurement of adsorption isotherms by the elution chromatographic technique and the application of a data logger and a computer for this purpose is described. A new method of calculation which commences from the top of the chromatographic peak and progresses downwards is developed. Long elution times are shown in the examples quoted but reproducible isotherms are obtained. Isosteric heats of adsorption calculated from these isotherms are found to be in better agreement with other methods of measurement than previous chromatographic results.

INTRODUCTION

The methods for the determination of adsorption isotherms by chromatographic techniques are divided into two types. Frontal analysis has been described by GREGG AND STOCK[1] and HABGOOD[2] and is based on the work of GLUECKAUF[3]. The slightly different technique of elution analysis was described by CREMER AND HUBER[4] and has also been reviewed by HABGOOD[2] and SAINT-YRIEIX[5]. The first method would seem to be the more popular from a study of the literature, but is more difficult to carry out due to the difficulty of keeping the vapour at a constant partial pressure with a bubbler system or gas mixing device. The second method involving simple injection of a liquid sample with a syringe or gas with a dosing valve does not seem to have produced such successful results. The suitability of the elution method was therefore examined in this study.

EXPERIMENTAL

A conventional Pye 104 chromatograph was used with a separately heated katharometer detector. The apparatus was adapted so that parallel 10-cm columns made from 0.4-mm-bore pyrex glass tubing could be fitted using knurled nut type connectors with 'O' ring seals. These columns held about 1 g of sample. The carrier gas was helium which was dried by passing it over molecular sieve type 4A. The

two columns were packed with different samples, one acting as a blank while the other was being tested. Mercury manometers were added so that the pressure drop across the columns could be monitored and this was always kept below 100 mm of mercury height. The temperature of the columns was measured with a chrome-alumel thermocouple and was found to be constant to 0.01° C.

The output of the katharometer bridge was fed to a Solartron compact data logger which consisted of a digital voltmeter used on 20-mV range, coding circuits and a paper tape punch. A manual entry unit was also included to allow digits to be punched independently of the logger.

The katharometer reading was taken every 2 sec, coded and punched out on tape. At the end of a run the data tape was processed on an English Electric KDF9 computer to obtain the isotherm. The katharometer signal was simultaneously fed to a Kent Mark III potentiometer recorder on a 0–20 mV range as a visual check on the peak produced.

The katharometer was initially balanced to give a reading of about 0.18 mV on the digital voltmeter with the chromatograph attenuator set to an appropriate sensitivity. With the data logger running, an injection of 10 μl was made in the normal type of septum cap and the polarity of output of the katharometer was reversed long enough for at least one negative reading to be punched and the polarity was then restored to positive. (This was to mark the injection time.) After the peak had appeared, ample time was allowed for the curve to return to the base line as seen on the chart paper and the digital voltmeter. The end of the data tape was then marked by punching the number 9999 using the manual entry unit. Certain control information for each run (described in the theory section) was also punched at the start of the tape using this unit. The data were logged as four digits (up to 2300 with the over-ranging allowed) without any decimal point indicated. A decimal point could, however, be entered in the control information section using the manual entry unit.

MATERIALS

In the results described here the adsorbent consisted of 1 g of a graphite occupying about 100 mm of the glass tube. The absorbate was benzene and the adsorbent was held at temperatures near the boiling point of benzene. Flow rates were in the region of 20 ml min^{-1}. The katharometer was kept at 100°C so as to be always hotter than the columns. In later results a carbon black called spheron 9 was used.

THEORY

The theory to be described is based on the details given by SAINT-YRIEIX[5] for results obtained on a normal chart recorder and is called here the old method.

Correction of diffusion

Following the method given by BECHTOLD[6] the diffusion in front of a peak was assumed to be equal to the diffusion to the rear of a peak at each concentration level. This is shown in Fig. 1, the distances x on the front of the peak being subtracted from the rear portion of the curve at each concentration level.

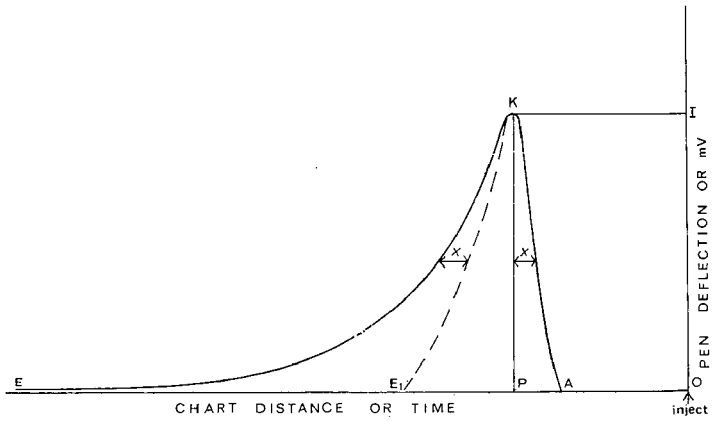


Fig. 1. The correction of the tailing edge of a chromatographic peak for diffusion.

A point on the adsorption isotherm $f(C_1)$ may then be calculated using the formula:

$$f(C_1) = \frac{WQ}{ug} \int_0^{n_1} (l - l_0)\,dn \qquad (1)$$

where W is the carrier gas flow rate ($cm^3\ sec^{-1}$), Q the sensitivity of the detector given by C/n and assumed to be a constant ($\mu mole\ cm^{-4}$), C the concentration of the adsorbate in the gas phase, usually in $\mu mole\ cm^{-3}$; n the deviation of the chart pen from the base line (cm), u the speed of the chart paper ($cm\ sec^{-1}$), g the mass of the adsorbent (g), l the distance on the chart paper from the injection point to a particular point on the curve (cm), l_0 the distance on the chart paper from the injection point to the appearance of a non-adsorbed gas such as air (cm).

With the size of columns being used in this experiment l_0 was found to be zero and was neglected. Fig. 2 shows the meaning of this integral on a typical experimental

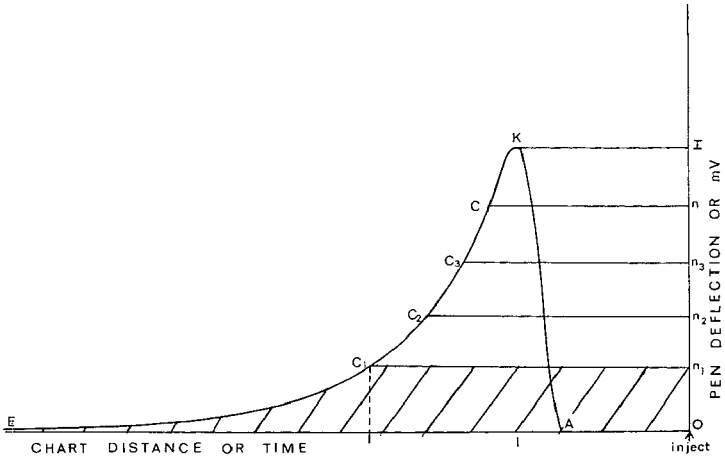


Fig. 2. The calculation of adsorption values from a chromatographic peak.

curve. The point with deviation n_1 from the base line is at a distance l from injection, thus the shaded area represents the integral and multiplication by the other factors of eqn. 1 gives one point on the isotherm. The concentration at which this amount is adsorbed is given by

$$C_1 = Qn_1 \tag{2}$$

Further points on the isotherm are then obtained by drawing more horizontal lines at say n_2, n_3 etc. and adding on the extra areas then included. The calculation is thus carried up the experimental curve from the base line to the peak. The number of such points obtained is governed by the tedium of dividing the diagram by a large number of lines and estimating areas. The areas may be found by counting squares or similar methods but more commonly the horizontal lines are assumed to be close enough together to assume that the portion of the curve cut off between two lines approximates to a straight line. The area is then a trapezium and its area is the difference between deviations n for the upper and lower lines times the average value of l. A reasonable number of lines to draw in this method would be about twenty. The value of Q is obtained by what is effectively a simultaneous calibration. The amount of solute injected is proportional to the area under the curve for any chromatographic peak. The area under the whole peak in Fig. 1 was found (before diffusion correction) and is called S. If the amount of solute injected is m μmoles, then:

$$Q = \frac{um}{WS} \tag{3}$$

where

$$S_1 = \int_{l_1}^{l_2} n \, \mathrm{d}l \tag{4}$$

Use of the data logger to investigate the old method of calculation

Eqn. (1) may be rewritten (dropping l_0) as

$$\mathrm{f}(C_1) = \frac{WQ}{g} \int_0^{n_1} \frac{l}{u} \, \mathrm{d}n = \frac{WQ}{g} \int_0^{n_1} t \, \mathrm{d}n \tag{5}$$

where t is the time of the particular point after injection and n now represents the digital voltmeter reading above the base line reading instead of cm of chart paper.

Similarly in eqn. 3 dividing S by u gives an area S_1 in terms of time $\times$ voltmeter reading,

$$Q = \frac{m}{WS_1} \tag{6}$$

where

$$S_1 = \int_{t_1}^{t_2} n \, \mathrm{d}t \tag{7}$$

The absence of the decimal point in the reading punched out does not matter

because the value of n appears in the calibration in eqn. (7) as well as in eqn. (5). Combining eqns. (6) and (5) gives

$$f(C_1) = \frac{m}{S_1 g} \int_0^{n_1} t\,dn = Q_2 \int_0^{n_1} t\,dn \tag{8}$$

where $Q_2 = m/S_1 g$ is a constant factor. The value of C_1 is obtained from eqn. 2 in μmole cm^{-3} (n_1 is now voltmeter reading).

Assuming ideal gas behaviour of the vapour

$$P = CRT$$

where P is pressure

$$\therefore P = RTQn_1 = 8.31 \times 10^{-3}\, TQn_1 \text{ in kN m}^{-2}$$

The quantities m, g, W and T were entered on the data tape at the start of a run.

The reading was logged at regular 2-sec intervals so as the data were read into the computer an integer representing time was increased by two as each piece of data was read in and stored as the time of that piece of data. This time sequence was only started from zero after the negative reading representing injection had been detected.

Since a large number of points on the experimental curve are read into the computer (several hundred) a more complete and more accurate isotherm results. It would be pointless to allow the computer to print out all these points, so since they were relatively closely spaced along the isotherm the points were used to interpolate for the pressure at which standard amounts of material are adsorbed. The steps were chosen this way round to ease the calculation of isosteric heats of adsorption. The points chosen were 5, 10 and 20 μmole g^{-1} and steps of 20 μmole g^{-1} up to the peak of the curve (highest point on the isotherm obtained from that experiment).

The main difficulty in conducting this experiment was to detect when the recording device had returned to the base line. Since base line drift could and did occur, this had necessarily to be a return to a constant reading and not to the original reading before the peak. The digital voltmeter proved to be more sensitive than the pen recorder but due to inherent noise in the output of the katharometer the last digit tended to wander up and down while reaching a final constant value. The shape of the curve for benzene on carbon showed a large degree of tailing (steep isotherm at low pressures) and a pronounced asymptotic approach to the base line. This made detection of the finishing point very difficult. Unfortunately this point needs to be known accurately to allow the start of the integrations implied in eqn. 5. A special numerical procedure to find this point had to be adopted.

If all the data logged in the asymptotic region are examined it is seen to involve a large number of readings changing only very slowly by one in the last digit, constant readings or even rising readings for a few steps. Thus a simple comparison of the digits of a pair of successive readings is of no use nor is a smoothing procedure trustworthy in this case (SAVITSKY AND GOLAY[7]). The processing program was arranged to store every separate reading until the rear portion of the peak was appearing and

a pair of readings remained constant. The first reading was stored normally with the previous ones, but the second was stored in a separate loop of the program with the first reading repeated as well. The following readings were stored in this sub-section and a running average of these readings was computed. This average value was compared with the last reading in the main storage array until twice the difference between them equalled or exceeded unity. This means that despite fluctuations in actual readings taken, on average a decrease of unity in the last digit has been detected. The last figure stored in the main array and the average were then used to calculate a new figure for the main array (twice the average minus last stored reading). The time continued to increase as each figure was read in but only the last time was stored to correspond to the new array figure. The figures stored in the array thus continued to decrease by about unity but the time scale stretched out more and more as the slope became less. This procedure economised on storage space and computing time by reducing the stored data to reasonable proportions. All the data stored were then used in subsequent calculations. The end of the data tape was detected when the 9999 previously added as a marker was reached.

The integration involved for eqns. 7 (calibration) and 8 (isotherm points) was then carried out numerically by the computer.

RESULTS AND DISCUSSION

To test the reproducibility of the method duplicate injections of 10 μl of benzene were made under fixed conditions. The results are shown in Table I and were obtained with elapses of 1 h and 24 h between injections. A second injection about 1 h after the first produced a higher peak (up to 10 % higher) as did injections at 2-h and 8-h intervals. The best reproducibility of peak heights as seen on the recorder was obtained after purging for 24 h. It was assumed that the last of the benzene requires 24 h to be purged off a column despite the apparent return of the trace to the baseline. Thus readings are best only taken at 24-h intervals.

TABLE I

REPEATABILITY OF AN ISOTHERM USING THE OLD METHOD OF CALCULATION

Adsorption of benzene on graphite at 345° K. Detector sensitivity setting 10. Flow rate at katharometer temperature, 28.0 ml min^{-1}. Flow rate at column temperature, 25.5 ml min^{-1}. Area under the curve, 3.207×10^5. Lowest point on the isotherm, 0.97 μmole g^{-1} at 0.0025 kN m^{-2}.

Adsorption (μ*mole* g^{-1})	*Pressure* (*kN* m^{-2})	*ln (pressure)*	*Difference between two injections*			
			Pressure		*ln (pressure)*	
			1 h	*24 h*	*1 h*	*24 h*
5	0.001	−4.312	0.011	0.003	0.595	0.259
10	0.003	−3.536	0.022	0.006	0.564	0.220
20	0.007	−2.673	0.004	0.010	0.493	0.153
40	0.185	−1.686	0.087	0.014	0.383	0.076
60	0.353	−1.041	0.128	0.013	0.310	0.038
80	0.577	−0.551	0.172	0.009	0.261	0.015
100	0.863	−0.147	0.220	0.001	0.227	0.001
120	1.225	0.203	0.277	0.012	0.204	0.009
140	1.682	0.520	0.348	0.032	0.188	0.018
160	2.267	0.819	0.435	0.061	0.175	0.026

The results in Table I show clearly the improvement in reproducibility of 24-h injections over 1-h interval injections at least at the upper end of the isotherm. Experiments at 354°K and 344°K gave the isosteric heats of adsorption shown in Table II. The heat of condensation of benzene is 30.7 kJ mole^{-1} so the low values for heat of adsorption shown at the low pressure end are unexpected and do not compare with results obtained by static adsorption measurements[8]. The results are, however, similar to those of CREMER AND HUBER[4]. It was then assumed from the slow elution shown above and the unreasonably low heats of adsorption produced that some benzene is eluted too slowly to be detectable in the katharometer. Since the calculation of the isotherm starts from the point of return to the base-line with the assumption of zero adsorption the whole of the isotherm must be seriously in error. It should be noted that the value of S_1 and thus of Q is also incorrect.

TABLE II

VARIATION OF ln (PRESSURE) WITH AMOUNT ADSORBED AT TWO TEMPERATURES AND HEAT OF ADSORPTION $-\Delta H$

Adsorption (μmole g^{-1})	*ln (pressure)*		$-\Delta H$ (kJ mole^{-1})
	at 354° K	*at 344° K*	
5	−3.771	−3.865	7.1
10	−2.989	−3.068	8.0
20	−2.043	−2.199	15.9
40	−0.912	−1.156	14.6
60	−0.160	−0.450	29.7
80	0.408	0.094	31.8
100	0.867	0.537	33.4
120	1.256	0.914	34.7
140	1.596	1.245	35.6
160	1.901	1.543	36.4

The new method of calculation

To overcome this difficulty a method of estimating the error at the bottom of the isotherm is required or the calculation of the isotherm from the top downwards must be made. The second of these alternatives was attempted firstly by obtaining an independent value of S_1 and thus of Q. Since the method assumes linearity of the detector the area under the peak S_1 is proportional only to the amount injected and is independent of peak shape. Thus other column packings may be used which produce a sharper return to base line. At first other solid adsorbents were tried, but the best results were obtained using a standard 1-m column packed with 15% Silicone SE-30 on Celite.

This was operated at the same temperature of detector and flow rate as in the adsorption experiment. The value of S_1 obtained was dependent upon flow rate, despite parallel flow in the detector, *i.e.* both halves at atmospheric pressure. Since, in the adsorption run, the flow rate could not be reset accurately, but had to be read each time, calibrations were carried out at various flow rates and a calibration graph was drawn to give the correct S_1 at any flow rate. The reproducibility of S_1 using the liquid column at constant flow rate is shown in Table III and compared with values obtained on solid columns with severe tailing and non-elution of residual

benzene. The variation of S_1 with flow rate used to produce a calibration graph is also shown in Table III.

In the adsorption calculations the correct S_1 is read in with the initial data and used to calculate Q, Q_1 and pressure values using eqn. 2. In Fig. 2 the maximum adsorption is given by the area EKIO, but it is now assumed that there is an error at E and that area EKA is incorrect and should be obtained from S_1.

In Fig. 1 the area E_1KA represents S_1 which is the required area EKA plus the strip E_1KE. Since the distances marked x are equal this strip is equal in area to PKA. Using the points along the curve AK and dividing into horizontal strips a numerical integration for PKA was obtained. This was subtracted from S_1 to obtain area EKA and to this was added area AKIO also obtained by numerical integration, the total being the required area EKIO.

TABLE III

REPEATABILITY OF AREA UNDER THE CURVE ON A LIQUID COLUMN

Liquid column, 15 % silicone SE-30 on Celite. Sample injected, 10 μl benzene. Detector sensitivity, 50. Column temperature, 364.7°K; katharometer temperature, 373.9°K. Flow rate at katharometer temperature, 14.4 ml min^{-1}. Calibration of katharometer at various flow rates. Flow rates given at katharometer temperature. Flow rates, 32.5, 34.4, 31.3, 24.7, 19.5, 17.9, and 15.8. Area $\times 10^{-4}$, 3.502, 3.242, 3.534, 4.515, 5.902, 6.485 and 7.253 (logged reading $\times$ sec).

No. of run	*Area* $\times 10^{-4}$ *(logged reading* $\times$ *sec) on liquid column*
1	6.938
2	6.907
3	6.913
4	6.912
5	6.708
6	6.844
7	6.889
8	6.926
9	6.959

TABLE IV

REPEATABILITY OF AN ISOTHERM USING THE NEW METHOD OF CALCULATION

Lowest point detected on the isotherm, 19.93 μmole g^{-1} at 0.002 kN m^{-2}. Amo untnot desorbed at the end of calculation (pressure appears to be zero), 19.13 μmole g^{-1}. Conditions, as in Table I. Area under curve taken from calibration data, 3.90×10^5.

Adsorption (μ*mole* g^{-1})	*Pressure* (*kN* m^{-2})	*ln (pressure)*	*Difference between two injections*			
			Pressure		*ln (pressure)*	
			1 h	*24 h*	*1 h*	*24 h*
5	—	—	—	—	—	—
10	—	—	—	—	—	—
20	0.0022	−6.109	—	0.0003	—	0.132
40	0.078	−2.545	0.027	0.007	0.298	0.097
60	0.214	−1.543	0.064	0.009	0.261	0.040
80	0.414	−0.882	0.102	0.005	0.220	0.012
100	0.688	−0.374	0.147	0.003	0.194	0.004
120	1.052	0.051	0.205	0.016	0.078	0.014
140	1.538	0.430	0.283	0.036	0.017	0.023

TABLE V

HEATS OF ADSORPTION OF CYCLOHEXANE ON SPHERON 9 CALCULATED FROM ISOTHERMS AT 348.7°K, 351.1°K, 355°K, 360°K, AND 365°K

Adsorption (μ*mole* g^{-1})	$-\Delta H$	
	kcal mole^{-1}	*kJ mole*$^{-1}$
5	11.4	47.9
10	9.6	40.3
20	10.2	42.6
40	8.5	35.7
60	8.5	35.7
80	8.5	35.7
100	8.5	35.7
120	8.5	35.7
140	8.5	35.7
160	8.5	35.7
180	8.5	35.7
200	8.5	35.7
220	8.5	35.7
240	8.5	35.7
260	8.5	35.7

The other points on the isotherm were then obtained by subtracting successive trapezia KInC until the base line was reached. The residual amount adsorbed at this point was found to be of the order of 10–20 μmole g^{-1}.

The reproducibility of the isotherm by this new method is shown in Table IV using exactly the same data as for Table I. The results are better or at least as good as the old method but terminate at about 20 μmole g^{-1} because this amount is not eluted at a measurable concentration. The more reasonable values of heat of adsorption are shown in Table V and further results in Figs. 3 and 4.

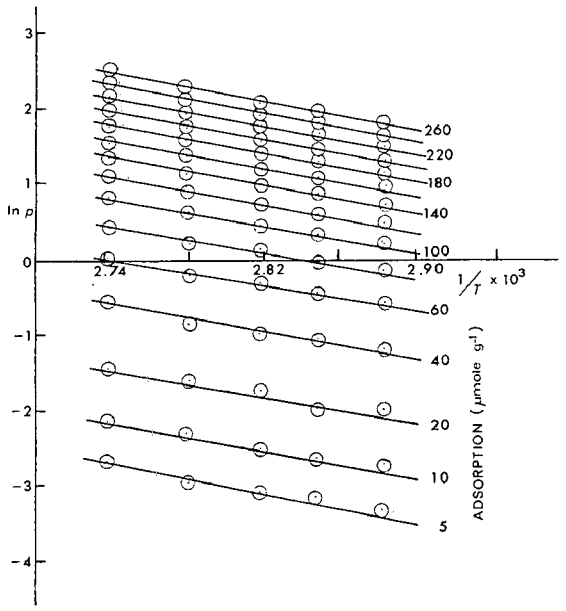


Fig. 3. Plots of ln P against $1/T$ at various coverages of cyclohexane on spheron 9.

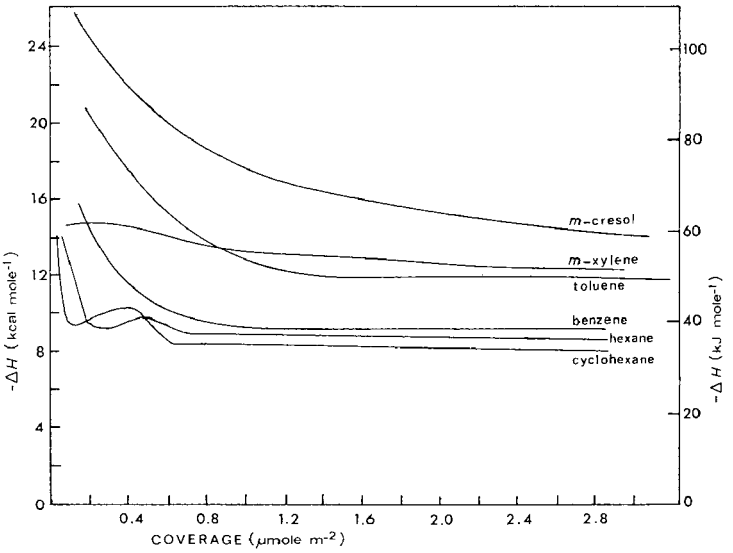


Fig. 4. Variation of $-\Delta H$ with coverage for spheron 9 and various adsorbates.

CONCLUSIONS

The elution technique for the measurement of the initial part of adsorption isotherms has proved to be very simple to carry out with standard chromatographic equipment. The tedious calculation involved is relieved by the use of a data logger and a computer. The change in the method of calculation produces more meaningful results for heat of adsorption at low coverage showing good correlation over a range of temperatures. A lower limit of adsorption measurements of 10–20 μmole g^{-1} is also shown for a sample of about 100 m^2g^{-1} surface area.

ACKNOWLEDGEMENT

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CHROM. 4795

GAS CHROMATOGRAPHIC ANALYSIS OF TRINITROTOLUENE FROM THE CONTINUOUS PROCESS

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SUMMARY

A rapid gas chromatographic method has been developed for determining the composition of the organic phase of the continuous TNT process. The method provides analyses for isomers of mono-, di-, and trinitrotoluenes in samples from nitration phases and for residual dinitrotoluenes and asymmetrical trinitrotoluenes from the TNT purification process and in the final product. The column used for separation was 10 % UC-W98 silicone rubber on 80–100 Diatoport-S. Detector response factors were determined and used to calculate relative percent components to eliminate time-consuming calibration and achieve better reproducibility.

INTRODUCTION

During 1968, the first continuous process plant in the U.S. for the manufacture of trinitrotoluene (TNT) began operation at Radford Army Ammunition Plant (RAAP), Radford, Va. In the continuous process, toluene is fed to the first nitrator and mixed nitrating acids to the latter nitrators. Nitric acid is fed at various stages of nitration to replenish acid used in the process. The flow is countercurrent and the toluene is nitrated to the mono-, di-, and trinitro products in a series of eight nitration vessels while the acid becomes progressively more dilute as the result of nitration. As TNT leaves the last nitrator, it enters the purification section of the process where the *meta* TNT isomers are removed by treatment with aqueous sodium sulfite. Following purification, the TNT is water-washed, dried, and flaked.

Rapid analytical methods were required to study the continuous change in composition of the organic phase throughout the process. GEHRING AND SHIRK[1] showed that gas chromatographic techniques could be applied to analyzing crude and refined TNT for TNT and DNT isomers of 0.02 to approximately 3.00 %. However, their method requires the availability of high-purity isomers for preparation of external standards and frequent checks on instrument calibration. The procedure also does not indicate that isomers of mononitrotoluene and other nitration components can be determined.

Others have reported methods on thin-layer chromatography[2,3], paper chroma-

tography[4], and infrared spectrometry[5,6]. Although these methods may be satisfactory for a specific purpose, none of the methods were satisfactory for samples taken throughout the continuous TNT process. Therefore, this paper describes a single, rapid, and accurate gas chromatographic procedure for the determination of toluene, trinitrobenzene, and isomers of mono-, di-, and trinitrotoluene throughout the continuous process. Flame ionization detector response factors were determined and used in calculating relative percentages thus eleminating daily time-consuming calibrations.

EXPERIMENTAL

Apparatus

All work was performed using a Hewlett-Packard Model 5750 dual-column gas chromatograph equipped with a flame ionization detector and a 1-mV recorder. The analytical columns were stainless steel, 12-ft. × 1/8-in. O.D. containing 10 % UC-W98 silicone gum rubber on 80–100 mesh Diatoport-S. (One was used as a reference column.) The columns were preconditioned at 280° for 18 h with a helium flow of 60 cc/min.

Reagents

The 2,5-, 2,3-, and 3,5-DNT isomers and the 2,3,5-, 2,3,4-, and 2,3,6-TNT isomers were furnished by Picatinny Arsenal, Dover, N.J. The 2,6- and 3,4-DNT isomers were purchased from K & K Laboratories Incorporated. The 2,4-DNT was military grade per MIL-D-204A from Joliet Army Ammunition Plant, Joliet, Ill. The 2,4,5-TNT was laboratory synthesized and purified by selective solvent extraction[7]. The 2-, 3-, and 4-MNT isomers were purchased from Aldrich Chemical Company, Incorporated, Milwaukee, Wisc. High-purity 2,4,6-TNT was received from Volunteer Army Ammunition Plant, Chattanooga, Tenn. and the 1,3,5-trinitrobenzene (TNB) from Eastman Organic Chemicals. All reagents were first tested individually by gas chromatography, thin-layer chromatography, and infrared to identify any traces of impurities and moisture was removed prior to use. Toluene was reagent grade.

Detector response factors

Since detector response will vary for each compound, it was necessary to determine correction factors for each component to be analyzed. The method used for this determination was as follows: Standard solutions were prepared containing a known weight (w) of each compound (approximately 4 mg/ml in acetone). Two microliters were injected into the gas chromatograph and a chromatogram was obtained. The area "A" of each peak was measured using the height of the peak times width at half height. The ratio A/w was calculated for each peak. The correction factor "F" was calculated by dividing the A/w of each peak by 2,4,6-TNT A/w thus arbitrarily setting 2,4,6-TNT equal to 1.00 and all other components relative to 2,4,6-TNT.

When separation interference occurred between components, each was prepared in individual standards with 2,4,6-TNT. Since 2,3,6-TNT elutes with 2,4,6-TNT, this comparison was made with 2,3-DNT which was previously standardized against 2,4,6-TNT. The relationship between 2,3,6-TNT and 2,4,6-TNT was then determined. Since 1,3,5-TNB does not completely separate from 2,4,6-TNT, its response factor was calculated two ways: (1) In direct comparison with 2,4,6-TNT and (2) in comparison with 3,4-DNT which was previously standardized against 2,4,6-TNT.

In sampling organic material from separator vessels, 1 ml was pipetted into a 25-ml volumetric flask containing approximately 5 ml of distilled water, hence quenching the reaction. The flask was then diluted to volume with reagent grade acetone. When nitrators were sampled, the acid and organic materials were allowed to first separate.

For analysis of organics in the acid phase, the organic material was separated by ether extraction. After the ether was evaporated, 1 g of organic material was dissolved in acetone and diluted to volume in a 25-ml volumetric flask.

Two microliters of the prepared samples were injected into the gas chromatograph at conditions given below. The instrument was attenuated prior to elution of each peak to obtain a satisfactory peak measurement.

Injection port:	230°
Detector:	260°
Column:	(A) 200° for samples containing only DNT and TNT isomers
	(B) 150° to 240° at 6°/min when toluene, MNT, DNT, and TNT isomers are present
Helium carrier gas flow:	60 cc/min
Hydrogen gas flow:	30 cc/min
Air flow:	500 cc/min

In calculating results, the area of each peak was obtained by multiplying instrument attenuation times peak height times width of peak at half height times "$1/F$". Total area was thus obtained, and percent by weight was calculated for each component by multiplying each individual area by 100 and dividing by total area.

TABLE I

FLAME IONIZATION DETECTOR RELATIVE RESPONSE FACTORS

Compound	*Weight factors* ($1/F$)	*Mole factors*[a]
Toluene	0.275	1.475
2-MNT	0.46	1.313
3-MNT	0.45	1.342
4-MNT	0.47	1.285
2,6-DNT	0.72	1.114
2,5-DNT	0.72	1.114
2,4-DNT	0.76	1.055
2,3-DNT	0.66	1.215
3,5-DNT	0.71	1.129
3,4-DNT	0.72	1.114
2,4,6-TNT	1.00	1.000
2,3,6-TNT	0.99	1.010
2,3,5-TNT	1.13	0.885
2,4,5-TNT	1.12	0.893
2,3,4-TNT	1.03	0.971
1,3,5-TNB	1.18[b]	0.795[b]
	1.53[c]	0.614[c]

[a] [(F) (molecular weight)] ÷ [(F for 2,4,6-TNT) (molecular weight for 2,4,6-TNT)].

[b] In the absence of 2,4,6-TNT.

[c] In the presence of large amounts of 2,4,6-TNT. This value is used for estimating TNB content in actual samples.

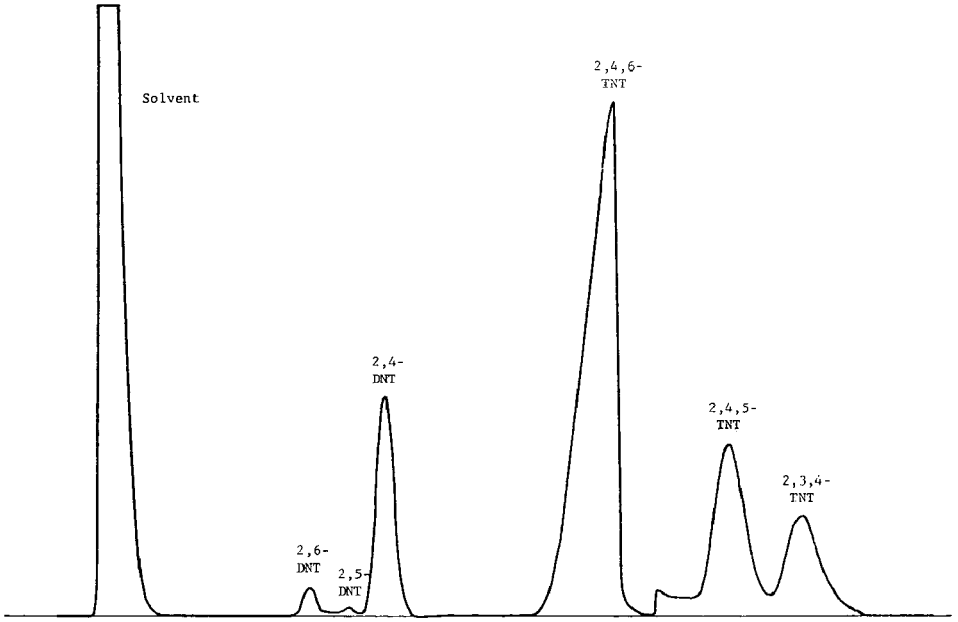


Fig. 1. Chromatogram of material from the last nitrator.

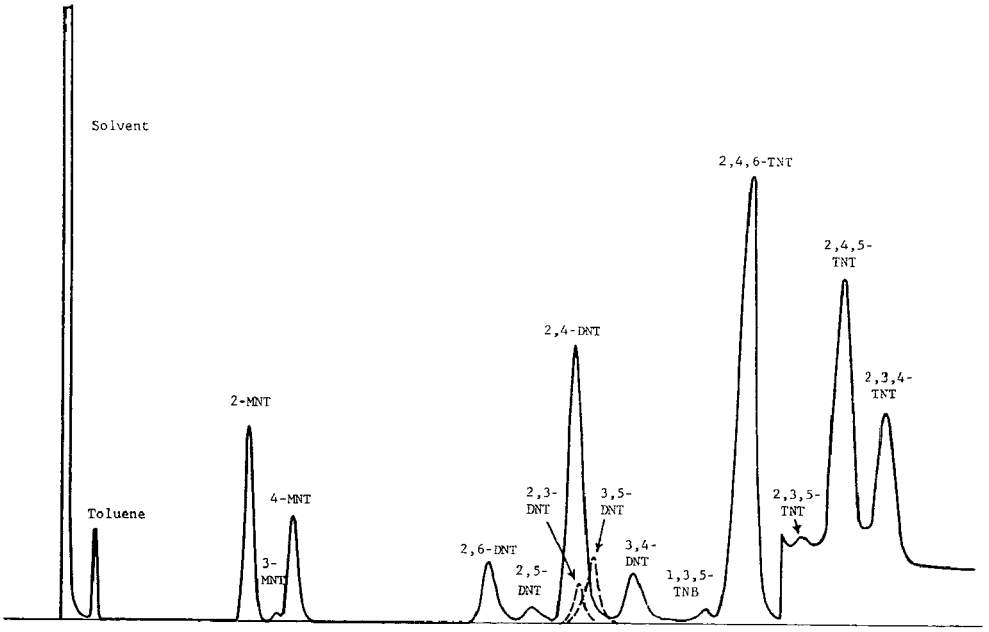


Fig. 2. Chromatogram of a synthetic mixture.

RESULTS AND DISCUSSION

Response factors were determined for nitration products found in various stages of nitration from the continuous process. These are shown in Table I. As might be expected, a relationship exists between the response factors and the number of unnitrated carbons. Fig. 3 shows this relationship where the average molar response factor is plotted against the number of unnitrated carbons in toluene.

During the course of this investigation, both programmed and isothermal gas chromatographic operating conditions were used. Chromatograms for these conditions are shown in Figs. 1 and 2 for isothermal and programmed, respectively. Isothermal conditions can be used to decrease the analysis time on samples containing only di- and trinitrotoluenes, whereas programming was required to get adequate separation of the various components when the samples contained aromatics with all degrees of nitration.

A number of the samples analyzed contained 2,4,6-trinitrobenzoic acid (TNBA) in addition to the nitrotoluenes. The TNBA is an oxidation product of 2,4,6-TNT.

TABLE II

PERCENT RECOVERY EVALUATION

Sample No.	*Components*	*Actual percent*	*Measured percent*	*Percent recovery*
I[a]	2-MNT	16.37	16.00	97.7
	3-MNT	0.05	—	—
	4-MNT	16.25	16.51	101.6
	2,6-DNT	16.83	17.06	101.4
	2,4-DNT	16.86	17.31	102.7
	2,4,6-TNT	16.66	16.85	101.1
	2,4,5-TNT	16.98	16.24	95.6
II[a]	2-MNT	17.40	17.89	102.8
	3-MNT	17.42	17.30	99.3
	4-MNT	0.03	—	—
	2,6-DNT	16.14	15.58	96.5
	2,4-DNT	17.08	16.94	99.2
	2,4,6-TNT	16.59	16.59	100.0
	2,4,5-TNT	15.34	15.67	102.2
III[b]	2,6-DNT	25.00	24.59	98.4
	2,4-DNT	25.05	24.89	99.4
	2,4,6-TNT	24.74	24.64	99.6
	2,4,5-TNT	25.21	25.88	102.7
IV[b]	2,6-DNT	24.77	24.26	97.9
	2,4-DNT	26.22	26.30	100.3
	2,4,6-TNT	25.46	25.95	101.9
	2,4,5-TNT	23.55	23.49	99.8
V[b]	2,6-DNT	24.77	25.59	103.3
	2,4-DNT	26.22	26.37	100.6
	2,4,6-TNT	25.46	24.92	97.9
	2,4,5-TNT	23.55	23.13	98.2

[a] Gas chromatographic oven programmed from 150° to 240° at 6°/min.
[b] Gas chromatographic oven operated isothermally at 200°.

With the injection port at 230°, the TNBA was converted to 1,3,5-TNB which shows up as a poorly resolved peak adjacent to the peak for 2,4,6-TNT, thus allowing only an estimation of total TNB content. The 3,5- and 2,3-DNT peaks are poorly resolved from the 2,4-DNT peak. However, the major DNT isomer present in all samples was the 2,4; therefore, the presence of the very small amounts of 2,3- and 3,5-DNT caused very little error in the determination of the 2,4-DNT; they were included as 2,4-DNT. The 2,3,6-TNT has the same retention time as 2,4,6-TNT; however, the amount of 2,3,6-TNT is so small as to cause a negligible error in the determination of the 2,4,6-TNT. The major unsymmetrical isomers produced in the continuous process are the 2,3,4- and 2,4,5-TNT. Both of these components are well resolved from 2,4,6-TNT permitting accurate analysis for these components.

Synthetic mixtures containing all of the nitration products were prepared to evaluate the GC method. The data for this study are shown in Table II. The results show very good recoveries for all components using the separation techniques described above and response factors in the calculations.

To further evaluate the method, duplicate samples were taken from several of

TABLE III

REPRODUCIBILITY STUDY OF SAMPLES FROM NITRATORS

	Average (%)		
	Nitrator No. 1	*Nitrator No. 3*	*Nitrator No. 6*
I. Components			
2-MNT	48.94	0.0023	
3-MNT	1.71	0.063	
4-MNT	27.86		
Total MNT	78.51	0.066	
2,6-DNT	2.47	2.37	0.0042
2,5-DNT	0.032	0.16	0.0010
2,4-DNT	13.55	19.81	0.058
3,4-DNT	0.114	0.28	
Total DNT	16.17	22.62	0.064
2,4,6-TNT	5.32	75.00	96.42
2,4,5-TNT	<0.01	1.60	2.19
2,3,4-TNT	0.018	0.72	1.31
Total TNT	5.34	77.32	99.92
	$S/\sqrt{\bar{X}}$		
	Nitrator No. 1	*Nitrator No. 3*	*Nitrator No. 6*
II. Variability			
Testing variability	0.075	0.043	0.060
Sample variability	0.093	0.055	0.047
Process variability	0.175	0.080	0.070

the nitrators at 10-min intervals. These samples were then analyzed in replicate to obtain a measure of testing, sampling, and process variability. The results of this study are given in Table III. Several methods were attempted for treating the data to obtain a measure for variability. As might be expected, the standard deviation was not constant for the different components at the various levels. This was also true of the coefficient of variation. A value for variability was obtained by dividing

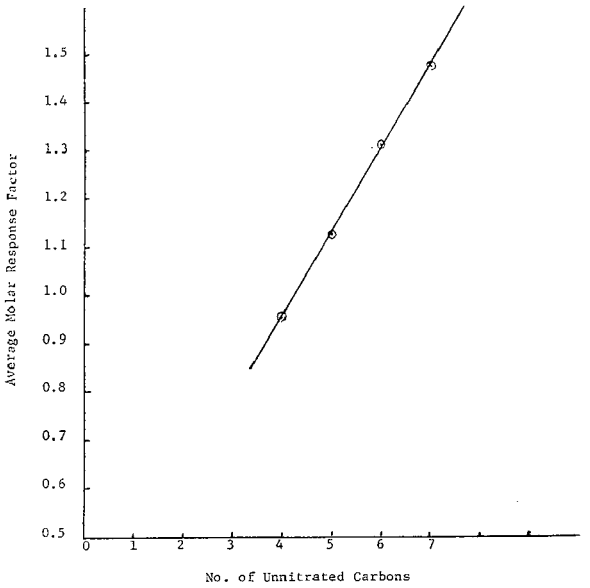


Fig. 3. Relationship between the response factors and the number of unnitrated carbons.

the standard deviation by the square root of the average value or the square root of 100 minus the average value for minor (< 50 %) and major (> 50 %) components, respectively. This value was found to be fairly constant for all components in a given nitrator and is also given in Table III for each of the nitrators for the three types of variability. The value for testing represents the reproducibility in the analysis of replicate aliquots from a given sample solution. The value for sampling includes the error involved in taking duplicate samples and in testing these samples. The value for process variability serves as a measure of the change in the process over a 30-min period. As can be seen from the data, there is very little difference in the testing and sampling variability indicating that a single sample provides an adequate measure of the composition of the organic phase at any given time. The values for process variability show that there is essentially no change in the composition of the organic phase in the No. 6 nitrator over a 30-min period. There is some change in the composition of the organic phase at nitrator No. 3, and a marked change in the composition of the organic phase in the No. 1 nitrator. Thus, by treating the data in the indicated fashion, it is possible to determine if changes in the composition of the organic phase as a result of changes in operating parameters are statistically significant.

Table III shows the types of nitration products present in several of the nitra-

tion vessels. In the first nitrator, toluene is nitrated to MNT with some DNT and TNT also being found. The presence of the TNT, and to some extent DNT, results from entrainment of these species in the acid being fed to the first nitrator rather than being a reaction product in this vessel. As the organic phase proceeds through the higher nitrators, the concentration of the MNT is reduced to nil. The concentration of TNT increases continuously while the concentration of DNT increases to a maximum in nitrator No. 2 and then decreases continuously through the higher nitrators.

From nitrator No. 6, the organic phase is washed with water to remove most of the entrained nitrating acids and is then reacted with a sodium sulfite solution in two successive vessels called sellite washers to remove the asymmetrical TNT isomers. A study of the purification process revealed that the extent of isomer removal was

TABLE IV

EFFECT OF pH IN THE NO. 2 SELLITE WASHER ON PURIFICATION

pH	*Percent by weight (2,4,5- plus 2,3,4-TNT)*
7.0	0.64
7.6	0.18
8.0	0.08
9.0	0.07

dependent upon pH. Table IV gives results on samples taken from the No. 2 sellite washer under different operating conditions. As can be seen, there is a marked difference in the amount of *meta* isomers remaining as a function of pH.

Table V shows another example of the application of the GC method in process studies. In this study, the temperature in nitrator No. 5 was adjusted from a low of 86.5° to a high of 99.0° to determine the magnitude of the effect of nitration temperature on the conversion of DNT to TNT. As can be seen, this roughly 10° increase in

TABLE V

EFFECT OF TEMPERATURE IN NO. 5 NITRATOR

Component	*86.5°*	*99.0°*
2,6-DNT	0.18	0.08
2,5-DNT	0.05	0.03
2,4-DNT	2.26	1.16
3,4-DNT	0.03	<0.01
Total DNT	2.52	1.27
2,4,6-TNT	94.75	95.99
2,3,5-TNT	0.01	0.02
2,4,5-TNT	1.72	1.78
2,3,4-TNT	0.98	0.90
Total TNT	97.46	98.69

reaction temperature reduced the concentration of residual DNT by approximately 50 %.

In another study, the concentration of the nitrating acid was varied to determine the magnitude of the effects of degree of nitration as a function of total acidity. Results from this study are given in Table VI. The results from this study show that the nitration products leaving the first nitration vessel are highly dependent upon total acidity.

TABLE VI

EFFECT OF SPENT ACID STRENGTH IN NO. 1 NITRATOR

	Normal total acidity (73.4 %)	*High total acidity (78.1 %)*
2-MNT	60.26	14.66
3-MNT	2.44	0.76
4-MNT	29.81	9.12
Total MNT	92.51	24.54
3,4-DNT	0.04	0.67
2,5-DNT	0.03	0.23
2,6-DNT	0.81	6.92
2,4-DNT	4.51	40.27
Total DNT	5.39	48.09
2,3,4-TNT		0.19
2,4,5-TNT	0.01	0.40
2,4,6-TNT	2.08	26.76
Total TNT	2.09	27.35

These are but a few examples of the information provided by the GC analysis of TNT samples in a process variable study. The results from these and other investigations will be used to optimize the process. Other process variables to be investigated are the ratio of nitrating acid to organic material in the various nitrators; the composition of the nitrating acid, *i.e.*, relative amounts of sulfuric and nitric acid and water; the temperature of nitration in all nitrators; and the rate of agitation.

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CHROM. 4770

STEROID MONO- AND DIHEPTAFLUOROBUTYRATES; PREPARATION, PURIFICATION AND ESTIMATION

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SUMMARY

Reaction conditions have been determined for the quantitative formation of steroid mono- and diheptafluorobutyrates with hydroxyl and 3-enol substituents. The percentage conversion at the low concentrations found in peripheral plasma (10^{-10} to 10^{-12} moles) was greater than 90%. Identification of these esters was established by gas chromatography on different stationary phases and by combined gas chromatography–mass spectrometry. Purification by gel filtration on small columns of Sephadex LH-20 permitted the separation of heptafluorobutyrates of oestrogens and 20β-dihydroprogesterone from the parent compounds without any detectable breakdown. These techniques, which are being employed for the assay of progesterone and oestradiol in peripheral plasma, are discussed in relation to other highly sensitive procedures.

INTRODUCTION

The preparation of steroid heptafluorobutyrates was described by CLARK AND WOTIZ[1] in 1963, and techniques have been developed subsequently for the estimation of steroids as these derivatives using gas–liquid chromatography with an electron capture detector[2–6]. In order to achieve high sensitivity and to assay circulating levels of ovarian steroids, we have studied in some detail the preparation and purification of diheptafluorobutyrate derivatives, which have greater electron-capturing properties than monoheptafluorobutyrates[3,7], pentafluorophenylhydrazones[8] or corresponding chloroacetates[9]. The diheptafluorobutyrates, unlike the monoheptafluorobutyrates, are relatively unstable. Chromatographic purification prior to assay has usually resulted in breakdown of these derivatives[9] but the use of gel filtration on Sephadex LH-20, swollen in organic solvents, has allowed their purification[10]. This paper describes the reaction conditions necessary to prepare high yields of steroid diheptafluorobutyrates compared with those for monoheptafluorobutyrates, the gel filtration of these derivatives on Sephadex LH-20, and their gas chromatographic behaviour on different liquid phases. Molecular weights have been determined by gas chromatography–mass spectrometry.

EXPERIMENTAL

Trivial names of steroids

Testosterone = 17β-hydroxyandrost-4-en-3-one; oestradiol-17β = oestra-1,3,5(10)-triene-3,17β-diol; oestradiol-17α = oestra-1,3,5(10)-triene-3,17α-diol; oestriol = oestra-1,3,5(10)-triene-3,16α,17β-triol; oestradiol-3-methyl ether = 3-methoxyoestra-1,3,5(10)-trien-17β-ol; dehydroepiandrosterone (DHA) = 3β-hydroxyandrost-5-en-17-one; oestrone = 3-hydroxyoestra-1,3,5(10)-trien-17-one; progesterone = pregn-4-ene-3,20-dione; 20α-dihydroprogesterone = 20α-hydroxypregn-4-en-3-one; 20β-dihydroprogesterone = 20β-hydroxypregn-4-en-3-one.

Chemicals

Sephadex LH-20 was obtained from Pharmacia Ltd., Uppsala, Sweden. All solvents were A.R. grade and were normally used within one week of redistillation. Nanograde benzene (Mallinckrodt Co.) was used directly. Heptafluorobutyric anhydride (HFBA) was prepared by refluxing a large excess (2 moles) of phosphorus pentoxide with 1 mole of heptafluorobutyric acid (Koch-Light Ltd.) overnight. A fraction that distilled at 108° was collected under anhydrous conditions, redistilled, and stored at 4° over silica gel where it was stable for several months.

Radioactive steroids

These were obtained from the Radiochemical Centre, Amersham, and had the following specific activities: [7α-^{3}H]progesterone, 8.5 Ci/mmole; [4-^{14}C]progesterone, 58.5 mCi/mmole; [6,7-^{3}H]oestradiol, 31.7 Ci/mmole; [6,7-^{3}H]oestrone, 500 mCi per mmole.

Radiochemical purity was checked at frequent intervals by radiochromatogram scanning after paper chromatography in the following systems: light petroleum–methanol–water (10:9:1) and petroleum ether–benzene–methanol–water (66:33:80:20); only those solutions which revealed a single radioactive peak corresponding to authentic steroid were used.

Labelled 20β-dihydroprogesterone was prepared from [7α-^{3}H]progesterone by conversion with 20β-hydroxysteroid dehydrogenase[11] and purified by paper chromatography[12].

Gas–liquid chromatography

Electron capture detection was performed on a Pye Series 104, Model 74 GLC apparatus, with a ^{63}Ni electron capture detector operated in the pulsed mode with an interval of 500 μsec and using a 1% XE-60 or 1% SE-30 column. The column oven temperature was 195°, and the detector oven temperature 235°. The carrier gas (argon–methane, 90:10) flow was 50 ml/min, and no purge gas was used. Samples applied in hexane to glass sample holders were also analysed using a Pye Series 104 Model 94 gas–liquid chromatograph equipped with an "Auto-Solids" injection system. The column oven was operated at 200°, and the flash heater 20–30° higher; carrier gas (argon–methane, 90:10) flow was 75 ml/min. GLC with flame ionisation detection was carried out on a Model 400 F and M Scientific Corporation gas chromatograph. Stationary phases used were 1% XE-60, 1% SE-30 and 1% QF-1. The 6-ft. columns were operated under the following conditions: column temperature 185–195°, detector

temperature 220°, flash heater temperature 240°; gas flows, argon (carrier) 50 ml/min, hydrogen 40 ml/min, and air 300 ml/min.

Radioactivity

Samples were counted on a Packard Tri-Carb liquid scintillation counter, Model 2003, in scintillation fluid (4 g PPO* + 0.1 g POPOP**/l toluene). The counting efficiency at optimum instrument settings was 38 % for tritium and 80 % for carbon-14. Quench corrections were found to be unnecessary.

Gas chromatography–mass spectrometry

This was performed on an LKB 9000 gas chromatograph–mass spectrometer in the Department of Chemistry, University of Glasgow. Chromatography was on a 1 % XE-60 column operated under conditions similar to those used in our own laboratory, and with 5α-cholestane as reference steroid.

Cleaning of glassware

New glassware was washed in chromic acid, acetic acid (10 %), hot tap water and finally distilled water. Subsequently it was washed in hot water and soaked overnight in 2 % Decon 75 and finally rinsed thoroughly with hot and distilled water and oven dried. In addition, tubes for heptafluorobutyrate esterification were rinsed in freshly double-distilled acetone, and allowed to air dry.

Preparation of steroid heptafluorobutyrates

The steroid solution was evaporated to dryness in small test tubes with ground glass joints under a stream of nitrogen at 53°. The optimal concentrations for the preparation of mono- and diheptafluorobutyrates were investigated by using different amounts of HFBA and benzene. The tubes were heated at 70° for 30 min on an aluminium block, then immediately placed in a desiccator at room temperature for 15 min. A fine stream of nitrogen was used to remove all traces of reagents from the tubes, and samples were taken up in a small volume (50 or 100 μl) of solvent. The percentage conversion of steroids to their heptafluorobutyrates was assessed chromatographically by GLC, and by gel filtration (Sephadex LH-20).

Purification

Sephadex LH-20 was equilibrated for at least 24 h in organic solvents (hexane, ethyl acetate, acetone or mixtures of these) and transferred to glass columns plugged with glass wool. Column dimensions were 200 × 2.5 mm (I.D.), 60 × 5 mm, 180 × 4 mm or 200 × 20 mm.

The effect of bed volume and solvent polarity on steroid and heptafluorobutyrate elution patterns was examined. Samples were applied to columns of known flow rates in three transfers of 100 μl solvent and timed fractions collected. After use, columns were stored at 4° under the appropriate solvent and were suitable for repeated use with negligible changes in flow rates for at least seven months.

Estimation

Heptafluorobutyrates were estimated by gas chromatography with electron

* PPO = 2,5-diphenyloxazole.
** POPOP = 1,4-bis[2-(5-phenyloxazolyl)benzene].

capture detection using a peak height ratio method after the addition of a suitable internal standard[13]. A known amount of labelled steroid added at the beginning of the experiment was used to correct for procedural losses.

RESULTS

Preparation of derivatives

The conditions required for the preparation of micro quantities of the derivatives are given in Table I. The substitution of a non-phenolic hydroxyl group required milder conditions of esterification (HFBA–benzene, 1–5:5–50) than those needed for the formation of an enol monoheptafluorobutyrate or of di- and tri-substituted compounds. The precise duration and temperature of the reaction was not critical and of the various conditions tested, most reproducible results were obtained by heating for 30 min at 70°.

TABLE I

CONDITIONS OF PREPARATION AND GAS CHROMATOGRAPHIC DATA OF STEROID HEPTAFLUOROBUTYRATES

Derivative	*Esterification* [a] *in HFBA (μl)–benzene (μl)*	*RRT*[b] *of steroid HFB*		ΔR_{mr}[c]	
		1% *SE-30*	1% *XE-60*	1% *SE-30*	1% *XE-60*
Monoheptafluorobutyrates					
(i) Hydroxyl group					
Testosterone	5:5	0.33	1.83	−0.16	−0.68
20β-Dihydroprogesterone	1:50	0.61	5.76	−0.15	−0.24
20α-Dihydroprogesterone	1:50	n.d.[d]	6.71	n.d.	−0.27
Oestradiol-17β	1:50	0.34	4.32	−0.16	−0.39
Oestradiol-17α	1:50	n.d.	3.95	n.d.	−0.41
Oestradiol-3-methyl ether	5:5	0.37	1.18	0.00	−0.42
Dehydroepiandrosterone	1:50	0.28	1.51	−0.08	−0.42
Oestrone	5:5	0.33	1.83	−0.16	−0.68
Pregnenolone	5:5	0.53	n.d.	−0.08	n.d.
(ii) Enol					
Progesterone	25:25	0.60	2.00	n.d.	−0.80
Diheptafluorobutyrates					
(i) Hydroxyl group					
Oestradiol-17α	5:5	n.d.	0.67	n.d.	−0.59
Oestradiol-17β	5:5	0.34	0.79	−0.08	−0.56
(ii) Hydroxyl and enol groups					
Testosterone	5:5	0.34	0.63	−0.07	−0.50
20α-Dihydroprogesterone	10:50	n.d.	0.81	n.d.	−0.59
20β-Dihydroprogesterone	25:25	0.60	0.83	−0.08	−0.55
Triheptafluorobutyrates					
Oestriol	10:5	0.49	1.24	−0.10	−0.33

[a] Reaction carried out at 70° for 30 min.

[b] RRT = relative retention time when 5α-cholestane = 1.0.

[c] $\Delta R_{mr} = \dfrac{\log r_{HFB} - \log r_{free}}{\text{number of substituted groups}}$, where r = relative retention time.

[d] n.d. = not determined.

The percentage conversion of microgram and submicrogram amounts was assessed by gas–liquid chromatography using flame ionisation detection and Sephadex LH-20 gel filtration. With the reaction conditions given above, greater than 95% conversion was observed for all steroids except oestriol, which gave a 75% yield. Mono- and di-substituted steroids were stable for up to six months if kept in acetone at $-15°$.

Gas chromatography

A better separation of steroid heptafluorobutyrates from the free compound was achieved on 1% XE-60 columns than on 1% SE-30 (Table I). The value ΔR_{ur} (see legend, Table I) was calculated and on XE-60 the following order of change was observed: progesterone ($\Delta R_{mr} = -0.80$), oestrone (-0.68), diheptafluorobutyrates of testosterone and the epimers of oestradiol and 20-dihydroprogesterone, monoheptafluorobutyrates of oestradiol and dehydroepiandrosterone (-0.39 to -0.42), the triheptafluorobutyrate of oestriol (-0.33), and the monoheptafluorobutyrates of testosterone and 20-dihydroprogesterone epimers (-0.22 to -0.27). Hence in compounds forming a diheptafluorobutyrate, the ΔR_{mr} values were very similar, the addition of a second group giving a relatively larger increase in ΔR_{mr} than was found in the mono-substituted compounds.

Gas chromatography–mass spectrometry

Definitive identification of steroid heptafluorobutyrates was ascertained by GC–MS, and the *m/e* values for the principal peaks of the spectrum are recorded in Table II. The base peak of all spectra was observed at *m/e* values which corresponded to the calculated parent molecular ion. For oestriol triheptafluorobutyrate (expected M^+, *m/e* 876) an accurate assignment was not possible under the conditions used. Only a small parent peak at *m/e* 484 was observed for DHA monoheptafluorobutyrate, a larger peak occurring at *m/e* 270, and corresponding to $[M\text{-}214]^+$, the loss of a heptafluorobutyryloxy residue (mass = 213) and hydrogen at C_3 of the parent molecule. For most steroids, peaks of *m/e* values were observed corresponding to the loss of one, two, or, for oestriol, three heptafluorobutyryloxy moieties.

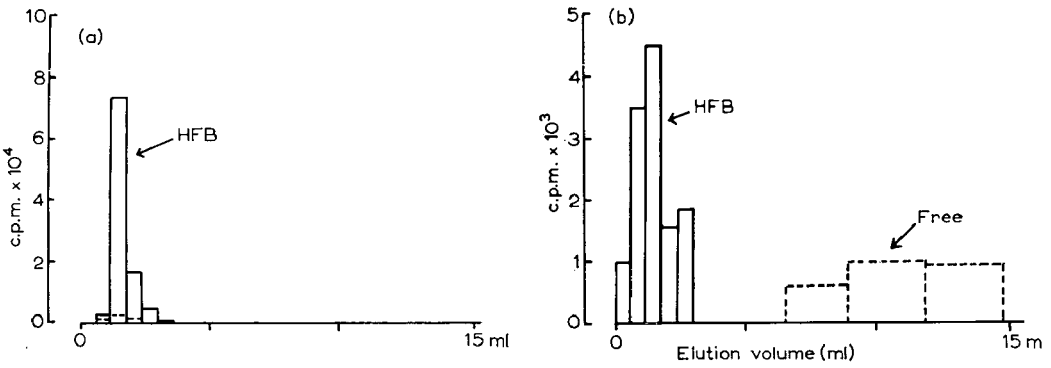


Fig. 1. Oestrogen elution pattern on Sephadex LH-20 swollen in hexane: (a) Oestradiol-17β, (b) oestrone. Column size, 180 × 2.5 mm. Flow rate, 0.62 ml/min. Solid bars, heptafluorobutyrate ester (HFB): dotted bars, parent steroid.

TABLE II

GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF STEROID HEPTAFLUOROBUTYRATES

Derivative	*Formula*	*Molecular weight*	*Mass spectrometry*		*Corresponds to*
			Main peaks (m/e)	*Mass loss*	
Monoheptafluorobutyrates					
(i) Hydroxyl group					
Oestrone	$C_{22}H_{21}O_3F_7$	466	466	—	molecular ion
			253	213	—HFB
Oestradiol-17β	$C_{22}H_{23}O_3F_7$	468	468	—	molecular ion
			254	214	—HFB + H
Oestradiol-3-methyl ether	$C_{23}H_{25}O_3F_7$	482	482	—	molecular ion
			269	213	—HFB
Testosterone	$C_{23}H_{27}O_3F_7$	484	484	—	molecular ion
			399	85	$-C_5H_9O$
Dehydroepiandrosterone	$C_{23}H_{27}O_3F_7$	484	(484)	—	molecular ion (very small)
			270	214	—HFB + H
20β-Dihydroprogesterone	$C_{25}H_{31}O_3F_7$	512	512	—	molecular ion
(ii) Enol					
Progesterone	$C_{25}H_{29}O_3F_7$	510	510	—	molecular ion
			495	15	$-CH_3$
Diheptafluorobutyrates					
(i) Hydroxyl groups					
Oestradiol-17β	$C_{26}H_{22}O_4F_{14}$	664	664	—	molecular ion
			451	213	—HFB
			237	427	—2 HFB + H
Oestriol	$C_{26}H_{22}O_5F_{14}$	680	680	—	molecular ion
			466	214	—HFB + H
(ii) Hydroxyl, enol					
Testosterone	$C_{27}H_{26}O_4F_{14}$	680	680	—	molecular ion
			467	213	—HFB
			253	427	—HFB + H
20β-Dihydroprogesterone	$C_{29}H_{30}O_4F_{14}$	708	708	—	molecular ion
			495	213	—HFB
Triheptafluorobutyrates					
Oestriol	$C_{30}H_{21}O_6F_{21}$	876	850	—	molecular ion
			663	213	—HFB
			450	426	—2 HFB
			237	639	—3 HFB
			234	642	—3 HFB + 3 H

Purification

Mono- and di-substituted compounds were purified and separated from unconverted steroid by gel filtration on Sephadex LH-20. Experiments were carried out with radioactive steroids to establish the elution patterns of free steroids and their separation from heptafluorobutyrate esters (Fig. 1). It was found with Sephadex columns (180 × 2.5 mm) swollen in hexane that nanogram amounts of oestrone and oestradiol heptafluorobutyrates were eluted immediately after the void volume (V_e values: oestrone, 1.5; oestradiol, 1.5; where V_e is elution volume, in ml, to position of maximum of peak). Recoveries of 17β-oestradiol diheptafluorobutyrate and oestrone monoheptafluorobutyrate were greater than 90 % in the first 3 ml while less than 3 %

of the free oestrogens eluted in the same volumes. Although free oestrone (V_e 10.8) was well separated from its heptafluorobutyrate (Fig. 1), free oestradiol, which is sparingly soluble in hexane, was eluted very slowly. On columns swollen in solvents of greater polarity (hexane–ethyl acetate, ethyl acetate, acetone) free oestradiol was more readily eluted, although its separation from the heptafluorobutyrate was less complete. A good separation of free and esterified oestradiol was achieved when the bed volume was increased (180 × 4 mm or 200 × 20 mm) but these columns were not suitable for routine assays.

In contrast, the mono- and diheptafluorobutyrates of progesterone and 20β-dihydroprogesterone, respectively, were less well resolved from the free compounds (Fig. 2). The best separation was obtained on Sephadex columns (180 × 4 mm) swollen in ethyl acetate from which the heptafluorobutyrates were eluted in a relatively discrete fraction (V_e 1.80–1.85) compared with the free compound (V_e 2.5–3.0). Recoveries of known amounts of free steroids and their esters were greater than 85 %.

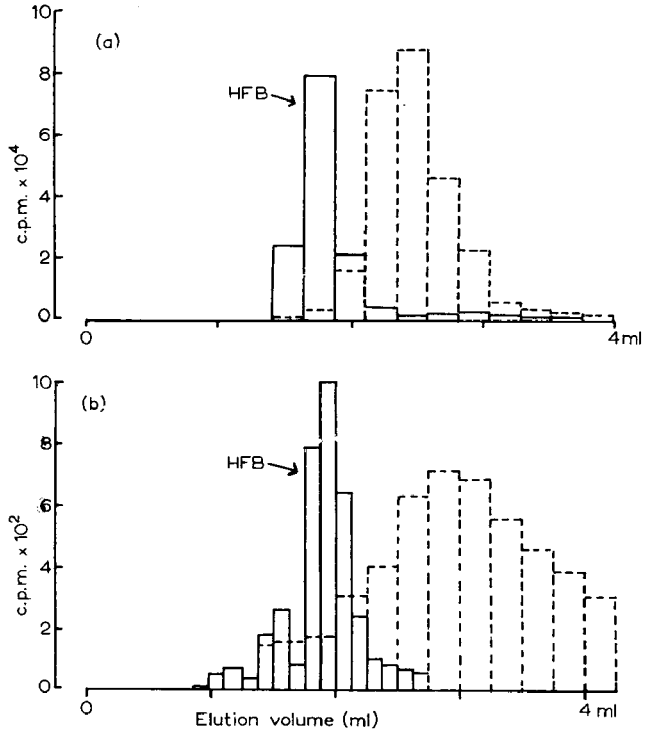


Fig. 2. Progesterone (a) and 20β-dihydroprogesterone (b) elution patterns on Sephadex LH-20 swollen in ethyl acetate. Column size, 180 × 4.0 mm. Flow rate, 0.12 ml/min. Solid bars, heptafluorobutyrate ester (HFB); dotted bars, parent steroid.

The amount of free steroid overlapping into the heptafluorobutyrate fraction was 14.6 % but since in assays at least 90 % of the steroid was usually esterified, the radioactivity derived from any unconverted free steroid would be negligible.

The stability of mono- and diheptafluorobutyrates during the purification

procedure was investigated. When a known amount (12.5 ng) of 17β-oestradiol was esterified, purified through Sephadex LH-20 and estimated by gas chromatography using dehydroepiandrosterone monoheptafluorobutyrate as internal standard, a recovery of 11.8 $\pm$ 1.2 ng was obtained. No additional peaks, indicative of breakdown, were observed on the GLC trace.

Estimation

Crystalline DHA and progesterone monoheptafluorobutyrates for use as internal standards were prepared by techniques previously described[9,14]. Purity was confirmed by melting point determinations, and by gas chromatography of high concentrations using flame ionisation and electron capture detectors. The derivatives were stored at $-15°$, and were stable for at least three months under anhydrous conditions. Solutions in acetone or benzene were stable for up to twelve months.

Calibration lines for progesterone, 20β-dihydroprogesterone and 17β-oestradiol were constructed. After esterification and gel filtration heptafluorobutyrates were taken up in hexane (100 μl) containing an internal standard, DHA heptafluorobutyrate (2 μg/ml) for 17β-oestradiol diheptafluorobutyrate and progesterone monoheptafluorobutyrates, or progesterone heptafluorobutyrate (13 μg/ml) for 20β-dihydroprogesterone diheptafluorobutyrate. There was a linear relationship between peak height ratios (steroid estimated:internal standard) and concentration for amounts up to 1 ng injected onto the column (Fig. 3).

The stability of esterified progesterone and 17β-oestradiol was investigated by

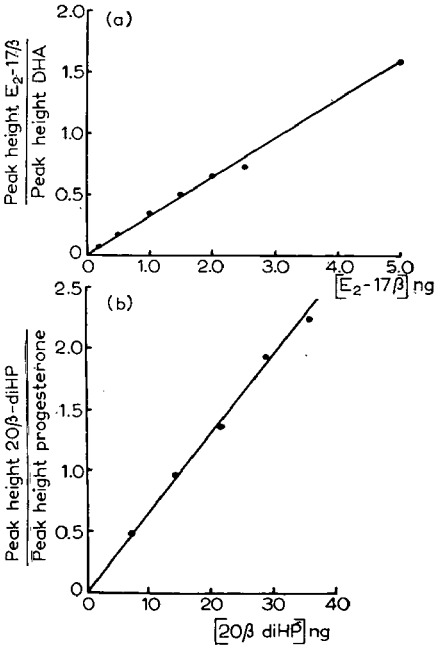


Fig. 3. Calibration lines for (a) oestradiol-17β (E_2-17β) and (b) 20β-dihydroprogesterone (20β-diHP). For GLC conditions, see text.

TABLE III

THE STABILITY OF PROGESTERONE MONOHEPTAFLUOROBUTYRATE

Samples of progesterone (5, 10, 25, 35, 50 ng) were esterified and applied in liquid injections to gas chromatography after taking up in hexane containing dehydroepiandrosterone monoheptafluorobutyrate (2 μg/ml). Day 1 = day of preparation.

Statistic	*Day*			
	1	*2*	*3*	*4*
Coefficient of linear regression, r	0.999	0.999	0.994	0.989
t-Value	79.923	47.973	16.230	11.949
Slope of linear regression, b	0.0321	0.0310	0.0301	0.0280
S.E.$_b$	0.0003	0.0006	0.0018	0.0023

repeated assays of the same samples stored at $-15°$ on four consecutive days (Table III). The coefficient of the linear regression was highly significant in all instances, but for progesterone heptafluorobutyrate there was evidence of slow hydrolysis judged by a decrease in slope of the line, and an increased variation. The calibration line for oestradiol 17β-diheptafluorobutyrate was constant for at least four days.

Standard solutions of all three steroids were also measured using a solid injection system for applying samples to the column. After the samples had been applied in hexane to glass "slugs" and allowed to evaporate in air at ambient temperature, repeated chromatography showed that the compounds were stable for at least 24 h in a dry state and in an inert atmosphere (progesterone, coefficient of linear regression for five standards: $r = 0.993 \pm 0.003$, S.E.$_b \pm 0.0032$, $n = 3$).

DISCUSSION

The reaction conditions required to form hydroxyl and 3-enol substituted mono- or diheptafluorobutyrates have been determined for several steroids at concentrations expected in peripheral plasma of different species (10^{-10} to 10^{-12} moles). These conditions are similar to those described for the preparation of the two testosterone heptafluorobutyrates[3]. With the exception of DHA monoheptafluorobutyrate, definitive identification of both mono- and di-substituted esters was obtained by GC–MS since the M^+ of the mass spectra was observed at an m/e corresponding with the theoretical molecular ion of the steroid heptafluorobutyrate. The GC–MS results for monosubstituted compounds confirm those of other workers[9].

An objection to the use of the high electron-capturing diheptafluorobutyrates for steroid assays has been the inability to purify these relatively unstable compounds by chromatography[7,9]. Consequently direct assay by gas chromatography may result in a false estimation of tracer recoveries[4]. We have found, however, that by the careful standardisation of anhydrous reaction conditions, high yields of these esters were obtained, and in instances where this was not achieved, gel filtration on Sephadex LH-20 allowed separation of the ester from unconverted steroid[15]. With regard to oestradiol diheptafluorobutyrate, gel filtration aids mainly in the removal of electron capturing impurities before GLC rather than in separation from the free compound, since unesterified steroid has a low solubility in the volume of hexane employed.

Stable crystalline preparations of DHA and progesterone monoheptafluorobutyrates were used as internal standards for GLC, but attempts to crystallise oestradiol diheptafluorobutyrate were unsuccessful. In assays, calibration lines were constructed from different concentrations of oestradiol esterified simultaneously with unknown samples. The slope of these standard lines remained constant for at least four days provided the samples were kept in hexane at −15°. Low concentrations of progesterone monoheptafluorobutyrate or 20β-dihydroprogesterone diheptafluorobutyrate, however, showed a gradual breakdown over a period of four days. It was significant that both mono- and diheptafluorobutyrates were stable for up to 24 h in the inert atmosphere of the loading magazine of the "Autosolid" injection system which should give considerable improvement in sensitivity and rapidity of such GLC assays.

Despite the preparation of derivatives with greater electron capturing properties than even the diheptafluorobutyrates[15,16] far greater sensitivity, necessary for the measurement of some steroids, may be achieved by competitive protein-binding assays[17,18]. Problems such as solvent impurities are common to both types of procedures, but although the technique of electron capture is both more expensive and time-consuming than that of protein binding, it is still essential for those steroids for which a specific receptor protein has not yet been isolated. In addition it is difficult by displacement analysis using radioisotopes to obtain precise estimations of mass in those kinetic experiments where the isolated steroids are already labelled. Moreover, the extensive chromatographic purification and derivative formation required to establish radiochemical purity offsets the advantages of protein-binding techniques.

In our laboratory, assays employing gas chromatography with electron capture detection have been developed for the routine estimation of plasma progesterone, oestradiol-17β and oestrone. Thin-layer chromatography has been used for purification, thereby achieving a 50–60% recovery of added radioactive oestradiol-17β. It is possible to measure this steroid as its 3,17β-diheptafluorobutyrate at concentrations as low as 30 pg/ml plasma.

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A SYSTEM FOR SIMULTANEOUS 25-COLUMN, CAPILLARY COLUMN LIQUID SOLID CHROMATOGRAPHY

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SUMMARY

A system for simultaneous 25-column, capillary column liquid solid chromatography is described. It uses compressed nitrogen to drive the eluant through the capillary columns. A comparison with a system using chromatographic pumps shows that the nitrogen system costs 80% less per channel, has better resolution and higher reproducibility and there are fewer maintenance problems. The problem of the more variable flow rate has been overcome by the addition of an internal standard.

INTRODUCTION

We first described a system for multi-column liquid chromatography in 1960[1,2]. This system has been gradually expanded in capacity and improved in the decade since then. The first multi-column system used gravity feed to drive the eluant through the columns and the colorimetry was done manually. An improved system[3] pumped the liquid through the columns with chromatographic pumps and at the same time an automated read-out system with computer calculation of data was introduced. The system gained much in practicality and overall performance characteristics when capillary columns, first glass[4] then teflon[5], supplanted the wider-diameter thermostatted glass columns previously used. A technique was described for the machine filling of the capillary columns[6] and an improved multi-column fraction collector using magazines[7] was constructed. This new type of collector formed the nucleus for a high-capacity multi-channel computerized read-out system[8] with a capacity of 100 chromatograms per day expandable to 400 chromatograms per day. Practical work with this system has made it clear that we would have to simplify the chromatographic technique to fully exploit the increased capacity made possible by the new read-out system.

The main obstacle to very high capacity multi-column systems are the chromatographic pumps used to pump the eluant through the capillary teflon columns[3]. These pumps are expensive, costing several hundred dollars per channel, and this for most laboratories would make it prohibitively expensive to consider systems incorporating for example a hundred or more of these pumps.

We have, consequently, explored the possibility of building a system using compressed nitrogen to drive the eluant through the capillary columns thereby greatly reducing the cost per channel in multi-column chromatography.

GENERAL DESCRIPTION OF SYSTEM

A general view of the system is given in Fig. 1. The capillary teflon columns used in the chromatography[5,6] are in the waterbath. Sample transfer is through the wider-diameter cartridges of teflon tubing shown above the waterbath. The elution solvent for gradient elution is mixed in the stainless steel cylinder shown to the left

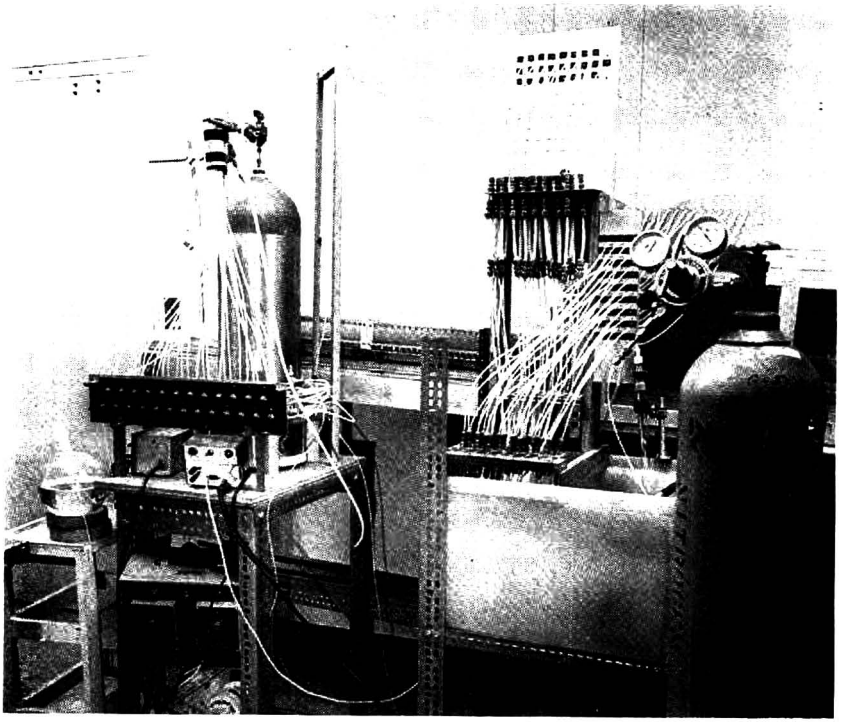


Fig. 1. Mixing and distributing cylinder for the formation of the gradient used for elution is to the left. The capillary columns are in the center with the filling cartridges on top. The nitrogen tank with its regulator is to the right and the multicollector is seen in the background.

and is then sent through the panel of needle valves located in front of the cylinder to the chromatographic columns. The pressure to drive the solvent through the capillary columns is supplied by the nitrogen tank with regulater positioned to the right in the figure. The effluent is collected in the 25-column multi-collector shown in the background. The gradient is formed by pumping a more polar solvent into a less polar solvent in the stainless steel pressure cylinder using a chromatographic pump positioned on the floor.

DETAILS OF CONSTRUCTION

Mixing and distributing cylinder

The mixing and distributing cylinder (Fig. 2) has been made from a stainless steel sampling cylinder (Hoke, Cresskill, N. J., No. 8LS5-G) of five gallons' capacity. The round bottom of this cylinder was cut off and a flat piece of 1-in.-thick stainless steel was welded on as a bottom. The stainless steel used has been of the non-magnetic type to make magnetic stirring of the solvent inside possible.

In the periphery of the bottom of the cylinder 25 L-shaped holes have been drilled and swagelock fixtures (Hoke No. 2CM2) have been fitted to each of these holes. Teflon capillary tubing leading the solvent to the needle valves have been placed inside the swagelock fittings. The teflon tubing from the outlets at the bottom of the cylinder are connected to the 25-valve needle valve (Hoke No. 1325GY)

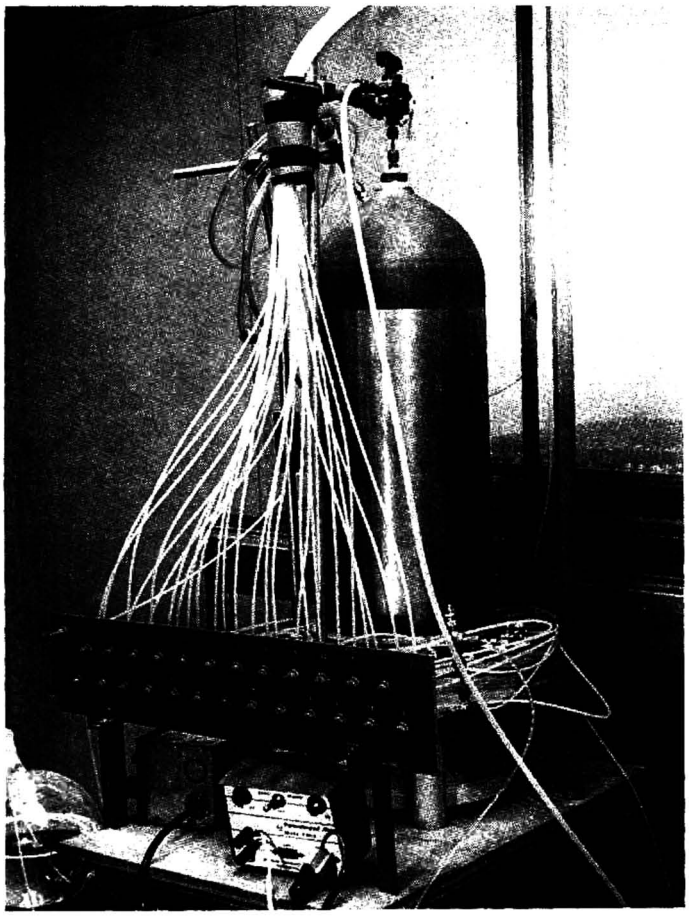


Fig. 2. The mixing and distributing cylinder. Teflon tubing goes from the outlets at the bottom of the cylinder to the needle valves in front to pass from here to the chromatographic columns. Two ball valves on top for solvent introduction and the application of nitrogen pressure to the system. A magnetic stirrer is placed under the cylinder for mixing of the gradient in the cylinder.

panel shown in the foreground of Fig. 2. From these valves solvent is passed through capillary teflon tubing to the chromatographic columns located in the waterbath.

Two ball valves (Hoke No. 7165 G25) lead to the inside on the top of the cylinder. One serves to enter the main solvent used in the chromatography into the cylinder before the run is started and to introduce the gradient forming solvent during a run. The other ball valve connects to the nitrogen tank through a reduction valve and regulator that allows the selection of the proper pressure for the chromatography.

The cylinder is placed on top of a magnetic stirrer and during operation a teflon-covered magnet on the bottom of the cylinder provides the stirring action needed to mix less polar and more polar solvents during the chromatographic run.

Chromatographic column arrangement

We have previously[7] described a very concentrated arrangement of capillary teflon columns spirally wound in a small space in a small waterbath that could be placed close to the multi-collector used in the fraction collection. This system had

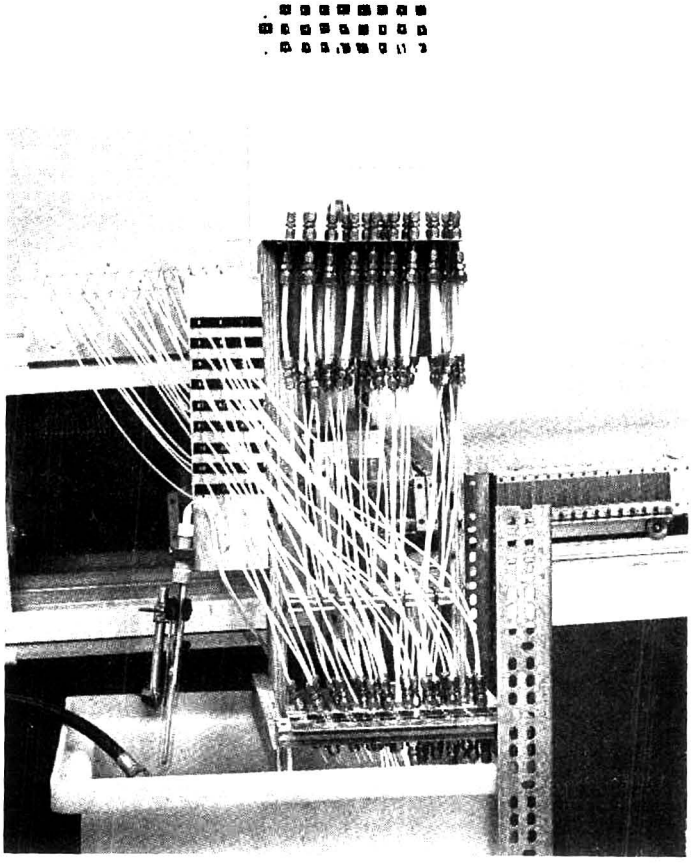


Fig. 3. The column arrangement. The capillary columns are in large loops in the waterbath. The tubing over the water level serves for transfer of the sample to the top of the capillary columns.

the undeniable advantage of giving very little mixing of the eluent as it leaves the columns. It was, however, found quite time-consuming to use the system in daily routine runs and we have therefore changed over to the system shown in Fig. 3. The sample is transferred in the proper solvent to the top portion of the capillary column and the wide-diameter teflon cartridges shown in the centre of the figure. The total transfer volume is about 3 ml, which allows one 1-ml transfer and four washes with 0.5 ml solvent to assure complete transfer to the column of a dry extract. The capillary column itself is loosely wound into a couple of circular loops held together with two pieces of plastic-covered wire as used in an ordinary household for the closing of plastic bags for freezer or refrigerator. The column is then placed in the waterbath.

The 25-column multi-collector

This (Fig. 4) is similar to the original design[1] only enlarged in capacity to accomodate 25 rows of test tubes. The reason we prefer the older desing over the magazine-type collector for high-capacity multi-column chromatography[7] is that this type of collector can be stacked one on top of each other so that for example four 25-column collectors can conveniently be combined into one 100-column collector.

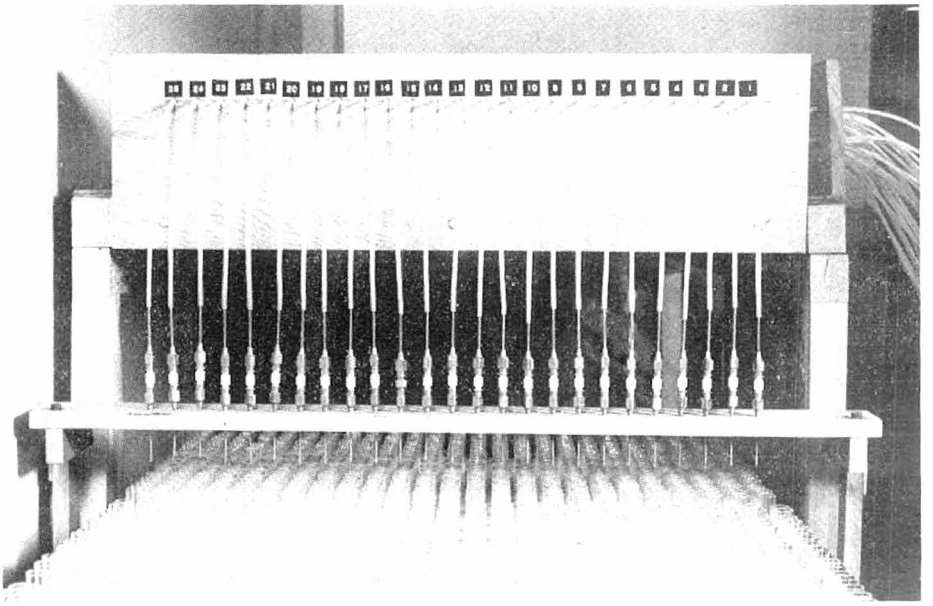


Fig. 4. This figure shows the 25-column multicollector and the way the effluents from the chromatographic columns are transferred to the collector tubes.

OPERATION OF THE SYSTEM

Preparing the columns for chromatography

The capillary teflon columns are dry-filled by machine in a one-batch operation as previously described[6]. They are loosely bound into large loops as described above

and arranged in the waterbath where they are connected to the wider-diameter teflon tubing on the cylinder side and the tubing leading to the fraction collector on the other side.

Non-polar solvent is now introduced into the stainless steel cylinder through the T-valve at the top of the cylinder with the needle valves closed. Pressure is applied to the cylinder through the valve connected to the nitrogen after closure of the other ball valve and the needle valves are kept open until all air has been flushed out of the tubing and the chromatographic columns. The valves are then closed and the solvent remaining in the cylinder is let out of the cylinder through a separate needle valve attached to one of the outlets.

Application of the sample to the columns

The swagelock fitting on top of the wider teflon tubing is opened up. The solvent down to the top layer of the chromatographic column is aspirated with a syringe to which has been attached a thin needle equipped with a piece of thin capillary teflon tubing at the end. The sample is introduced using the same small syringe with teflon tubing attached .Because of the wider teflon tubing attached to the capillary tubing a transfer of an extract from a test tube can be done with an initial 1-ml transfer of sample in solvent followed by four ½-ml washes to ensure complete transfer of the extract. The system is connected up again after the transfer of the sample and the columns are now ready for chromatography.

Filling the cylinder

The appropriate amount of non-polar solvent is introduced through one of the ball valves located at the top of the cylinder. This is done with the needle valves closed. The solvent feed valve, a T-valve, is turned towards the gradient pump that pumps the more polar solvent into the mixing cylinder. The system is now started up by turning the magnetic stirrer on, starting the gradient pump pumping, opening the needle valves to the chromatographic columns and opening the valve leading to the nitrogen tank. The nitrogen pressure is set to an appropriate value and the fraction collector is started up.

PERFORMANCE

Resolution

We were somewhat surprised to find in initial runs with the system that the resolution in the nitrogen pressure system seemed to be better than in a system using pumps to get the solvents through the capillary columns. However, this was repeatedly found to be so and was proven by the following experiment.

Two sets of twelve batches of standards each containing the following seven common urinary 17-ketosteroids, *viz.* dehydroepiandrosterone (DHEA), androsterone (A), etiocholanolone (E), 11-ketoandrosterone (OA), 11-ketoetiocholanolone (OE), 11-hydroxyandrosterone (OHA) and 11-hydroxyetiocholanolone (OHE), were run in a 12-column system using the new nitrogen pressure system.

Identical conditions were maintained throughout. The packing of the columns were done in the same manner, the gradient was the same, the temperature was kept the same and so on. Approximately 50-μg amounts of each of the standards were applied to each column.

The conditions for the chromatography were: 6-ft.-long capillary columns with an I.D. of 1.5 mm were used. Neutral alumina for chromatography, Merck, deactivated with 6 % water was the substrate. The gradient was ethanol in benzene. The pressure was approximately 8 atm. in the nitrogen system. Total run time was 20 h.

Theoretical plate numbers were calculated after the standard formula[9]: $N = 16(R'/w)^2$, where R' is the corrected retention volume and w is the width of the peak defined as that part of the peak that is within two standard deviations on either side of the peak maximum assuming that the peak follows a Gaussian curve. Since we do not have a continuous curve in our system we have based the width calculation on the fact that 95.44 % of the area of a normal curve is within two standard deviations on each side of the curve maximum and we have consequently included in the calculation of peak width enough fractions to give 95.44 % or more of the total area of a given peak.

A comparison of the resolutions found in the system using chromatographic pumps as compared with the nitrogen pressure system is given in Table I. It can be seen from this table that the theoretical plate numbers are consistently higher, on an average 50 % higher, in the system using compressed nitrogen.

TABLE I

AVERAGES FOR THEORETICAL PLATE NUMBER CALCULATIONS FOR TWELVE SETS OF SEVEN 17-KETOSTEROIDS RUN IN A NITROGEN PRESSURE SYSTEM AS COMPARED WITH A CHROMATOGRAPHIC PUMP SYSTEM

For abbreviations used for 17-ketosteroids, see text.

	DHEA	*A*	*E*	*OA*	*OE*	*OHA*	*OHE*	*Average for all seven 17-ketosteroids*
Nitrogen system	3730	5133	5737	6893	7700	7503	7923	6374
Pump system	2664	2951	3637	4739	5077	4692	5324	4155

The difference in the performance is shown in Fig. 5 that compares the best of the chromatograms from the pump and the nitrogen runs. It can be seen that there is considerable less tailing in the nitrogen system.

We believe that the difference in resolution between the two systems is due to the segmentation of the eluant stream produced by the release of dissolved nitrogen as it passes from the column as shown in Fig. 6. The deliberate introduction of gas bubbles to obtain sample separation in continuous stream analysis is one of the important features of the widely used Technicon Autoanalyzer system and it seems probable that the higher resolution in the pressurized system is due to the nitrogen bubbles serving as separating segments between each stretch of eluant solvent.

Reproducibility

The reproducibility of the two systems was compared by calculating coefficients of variation for the two 12-column runs for the seven 17-ketosteroids estimated. As can be seen from Table II somewhat better reproducibility is obtained overall in the nitrogen system.

Application of the nitrogen system to routine urinary steroid analyses

Use of an internal standard and R_F values relative to this standard. One of the drawbacks the nitrogen system has relative to the pump system is that whereas in the pump system elution volume per hour of elution time is quite constant (within 1 %) from column to column because of the high reproducibility of the chromato-

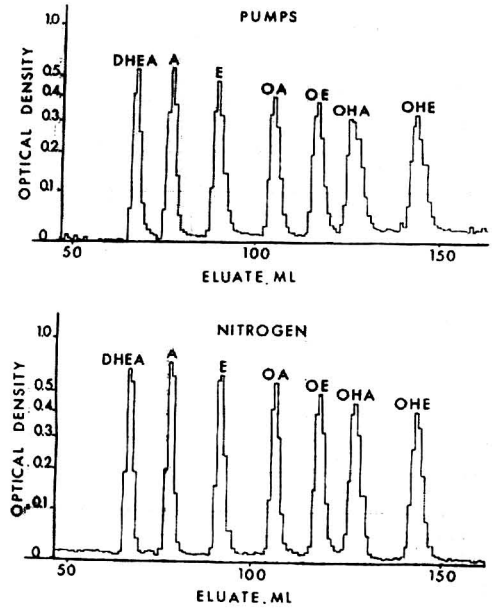


Fig. 5. Two chromatograms of seven 17-ketosteroids from the 12-batch runs mentioned in the text. On top the best of the chromatograms form the pump run, at the bottom the best of the chromatograms from the nitrogen system. For abbreviations used for 17-ketosteroids, see text.

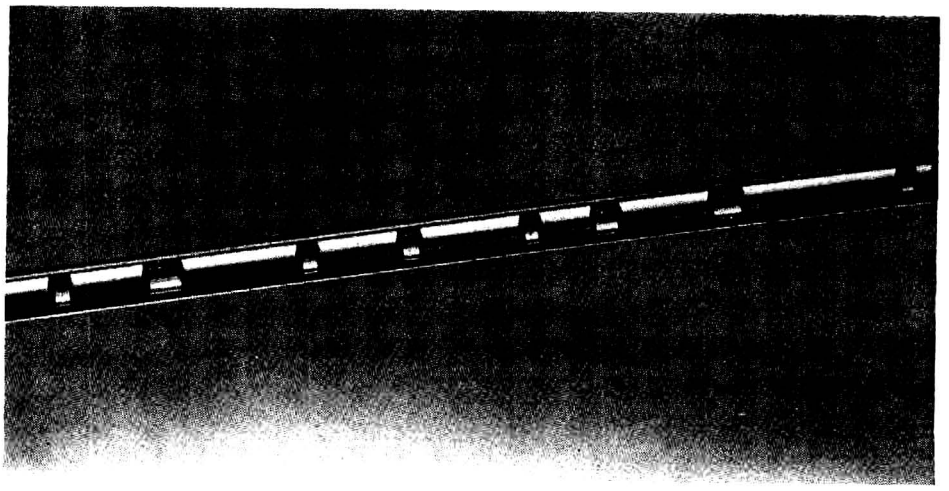


Fig. 6. A photograph of the effluent stream in the teflon tubing leading from the chromatographic column. The segmentation of the stream by nitrogen bubbles is clearly visible.

TABLE II

AVERAGES WITH COEFFICIENTS OF VARIATION FOR TWO 12-COLUMNS RUNS OF BATCHES OF SEVEN 17-KETOSTEROIDS RUN USING THE NITROGEN SYSTEM AND THE CHROMATOGRAPHIC PUMP SYSTEM

Approximately 50-μg amounts were used. Values refer to a DHEA standard and are uncorrected for differences in chromogenicity. Coefficient of variation is the standard deviation in per cent of the mean. For abbreviations used for 17-ketosteroids, see text.

	DHEA	*A*	*E*	*OA*	*OE*	*OHA*	*OHE*	*Average*
Nitrogen system								
Average (μg)	48.5	50.2	47.7	44.9	46.2	47.2	43.4	
Coefficient of variation	1.6%	2.4%	2.1%	2.5%	2.8%	2.6%	2.3%	2.3%
Pump system								
Average (μg)	47.1	49.8	47.5	44.7	46.1	48.9	45.9	
Coefficient of variation	3.7%	1.5%	2.0%	2.8%	4.4%	3.8%	5.2%	3.8%

graphic pumps this is not the case in the nitrogen pressure system. Differences in elution volume from column to column generally may vary as much as 10% around a mean if no attempt is made to adjust the flow through the needle valves. This of course is possible, but generally much too time consuming to be practical in a routine run.

To get around this problem in our routine analysis for urinary 17-ketosteroids we have added an internal standard to each urinary extract before transfer to the chromatographic column. We have for this particular analysis chosen to add 11-keto-androsterone (OA). It is the center peak in the chromatogram and is of little physiological and biological interest and besides it is usually the smallest peak in a chromatogram of urinary 17-ketosteroids. The addition of such an internal standard makes it possible to calculate relative R_F values for the other main 17-keto-steroids when compared with the internal standard. One prerequisite for this is that so much of this standard is added that it can easily and unequivocally be identified from the chromatogram.

As can be seen from Fig. 7, showing the chromatogram of a urinary extract with OA added compared with a standard chromatogram, such a strategy is quite feasible. The important question is now how reproducible relative retention times for the different 17-ketosteroids as compared to OA are in daily routine use.

Constancy of R_{OA} values. To get retention values for the various 17-ketosteroids relative to OA twelve sets of standards each containing the seven 17-ketosteroids commonly present in urine were chromatographed and average values for the retention time relative to OA (R_{OA}) were determined for all the steroids together with the range. R_{OA} intervals for each compound were calculated by making the limits between intervals equal to the mean of the two adjoining R_{OA} values.

The values are given in Table III. Many subsequent routine determinations of urinary 17-ketosteroids have proven that R_{OA} values for a given steroid always have fallen within the R_{OA} intervals given in this table. It should be emphasized that we always in multi-column work include a set of standards with any given run. This will protect against misinterpretations should major errors in the chromato-graphic technique occur, for example errors in the deactivation of the alumina used or in the gradient formation. Such errors have not been made in the chromato-

graphic runs during the last half year when the method has been in routine use. However, the inclusion of a set of standards with any given run protects against misinterpretations should errors occur.

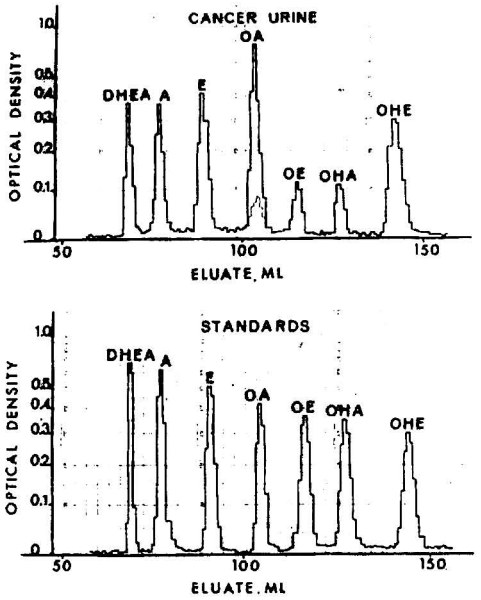


Fig. 7. From a routine run of urinary steroids. A chromatogram of an extract from a cancer patient is on top. OA has been added to this urine as an internal standard. The amount of OA present in the same urine without addition of internal standard is shown with dotted lines. At the bottom a chromatogram of standards as run in the same multi-column run. For abbreviations used for 17-ketosteroids, see text.

TABLE III

RETENTION VALUES OF DIFFERENT 17-KETOSTEROIDS RELATIVE TO THE INTERNAL STANDARD OA (R_{OA} VALUES)

For abbreviations used for 17-ketosteroids, see text.

	DHEA	*A*	*E*	*OA*	*OE*	*OHA*	*OHE*
Average	0.61	0.73	0.86	1.00	1.12	1.21	1.38
Range	0.60–0.64	0.72–0.74	0.85 0.86		1.10–1.13	1.20–1.23	1.35–1.46
Interval	0.55–0.67	0.67–0.79	0.79 0.93		1.06–1.16	1.16 1.29	1.29–1.50

Value of the R_{OA} values for computer programming. Very high capacity work with the multi-column system is not possible without computer calculation of the chromatographic data as previously discussed[8] since, if manual calculations are used, a point is reached as the capacity increases where more personnel is needed for the calculation of data than for performance of the analytical work. The use of an internal standard in the chromatographic analysis has proven of great value for the computer calculations. Relating to this internal standard in the computer

programming by using R_{OA} intervals makes the identification and labeling of individual steroids in a urinary extract more reliable than just comparing with a set of standards run simultaneously in a multi-column run although this in a system using pumps generally is quite adequate. The internal standard becomes a necessity, however, in a system using compressed nitrogen to drive the solvent mixture through the chromatographic columns.

DISCUSSION

The main reason for building the multi-column system using nitrogen to move the solvent through the capillary columns has been to cut the cost of high-capacity multi-column chromatography. A considerable saving has been achieved in this respect. The total cost of cylinder and needle valves used in the nitrogen system is approximately $ 1000 for a 25-column system, whereas the cost of pumps and mixing cylinder in a 25-column pump system is approximately $ 5000. The cost per channel has therefore been cut by 80 %.

This saving per chromatographic channel has not been obtained at the cost of reduced performance. Actually because of the better resolution in the nitrogen system performance is significantly improved in the nitrogen system as compared with the pump system both as regards resolution and reproducibility.

The use of an internal standard has been found to be a necessity in the nitrogen system where the flow rate varies considerably more from column to column than in a pump system even though columns are made of the same diameter tubing of the same length and other parameters are kept as similar as possible.

Extra amounts of an already present compound added to the chromatogram as an internal standard as in the chromatographic system for urinary 17-ketosteroids described here can be subtracted from the total value of the peak and an estimate of the amount of this compound present before addition of internal standard can thus be arrived at. This estimate will clearly be less accurate than for directly estimated compounds but will probably be adequate in most cases. Such an addition of extra amounts of an already present compound in an insensitive area of the chromatogram would appear to be a possibility for the addition of an internal standard in many situations. In other cases a compound with a retention time different from that of the compounds being determined can be used or it may be possible to use a colored substance with a proper retention time as an internal standard.

The addition of an internal standard has been found of considerable value for the computer calculation of compounds in extracts of unknown composition since retention times relative to such an internal standard make it possible to get compound identification and labeling performed by the computer with greater certainty than if no internal standard is used.

The compressed nitrogen system has been used for the routine assay of urinary steroids for the last half year. It has been found convenient and practical in use. A definite advantage this system has when compared with a system using chromatographic pumps it that the maintenance problem that is considerable when a battery of 25 pumps has to be used has been eliminated almost entirely.

This system overall evaluated and compared with the previously used pump

system is much cheaper per chromatographic channel, has higher resolution and better reproducibility and very little maintenance is needed to keep the system going. The disadvantage of a more variable flowrate has been overcome by the addition of an internal standard to the extracts to be chromatographed. This internal standard has proven of considerable value in the computer calculation of the chromatographic data.

It is our conviction that multi-column capillary column chromatography has much to offer the analytical chemist. It combines high capacity with high chromatographic resolution. With the current rapid developments in fast liquid chromatography it can also be expected over the coming few years to become an increasingly fast technique in many applications.

We hope in future developments of the system to improve the techniques further. A greatly simplified read-out system with computer calculation of data is currently under development. It will use a small on-line laboratory computer for direct calculation of data and should cut the cost of multi-column chromatography further.

ACKNOWLEDGEMENTS

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CHROM. 4749

CHROMATOGRAPHY OF BIOGENIC AMINE METABOLITES AND RELATED COMPOUNDS ON LIPOPHILIC SEPHADEX

I. THE PHENOLIC ACIDS AND ALCOHOLS

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SUMMARY

Liquid–gel chromatography on Sephadex LH-20 in solvent systems of 1,2-dichloroethane–methanol was used to separate the aromatic acids and neutral compounds derived from catechol- and indole amines. The system is characterised by high efficiency (1600–2500 theoretical plates/m), speed (2–6 h) and high recovery in the submicrogram range. The method has been found useful in *in vivo* and *in vitro* studies on the metabolism of monoamines, as well as for isolation of metabolites prior to gas chromatographic–mass spectrometric analysis.

INTRODUCTION

Recently, new derivatives of Sephadex have extended the use of gel chromatography for lipophilic substances[1]. The methyl ethers and 2-hydroxypropyl ethers of Sephadex have been investigated by SJÖVALL *et al.*[1], who found them highly efficient for the separation of closely related substances of biological origin, such as steroids, peptides and vitamins. The separations involve liquid–gel partition and molecular sieving whereas adsorption in most cases seems to be negligible. It occurred to us that similar systems might be useful in the analysis of aromatic acids and alcohols. Some of these compounds are metabolites of the biologically important catechol- and indole amines. The present report shows that excellent separation of these substances can be achieved using Sephadex LH-20 in solvent mixtures of methanol and 1,2-dichloroethane.

EXPERIMENTAL

Materials

Sephadex LH-20 was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. DL-3-Methoxy-4-hydroxymandelic acid (VMA); DL-3,4-dihydroxymandelic acid (DOMA); 3-methoxy-4-hydroxyphenylacetic acid (HVA); 3,4-dihydroxyphenyl-

acetic acid (DOPAC); 3-methoxy-4-hydroxyphenylethylene glycol (MOPEG) and 3,4-dihydroxyphenyl-ethylene glycol (DOPEG) were purchased from Calbiochem AG, Luzern, Switzerland. 3,4-Dihydroxyphenylethyl alcohol (DOPET) and 3-methoxy-4-hydroxyphenylethyl alcohol (MOPET) were from Kistner Lab.tjänst, 40224 Göteborg 5, Sweden.

5-Hydroxy-3-indoleacetic acid (5-HIAA) was supplied as the dicyclohexyl ammonium salt from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. The 3-indoleacetic acid was from E. Merck AG, Darmstadt, G.F.R. The 5-hydroxytryptophol was a gift from Hoffman-LaRoche AG, Basel, Switzerland. All solvents were of reagent purity as supplied by E. Merck AG. ^{3}H-labelled DL-noradrenaline (spec. act. 2.1 Ci/mmole) was from the Radiochemical Centre, Amersham, Bucks., Great Britain). The radiopurity was checked by paper chromatography and was found to be better than 95 %. The tritiated dopamine had a specific activity of 2.5 Ci/mmole. The radiopurity of this compound was checked by paper chromatography and found to be within specification (> 99 %).

Preparation of the column

Sephadex was allowed to swell in methanol and was then refluxed three times for several hours with a large excess of methanol. After drying a suitable quantity was equilibrated with the solvents (1,2-dichloroethane–methanol, 9:1, 8:2 or 7:3) for a minimum of 2 h in a filtering flask. The gel was de-aerated by brief application of vacuum (*ca.* 15 mm Hg) and then immediately used for packing the chromatography column. A glass container serving as a reservoir for the solvent was fitted to the top of the column (750 × 10 mm), while the bottom of the column had a narrow stopcock. Care was taken to keep dead space to a minimum.

The gel was poured into the column on top of a tightly packed wad of glass wool and allowed to settle under gravity. A small circular plug of porous teflon was placed on the top of the gel surface to prevent disturbances to the fragile gel surface. Since the gel had a lower specific gravity than some of the solvents the plug also kept the gel from floating. The columns were conditioned by running the solvent mixture through the column for 6–12 h. The sample was applied in the smallest possible volume of the solvent mixture and allowed to sink into the gel. The sample was rinsed from the teflon disc by several 0.5 ml volumes of solvent mixture. The column and top reservoir were filled with about 300 ml of solvent mixture. The total bed volume of the columns was about 45 ml. The flow rate under the stated conditions was about 0.15 ml/cm^2/min. Fractions of about 0.8 ml were collected.

Aromatic amine metabolites were detected in the effluent by measuring the absorption at 280 nm in a Zeiss spectrophotometer Model PMQ II. Radioactivity was measured in a gas flow counter (Frieseke Hoepfner Model FH 51) after drying an aliquot on aluminium planchets. The void volume (V_0) was found to be about 33 % of the bed volume. This was determined by application of 10 mg of polyvinylpyrrolidone and gravimetric determination of its appearance in the eluting medium. β-Carotene, which appears at $1.3 \times V_0$ in the solvent system 1,2-dichloroethane–methanol (7:3) was used as an internal standard.

Preparation of derivatives

Aromatic acids or acidic extracts were esterified by the use of freshly prepared

diazomethane. The sample was dissolved in a small amount of ethyl acetate and sufficient methanol to act as a catalyst added. After addition of an excess of diazomethane in cold ether (−20°), the reagent was immediately removed by a stream of nitrogen. Esterification under these conditions is instantaneous. Longer exposure to diazomethane, when methanol is used as solvent, leads to the methylation of phenolic groups[2–4]. This tendency is especially noticeable with compounds having a catechol structure. The structures of all derivatives were verified by gas chromatography–mass spectrometry[4].

Preparation of urine and tissue extracts

An amount of 10 ml of rat urine was subjected to enzymatic hydrolysis for 16 h at room temperature, at pH 5.5, using 0.05 volumes of Glusulase (Endo Lab., New York, U.S.A.). The urine was acidified to pH 2–3 and extracted 5 times with 25 ml of ethyl acetate. The combined organic phases were washed repeatedly with small volumes of distilled water and then evaporated *in vacuo* to dryness. The dried extract, containing the acidic and neutral amine metabolites, was treated with diazomethane as described above and subjected to chromatography on a Sephadex LH-20 column.

Rabbits were used for the study of the brain metabolism of ^{3}H-dopamine. The animals were stunned by a blow on the neck and exsanguinated from the large vessels of the neck. The whole brain was taken out, immersed in ice cold Krebs Henseleit solution containing glucose 0.2 %, ascorbic acid 0.02 % and disodium EDTA 0.015 % (ref. 5). The whole brain was rapidly chopped with scissors into smaller fragments weighing about 10 mg each. 2 g (wet weight) of chopped rabbit brain was combined with 5 ml of the Krebs Henseleit solution and 2 μCi of ^{3}H-dopamine. Incubation was carried out at 37° with shaking. After 60 min, 2 ml of 2 *M* HCl was added together with carrier metabolites (about 0.5 mg each) and the sample was homogenised using an Ultraturrax model 45/2 homogeniser. The sample was centrifuged and the supernatant was adjusted to pH 4 with 1 *M* NaOH and applied on a short Dowex 50 4X (H) 200–400 mesh column. The effluent and two 10 ml washes were combined; after adjusting the pH to 2 with 1 *M* HCl, it was extracted twice with 50 ml of ethyl acetate and twice with 50 ml of ether. The combined organic phases were washed with small volumes of water until neutral and evaporated to dryness *in vacuo*. The residue was dissolved in methanol and transferred to a small test tube and reduced to dryness under nitrogen. After conversion of the acid to the methyl esters by diazomethane as described above, the sample was dissolved 0.15 ml methanol and 0.35 ml of 1,2-dichloroethane and applied on the Sephadex LH-20 column.

Preparation of labelled DOPAC, HVA and VMA

^{3}H-labelled DOPAC was prepared by incubating 230 μCi^{3}H-dopamine with liver mitocondria in 40 ml 0.15 *M* potassium phosphate buffer, pH 7. This solution also contained 0.5 m*M* ascorbic acid, 5 m*M* pyrogallol, 0.05 m*M* ethanethiol and 0.5 m*M* nicotinamide-adenine dinucleotide (NAD^{+}). The incubation (37°) was interrupted after 60 min by adding 360 ml of chloroform–ethanol (1:1). The protein precipitate was removed by filtration. The filtrate was evaporated *in vacuo*. A small amount of water, pH 3, was added and the mixture was extracted with ethyl acetate three times. The combined ethyl acetate extracts were washed repeatedly with small amounts of water and evaporated *in vacuo*. ^{3}H-labelled DOPAC was isolated by preparative

thin-layer chromatography (toluene–ethyl acetate–water (70:36:1.5). Yield 20 μCi.

The preparation of ^{3}H-labelled HVA was performed in the same manner as for DOPAC, but in this case 2 mg S-adenosyl-L-methione chloride was added and the supernatant from centrifugation at 15000 *g* was used as solvent.

For preparation of ^{3}H-VMA, [^{3}H]noradrenaline was incubated with chopped pieces of whole rabbit brain essentially as described above for preparation of ^{3}H-HVA and DOPAC.

Recovery

To calculate the recoveries in the submicrogram range the methyl esters of the ^{3}H-labelled acids, prepared as described above, were put on Sephadex LH-20 columns (4 × 400 mm). Each compound was put on four replicate columns. The radioactivity in the eluate was followed. The fractions in the peak corresponding to the methyl ester of the radioactive compound were pooled and evaporated to dryness unter nitrogen and counted in a liquid scintillation spectrometer. The recovery of 3–5 μg of 5-HIAA-Me was determined on columns (4 × 400 mm) of LH-20 using fluorometry (Aminco-Bowman Spectrofluorometer).

RESULTS

Metabolites of catecholamines

Three solvent systems, using different concentrations (10, 20 and 30 vol. %) of methanol in 1,2-dichloroethane, were used to separate the amine metabolites. The elution volumes relative to β-carotene are given in Table I. The shape of the peaks for these compounds was narrow and symmetrical indicating high efficiency of the separations. A chromatogram showing the separation of the methyl esters of HVA, VMA, DOPAC and DOMA as well as the glycols MOPEG and DOPEG is shown in Fig. 1. The number of theoretical plates/m was 1700 for HVA-Me, 2100 for VMA-Me and 2300 for MOPEG.

It proved difficult to analyse these compounds as the free acids because the

TABLE I

ELUTION VOLUMES RELATIVE TO β-CAROTENE

Compound[a]	*1,2-Dichloroethane–methanol*		
	9:1	*8:2*	*7:3*
3-Methoxy-4-hydroxyphenylacetic acid (HVA)	1.7	1.7	1.7
3,4-Dihydroxyphenylacetic acid (DOPAC)	—	2.5	2.3
3-Methoxy-4-hydroxymandelic acid (VMA)	2.6	2.1	2.1
3,4-Dihydroxymandelic acid (DOMA)	—	3.5	2.9
3-Methoxy-4-hydroxyphenylethylene glycol (MOPEG)	5.8	3.3	2.7
3,4-Dihydroxyphenylethylene glycol (DOPEG)	—	5.4	4.5
3-Methoxy-4-hydroxyphenylethanol (MOPET)	—	2.4	2.1
3,4-Dihydroxyphenylethanol (DOPET)	—	4.7	3.6
5-Hydroxy-3-indole acetic acid (5-HIAA)	—	—	2.3
3-Indoleacetic acid (IAA)	—	—	1.8
5-Hydroxytryptophol	—	—	3.3

[a] All acids were run as the methyl esters.

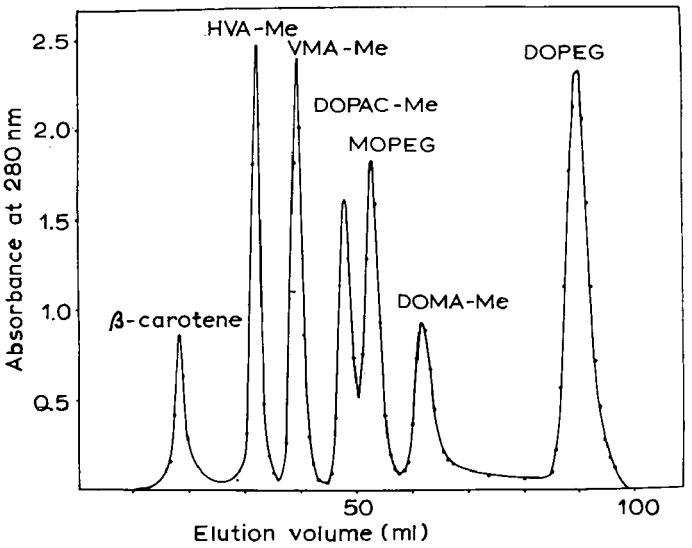


Fig. 1. Separation of 3-methoxy-4-hydroxyphenylacetic acid methyl ester (HVA-Me); 3-methoxy-4-hydroxymandelic acid methyl ester (VMA-Me); 3,4-dihydroxymandelic acid methyl ester (DOMA-Me); 3,4-dihydroxyphenylacetic acid methyl ester (DOPAC-Me); 3-methoxy-4-hydroxy phenylethylene glycol (MOPEG); and 3,4-dihydroxyphenylethylene glycol (DOPEG) on Sephadex LH-20. Solvent systems: 1,2-dichloroethane–methanol (7:3).

peaks were broad and showed pronounced tailing. This was not altered by the addition of 1 % formic acid to the elution medium.

For various analytical purposes it could be desirable to hydrolyse the methyl esters to the free acids. Acidic hydrolysis (2 *M* HCl, 60° for 2 h) gave quantitative conversion of the methyl esters of HVA, DOPAC, VMA, but not of DOMA, to the acids. The glycols are probably dehydrated under these conditions. Mild alkaline hydrolysis (0.1 *M* NaOH in EtOH, 60° for 2 h) was efficient for HVA-Me but the catecholic and β-hydroxylated structures were labile in alkali, as expected.

The recoveries for the methyl esters of labelled HVA and VMA from four determinations were 99.1 ± 0.6% (S.D.) and, 97.5 ± 5.8% (S.D.), respectively, while the ^{3}H-DOPAC-methyl ester in these small amounts gave lower and more variable figures. The recovery of 1 mg of DOPAC methyl ester was found to be 92 %, while 5 HIAA-Me in the microgram range from six determinations gave 97.0 ± 6.2 % (S.D.).

The practical use of the chromatography system was demonstrated by two experiments. A rat was given an intravenous injection of ^{3}H-labelled noradrenaline and the urinary acids and alcohols were extracted as described under EXPERIMENTAL and chromatographed on Sephadex LH-20 in 1,2-dichloroethane–methanol (8:2), after the extract had been treated with diazomethane. The chromatogram is shown in Fig. 2. Two major radioactive peaks corresponding to VMA-Me and MOPEG, respectively, were observed. A great deal of material absorbing at 280 nm was also present in various parts of the chromatogram indicating other acids and neutral compounds present in the urine.

In another experiment, ^{3}H-labelled dopamine was incubated with chopped pieces of whole rabbit brain. After termination of the incubation, carrier metabolites

were added and the acidic and neutral metabolites were extracted as described under EXPERIMENTAL. After treatment of the extract with diazomethane as described above, the extracted material was chromatographed on Sephadex LH-20 in 1,2-dichloroethane–methanol (7:3). The chromatogram is shown in Fig. 3. Two peaks of radioactivity are visible, the major one coinciding with the carrier DOPAC-Me and the minor peak with the carrier HVA-Me.

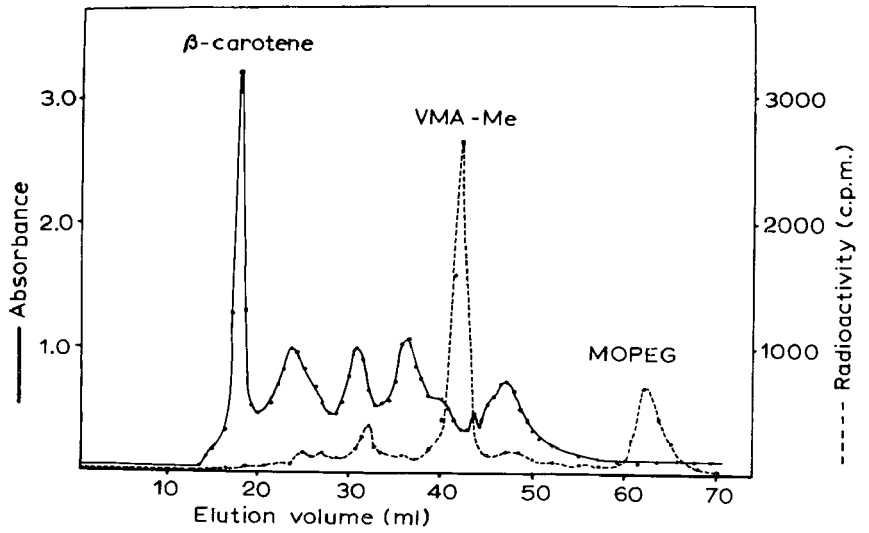


Fig. 2. Chromatography of urinary acidic and neutral metabolites from a rat given [^{3}H]noradrenaline. The solid line represents absorption at 280 nm and the broken line represents radioactivity. Sephadex LH-20, solvent system 1,2-dichloroethane–methanol (4:1).

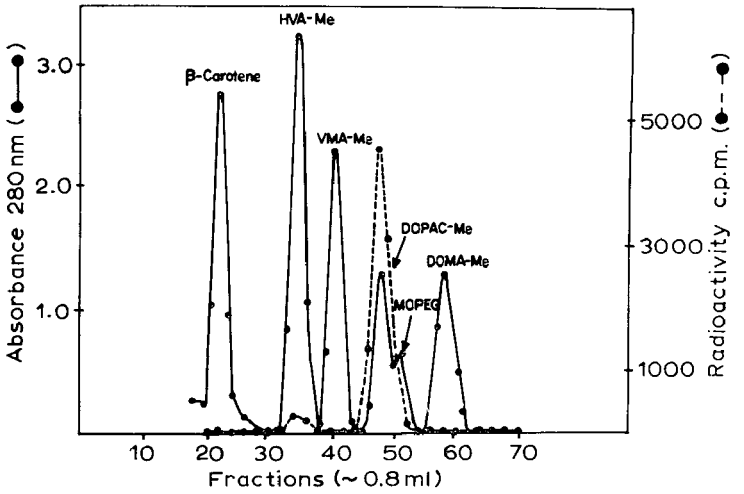


Fig. 3. Chromatography of acidic metabolites of [^{3}H]dopamine after incubation with chopped rabbit brain. The solid line represents absorption at 280 nm due to carrier metabolites and the broken line represents radioactivity. Sephadex LH-20, solvent system: 1,2-dichloroethane–methanol (7:3).

Metabolites of indoleamines

The columns also proved suitable for the separation of indoleacetic acid (IAA), 5-hydroxyindoleacetic acid (5-HIAA) and 5-hydroxytryptophol (5-HIOL). The acids were quantitatively converted to the methyl ester using diazomethane as described under EXPERIMENTAL.

The elution volumes of the methyl esters and of 5-HIOL are given in Table I. Excellent separation was obtained with 1,2-dichloroethane–methanol (7:3) (Fig. 4). The peaks were symmetrical. The number of theoretical plates/column was 900 for IAA methyl ester, 1400 for 5-HIAA methyl ester and 1600 for 5-HIOL.

The methyl ester of 5-HIAA could not be hydrolysed in acid or alkali without degradation.

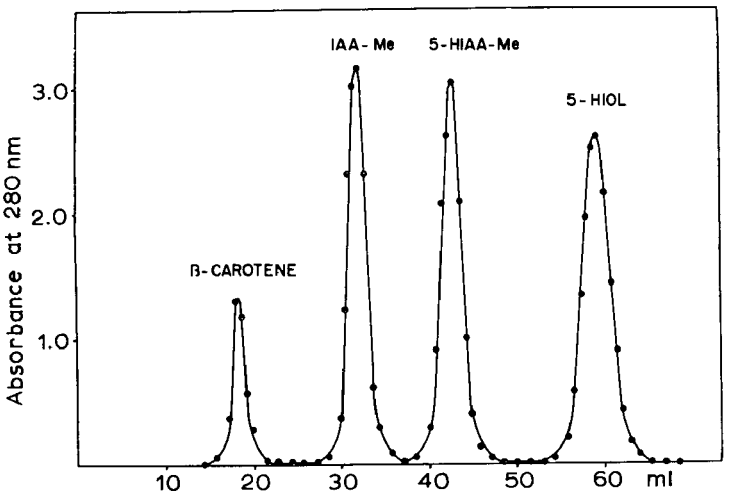


Fig. 4. Chromatography of 5-hydroxytryptophol (5-HIOL) and the methyl esters of 5-hydroxy-3-indoleacetic acid (5-HIAA) and indoleacetic acid (IAA) on Sephadex LH-20. Solvent system: 1,2-dichloroethane–methanol (7:3).

DISCUSSION

The separation and analysis of aromatic acids and alcohols by nondestructive methods generally include the principles of ion exchange, adsorption and electrophoresis[6,7]. None of these methods have the efficiency inherent in liquid–liquid and gas–liquid partition systems. In the present report, chromatography on Sephadex LH-20 was found to give excellent separations of the above-mentioned phenolic acids and alcohols in solvent systems of 1,2-dichloroethane and methanol.

Sephadex LH-20 is prepared from Sephadex G-25 in bead form by hydroxypropylation of the hydroxyl groups in the dextran chain. In a mixed solvent system the beads swell, possibly incorporating one of the solvent components preferentially in the gel phase. The molecules solvating and surrounding the substituted dextran will therefore have a different composition from those of the mobile phase. Solutes

may therefore be separated according to their distribution between the mobile phase and the stationary gel phase. They may also be distributed according to their size by molecular sieving mechanisms. These separations have been designated liquid–gel chromatography and liquid–gel filtration chromatography respectively by SJÖVALL *et al.*[1]. For a thorough discussion of the principles and practice of liquid–gel chromatography in organic solvents the reader is referred to the review by these authors.

The compounds separated in the present investigation are roughly of the same size. Therefore liquid–gel chromatography rather than liquid–gel filtration seems to be the separating mechanism. Since the peaks are symmetrical adsorption seems to be minimal. The compounds appear in order of increasing polarity. The partition chromatography is therefore of "straight-phase" type. Since more lipophilic Sephadex gels are now available[8–10], it appears possible to construct "reversed-phase" liquid–gel chromatographic systems for the aromatic acid and alcohols as has been done for cholesterol esters, bile acids and other lipids[9,10].

Liquid–gel chromatography of the aromatic acids and alcohols possesses advantages over other methods based on adsorption, ion-exchange and paper chromatography. In many cases, the efficiency of the separation approaches those of the analytical gas–liquid chromatography systems. The recovery is high and for the O-methylated compounds practically quantitative. The number of theoretical plates/m was around 2000 for most of the compounds studied. Thus the compounds appear separated from each other in small volumes of organic solvent, which are easily removed prior to analysis by other methods. As in gas chromatography, the column is used repeatedly without any alteration of the composition of the mobile phase although possibilities for programmed variations exist. Furthermore, the elution volume of a given compound seems very reproducible. It is therefore possible in routine analyses to discard most of the effluent while collecting only those fractions containing the desired compounds, thus rendering expensive fraction collectors unnecessary.

With the compounds studied under the conditions described a separation takes 2–6 h. The chromatogram shown in Fig. 1 was run in about 6 h. The virtual absence of column bleed from a well washed and conditioned column, and the absence of adsorption effects, is of value in isolation of submicrogram amounts of monoamine metabolites.

The application of this liquid–gel chromatography system to *in vivo* and *in vitro* studies on the metabolism of catecholamines was demonstrated. Another obvious use of the method — and the use for which it was designed — is for isolation of individual metabolites prior to analysis by gas chromatography–mass spectrometry. Here the conversion of the acids to the methyl esters represents no problem since these or similar derivatives have to be prepared anyway. For fluorimetric measurement some of the methylesters may be quantitatively hydrolysed.

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CHROM. 4741

AUTOMATIC ION-EXCHANGE CHROMATOGRAPHY OF AMINO ACIDS

EXPERIMENTAL STUDIES FOR OPTIMISING RESIN COLUMN DIMENSIONS

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SUMMARY

Experimental studies are reported concerning the optimisation of two fundamental column parameters in automatic ion-exchange chromatography of amino acids, namely the amount of resin and the cross sectional area of the column. The experiments have been performed employing a crushed Amberlite IR 120 resin, and the amount of resin has been considered disregarding the height of the resin bed. From the results obtained some practical rules have been derived, which permit a better control of the chromatographic working conditions; consequently the solution of the problems of sensitivity and time is facilitated. Regarding the speed of the chromatogram, it is demonstrated how the problem of the overpressure can be avoided without employing spherical resins, which from a theoretical viewpoint are less suitable for the chromatographic process.

INTRODUCTION

Independently of the various theoretical studies on chromatographic separation[3,6,11,12,15], many of which have been followed and investigated by HAMILTON *et al.*[5] in their work dealing with the analysis of the mass transfer mechanisms for amino acids through ion-exchange columns, it has been possible to obtain some practical rules which permit a better control of the ion-exchange chromatography of amino acids starting from the results of the experimental studies reported here.

As a consequence, the operating capabilities and limitations of the resin columns can be more easily anticipated and the problems of resolution, sensitivity, speed of analysis and operating pressure can be solved more easily.

EXPERIMENTAL

The experiments were performed on a new analytical system whose advantages over the traditional amino acid analysers have been reported in a series of earlier papers[8–10] concerning the analysis of protein and biological fluid amino acids and related compounds. It should be mentioned that this new system allows peak eval-

uation simply by measurement of the height in mm above the base-line, with a precision higher than that achieved by measurement of their areas[10]. The representation on the recorder chart is linear with respect to the optical density and for all the experiments reported in this paper, sensitivity was set at an optical density of 1 for a full scale deflection, employing flow-cuvettes having a 10-mm light path and an internal diameter of 2 mm, provided with plane and parallel windows. The chart speed was set at 3 in./h.

The resin employed was a crushed Amberlite IR 120 prepared and conditioned in our laboratory according to the method previously described[8,9], having an average particle size of 35 μ and corresponding to the harvest obtained in HAMILTON's system of classification[4] at a water flow-rate of 50–100 ml/min.

A theoretical interpretation of the experimental observations was not sought intentionally. An experimental solution to the analytical problems was preferred to the development of theories made too complex by the large number of parameters that must be taken into consideration, too many of which are outside the possibility of measurement if not of detection.

THE CHOICE OF THE CORRECT AMOUNT OF RESIN

It is easy to anticipate that in a chromatogram it will not be possible to obtain a good resolution pattern, if, for a given set of conditions (including resin mesh-size, degree of resin cross-linking, buffer flow-rate, buffer pH, temperature, etc.), the quantity of resin is inadequate for the separation of a given mixture of amino acids.

It is likewise easy to foresee, given the same set of conditions as before, that the side effects of diffusion and non-equilibrium, when the quantity of resin is too great, can be undesirable.

Like HAMILTON *et al.*[5] we had observed that peak spreading increased, in a given glass column, when the height of the resin bed (*i.e.* the amount of resin) was increased. We therefore wished to find experimentally a law of variation of the peak height as a function of the apparent volume of resin in the column for our system.

Unlike HAMILTON, who in his experimental work[5] collected fractions emerging from the column and subsequently submitted them to analysis to determine the peaks, automatic colorimetry was applied to the column effluent in order to take into account, also, the effect of the reaction coil and the colorimetric system in deforming peaks, just as happens in every-day analytical conditions. Moreover, while HAMILTON for his purposes was chiefly interested in observing the peak width variation as a function of the column length, we were mainly concerned with studying the peak height behaviour on the recorder chart by varying the amount of resin, since in our system the peaks can be evaluated by measuring their height over the base-line.

For this purpose a jacketed column of pyrex glass, 40 cm high, having an I.D. of 1.2 cm, was set up on the apparatus previously described[8] and thermostated at 40°. According to our method[9] the column was packed four times with four different volumes of crushed Amberlite IR 120 resin. For each amount of resin a chromatogram was performed by loading the column with 100 nmoles of aspartic acid contained in 0.2 ml of 0.1 *N* HCl, which were carefully placed on the drained surface of the column and then forced in under air pressure. The column was then refilled with buffer, pH 3.26, which thereafter was pumped for the elution at a flow-rate of 2 ml/min. At

the same time the ninhydrin colour reagent, prepared as previously referred to[8,9], was pumped at a flow-rate of 1 ml/min. These flow-rates were kept constant for all the chromatograms performed in this experiment. After each chromatogram the resin was taken out of the column and the packing of the subsequent new amount of resin was performed.

It was necessary to operate with only one amino acid in the feed pulse; if an amino acid mixture were loaded on to the column, separation could not be achieved on the smallest amounts of resin at the speed of elution used and which was kept constant for all the chromatograms.

The height of the resin bed, the apparent volume of the resin and the height of the peaks expressed in millimeters are reported for each chromatogram in Table I.

TABLE I

RESULTS OBTAINED FROM FOUR CHROMATOGRAMS OF ASPARTIC ACID RUN ON FOUR DIFFERENT VOLUMES OF RESIN PACKED IN THE SAME COLUMN

Resin bed height (cm)	34.5	27.0	21.5	16.5
Apparent volume of resin (ml)	40.0	30.5	24.3	18.7
Peak height (mm)	101.0	117.5	133.0	151.7

From the diagram shown in Fig. 1 it can be seen that if the logarithms of the values of the apparent resin volumes employed are placed on the ordinates and the values of the corresponding peak heights expressed in millimeters are on the abscissae,

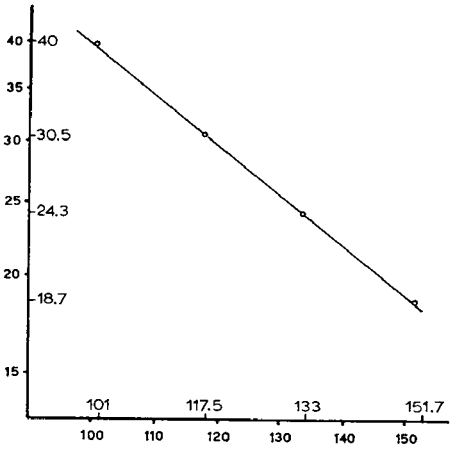


Fig. 1. Variation of the peak height as a function of the amount of resin in the column. On the abscissae are reported the peak heights in mm and on the ordinates the corresponding values of the resin volumes, in ml, on a logarithmic scale, are given.

a straight line is obtained with a negative slope. Thus, it can be deduced that by increasing the volume of resin, at a constant elution flow-rate, the peak height will

decrease according to an exponential function, which can be expressed by the following equation:

$$H_p = A - K \log V_r$$

where

A = constant
H_p = peak height
V_r = resin volume
K = constant

This behaviour may be related to diffusion and/or mass transfer resistance phenomena relating to the amino acids as they travel through the column, and since the diffusion and distribution coefficients are different from one amino acid to another, the value of K (the slope of the line) will be peculiar to each amino acid.

Taking this equation into account some conclusions can be drawn.

Concerning the sensitivity, it must be pointed out that it will vary as an inverse function of the amount of resin employed and specifically according to the above equation. It will then be very convenient to employ the minimum amount of resin to obtain separation of the components of the mixture under examination, avoiding any excess resin.

Furthermore, when a doublet gives an overlap which must be decreased, it will generally be preferable to decrease the elution flow-rate, in order to solve the problem, instead of increasing the amount of resin. As it will be shown later, slowing the flow-rate favours an increase in the peak height with a consequent narrowing of the peak due to a diminution of the band spreading, as a consequence of the diminished diffusional and/or non-equilibrium effects. In this way the overlap will be reduced and, since the separation will be improved, there will be a gain in sensitivity also.

Furthermore, since in our system the peaks can be evaluated by merely measuring their height and consequently the overlapping of peaks has much less influence on the precision and accuracy of the analysis results, it is generally convenient to reduce the amount of resin, augmenting in this way the degree of sensitivity and reducing the total elution volume of the chromatogram, which also involves a reduction of the analysis time.

The following experiment will illustrate these rules more extensively.

A pyrex glass column having an inner diameter of 0.7 cm was set up on the amino acid analyser and thermostated at 58°. An apparent volume of resin of about 7 ml was packed into the column according to the method previously described; then, after equilibration with buffer, pH 5.28, 0.1 ml of a mixture containing 100 nmoles of the following amino acids: tryptophan, lysine, histidine, ammonia and arginine, were loaded on the column. Buffer and colour reagent flow-rates were set at 1.4 ml/min and 0.7 ml/min, respectively, and a chromatogram was performed.

After this run, half of the apparent volume of the resin was withdrawn from the column and a chromatogram was then run under the same conditions as before.

On this new amount of resin and still in the same glass column, another chromatogram was next performed at a buffer flow-rate of 1 ml/min, while the colour reagent was proportionally decreased. The length of the reaction coil was also proportionally decreased.

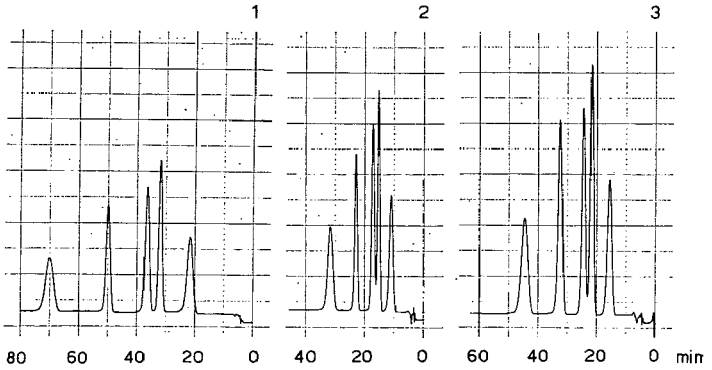


Fig. 2 Each chromatogram shows the separation of the following amino acids (from right to left): tryptophan, lysine, histidine, ammonia and arginine, under the same conditions of colour development and photometry, and employing the same glass column. Chromatograms No. 1 and No. 2 were eluted at 1.4 ml/min, but chromatogram No. 2 was obtained after removing half the apparent volume of resin from the chromatographic column. Chromatogram No. 3 was obtained on this reduced quantity of resin with a buffer flow-rate of 1 ml/min.

TABLE II

PEAK HEIGHTS IN MM AND PEAK HEIGHT-TO-VALLEY RATIOS OF THE CHROMATOGRAMS REPORTED IN FIG. 2

No.	*Tryptophan*	*Lysine*	*Histidine*	*Ammonia*	*Arginine*	*Peak height-to-valley ratio*[a]
1	33.5	67.5	56.0	47.5	23.5	0.5/56.0 = 0.009
2	52.5	99.0	83.5	70.5	37.5	11.5/83.5 = 0.14
3	61.0	112.5	93.0	87.5	43.0	85.0/93.0 = 0.09

[a] These are the ratios of the distance of valley between the peaks of lysine and histidine to the base line to the height of the histidine peak (the lower of the two peaks). The lower the ratio, the higher the resolution.

The three chromatograms are shown in Fig. 2 and the relative data are reported in Table II. Comparing the first and second chromatograms, the increase of sensitivity obtained by reducing the amount of resin for a given flow-rate can be seen. The amount of resin used for the first chromatogram could be considered excessive for the separation of the components in question, particularly at that preselected flow-rate. The resolution of the lysine-histidine doublet, in the second chromatogram, is still fully acceptable, particularly in our system where the peaks are evaluated by simply measuring their heights. Comparing the third and second chromatograms, the effect of reducing the elution flow-rate on sensitivity and separation, for a given amount of resin, can be seen.

From these examples and from the other considerations previously reported, it can be stated that the correct amount of resin can be defined as the least apparent bed volume, which, for a given flow-rate, allows the separation of the components of the mixture with a resolution pattern which does not impair the degree of accuracy and precision required.

THE CHOICE OF THE CORRECT COLUMN DIAMETER

So far an experimental study concerning the effect of the variation of the column cross sectional area on the chromatographic resolution, when the apparent bed volume of the resin and the elution flow-rate (buffer volume/time) are maintained constant, has not yet appeared in the literature.

The results of the experiments reported here checking this variation have led to the solution of many problems in this kind of analysis, mainly regarding the acceleration of the chromatogram running time.

A pyrex glass column having an inner diameter of about 0.7 cm and a height of 100 cm was packed by sections, as reported in a preceding paper[9], with an apparent volume of about 35 ml of the usual crushed Amberlite IR 120 resin.

A chromatogram was performed as previously described[8] for each of the following flow-rates: 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 ml/min. The resin was then transferred quantitatively to another column having an inner diameter of about 0.9 cm and a height of 60 cm. A second series of chromatograms was performed at the same flow-rates as before on this new column. The variation of the flow-rates was obtained by changing the gear transmitting motion from the driving synchronous motor to the pump piston, and in order to get the flow-rates of 0.6 and 0.8 ml/min, a motor giving 1 r.p.m. on the buffer feeding pumps and a motor giving 1/2 r.p.m. on the ninhydrin pump were used in place of the usual motors.

The temperature change for the slowest chromatogram was made at 75 min; for the subsequent chromatograms it was made at proportionally shorter times.

The buffer change took place for all the chromatograms after elution of the same buffer volume and after precisely 119 ml had been pumped. In other words the preset volumetric output of the first buffer pump was not varied for all the chromatograms run on the same column.

Regarding the times of colorimetric reaction with the ninhydrin colour reagent, the length of the reaction coil was varied in order to assure an equal residence time of the reaction mixture in the 100° bath for all the chromatograms.

The results of these series of chromatograms are reported in Table III. In order to compare the variation of resolving power, the peak height-to-valley ratio for all the doublets was computed; that is, the ratio of the absorbance measured in millimeters at the minimum of the valley between two juxtaposed peaks to the absorbance measured in millimeters at the maximum of the lower of the two peaks, which is indicative of the resolving power[1].

It can be observed that on increasing the flow-rate the peak heights decrease, since, due to the augmenting diffusion and mass transfer resistance, the peaks spread more and more as the chromatograms are accelerated. This is confirmed by the fact that the resolving power also becomes lower. But even though the spread gets broader and broader on increasing the elution flow-rate, the elution volume of phenylalanine (measured as the number of ml of buffer pumped at the moment when this peak reaches its highest value, *i.e.* the elution volume of the whole chromatogram) remains constant regardless of the flow-rate and the column diameter. This shows that the elution volume of a peak, under these conditions and for a same feed pulse concentration and volume, is only a function of the quantity of resin employed.

Pressures were reaching very high values at 1.6 ml/min flow-rate on the smaller

TABLE III

COMPARISON OF THE RESOLVING POWERS OBTAINED ON TWO COLUMNS OF DIFFERENT CROSS SECTIONAL AREA FILLED WITH THE SAME AMOUNT OF RESIN, AT SIX DIFFERENT FLOW-RATES

The following doublets: hydroxyproline–aspartic acid, threonine–serine, glutamic acid–proline, isoleucine–leucine and tyrosine–phenylalanine were considered. In columns 3, 4, 5, 6 and 7 the first figure of the ratio is the height of the valley between the two juxtaposed peaks from the base line, and the second is the height of the lower peak of the doublet, both expressed in millimeters. The lower this ratio (represented by the third figure), the better is the resolving power.

Flow-rate	*Column diameter*	*Hydroxyproline/ aspartic acid*		*Threonine/serine*		*Proline/glutamic acid*		*Isoleucine/leucine*		*Tyrosine/ phenylalanine*	
1.6	0.7	10.0/31.0	0.323	17.0/126.0	0.135	8.0/48.0	0.166	16.5/142.0	0.116	9.0/83.0	0.108
1.6	0.9	9.5/31.5	0.302	18.0/123.0	0.146	7.5/45.0	0.167	17.5/139.0	0.126	9.0/80.0	0.113
1.4	0.7	8.0/31.5	0.254	17.0/132.0	0.129	5.5/53.0	0.104	15.0/155.0	0.097	7.0/88.0	0.079
1.4	0.9	7.5/31.0	0.242	17.0/129.0	0.132	5.5/50.0	0.110	13.0/154.0	0.085	6.5/85.5	0.076
1.2	0.7	7.0/37.0	0.189	16.0/141.5	0.113	3.0/59.0	0.051	12.0/168.0	0.071	6.0/91.0	0.066
1.2	0.9	6.5/37.0	0.176	15.5/140.0	0.111	3.0/53.0	0.057	11.0/158.0	0.070	5.0/90.0	0.056
1.0	0.7	7.0/38.0	0.184	13.5/144.0	0.094	2.5/62.0	0.040	8.0/165.0	0.049	4.0/96.0	0.042
1.0	0.9	6.5/37.0	0.176	12.0/142.0	0.085	2.0/60.0	0.033	8.0/168.0	0.048	4.0/99.0	0.040
0.8	0.7	5.0/38.0	0.131	12.0/153.0	0.079	3.0/67.0	0.045	6.5/182.0	0.036	2.0/107.0	0.019
0.8	0.9	5.0/38.0	0.131	12.0/154.0	0.078	2.5/67.0	0.037	6.0/178.0	0.034	2.5/106.0	0.024
0.6	0.7	6.0/36.5	0.165	12.0/165.0	0.073	1.5/71.5	0.021	5.0/196.0	0.025	1.5/118.0	0.013
0.6	0.9	6.0/37.0	0.162	12.0/163.0	0.073	1.5/75.0	0.020	4.0/196.0	0.020	1.5/120.0	0.013

TABLE IV

SEPARATION OF THE LYSINE–HISTIDINE DOUBLET PERFORMED ON TWO COLUMNS HAVING A DIFFERENT CROSS SECTIONAL AREA, WITH THE SAME VOLUME OF RESIN

$H_{lys.}$ = height in mm of the lysine peak; $H_{his.}$ = height in mm of the histidine peak; V = height in mm of the valley between the peaks; V/H = peak height-to-valley ratio; P = operating pressure; $H_{0.292}$ = height in cm of the apparent resin bed in the 0.292 cm² column; $H_{0.608}$ = height in cm of the apparent resin bed in the 0.608 cm² column.

Chromatogram No.	*Column cross sectional area 0.292 cm²*						*Column cross sectional area 0.608 cm²*					
	$H_{lys.}$	$H_{his.}$	V	V/H	P	$H_{0.292}$	$H_{lys.}$	$H_{his.}$	V	V/H	P	$H_{0.608}$
1	177.0	162.5	12.0	0.074	5	21.6	176.5	161.0	11.9	0.074	0.35	11
2	175.0	160.0	12.0	0.076	5	21.6	178.0	163.0	11.8	0.072	0.35	11
3	179.0	161.0	11.9	0.074	5	21.5	175.0	160.0	11.8	0.074	0.35	11
4	175.0	159.5	12.1	0.075	5	21.5	177.0	163.0	11.9	0.073	0.35	11
5	176.0	160.0	12.0	0.075	5	21.6	175.0	161.0	11.8	0.073	0.35	11
6	178.0	163.0	11.9	0.073	5	21.6	175.5	162.0	11.9	0.073	0.35	11
Mean	176.6	161.0	12.0	0.074	5	21.6	176.1	161.6	11.85	0.073	0.35	11
Coefficient of variation	±0.92	±0.90	±0.74	±1.4	—	—	±0.80	±0.75	±0.50	±1.0	—	—
	Apparent resin volume 6.2072 ml						Apparent resin volume 6.688 ml					

column and it must be stressed that this experiment would not be possible if traditional reciprocating pumps were used, since in this case the pressure values would be higher. But the most important observation which can be made by examining the reported data is that, even on different column cross sectional areas, provided that the quantity of resin is kept constant, equal flow-rates (buffer volume/time) correspond to equal resolving powers.

As it will be shown later, this observation has been of valuable help in solving the problem of ultrarapid chromatograms, because the overpressure resulting from the acceleration of the elution feed can be diminished by employing a column having a larger cross sectional area but the same volume of resin.

These results have been confirmed by means of a simpler experiment, easily repeated many times for statistical purposes, which is described below.

Two columns were chosen so that the cross sectional area of the first was half the cross sectional area of the second. To be more precise, one column had an I.D. of 0.61 cm, corresponding to a cross sectional area of 0.292 cm^2, and a height of 30 cm; the other column had an inner diameter of 0.88 cm, corresponding to a cross sectional area of 0.608 cm^2 and a height of 15 cm. Both columns were jacketed. They were set up on our amino acid analyser and thermostated at 50°, then an apparent volume of about 6.5 ml of the usual crushed Amberlite IR 120 resin suspended in 0.2 *N* NaOH was packed in one of them by sections. After equilibration with pH 5.30 buffer, 0.1 ml of a solution containing 1 μmole per ml of lysine and histidine was loaded on the column which was then chromatographed at a flow-rate of 1 ml/min. The ninhydrin colour reagent was pumped at 0.5 ml/min. When the chromatogram was completed, the resin was withdrawn from the column, converted to Na form and then, after repacking the column, a subsequent chromatogram was performed. A total of 6 chromatograms were run in this way and extreme care was taken in order not to loose any of the resin in all the operations. After that, the same resin was transferred to the second column, where a further 6 chromatograms were run operating in the same way as before.

Table IV reports the results obtained in both series of chromatograms. Even in this case it appears that, on keeping the volume of resin constant, *i.e.* it was exactly the same for both columns, the resolving power remained constant, in spite of the fact that the cross sectional area from column to column as well as the height of the resin bed varied by a factor of two.

Regarding the operating pressure, some interesting comments can be made. We know that the pressure is a direct function of the resin bed height (H) and of the flow-rate ($cm^3 t^{-1}$) and is an inverse function of the cross sectional area (A) of the column. This relationship can be written according to the equation

$$P = K \frac{H \cdot cm^3 t^{-1}}{A}$$

Consequently, if $P_{0.292}$ is the pressure of the column having a cross sectional area of 0.292 cm^2, and $P_{0.608}$ that of the column having a cross section of 0.608 cm^2, when they are eluted at the same flow-rate and contain the same volume of resin, we can say:

$$\frac{P_{0.608}}{P_{0.292}} = \frac{H_{0.608} \cdot 0.292}{0.608 \cdot H_{0.292}}$$

which is equivalent to

$$P_{0.608} = \frac{H_{0.608} \cdot 0.292 \cdot P_{0.292}}{0.608 \cdot H_{0.292}}$$

By inserting in this equation the experimental values of $H_{0.608}$, $H_{0.292}$ and of $P_{0.292}$, which are reported in Table IV and the values of the cross sectional areas of both columns, the theoretical value of $P_{0.608}$ should give 1.22 kg/cm^2. In practice the observed value of this pressure was equal to 0.35 kg/cm^2. Consequently it can be stated that the diminution of pressure which can be achieved by enlarging the column cross sectional area, for a constant quantity of resin, is much greater than that expected.

On the other hand, if a same height of resin, 10 cm, is packed on both columns, thermostated at 50°, and the buffer is pumped through the one having 0.608 cm^2 cross sectional area at 2 ml/min, whereas the buffer is pumped at 1 ml/min through the other of 0.292 cm^2 (that means the same linear flow-rate for both columns), the pressures observed, instead of being equal, were 3.6 kg/cm^2 for the larger column and 2.5 kg/cm^2 for the smaller one. This would seem to be in apparent contradiction with what has been observed before.

As a matter of fact, the behaviour of both can be explained by taking into account the variations of the apparent density of the resin, which increases with the resin bed diameter, with the height of the resin bed, with the flow-rate and with the resin particle size, as has already been observed by KUNIN on spherical resins[7].

In the first case, as can be seen from the data reported in Table IV, the apparent density of the resin, that is the ratio between the resin weight and the apparent bed volume, was higher in the smaller diameter column.

In the second case, considering the pressure values observed on the two columns, it can be deduced that the apparent density was higher in the wider column. In other words, in this column the amount of resin was greater than that expected. Consequently it can be stated that, if two columns of different cross sectional area are poured and packed with resin to the same height, the actual amounts of resin are not geometrically proportional.

However, it is not within the scope of this work to discuss the theoretical interpretation of this behaviour or the fact that the resolving power does remain constant for a given quantity of resin when this is transferred to columns of different diameters. We will merely state that this is governed by numerous factors and is the result of many concomitant causes.

However, from these observations practical guidance can follow concerning the choice of the correct column diameter. *E.g.*, the quantity of resin in a given column was chosen in order to obtain, in a given time of analysis, and at a given flow-rate, a satisfactory degree of resolution of the components which are to be separated (and under these operating conditions the requirements of sensitivity as well as of precision and accuracy are satisfied). The operating pressure can, under these conditions, reach excessive values almost at once or packing down of the resin from run to run can take place with a consequent progressive pressure rise[2]. In order to avoid this, instead of reducing the flow-rate of the eluting buffers and consequently increasing the time of analysis, the resin can be transferred quantitatively into a column of larger diameter, on which all the other operating parameters are kept as before. The chromatogram obtained on the new column, as shown from data reported in Table

III, is just the same from every point of view as that obtained on the smaller column without any sacrifice of analysis speed.

It can be asked to what extent this can be done. It has already been shown that a diminution of the resin bed height, for a constant weight of resin and for a constant flow-rate, is accompanied by a reduction of the operating pressure to values which are lower than those expected. If the cross sectional area of the column is enlarged excessively (with a consequent excessive height reduction), the operating pressure will decrease to values which are too low to permit a good packing of the resin in the column; the void fraction will become too large and uneven, so that the chromatographic resolution will be consequently affected.

Concluding we can say that, for a given quantity of resin, by varying the elution flow-rate and the column cross sectional area, optimum conditions of working pressure can be found at which the progressive packing of the resin and the consequent pressure rise from chromatogram to chromatogram does not take place.

It should be mentioned that the volume of the feed pulse should be proportional to the cross sectional area. For example, we can load a feed pulse having a 0.1 ml volume on a 0.9 cm diameter column; on a 1.2 cm diameter column, the volume of the feed pulse must be at least 0.2 ml. Otherwise uneven distribution of the solute on the resin surface may occur with the consequent alteration of the shape of the chromatogram peaks and thus of the resolving power.

PRACTICAL APPLICATIONS AND CONCLUSIONS

Taking into account the practical rules emerging from the experimental observations previously reported, the problem of obtaining operating conditions which will give a rise in sensitivity as well as acceleration of the chromatographic procedure can be more easily resolved, since the operating difficulties occurring in both cases can be more easily overcome.

The solution to the sensitivity problems will be dealt with in a forthcoming paper, where a high sensitivity methodology suitable for 10 nmole loads will be fully discussed. Here it will be only mentioned that high sensitivity conditions are obtained by a simple dramatic reduction of the amount of the resin employed, which is followed by lowering the eluting buffer flow-rate until the separation and resolution of the chromatographed components is achieved. The choice of the column cross sectional area only takes into account the value of the operating pressure which must be appropriate to the instrument used and must remain constant from chromatogram to chromatogram. Since the operating column pressure can be very easily controlled in this way, for ultrasensitive chromatograms, the employment of a resin having a smaller particle size should also be taken into consideration.

Concerning the problem of accelerating the chromatographic separation, we have found that by applying the rules previously reported, it becomes a matter of routine to employ a procedure which gives, on our analytical system, a full chromatogram in 90 min with a crushed Amberlite IR 120 resin. With a possible sacrifice of accuracy, but still with a high degree of precision, it is also very easy to obtain the same chromatogram in 60 min. Figs. 3 and 4 show chromatograms run in 90 and 75 min, respectively. The acidic and neutral amino acids were separated on a column having an inner diameter of about 1.2 cm with an apparent resin bed height of 31 cm,

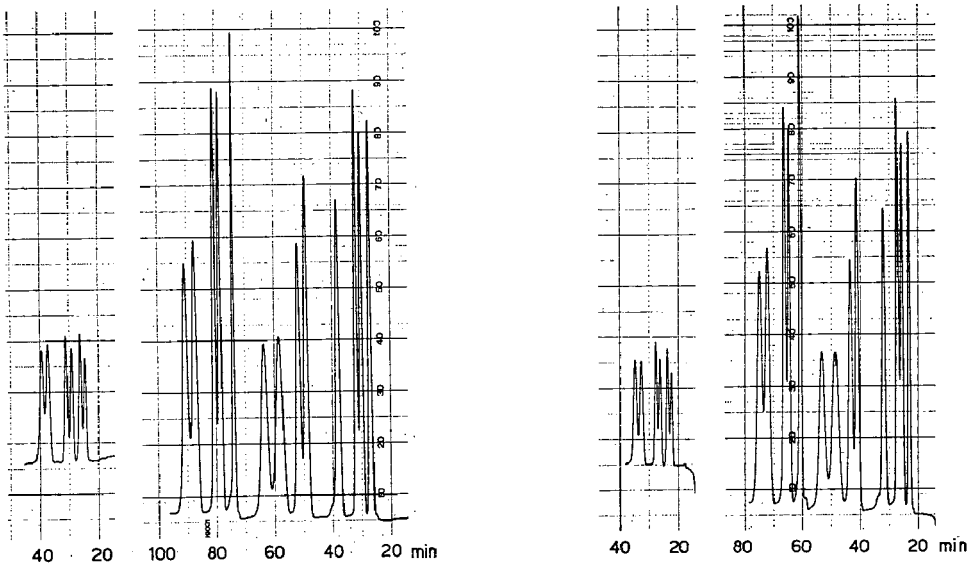


Fig. 3. Chromatogram run in 90 min on a 1.2-cm diameter column at a flow-rate of 2 ml/min. The feed pulse of 0.2 ml contained 200 nmoles of each amino acid. On the right hand side of the figure, the 570 mμ reading is reported and shows the separation, from right, to left of aspartic acid, threonine, serine, glutamic acid, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine. On the left hand side of the figure, the 440 mμ reading shows the separation of hydroxyproline, aspartic acid, threonine, serine, glutamic acid and proline. The temperature was kept at 40° for the first 19 min and then raised to 58°.

Fig. 4. Chromatogram run in 75 min on a 1.2-cm diameter column at a flow-rate of 2 ml/min. The feed pulse of 0.2 ml contained 200 nmoles of each amino acid. On the right hand side of the figure, the 570 mμ reading is reported and shows the separation, from right to left, of aspartic acid, threonine, serine, glutamic acid, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine. On the left hand side of the figure, the 440 mμ reading shows the separation of hydroxyproline, aspartic acid, threonine, serine, glutamic acid and proline. The temperature was kept at 40° for the first 15.8 min and then raised to 58°.

that is an apparent resin bed volume of about 35 ml. The 90-min chromatogram was obtained at a flow-rate of 2 ml/min against a total pressure (column plus coil) of 13 kg/cm^2 at the lower temperature, while the 75-min chromatogram was run at 2.4 ml/min with a total pressure of 17 kg/cm^2 at the lower temperature. The basic amino acids were separated on a 0.7 cm inner diameter column with an apparent resin volume of about 3.5 ml at a flow-rate of 1 ml/min; since this chromatogram is completed in 30 min (see Fig. 2/2), if recording is started on the second channel just after the breakthrough of the proline from the long column, both recordings will be finished at the time at which the phenylalanine is over on the long column chromatogram. Consequently the times of 90 min and of 75 min for the acidic and neutral amino acids which are chromatographed on the first channel, become the total chromatogram times[8].

It must be stressed that these results can be obtained with crushed Amberlite IR 120 resin, of the same type and mesh as that employed by SPACKMAN *et al.*[13]. At the same time we would like to mention that this type of resin has also been generally abandoned because it was thought unsuitable for obtaining a protein amino acid chromatogram in less than 4 h (ref. 14). The crushed resin has been generally sub-

stituted by much more sophisticated specially prepared spherical resins; in this way it was hoped to overcome the problems of overpressure when high flow-rates of buffer are pumped through the column. As a matter of fact, this expectation was not realised[2], because, in order to get resolution when spherical resin is employed, a smaller particle size has to be adopted. We agree with HAMILTON, who reported[4] that irregular 30-μ particles gave resolution comparable to that obtained with spherical 25-μ particles. A possible explanation of this fact may be that equilibrium can take place more rapidly in nonspherical particles than in spheres of the same mass, as the time necessary for diffusion to their centres is governed by the smallest of the three dimensions.

Consequently, since the ion exchange kinetics are strictly related to the extent of available surface presented by a given amount of solid phase, it is to be recommended that the irregular crushed resin should be retained as much more suitable for chromatographic purposes than the spherical resins, considering that the sphere is characterised by the smallest surface area per mass unit.

The experimental results reported here demonstrate that in order to avoid the overpressure, which occurs when the chromatographic elution is accelerated, it is not necessary to change the geometrical form of the resin, since this problem can be solved perfectly by a suitable choice of column parameters (that is the amount of resin and cross sectional area), and by abandoning the single reciprocating pumps usually employed to feed the columns. During the positive stroke, these pumps supply a pumped volume which is the double of that of the preset output, and consequently the pulsing pressure at the top of the resin bed reaches values which are higher than those obtained with our syringe-type pumps for the same flow-rate.

It has only to be stressed that when working with a crushed resin, particular care must be taken concerning the packing operation.

As a matter of fact, the dispersion of the particle classification which occurs in a pulverised resin is, of course, always much greater than that which can be achieved by regrading of spherical shapes.

Consequently, the column packing must be accomplished according to the method described in a preceding paper[9] in order to get an evenly dispersed column.

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CHROM. 4755

DE LA POSSIBILITÉ DE DOSAGE SANS ÉLUTION DE PRODUITS SÉPARÉS PAR CHROMATOGRAPHIE SUR COUCHES MINCES

PARTIE II. DU DOMAINE DE VALIDITÉ D'UNE FORMULE DEVANT PERMETTRE CE DOSAGE

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SUMMARY

The possibility of quantitative analysis of products separated by thin-layer chromatography without eluting the products. Part. II. The range of validity of a formula essential to permit this determination

The validity of a mathematical formula correlating the amount spotted of a compound and the sole area of the spot obtained after migration on a thin layer has been investigated. The experimental technique and the reasons which have led to its utilisation are explained and the results obtained are discussed.

INTRODUCTION

À la fin de la première partie de cet article[1] où nous concluions que la méthode envisagée semblait nettement insuffisante, nous disions que nos recherches nous amenaient à étudier la validité de la formule donnée par PURDY ET TRUTER[2]. La deuxième partie de ce travail traite de ce problème.

RAPPEL DE LA FORMULE DE PURDY ET TRUTER

Ces auteurs ont fait migrer, en chromatographie sur plaque, des quantités données, Q_i, de produits à étudier et ont mesuré les surfaces, S_i, correspondantes. Ils ont alors constaté que la relation entre Q_i et S_i était linéaire, donc de la forme $y = ax + b$, entre log. Q_i et $\sqrt{S_i}$ (bibl. 3).

À partir de ces résultats expérimentaux, ils ont proposé l'application suivante: (a) Migration sur une même plaque d'une quantité X inconnue d'un produit, d'une quantité D dilution de cette quantité et d'une quantité Q d'un produit standard. (b) Application de la relation $\sqrt{S_i} = a \log Qi + b$ à ces trois cas. L'élimination des

constantes a et b, considérées comme identiques, entre les trois relations ainsi obtenues, amène à la formule suivante:

$$\log X = \log Q + \frac{\sqrt{S_X} - \sqrt{S_Q}}{\sqrt{S_D} - \sqrt{S_X}} \log d$$

dans laquelle, on a:

X = quantité déposée inconnue du produit à doser,
Q = quantité déposée connue du produit standard,
S_Q = surface, après migration, de la tache correspondant à Q,
S_X = surface, après migration, de la tache correspondant à X,
S_D = surface, après migration, de la tache correspondant à D,
d = dilution de D par rapport à X.

C'est donc cette formule que nous avons appliquée et tentée de vérifier.

TECHNIQUE EXPÉRIMENTALE

Il a souvent été signalé, dans la littérature, les difficultés d'obtention de reproductibilité à la fois des spots de départ, et des R_F des taches correspondantes. DALLAS[4] et FAIRBAIRN[5], entre autres, ont passé en revue tous les points qu'ils pensaient être la cause de ces inconvénients et ils ont proposé des méthodes et des techniques qui, d'après leurs travaux, permettraient d'éviter ces écueils. L'étude de ces articles nous a permis d'adopter le processus suivant.

Préparation des plaques

Nous avons utilisé deux sortes de plaques: (1) plaques de Gel de Silice GF_{254} (Merck), épaisseur 0.250 mm, préparées suivant la méthode donnée précédemment[1] et (2) plaques de Gel de Silice F_{254} (Merck) pré-étalées sur feuilles d'aluminium. Dans les conditions expérimentales décrites ici, les résultats obtenus étaient comparables.

Solvants

Les solvants utilisés etaient comme suit: solvant No. 1, phase supérieure du mélange de PARTRIDGE[6]; solvant No. 2, H-$COOC_2H_5$–H-COOH–toluène (4:1:5) en volume.

Révélateurs

Révélateur pour les amines: réactif de Dragendorf modifié par MUNIER ET MACHEBOEUF[7]; pour les flavonoïdes réactif au Cl_3Al suivi d'un passage aux UV à 254 et à 365 mμ (bibl. 8).

Mise en place des spots de départ

Pour les deux sortes de produits étudiés (amines et flavonoïdes) nous nous sommes servis de solutions éthanoliques aux concentrations nécessaires pour pouvoir déposer chaque fois le même volume de 4 μl. Ce dépôt a été fait au moyen de capillaires calibrés, microcaps Drummond, et le solvant a été évaporé sous courant d'air froid.

Mesure des surfaces

Nous avons utilisé la méthode la plus simple donnée à la fois par PURDY ET

TRUTER[3] et par NYBOM[9] et aussi employée par nousmêmes précédemment[1]. On a décalqué, avec une mine de crayon très fine, la tache colorée sur du papier millimétré transparent; puis on a compté le nombre de millimètres carrés des surfaces immédiatement supérieures et immédiatement inférieures au contour ainsi obtenu et la moyenne des deux chiffres trouvés sera la valeur utilisable de la surface.

Cuves et migration

Après avoir étudié les travaux de HONNEGER[10] nous avions construit un dispositif permettant à la fois une saturation préliminaire des cuves en vapeurs de solvant et une pré-saturation des plaques après mise en place des spots[11]. Depuis, la littérature nous a, de nouveau, montré l'influence de ces facteurs, mais alliés à d'autres[4,12-14]. En fonction de ces données, nous avons conçu les dispositifs et la technique suivante: Pour chaque solvant, la cuve employée durant toute l'expérimentation fut la même, trois de ses parois étant recouvertes de papier Whatman No. 3. La saturation de la cuve en vapeur de solvant et la présaturation de la plaque furent réalisées avec notre précédent appareillage[11]. De plus, nous avons placé tout ce dispositif dans une enceinte construite spécialement afin de maintenir, d'un part des températures les plus proches possible d'une migration à l'autre et, d'autre part, une température constante durant tout le temps de l'expérience. Cet appareillage est schématisé dans la Fig. 1. L'isolant des parois est un revêtement de polystyrène expansé; le couvercle de cette enceinte est en plexiglass de un centimètre d'épaisseur; un dispositif à tirette permet de redresser doucement la cuve.

Finalement, afin de conserver, pour chaque dépôt envisagé, des formes de taches comparables et de surface constante, nous avons adopté la technique de "l'overrun-

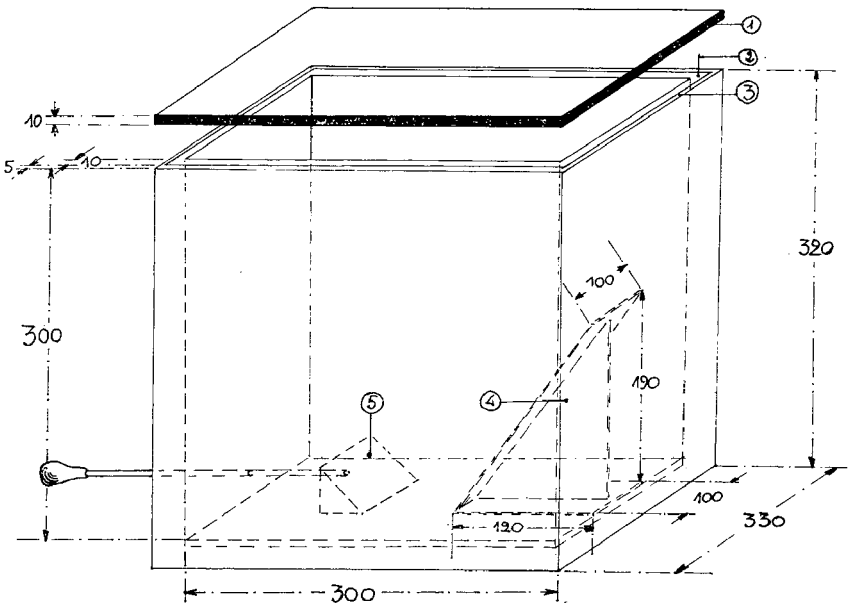


Fig. 1. Enceinte isothermique avec support de cuve. (1) couvercle de plexiglass; (2) isolant en polystyrène expansé; (3) revêtement extérieur en contre-plaqué; (4) support pour la cuve inclinée; (5) cale support et tirette. Les dimensions sont en millimètres.

ning" décrite par DALLAS[4]. La technique utilisée est donc la suivante: Dépôt des spots à 2 cm du bord inférieur de la plaque. Coupure dans la couche de gel de silice à 12 cm de ce même bord; par conséquent, la longueur de migration est de 10 cm. Mise en place de la plaque dans la cuve inclinée sur le support, la face recouverte de gel de silice tournée en dessous comme le montre la Fig. 2. Le tout est placé dans l'enceinte isothermique et laissé en position de pré-saturation durant 60 min, ce qui permet aussi d'atteindre un équilibre thermique intérieur. À la fin de ce temps, la cuve est redressée et la migration se fait. Lorsque le solvant a atteint la coupure de la couche adsorbante, on laisse encore le solvant avancer durant 15 min. Cette période étant écoulée les plaques sont sorties de la cuve, séchées, révélées et les surfaces sont mesurées comme il a été décrit au paragraphe précédent.

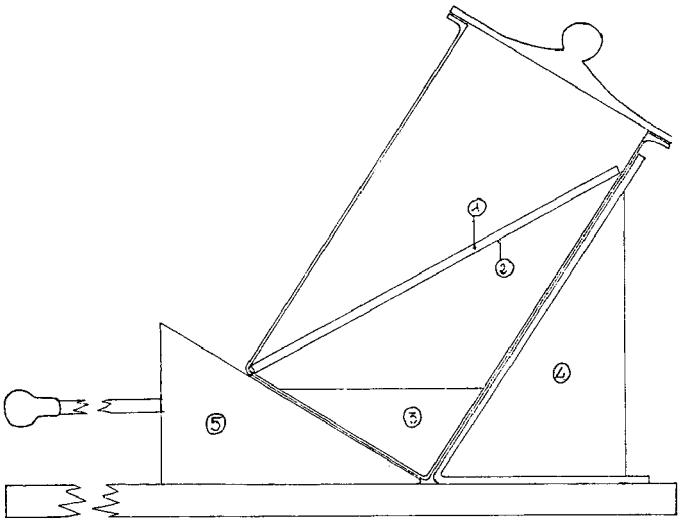


Fig. 2. Cuve en position de pré-saturation. (1) plaque de chromatographie; (2) couche d'adsorbant; (3) solvant; (4) support de cuve; (5) cale support et tirette.

RÉSULTATS

Essai sur une révélation par réaction colorée

Pour étudier la formule de PURDY ET TRUTER, dans ce cas, nous avons employé la trihexylamine. Le solvant utilisé était le solvant No. 1 et le révélateur, celui de Dragendorff modifié par MUNIER ET MACHEBOEUF.

Nous avons procédé à plusieurs sortes d'essais: (a) Essai où, sur une plaque, migraient la solution standard Q, la solution considérée comme inconnue X et sa dilution D et (b) essai où nous avons placé au départ les quantités 10, 20, 30, 40 et 50 μg.

(*a*) *Essai ou migraient les solutions Q, X et D.* Sur chaque plaque, nous avons placé les spots de départ dans des ordres variés: Q, X, D; Q, D, X; X, D, Q; D, Q, X. Nous avons pris les trois séries de valeurs données dans le Tableau I. Pour chacune de ces séries dix plaques ont été faites si bien que les résultats donnés sont la moyenne de 40 mesures pour chaque spot obtenu.

(*b*) *Essai où nous avons placé au départ les quantités 10, 20, 30, 40 et 50 µg.* Sur chaque plaque ont migré ensemble trois séries de ces quantités et dans chacune d'elles nous avons fait varier les ordres de voisinage des spots. Nous avons fait vingt plaques de cette manière si bien que les résultats donnés sont la moyenne de 60 mesures pour chaque tache obtenue. Sur le Tableau II figurent les résultats complets relatifs à la première série, sur le Tableau III, les résultats des mesures de la deuxième série et sur le Tableau IV, les résultats complets se rapportant aux différentes combinaisons possibles de D, X et Q acquises à partir des résultats du Tableau III.

TABLEAU I

QUANTITÉS Q, D, X PRISES POUR LES 3 SÉRIES ÉTUDIÉES

	1ère série (*µg*)	*2ème série* (*µg*)	*3ème série* (*µg*)
Q	20	12	30
D	30	10	15
X	40	20	20

TABLEAU II

RÉSULTATS COMPLETS DES ESSAIS DE LA PREMIÈRE SÉRIE

Quantité déposée (*µg*)				*Pour la quantité inconnue X*		*X trouvé* (*µg*)	*Écart entre les quantités déposées et trouvées* (%)
Q	D	X	d	$(\bar{S} \pm \Delta\bar{S})$ (mm^2)	$(\sqrt{\bar{S}} \pm \Delta\sqrt{\bar{S}})$ (mm^2)		
20	30	40	3/4	107.66 ± 7.75	10.38 ± 0.30	35.20	−12.0
12	10	20	1/2	84.90 ± 6.60	9.20 ± 0.35	17.69	−11.5
30	15	20	3/4	91.60 ± 2.30	9.56 ± 0.12	26,01	−30.0

Essai sur une révélation par fluorescence

Dans le cas envisagé, nous avons utilisé des produits dont la fluorescence sous UV à 365 mμ est connue et aussi pour lesquels cette fluorescence pouvait être changée ou augmentée par pulvérisation d'une solution de chlorure d'aluminium.

L'étude de la formule de PURDY ET TRUTER a donc porté, ici, sur les trois flavonoïdes suivants: galangine, kaempférol et quercétine. Les résultats ont été obtenus à partir de la chromatographie de spots correspondant aux quantités 10, 20, 30, 40 et 50 μg.

Le solvant employé était le solvant No. 2 et, après migration, les plaques furent séchées, examinées sous 365 mμ et la superficie des spots relevée. Puis on a pulvérisé du réactif et de nouveau relevé les surfaces sous UV. Dans ce cas là, les taches étaient plus nettes mais les différences constatées avec les mesures faites directement étaient minimes.

Nous avons opéré, pour chacun de ces flavonoïdes, d'une manière semblable à celle de la deuxième série des mesures de la trihexylamine, si bien que nous aurons, pour chaque composé, une série de tableaux de résultats donnant: le premier les

TABLEAU III

RÉSULTATS DES MESURES SUR LA DEUXIÈME SÉRIE

Quantité déposée (μg)	$\bar{S} \pm \Delta\bar{S}$ (mm^2)	$\sqrt{\bar{S}} \pm \Delta\sqrt{\bar{S}}$ (mm^2)
10	77.4 ± 2.9	8.79 ± 0.16
20	99.4 ± 1.9	9.96 ± 0.10
30	104.9 ± 1.8	10.18 ± 0.10
40	110.6 ± 1.6	10.51 ± 0.78
50	121.7 ± 2.6	11.02 ± 1.28

TABLEAU IV

RÉSULTATS COMPLETS DES DIFFÉRENTES COMBINAISONS DE LA DEUXIÈME SÉRIE

Quantité déposée (μg)			$D < X < Q$			$D < Q < X$			$Q < D < X$		
			d	*Quantité trouvée* (μg)	% *écart*	*d*	*Quantité trouvée* (μg)	% *écart*	*d*	*Quantité trouvée* (μg)	% *écart*
10	20	30	1/2	26.3	+30.0	1/3	23.8	−21.0	2/3	13.0	−56.6
10	30	40	1/3	31.4	+4.7	1/4	39.1	− 2.3	3/4	44.8	+12.0
10	30	50	1/3	25.7	−14.0	1/5	55.2	+10.4	3/5	38.7	−22.6
20	30	40	2/3	21.8	−27.0	1/2	45.5	+13.7	3/4	32.8	−19.5
20	50	50	2/3	26.3	−34.0	2/5	62.1	+24.2	3/5	31.8	−36.4
30	40	50	3/4	32.1	−20.0	3/5	54.5	+ 9.0	4/5	43.4	−13.2

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TABLEAU V

MESURES DES SURFACES DES TACHES

Quantité déposée (μg)	$\bar{S} \pm \Delta\bar{S}$ (mm^2)	$\sqrt{\bar{S}} \pm \Delta\sqrt{\bar{S}}$ (mm^2)
10	54.3 ± 1.9	7.39 ± 0.12
20	82.3 ± 0.7	9.09 ± 0.05
30	93.3 ± 1.7	9.64 ± 0.07
40	104.0 ± 0.3	10.10 ± 0.03
50	120.0 ± 1.3	10.95 ± 0.06

TABLEAU VI

RÉSULTATS COMPLETS DES DIFFÉRENTES COMBINAISONS RELATIVES À LA GALANGINE

Quantité déposée (μg)			$Q < D < X$			$D < Q < X$			$D < X < Q$		
			d	*Quantité trouvée* (μg)	% *écart*	*d*	*Quantité trouvée* (μg)	% *écart*	*d*	*Quantité trouvée* (μg)	% *écart*
10	20	30	2/3	52.51	>60	1/3	26.10	−13.00	1/2	24.00	+20.00
10	20	40	1/2	42.87	+ 7.15	1/4	33.54	−16.15	1/2	26.25	+31.25
10	20	50	2/5	57.65	+15.30	1/5	46.39	− 7.22	1/2	23.45	+17.25
10	30	40	3/4	51.38	+28.45	1/4	38.39	− 4.00	1/3	31.70	+ 5.93
10	30	50	3/5	40.08	−19.80	1/5	54.33	− 8.66	1/3	26.44	−11.80
10	40	50	4/5	26.05	−47.90	1/5	58.11	+16.22	1/4	33.37	−16.55
20	30	40	3/4	37.10	− 7.25	1/2	41.90	+ 4.25	2/3	28.10	− 6.30
20	30	50	3/5	41.22	−17.56	2/5	57.10	+14.20	2/3	19.04	−36.50
20	40	50	4/5	32.97	−34.20	2/5	60.32	+20.64	1/2	28.56	−28.60
30	40	50	4/5	43.67	−14.60	3/5	55.24	+10.48	3/4	30.40	−24.00

TABLEAU VII

MESURES DES SURFACES DES TACHES

Quantité déposée (μg)	$\bar{S} \pm \Delta\bar{S}$ (mm^2)	$\sqrt{\bar{S}} \pm \Delta\sqrt{\bar{S}}$ (mm^2)
10	39.7 ± 1.4	6.29 ± 0.14
20	54.0 ± 2.3	7.34 ± 0.11
30	61.3 ± 2.7	7.65 ± 0.20
40	69.3 ± 1.9	8.34 ± 0.11
50	82.0 ± 2.3	9.05 ± 0.15

TABLEAU VIII

RÉSULTATS COMPLETS DES DIFFÉRENTES COMBINAISONS RELATIVES AU KAEMPFÉROL

Quantité déposée (μg)			$Q < D < X$			$D < Q < X$			$D < X < Q$		
			d	*Quantité trouvée* (μg)	% *écart*	*d*	*Quantité trouvée* (μg)	% *écart*	*d*	*Quantité trouvée* (μg)	% *écart*
10	20	30	2/3	59.27	>60	1/3	25.57	−14.70	1/2	24.42	+22.10
10	20	40	1/2	41.41	+ 3.5	1/4	39.36	− 1.60	1/2	20.69	+ 3.45
10	20	50	2/5	43.80	−12.40	1/5	54.23	+ 8.46	1/2	16.17	+19.15
10	30	40	3/4	26.40	−43.50	1/4	48.00	+20.00	1/3	22.87	−23.40
10	30	50	3/5	27.42	−45.16	1/5	68.41	+36.42	1/3	16.13	−44.93
10	40	50	4/5	23.80	−52.40	1/5	60.50	+21.00	1/4	30.85	−22.90
20	30	40	3/4	30.97	−22.55	1/2	48.40	+21.00	2/3	16.21	−42.60
20	30	50	3/5	37.30	−25.40	2/5	63.56	+27.12	2/3	80.12	>60
20	40	50	4/5	32.45	−37.10	2/5	58.39	+17.78	1/2	30.57	−23.6
30	40	50	4/5	46.50	− 7.0	3/5	51.86	+ 3.72	3/4	37.14	− 7.15

TABLEAU IX

MESURES DES SURFACES DES TACHES

Quantité déposée (μg)	$\bar{S} \pm \Delta\bar{S}$ (mm^2)	$\sqrt{\bar{S}} \pm \Delta\sqrt{\bar{S}}$ (mm^2)
10	31.6 ± 2.2	5.61 ± 0.19
20	46.3 ± 4.2	6.78 ± 0.32
30	55.3 ± 5.4	7.31 ± 0.40
40	61.3 ± 5.1	7.82 ± 0.33
50	68.3 ± 1.1	8.26 ± 0.06

TABLEAU X

RÉSULTATS COMPLETS DES DIFFÉRENTES COMBINAISONS RELATIVES À LA QUERCÉTINE

Quantité déposée (μg)			$Q < D < X$			$D < Q < X$			$D < X < Q$		
			d	*Quantité trouvée* (μg)	% *écart*	*d*	*Quantité trouvée* (μg)	% *écart*	*d*	*Quantité trouvée* (μg)	% *écart*
10	20	30	2/3	36.90	+23.00	1/3	28.17	− 6.20	1/2	21.99	+ 9.95
10	20	40	1/2	43.70	+ 9.40	1/4	38.40	− 4.00	1/2	21.60	+ 8.00
10	20	50	2/5	50.98	+ 1.90	1/5	49.20	− 1.60	1/2	21.08	+ 5.40
10	30	40	3/4	34.40	−14.00	1/4	41.10	− 2.80	1/3	28.82	− 3.90
10	30	50	3/5	41.59	−18.82	1/5	53.49	+ 6.98	1/3	27.04	− 9.90
10	40	50	4/5	38.37	−23.20	1/5	52.43	+ 4.76	1/4	37.94	− 5.15
20	30	40	3/4	36.46	− 9.00	1/2	35.68	−11.05	2/3	27.09	− 9.70
20	30	50	3/5	44.32	−11.40	2/5	53.97	+ 7.94	2/3	24.17	−16.10
20	40	50	4/5	42.40	−14.60	2/5	52.52	+ 5.04	1/2	37.33	− 7.15
30	40	50	4/5	48.58	− 2.64	3/5	50.40	+ 0.80	3/4	39.05	− 2.35

valeurs de $\bar{S}$ et de $\sqrt{\bar{S}}$ pour chaque spot de départ; les trois autres, les différentes séries de combinaisons possibles de D, Q et X entre ces valeurs.

Galangine. Voir les Tableaux V et VI.

Kaempférol. Voir les Tableaux VII et VIII.

Quercétine. Voir les Tableaux IX et X.

DISCUSSION ET CONCLUSION

Cas de la trihexylamine

L'étude de tous ces tableaux montre que les trois différentes combinaisons examinées se succèdent dans l'ordre suivant: $D < Q < X$, $D < X < Q$ et $Q < D < X$ par rapport au nombre de résultats acceptables. De toute manière, la proportion des résultats où le pourcentage des écarts de récupération par rapport à la quantité déposée est inférieur ou au plus égal à 10 % n'est que de quatre cas sur dix-huit envisagés, ce qui n'est absolument pas satisfaisant.

La formule de PURDY ET TRUTER a été établie après avoir constaté expérimen-

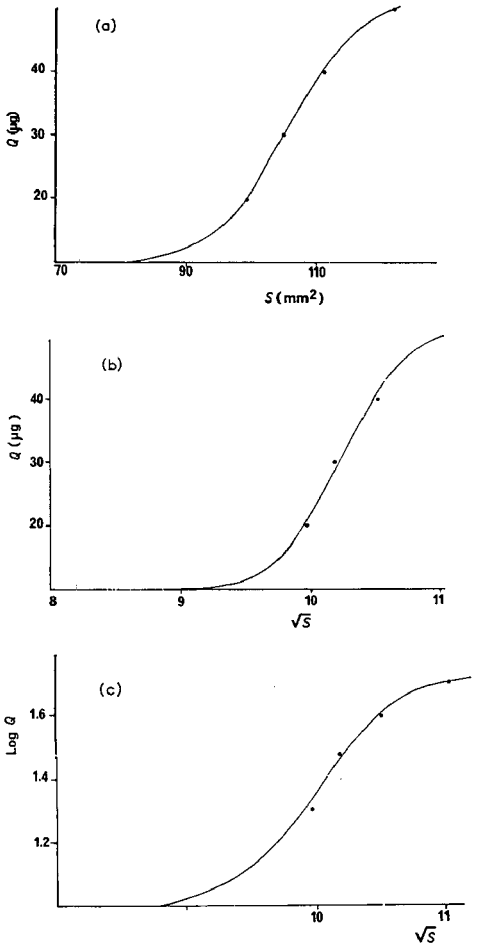


Fig. 3. Courbes relatives à la trihexylamine. (a) $S = f(Q)$; (b) $\sqrt{S} = f(Q)$; (c) $\sqrt{S} = f(\log Q)$.

talement que la courbe $\sqrt{S} = f(\log Q)$ était une droite. En outre, nos résultats précédents[1] ont montré que ceci n'était pas toujours obtenu de façon rigoureuse. Nous avons alors réalisé, pour la trihexylamine, les courbes: $S = f(Q)$ (Fig. 3a), $\sqrt{S} = f(Q)$ (Fig. 3b) et $\sqrt{S} = f(\log Q)$ (Fig. 3c). Sur ces courbes, nous remarquons que: la courbe la plus rigoureuse est $S = f(Q)$; la courbe $\sqrt{S} = f(Q)$ est linéaire dans le même domaine de quantité que $S = f(Q)$ mais que la sensibilité est réduite par augmentation de la pente de sa partie linéaire; la courbe $\sqrt{S} = f(\log Q)$ est celle où la région de linéarité par rapport à la quantité Q déposée est la plus petite et où la pente de la partie linéaire est la plus forte. La relation entre l'existence d'une très petite partie linéaire de cette dernière et la très faible quantité de résultats acceptables donnée par la formule de PURDY ET TRUTER est ici montrée.

Cas des flavonoïdes étudiés

Pour ces trois produits, la proportion de résultats où le pourcentage des écarts de récupération par rapport à la quantité déposée est inférieur ou au plus égal à 10 %,

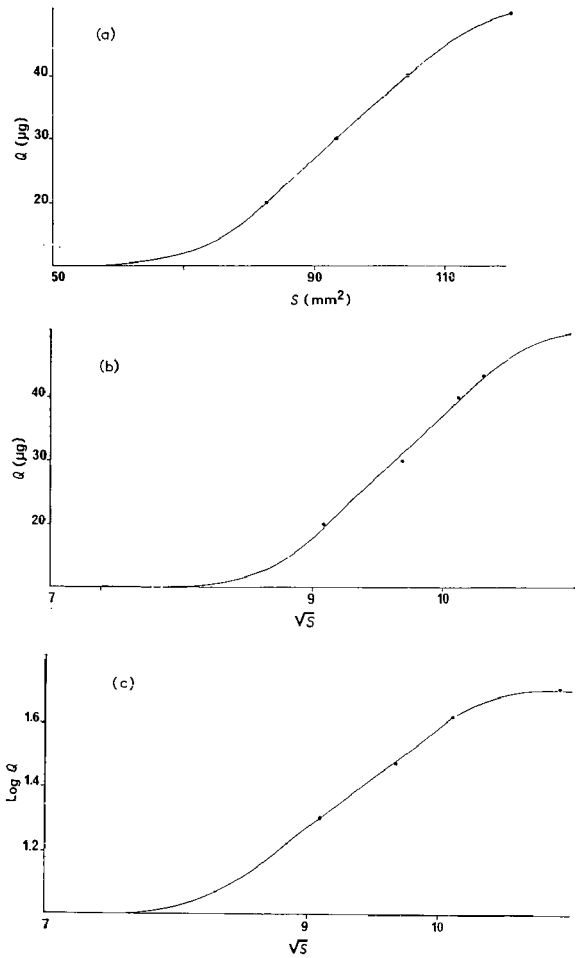


Fig. 4. Courbes relatives à la galangine. (a) $S = f(Q)$; (b) $\sqrt{S} = f(Q)$; (c) $\sqrt{S} = f(\log Q)$.

est plus forte que pour la trihexylamine. Le nombre le plus grand de cas favorables est trouvé pour la quercétine, puis se suivant par ordre décroissant, galangine et kaempférol. Nous avons fait, pour ces trois produits, les mêmes courbes que précédemment:

Quercétine: $S = f(Q)$ (Fig. 4a), $\sqrt{S} = f(Q)$ (Fig. 4b) et $\sqrt{S} = f(\log Q)$ (Fig. 4c).
Galangine: $S = f(Q)$ (Fig. 5a), $\sqrt{S} = f(Q)$ (Fig. 5b) et $\sqrt{S} = f(\log Q)$ (Fig. 5c).
Kaempférol: $S = f(Q)$ (Fig. 6a), $\sqrt{S} = f(Q)$ (Fig. 6b) et $\sqrt{S} = f(\log Q)$ (Fig. 6c).

L'examen de ces courbes nous permet de constater que: pour la quercétine, la courbe $\sqrt{S} = f(\log Q)$ possède la partie linéaire la plus longue; pour la galangine, cette partie est réduite; pour le kaempférol, on ne peut pas considérer cette courbe comme linéaire. Ces résultats recoupent la conclusion déjà établie pour la trihexylamine.

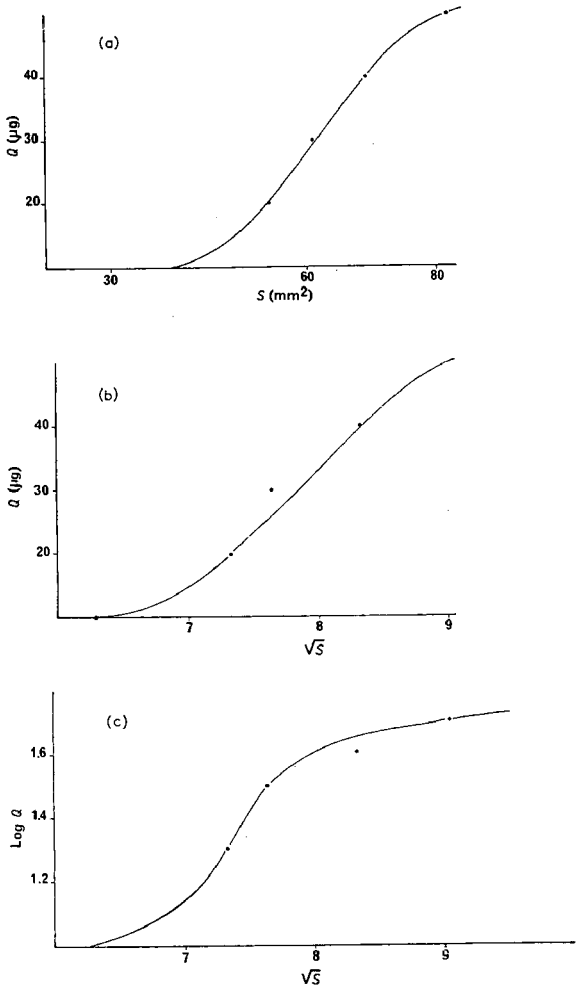


Fig. 5. Courbes relatives au kaempférol. (a) $S = f(Q)$; (b) $\sqrt{S} = f(Q)$; (c) $\sqrt{S} = f(\log Q)$.

Conclusions relatives au quatre produits étudiés

D'abord, du point de vue répartition des quantités déposées, on s'aperçoit que l'ordre des spots qui donnent le plus de résultats acceptables est, pour les quatre produits étudiés, la combinaison $D < Q < X$. De plus, les combinaisons envisagées, toujours pour les quatre produits, se suivent dans le même ordre: $D < Q < X$; $D < X < Q$ et $Q < D < X$. Par conséquent, il semblerait bien que cette méthode puisse donner des résultats relativement acceptables si la quantité standard Q est placée entre la quantité inconnue X et sa dilution D.

L'application de la formule de PURDY ET TRUTER devrait être une méthode générale et pourtant il apparaît que, seul, un mode de répartition donné soit privilégié, ce qui est une limitation très substantielle de ce système de dosage.

Si nous rapprochons les résultats exposés ici et ceux que nous avons établis dans la première partie de ce travail, nous pouvons conclure que, à de rares exceptions

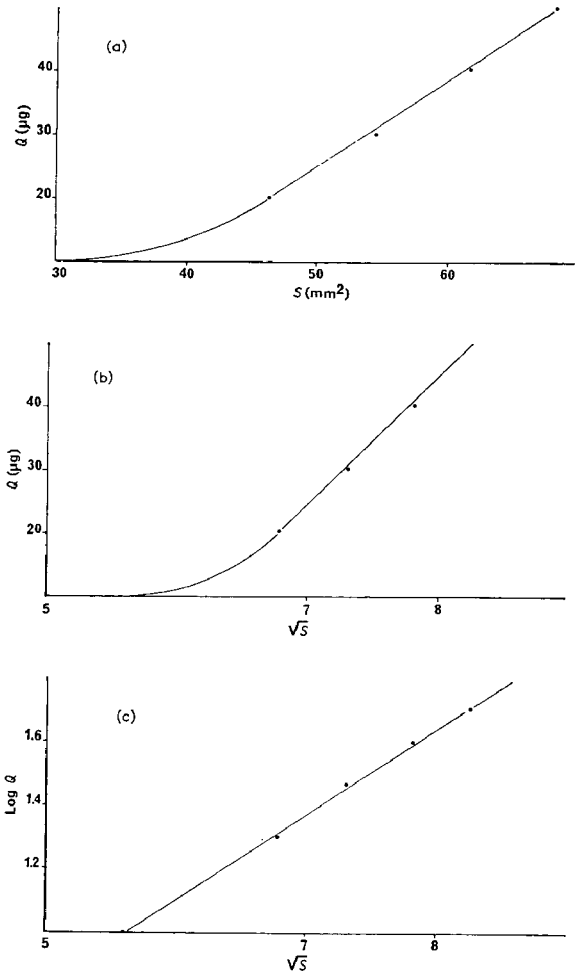


Fig. 6. Courbes relatives à la quercétine. (a) $S = f(Q)$; (b) $\sqrt{S} = f(Q)$; (c) $\sqrt{S} = f(\log Q)$.

et dans des conditions d'expérimentation extrêmement serrées, on ne peut envisager de réaliser une technique générale de dosage en chromatographie sur couches minces par mesure directe des surfaces sur les plaques au moyen de la technique très simple employée ici d'après PURDY ET TRUTER[2] et NYBOM[9]. Ceci rejoint, d'ailleurs, les conclusions de FAIRBAIRN[5] qui a dû utiliser des conditions expérimentales draconiennes et mettre au point un appareil fort coûteux pour la mise en place des échantillons. Quant à la lecture des surfaces, il semble aussi qu'il faille employer des techniques très élaborées afin d'obtenir des résultats satisfaisants.

Malgré une apparente rapidité, la technique par "superficimétrie" décrite ici et se terminant soit par l'établissement d'une courbe étalon, soit par l'application d'une formule mathématique, ne fournit ni la précision souhaitée, ni les résultats attendus. La détermination quantitative de produits séparés en chromatographie sur plaques, nécessitant le minimum de manipulation et d'appareillages et donnant le maximum de résultats acceptables, semblerait être soit l'élution suivie d'une colorimétrie ou d'un dosage UV, soit la densitométrie des taches après coloration[15]. En effet, ces méthodes tiennent compte des facteurs intensité de la coloration ou de la fluorescence et donnent des résultats obtenus par des appareils de mesure et non par des mesures subjectives.

REMERCIEMENTS

Nous tenons à remercier Monsieur le Professeur DUCHENE-MARULLAZ, Directeur Scientifique du C.E.R.M., pour la bienveillante attention qu'il a apportée à ce travail ainsi que Madame CLAUDE DUSSAP et Mademoiselle FRANÇOISE LOMBARD dont l'aide technique et les calculs nous ont été précieux.

RÉSUMÉ

Dans cet article, on a tenté d'appliquer pour un produit séparé en chromatographie sur plaque, une formule liant la quantité déposée à la seule surface obtenue après migration. La technique employée et les raisons qui nous ont amenés à son utilisation ont été exposées, suivie d'une discussion des résultats et les conclusions.

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QUALITATIVE AND QUANTITATIVE *IN SITU* FLUORIMETRY OF CITRUS OIL THIN-LAYER CHROMATOGRAMS

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SUMMARY

The development of direct fluorimetric scanning for thin-layer chromatogram analysis has introduced a versatile tool which has diversified applications in the analysis of citrus oils and potential for analysis of a wide variety of other natural products. The characterization of citrus oils from fluorescence and fluorescence quenching profiles which result from the presence of crystalline components in the oils can be accomplished. Quantitative determination of two coumarin derivatives, 5-geranoxy-7-methoxycoumarin and 5,7-dimethoxycoumarin, by *in situ* fluorimetry of lemon, lime, and bergamot oil thin-layer chromatograms provides an accurate and rapid procedure for quality control based on coumarin derivative content.

INTRODUCTION

Recently the application of direct fluorimetric scanning of thin-layer chromatograms with commercially available instruments has received considerable attention. In an early stage of development, *in situ* fluorimetry has been utilized primarily in bio-medical analysis. This development has paralleled that of fluorimetry which also is used extensively in bio-medical analysis and research[1]. Application of *in situ* fluorimetry to complex natural product mixtures such as citrus oils has not been explored. The application of fluorimetry in combination with thin-layer chromatography indicates that many widely diversified and thus far unexplored applications of fluorimetric scanning of thin-layer chromatograms are possible. An early application of *in situ* fluorimetry was made to demonstrate the usefulness of the technique in air polution studies[2]. KLAUS[3] has investigated the effect of spot shape in direct chromatogram analysis. JÄNCHEN AND PATAKI[4] summarized the various procedural features available for fluorimetric scanning of thin-layer chromatograms and demonstrated the methods with applications to amino acid derivative mixtures and nucleotide mixtures. ZÜRCHER *et al.*[5] have investigated reproducibility in quantitative thin-layer chromatography when direct spectrophotometry and fluorimetry are employed. This versatile technique has a great potential for both qualitative and quantitative analysis.

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Expressed citrus oils contain considerable amounts of crystalline components which can be separated by thin-layer chromatography and which exhibit fluorescence upon excitation by UV radiation[6–9]. An extensive examination of the luminescence properties of expressed lime oil and individual coumarin derivatives isolated from expressed lime oil[10] has shown that the presence of 5-geranoxy-7-methoxycoumarin (I) and 5,7-dimethoxycoumarin (II) are responsible for the fluorescence characteristics of lime oil. The amounts of I and II present in expressed lime oil are responsible for the variations in fluorescence emission intensity observed from ethanol solutions of lime oil samples from different geographical regions. Quantitative fluorimetric scanning of thin-layer chromatograms results in a rapid and accurate method for the determination of the amounts of I and II present in lime oil and other citrus oils.

The characterization of citrus oils from fluorescence and fluorescence quenching profiles obtained from thin-layer chromatograms resulting primarily from the presence of crystalline components in the oils can be accomplished. *In situ* fluorimetry combines the advantages of thin-layer chromatography with those of fluorimetry which include greater selectivity and sensitivity when compared to absorption spectrophotometry and densitometry.

EXPERIMENTAL

Apparatus and materials

An Aminco Bowman Spectrophotofluorometer (SPF) with a high-pressure mercury–xenon arc source lamp, RCA 1P28 multiplier phototube, motorized thin-film scanner (American Instrument Co., Silver Springs, Md.) and a Speedomax G Model S recorder, equipped with a Disc Chart Integrator Model 203 (Leeds and Northrup Co., Philadelphia, Pa.) was used to obtain all emission and quenching profiles and quantitative data. The photomultiplier microphotometer (PMP) supplied with the SPF is designed to be connected with a 50-mV, 1-sec response recorder. Therefore, to operate the recorder with a 0-mV to 5-mV span, it is necessary to apply one-tenth of the PMP output voltage to the recorder. This was accomplished by connecting a 500 Ω variable potentiometer in parallel with the output resistance of the PMP which allows the desired signal range to be applied to the recorder.

Precoated analytical layer chromatoplates, Silica Gel F-254 (Merck) (supplied by Brinkmann Instruments Inc., Westbury, N.Y.), were used to obtain thin-layer chromatographic separations in a Brinkmann sandwich developing chamber. Singly redistilled solvents were used to develop all chromatograms.

Thin-layer chromatography and direct chromatogram analysis

The following procedure was used to obtain thin-layer chromatograms and profiles from the chromatograms in the subsequent studies. Samples were applied 2.0 cm from the bottom of a thin-layer plate. Eight or nine samples were applied to each plate. The plate was then developed to 15 cm above the origin spot line with ethyl acetate–*n*-hexane–acetic acid (50:150:1). The developed chromatogram was then dried in the dark for at least 2 h.

Direct chromatogram analysis is based on the measurement of emission from components which exhibit fluorescence on the thin-layer plate. When the chromatoplate is placed on the thin-film scanner with the adsorbent layer face down, radiation

from the source lamp is carried through the excitation monochromator of the SPF and appropriate optics and is directed onto the adsorbent layer. This radiation can cause excitation of components chromatographed on the thin-layer plate or can cause excitation of a fluorescent indicator added to the adsorbent layer prior to preparation of the chromatoplate. In either case a portion of the visible fluorescence emission, which is transmitted through the glass plate backing of the adsorbent layer, is carried by a fiber optic to the emission monochromator of the SPF then to the multiplier phototube. If the components under investigation exhibit native fluorescence then an emission profile is obtained by scanning the thin-layer chromatogram in the direction of plate development using the excitation wavelength which will excite the greatest number or most prominent components and an emission wavelength which will monitor fluorescence from the greatest number or most prominent components. If the components under investigation do not exhibit native fluorescence but do absorb UV radiation, their presence can be detected in an adsorbent layer which contains a fluorescent indicator. The components which absorb UV radiation will quench the emission of the fluorescent indicator and a quencing profile is obtained when the chromatogram is scanned utilizing excitation and emission wavelengths which correspond to maximum excitation and emission respectively for the fluorescent material previously added to the adsorbent layer.

The plates were scanned in the same direction as development to obtain qualitative profiles. Quantitative data were obtained by scanning individual chromatogram spots in directions parallel to and perpendicular to the direction of plate development. Scanning was accomplished by orientating the plate on the scanner with the adsorbent layer face down so the scan passed through the center of the chromatogram spot(s) of interest.

Direct qualitative thin-layer chromatogram analysis

Emission monochromator wavelength settings of 403 nm, 440 nm, and 490 nm were used to obtain the various fluorescence emission profiles. Excitation at 313 nm or 365 nm produces the most intense emission when a mercury–xenon lamp is employed. An excitation wavelength of 272 nm and an emission wavelength of 520 nm were used to obtain the various fluorescence quenching profiles. This wavelength combination makes it possible to measure the maximum emission from the fluorescent indicator in the adsorbent layer with the described instrumental set-up. The slit program employed when quenching profiles were recorded was 5–4–0.5–5–5 where the slit widths expressed in millimeters are for the excitation monochromator slit, excitation side of the lens and mirror assembly slit, exit aperture of the lens and mirror system slit, emission monochromator slit, and multiplier phototube shutter slit, respectively. Emission profiles were obtained with slit program 5–4–0.1–5–5. This slit arrangement gives a small improvement in resolution for emission profiles, however, equivalent quenching profiles are obtained with either slit program. The wider exit aperture slit is favored in quenching studies because it allows measurements to be made at a higher PMP meter multiplier setting which reduces instrumental noise and also is not as sensitive in recording minor variations due to "grain" in the adsorbent layer.

Thin-layer chromatograms for the expressed citrus oil samples were obtained from 0.2 to 0.5 μl of lime oils, 1.0 μl of lemon, bergamot, and grapefruit oils, and

2.0 μl of sweet and bitter orange oils. Profiles for mixtures of citrus oils were obtained from chromatograms of 1.0 μl of each oil mixture. Lemon oil–grapefruit oil mixtures, 1:1, 2:1, and 4:1; lemon oil–lime oil mixtures, 1:1 and 4:1; and grapefruit oil–lime oil, 4:1 were investigated.

Direct quantitative thin-layer chromatogram analysis

The influence of time on fluorescence intensity for I and II was investigated under conditions to be used for quantitative determinations. The thin-layer chromatograms were obtained from appropriate amounts of I and II. The first scans of the chromatogram spots were started immediately after completion of plate development. Chromatogram spots were then scanned at approximately 15-min intervals for 5 h. The plate was aligned so that each scan would pass through the center of the individual spots. The center of the spot was located by manual adjustment of the plate position until a maximum signal was registered by the PMP. Quantitative *in situ* fluorescence emission and quenching were measured with slit program 5–4–0.5–5–5.

Known amounts of I and II were subjected to thin-layer chromatography. *In situ* fluorescence analytical curves were obtained from the resulting chromatograms. Fluorescence quenching analytical curves were obtained for 5-geranoxypsoralen (III) and 5,8-dimethoxypsoralen (IV) using a similar procedure. The quenching analytical curves were obtained using 272-nm excitation and 520-nm emission monochromator wavelength settings. The emission analytical curves were obtained with 335-nm excitation and 440-nm emission monochromator settings. While maximum excitation is not obtained from the mercury–xenon lamp at 335 nm this is a more suitable wavelength than 313 nm or 365 nm for quantitative analysis because photodecomposition due to the intense radiation does not become significant.

Determination of the amounts of I and II present in citrus oils was accomplished by analyzing up to five samples on the same thin-layer plate with four or five standards. Standard solutions which contain both I and II were prepared. The concentration of each component was such that an analytical curve for each could be obtained which is applicable for analysis of I and II in the particular citrus oil being analyzed. Standard solutions with the following concentrations of I and II, respectively, in ethanol were prepared: (A) 25.0 μg/ml, 10.9 μg/ml; (B) 49.9 μg/ml, 10.9 μg/ml; and (C) 8.32 μg/ml, 21.8 μg/ml. Ethanol solutions of the citrus oils which contained 2.0 mg/ml, 10 mg/ml, 20 mg/ml and 500 mg/ml of expressed lime, bergamot, lemon, and distilled lime oils, respectively, were prepared. Four aliquots ranging from 1.0 to 4.0 μl of solution A or B were spotted to obtain an analytical curve for the analysis of the amounts of I and II present in lemon or expressed lime oils. The amounts of each lemon or lime oil solution to be spotted were determined from a preliminary analysis on each sample. Depending on the approximate amounts of I and II present in each sample, 2.0, 2.5, or 3.0 μl of each lemon oil solution and 1.0, 1.5, 2.0, or 4.0 μl of each expressed lime oil solution were spotted. Five aliquots ranging from 1.0 to 5.0 μl of solution C were spotted along with 3.0 μl of each distilled lime oil solution and 1.5 μl of each bergamot oil solution for the analysis of I and II in these oils.

Relative fluorescence emission intensity (*RE*) determined from chromatogram profile peak areas was obtained as follows. A baseline was drawn tangent to the minima which occur between successive peaks in each profile. The geometric area (*GA*) expressed in square chart units, under the baseline was converted to integrator counts.

The conversion factor is 3.02 integrator counts per square unit of chart paper. The total integrator count (IC) for each peak includes this background or blank area. The relative emission intensity was determined using the following expression

$$RE = [IC - GA(3.02)]MM \quad (1)$$

where MM is the PMP meter multiplier setting. Recorder chart speed was two inches per minute, which represents approximately one inch of chart paper per inch of thin-layer chromatogram scanned. Peak areas as small as one square centimeter and as large as twenty square centimeters were obtained under the described conditions.

Relative fluorescence quenching intensity (RQ) from chromatogram profile peak areas was determined by triangulation. A baseline was drawn tangent to the maxima which exist when no absorbing component is present on the adsorbent layer. Peak height (PH) to the baseline was determined. The peak width at one-half peak height (WHP) and peak height were used to determine the peak area. The relative quenching was determined using the following expression

$$RQ = (PH)\,(WHP)\,(3.02)MM \quad (2)$$

where all terms have been previously defined. The 3.02 factor is introduced to convert peak area into integrator counts which allows RQ to be compared to RE.

Relative fluorescence emission and fluorescence quenching intensity from chromatogram profile peak heights (RH) were determined as follows

$$RH = (PH)MM \quad (3)$$

where the peak height, expressed in chart units, is measured from the baseline drawn as previously described to the peak maximum for emission or peak minimum for quenching.

RESULTS AND DISCUSSION

Direct qualitative fluorimetry of citrus oil thin-layer chromatograms

Fluorescence emission and fluorescence quenching profiles for various citrus oils are presented in Figs. 1 to 4. The fluorescence emission profiles in Figs. 1 and 2 represent typical but not necessarily the most characteristic profiles for each oil. Comparison of two emission profiles of grapefruit oil, Fig. 1A, and two emission profiles of bitter orange oil, Fig. 2A, reveals that the presence of the greatest number of components is detected when two or more profiles are obtained for each oil using different characteristic emission monochromator settings. The variations obtained are generally limited to increases or reductions in the relative emission intensity emanating from each component spot. It is not intended that these profiles reflect any correlation with respect to intensity and therefore to the amounts of individual components present. In extreme cases it may be possible to completely resolve one component from another in the emission profile through the judicious choice of both excitation and emission wavelengths. This technique has been investigated in studies related to air pollution[2]. An emission maximum of 440 nm for two spots predominates in lemon, lime, and bergamot oil thin-layer chromatograms. This emission is due to the presence

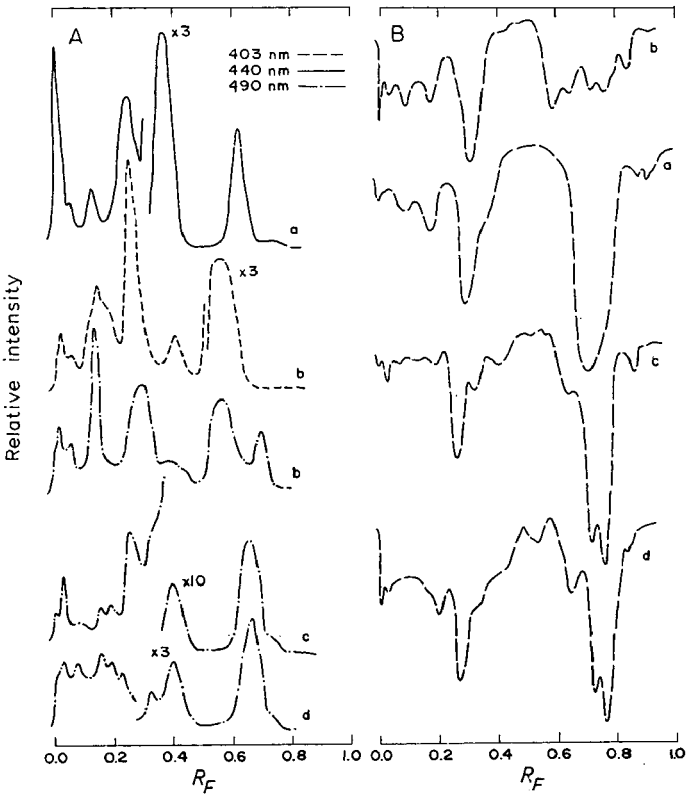


Fig. 1. Fluorescence (A) and fluorescence quenching (B) profiles of expressed citrus oils. (a) Bergamot; (b) California grapefruit; (c) Persian lime; (d) West Indian lime.

of I and II. The much lower relative fluorescence intensities of other types of coumarin derivatives result in less prominent emission from these components. Expressed citrus oils can be characterized to some extent by measuring emission profiles which result from the use of various emission wavelengths because of the presence of a number of 5-alkoxypsoralens, 5,7-dialkoxycoumarins, and 7-alkoxycoumarins. Each shows a characteristic emission spectrum with maxima at 490 nm, 440 nm, and 403 nm, respectively.

The citrus oils in turn exhibit profiles which are generally characteristic of each particular kind of oil. In addition, minor variations are observed in profiles obtained from different samples of each kind of citrus oil. Expressed lime oil samples show the greatest variations. Three distinctly different profiles, Figs. 1A, c and d and 4A, were obtained from samples of lime oil. However, classification into subgroups based on individual profiles after examination of only one sample from various geographical regions cannot be justified. Even classification of each lime oil sample into the Mexican or Tahitian group[11] cannot be made without reservation. Only two samples of expressed grapefruit oil and of expressed bergamot oil were examined. However, characteristic profiles are obtained for each oil. Only minor variations are observed in the profiles obtained for samples of lemon oil. The only sample of bitter

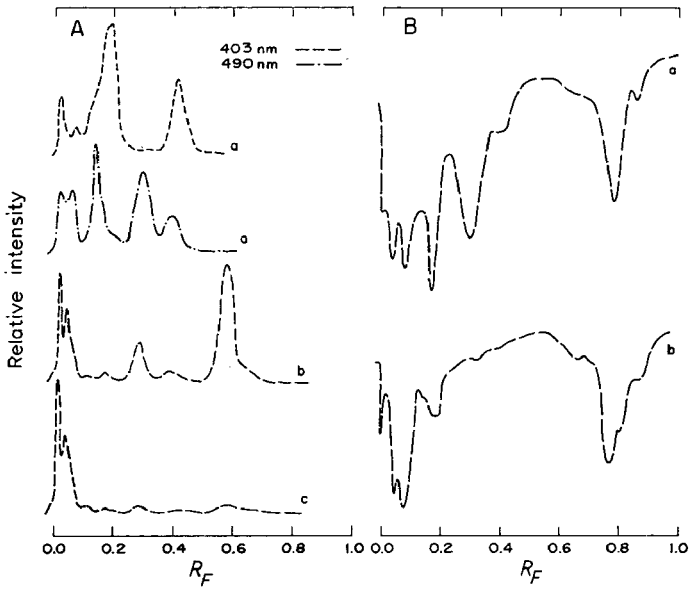


Fig. 2. Fluorescence (A) and fluorescence quenching (B) profiles of expressed orange oils. (a) West Indian bitter orange; (b) orange, North American standard; (c) Florida orange.

orange oil investigated has emission and quenching profiles which are greatly different from those obtained from various samples of sweet orange oil.

Comparison of the various emission and quenching profiles of the citrus oils reveals an increased complexity of expressed lemon and lime oils compared to the other oils. Lemon and lime oils while exhibiting similar profiles do show variations which allow for differentiation between the two. The emission and quenching profiles of expressed lemon oil samples are characterized by the presence of two well-resolved peaks at R_F 0.00 and 0.03. In the same region of expressed lime oil thin-layer chromatograms only two of five samples investigated yielded a profile similar to the lemon oil profile. Lime oil at R_F 0.70 and 0.75 exhibits two partially resolved peaks in the quenching profile. These can be attributed to the presence of III and citral and/or other oxygenated terpenes which are capable of absorbing short-wavelength UV radiation. In the corresponding region of the lemon oil quenching profile only a shoulder due to the presence of III is observed on the prominent peak which again results from the presence of citral and/or other oxygenated terpenes. This difference may be due to the larger amount of III present in lime oil compared to lemon oil. Prominent peaks which correspond to the presence of I and II do not occur in the quenching profiles. The fluorescence of these two components upon excitation with short-wavelength UV light apparently is great enough that emission from these components will compensate for loss of emission from the fluorescent indicator in the adsorbent layer. In the emission profiles of both lemon oil and lime oil the presence of III is indicated by a shoulder at R_F 0.7 on the very intense peak due to the presence of I. The peak for III is most apparent when measurement of the emission profile at 490 nm instead of 440 nm is employed. These are prominent differentiating features observed in the emission and quenching profiles for expressed lemon and lime oils.

A general feature which distinguishes expressed lime oil emission profiles from other citrus oils is present in the first quarter of the chromatogram scan. A relatively high background emission is observed. This background emission is not as pronounced in the emission profiles of other citrus oils. The thin-layer chromatograms of expressed lime oils, when examined under 365 nm UV light, show considerable streaking in this region while the other citrus oil thin-layer chromatograms are relatively free from streaking when the same plate development procedure is used.

Expressed bergamot and orange oils exhibit profiles of less complexity presumably because of the smaller number of coumarin derivatives present[6]. The presence of at least seven well-defined peaks or shoulders on peaks in the emission profiles for the samples of orange oil and bergamot oil indicates that the coumarin composition of these oils may be much more complex than has been reported.

The emission and quenching profiles obtained can be of considerable value in detecting mixtures of two oils. Lemon oil–grapefruit oil mixtures can be detected from the quenching profile, Fig. 3, where at R_F 0.4 a strong absorption appears at a 1:1 mixture of the two oils and becomes progressively stronger as the relative amount of lemon oil is increased. The value of this observation is limited to situations where grapefruit oil is adulterated with a large amount of lemon oil. A more meaningful relationship exists when comparisons are made of the peaks which appear at R_F 0.3 and 0.4. Both peaks are present in lemon oil profiles where the latter is generally more intense. However, only the peak at R_F 0.3 is present in grapefruit oil profiles. Therefore, the presence of even small amounts of grapefruit oil in lemon oil can be detected by observing an increase in intensity of the peak at R_F 0.3 with respect to the peak at 0.4.

The emission profiles of lemon oil–grapefruit oil mixtures are characterized by variations which occur over the 0.5 to 0.7 R_F region. These profiles, obtained with emission monochromator settings of 490 nm, 440 nm, and 403 nm, reflect the presence

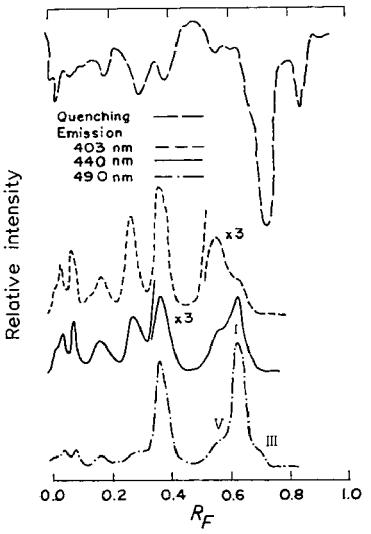


Fig. 3. Fluorescence and fluorescence quenching profiles of a California lemon oil and California grapefruit oil mixture, 4:1.

of III, I, and 7-geranoxycoumarin (V) in the mixture. As shown in Fig. 3 the presence of V is readily detected in the presence of I when a comparison is made of the profiles obtained at 403 nm and 440 nm.

Quenching profiles of lemon oil–lime oil mixtures cannot be used readily to detect the presence of a mixture. This is because of the similarity in profiles obtained for the individual oils and because samples of lime oil have been shown to exhibit variations in individual profiles. Some resemble lemon oil more closely than others. However, a mixture of lemon oil and lime oil can be suspected if the characteristic quenching profiles from R_F 0.6 to 0.8 are not observed. The emission profiles of lemon oil and lime oil are also very similar and no truly distinct characteristics are observed. The detection of lemon oil–lime oil mixtures is best evaluated from quantitative data obtained for I and II, which are present in both oils. These data are presented and evaluated in the section which considers quantitative applications of the technique.

The lime oil–grapefruit oil mixture was not extensively investigated. However this mixture can be detected from observations similar to those already discussed for lemon oil–grapefruit oil mixtures.

Thin-layer chromatography of the coumarin derivatives isolated from expressed lime oil[10] and run simultaneously with samples of expressed lemon and lime oils makes possible the tentative identification of the crystalline components responsible for many of the peaks in the emission and quenching profiles of the two oils. Table I identifies some of the coumarin derivatives which are responsible for peaks in expressed lemon and lime oil profiles and designates peaks in Fig. 4 which have been attributed to the presence of these components. Identifications were based on results obtained from *in situ* fluorimetry and by visualization of the citrus oil and crystalline component thin-layer chromatograms under UV light. A number of less prominent peaks appear which are possibly due to the presence of other not yet identified coumarin derivatives. Some components may not yield characteristic peaks in the emission and/or quenching profiles because of the small amounts present or weak native fluorescence.

An improvement in resolution of the individual components on the citrus oil

TABLE I

IDENTITY OF COUMARIN DERIVATIVES RESPONSIBLE FOR THE EMISSION AND QUENCHING PROFILES FOR EXPRESSED WEST INDIAN LIME OIL AND EXPRESSED CALIFORNIA LEMON OIL

Coumarin derivative	*Peak number*	
	Lime oil (Fig. 4A)	*Lemon oil (Fig. 4B)*
5-Geranoxypsoralen (III)	1	1
5-Geranoxy-7-methoxycoumarin (I)	2	2
5-Geranoxy-8-methoxypsoralen	3	3
5-Isopentenoxy-8-methoxypsoralen	4	
8-Geranoxypsoralen		5
5,7-Dimethoxycoumarin (II)	6	6
5-Methoxy-8-isopentenoxypsoralen 8-Isopentenoxypsoralen	7	
5-Methoxypsoralen	8	
5,8-Dimethoxypsoralen (IV)	9	
5-Dihydroxyisopentanoxypsoralen	10	10
5-Methoxy-8-dihydroxyisopentanoxypsoralen	11	11

thin-layer chromatograms can be accomplished using a developing solvent such as ethyl acetate–*n*-hexane–acetic acid 30:270:1 and developing the plate three times instead of only the single development with 50:150:1 solvent. The improvement is most apparent in the quenching profile. However, a single development is adequate to characterize the different citrus oils.

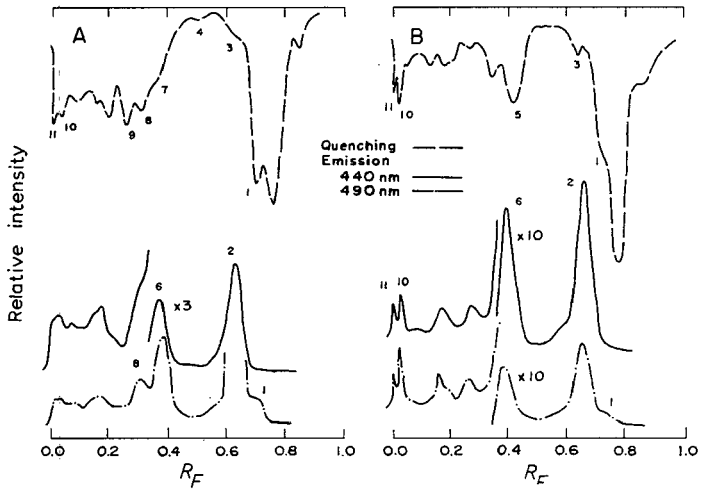


Fig. 4. Fluorescence and fluorescence quenching profiles of Florida lime oil (A) and California lemon oil (B). The numbered peaks are identified in Table I.

The same peaks appear in emission profiles obtained with excitation from a xenon lamp at 330 to 350 nm and from the mercury–xenon lamp at 313 nm or 365 nm. However, the intensity of various peaks is changed when different lamps and different excitation wavelengths are used. The mercury–xenon lamp is of greatest value for characterization purposes because the high intensity at 313 nm and 365 nm makes it possible to detect trace components. This advantage is limited if measurements are made on thin-layer chromatograms with high fluorescent backgrounds. Both lamps give identical quenching profiles using 272-nm excitation. However, the mercury–xenon lamp makes the use of a higher meter multiplier setting on the PMP possible.

Under the conditions employed to develop the chromatograms, the emission profiles can be used only for qualitative characterization of each individual oil. No extensive significance should be applied to the relative intensity of the various peaks in the profile because the emission from many of the components is highly quenched due to the relatively high concentration of the various components present. In addition, the fluorescence efficiency for individual coumarin derivatives has been shown to vary over a wide range. Variations in intensity observed when emission profiles are recorded with different emission monochromator settings can be significant for determination of the type of coumarin derivative present. For example, in the lemon oil–grapefruit oil mixture profiles, Fig. 3, recorded at 403 nm and 440 nm, the relative intensity for the peaks of R_F 0.5 and 0.6 is reversed. This indicates the presence of two components which are characterized by different emission wavelength maxima.

Evaluation of experimental parameters for direct quantitative fluorimetry of citrus oil thin-layer chromatograms

An initial investigation of various parameters which can affect quantitative *in situ* fluorimetry of thin-layer chromatograms was conducted. These parameters include the influence of time on fluorescence intensity, component spot alignment with respect to the exit aperture slit, the relationship of profile peak area and peak height to concentration for purposes of obtaining linear analytical curves, and methods of area determination for the profile peaks obtained from both emission and quenching measurements.

An investigation of the influence of time on fluorescence intensity for I and II revealed an initial decrease in intensity followed by an essentially constant intensity up to approximately 5 h when measurements were discontinued. This indicates that quantitative measurements should be made only after allowing the developed thin-layer chromatogram to dry in the dark for a minimum of 1 h. Apparently the "wetness" of the adsorbent layer exerts an influence on fluorescence emission intensity. The precaution of drying the plate in the dark is to prevent any possible photodecomposition of the components.

The length of the exit aperture slit of the lens and mirror assembly, which is approximately 5 mm, and the dimensions of the source lamp radiation covering this slit, which are determined primarily by the excitation side of the lens and mirror assembly slit, limits the area of the chromatogram spot that is subjected to the excitation radiation. This plus the small area monitored by the fiber optic does not allow measurement of the entire emission from one chromatogram spot during a single scan. Therefore individual spots should be aligned manually with respect to the exit aperture slit before an actual scan is made. This alignment should be such that maximum emission intensity can be measured. While some variation is observed when the same spot is consecutively rescanned, this does not appear to be a serious disadvantage. Best results are obtained from an average of duplicate or triplicate scans of each chromatogram spot.

A restricted but usable linear range for the relationship of peak area to concentration has been reported using the Aminco thin-film scanner[12]. These authors were concerned with lateral diffusion of the spots during plate development. They used scored thin-layer plates which limited the lateral diffusion to the length of the exit aperture slit. In the present investigation it was found that it is not necessary to use scored thin-layer plates. Fig. 5 shows typical analytical curves for I and II. Both peak

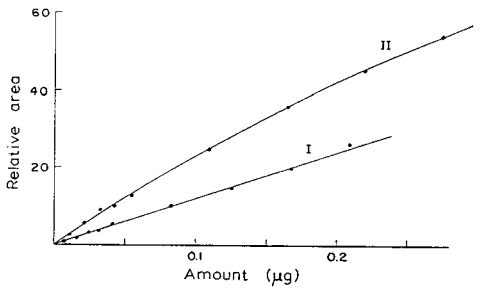


Fig. 5. Fluorescence analytical curves for 5-geranoxy-7-methoxycoumarin (I) and 5,7-dimethoxycoumarin (II).

area and peak height can be used to monitor emission intensity for analysis. These curves represent relationships of emission intensity to amount of component present where only part of the total chromatogram spot emission is measured. If care is taken to scan the chromatogram spot so the maximum emission intensity is measured then analytical curves are obtained which are capable of yielding accurate results in the analysis of unknowns.

The utility of fluorescence quenching in analysis of III and IV was investigated. A usable linear analytical curve can be obtained using either peak area or peak height to monitor the amount of quenching. Fig. 6 shows the typical behavior which is observed. The sensitivity of fluorescence quenching is not as great as for fluorescence emission measurements in many cases. However, in this case the emission intensity of III is quite low and that of IV undetectable at the concentrations where quenching is applicable.

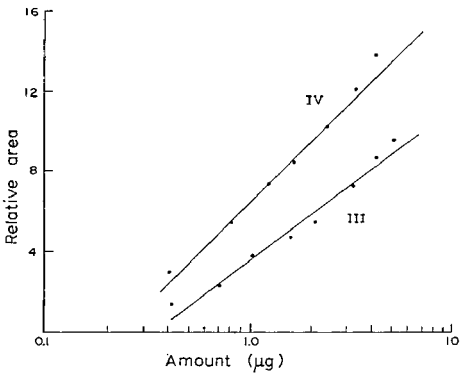


Fig. 6. Fluorescence quenching analytical curves for 5-geranoxypsoralen (III) and 5,8-dimethoxypsoralen (IV).

Comparison of profile peak areas determined by triangulation with values obtained from the integrator indicate that for profile peaks which result from measurement of emission the areas obtained from the integrator are more consistent. However, because a small decrease in a large integrator response is usually observed when quenching of plate fluorescence is measured, the triangulation procedure is favored.

Determination of 5-geranoxy-7-methoxycoumarin and 5,7-dimethoxycoumarin in citrus oils

The fluorescence emission profiles of expressed lemon, lime, and bergamot oils show two prominent peaks which are due to the presence of I and II. In addition, distilled lime oil thin-layer chromatograms exhibit two blue weakly fluorescent spots which correspond to those in the previously mentioned oils. An adjustment in the amount of citrus oil spotted on the thin-layer plate is necessary before quantitative determination of I and II can be accomplished because quenching of the measured emission is observed at the concentrations of I and II present when the whole oils are chromatographed. Application of a more dilute sample to the thin-layer plate,

while not significantly decreasing the intensity of the emission due to I and II, results in a decrease in the emission from other component spots. Therefore, the emission due to the presence of I and II is essentially free from interference of other components even in situations where complete separations from other components in the thin-layer chromatogram have not been accomplished. Only in the analysis of bergamot oil does appreciable interference arise due to the relatively large amount of III present.

Attempts to duplicate fluorescence emission analytical curves for I and II were of only limited success. Apparently minor variations in adsorbent layer thickness and conditions of plate development cause variations in chromatogram spot shape which make it difficult to obtain reproducible results from day to day and from plate to plate. This difficulty may be due to the fact that only a portion of the total potential emission can be measured with the instrument used. However, relationships similar to those shown in Fig. 5 are easily obtained. The slope of the analytical curves vary by as much as 25 % from plate to plate. For this reason quantitative determination of I and II was performed by spotting up to five citrus oil samples and four or five standards on each plate. This procedure makes it possible to obtain an analytical curve directly from the thin-layer chromatogram used in the analysis.

Chromatogram spots were scanned parallel to and perpendicular to the direction of plate development. Peak areas obtained by scanning in the direction of plate development are somewhat greater than those obtained by scanning perpendicular to the direction of plate development. This behavior is observed because the chromatogram spots of both I and II are elliptical in shape with the major axis in the direction of plate development. Peak heights obtained by parallel and perpendicular scanning of the same spot exhibit insignificant differences.

The amounts of I and II present in the citrus oil samples can be determined from peak area or peak height obtained either by scanning the individual chromatogram spots in a direction parallel to or perpendicular to plate development. Results obtained by these four procedures are presented for two selected citrus oils in Table II. The results obtained by scanning the chromatograms perpendicular to the direction of plate development are generally somewhat lower than those obtained from parallel scanning. A slight improvement is observed in the comparison of experimental and

TABLE II

A COMPARISON OF THE AMOUNT OF 5-GERANOXY-7-METHOXYCOUMARIN AND 5,7-DIMETHOXYCOUMARIN DETERMINED BY *in situ* FLUORIMETRY FROM CHROMATOGRAM PEAK AREA AND HEIGHT AND FROM DIFFERENT DIRECTIONS OF SCANNING

	5-Geranoxy-7-methoxycoumarin (%)[a]		*5,7-Dimethoxycoumarin* (%)[a]	
	California lemon	*Florida lime*	*California lemon*	*Florida lime*
Parallel scan				
Peak area	0.152 ± 0.002	0.690 ± 0.014	0.0606 ± 0.0020	0.203 ± 0.006
Peak height	0.152 ± 0.002	0.709 ± 0.012	0.0610 ± 0.0015	0.179 ± 0.002
Perpendicular scan				
Peak area	0.130 ± 0.003	0.667 ± 0.027	0.0568 ± 0.0005	0.185 ± 0.002
Peak height	0.131 ± 0.004	0.662 ± 0.012	0.0561 ± 0.0006	0.178 ± 0.003

[a] Average of triplicate analysis.

TABLE III

EVALUATION OF *in situ* FLUORIMETRY FOR THE DETERMINATION OF 5-GERANOXY-7-METHOXYCOUMARIN AND 5,7-DIMETHOXYCOUMARIN IN CITRUS OILS

	5-Geranoxy-7-methoxycoumarin		*5,7-Dimethoxycoumarin*		*Solution fluorescence relative intensity*	
	Av. (%)[a]	*RD*[b] (%)	*Av.* (%)[a]	*RD*[b] (%)	*Experimental*	*Calculated*
Expressed lime oil						
Oil lime	1.58	0.7	0.455	0.7	5.50	4.53
Persian	2.95	2.4	0.465	2.6	7.97	7.36
Mexican	3.54	2.5	0.656	1.5	10.7	9.12
West Indian	4.66	7.3	0.842	12.7	13.2	11.92
Florida	0.690	2.0	0.203	3.0	2.14	1.99
Expressed lemon oil						
Italian	0.134	2.2	0.0585	1.2	0.486	0.442
California	0.152	1.3	0.0606	3.3	0.501	0.485
California (dewaxed)	0.129	0.8	0.0578	1.4	0.455	0.429
North American standard (sample 1)	0.152	1.3	0.0297	3.7	0.400	0.396
North American standard (sample 2)	0.158	1.9	0.0471	1.5	0.506	0.458
Expressed bergamot oil						
Italian	0.100	4.0	0.258	1.2	1.10	0.948
Extra	0.0754	5.8	0.155	3.9	0.761	0.601
Distilled lime oil						
West Indian natural	0.00040	7.5	0.00319	6.5	0.0138	0.0100
Extra	0.00087	27.6	0.00365	1.7	0.0124	0.0123

[a] Average of triplicate analysis.
[b] Relative deviation.

calculated fluorescence intensities of the citrus oils discussed below when the amounts of I and II are determined from chromatogram scans parallel to the direction of plate development. Results obtained for other citrus oils from peak areas and chromatogram scanning in the direction of plate development are presented in Table III. The amounts of coumarin derivatives I and II present in the citrus oil samples were calculated as follows:

$$\%\text{I} = \frac{\mu\text{g I}}{\mu\text{g oil}}(100\,\%) \tag{4}$$

$$\%\text{II} = \frac{\mu\text{g II}}{\mu\text{g oil}}(100\,\%) \tag{5}$$

where μg I and μg II are determined directly from the appropriate analytical curve obtained from the same thin-layer plate.

The quantitative analysis of individual coumarin derivatives has not been the subject of extensive investigations[13,14]. The amounts of the individual components present in citrus oils are not accurately known and can vary from sample to sample.

A comparison can be made between the relative fluorescence intensity from ethanol solutions of citrus oils, determined as previously described[10], and ethanol solutions of components primarily responsible for the citrus oil emission. The fluorescence from ethanol solutions of lemon, lime, and bergamot oils can be attributed to the presence of I and II in each oil. The contribution of fluorescence intensity due to I and II can be determined if the relative fluorescence intensity of the two components in ethanol is known and if the concentrations of the two components in the oil sample under investigation are known. Calculation of a relative fluorescence intensity (*RI*) for citrus oils is based on the following equations:

$$RI_{\mathrm{I}} = \frac{20.4\ (\%\,\mathrm{I})}{10} \tag{6}$$

$$RI_{\mathrm{II}} = \frac{28.8\ (\%\,\mathrm{II})}{10} \tag{7}$$

$$RI_{\mathrm{I}} + RI_{\mathrm{II}} = RI_{\text{citrus oil}} \tag{8}$$

where the experimental numerical factors 20.4 and 28.8 are the relative fluorescence intensities from ethanol solutions of I and II (ref. 10). The factor of 1/10 is introduced in eqns. 6 and 7 to relate the relative fluorescence intensities of I and II measured for 0.1 μg/ml solutions and the percent of I and II to the relative intensity of 1.0 μg/ml citrus oil solutions. Agreement between the calculated relative fluorescence intensity for each citrus oil calculated from eqns. 6, 7, and 8 and an experimentally measured relative intensity is used to determine if the method for determination of the concentrations of I and II in each citrus oil is indeed reasonable and accurate. An evaluation of the method using the above comparison is presented in Table III, where agreement between calculated and experimental relative intensities is observed to be quite good. The assumption made prior to making this comparison is that the measured solution fluorescence characterized by excitation at 335 nm and emission at 420 nm of the four citrus oils is due primarily to the fluorescence of I and II. The results presented in Table III in addition to verifying this assumption have also established the reliability and accuracy of the method.

Determination of the concentrations of I and II in this limited number of samples has revealed that these components are present in widely varying amounts in expressed lime oil. This variation is not observed for expressed lemon oil. In no sample of lime oil did the amount of I or II represent less than three times the corresponding component in lemon oil. This observation indicates that *in situ* fluorimetry can be valuable for detecting mixtures of lemon oil with expressed lime oil simply by determination of the concentrations of I and II. It will be necessary to investigate a much larger number of samples before definite concentration limits can be formulated.

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DETERMINATION OF THE MOLECULAR-WEIGHT DISTRIBUTION OF POLYETHYLENE GLYCOLS BY THIN-LAYER CHROMATOGRAPHY COUPLED WITH DENSITOMETRY

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SUMMARY

Thin-layer chromatography with multiple one-dimensional elution was used to determine the molecular-weight distribution of polyethylene glycols of the general formula $HO(CH_2CH_2O)_nH$ after conversion into their dichloro derivatives. The adsorbent was silica gel, the developer was based on a mixture of 2-butanone and water, and the spots were visualised with iodine vapour, and then evaluated with the aid of an optical densitometer. This method permitted the quantitative fractionation of polyethylene glycols with degrees of polymerisation (n) of up to 21.

INTRODUCTION

Chromatographic techniques have proved particularly useful for the determination of the molecular-weight distribution of polyethylene glycol (PEG) with the general formula $HO(CH_2CH_2O)_nH$, where n is the degree of polymerisation. The molecular-weight distribution is an essential part of the characterisation of polyethylene glycol products, which are of growing industrial importance.

Temperature-programmed gas chromatography (GLC) of the trimethylsilyl derivatives permits the fractionation of the lower members extending up to $n = 18$ (refs. 1–3), but the results are accurate only for $n < 14$ (ref. 4).

The limitations of gas chromatography, which are imposed by the low volatility of the compounds, do not apply to liquid–solid chromatography, and, indeed, thin-layer chromatography (TLC) has given very good results in the fractionation of surface-active polyethylene glycols[5–9]. The present authors[10] have recently used TLC in conjunction with the direct densitometric evaluation of the spots. The latter were visualised with iodine vapour, which has been recommended[11,12] before in the qualitative detection. As n increases, the compound absorbs more iodine, and thus the photometric determination is progressively improved[10].

In the thin-layer chromatographic analysis of polyethylene glycol derivatives, the adsorbent is activated silica gel and the eluent is a mixture based on 2-butanone and water. This system is highly selective for n, but cannot be used for the direct fractionation of polyethylene glycols. This is because the energy of adsorption of the

primary OH groups on the silica gel is high, and so the sample does not migrate ($R_F < 0.1$).

In the present work, the distribution of the molecular weights or — what amounts to the same thing — the distribution of the degrees of polymerisation was determined after conversion of the polyethylene glycols into the dichloro derivative $Cl(CH_2CH_2O)_{n-1}CH_2CH_2Cl$ (n is invariably the degree of polymerisation of the parent polyethylene glycol). This was carried out with the aid of thin-layer chromatography and by the direct photometric evaluation of the spots, which were visualised by means of iodine vapour. Use was also made of multiple one-dimensional elution, whereby one can separate high-n and low-n members on the same plate, at least within the peak capacity of the chosen migration distance. The advantages of this technique have been discussed before in detail, together with its theoretical and practical aspects[13,14].

EXPERIMENTAL

The four commercial polyethylene glycol samples used had the following stated mean degrees of polymerisation: $\bar{n} = 8.2$, 13.2, 17.8 and 22.3.

We synthetised homogenous ethylene glycol oligomers with $n = 6$, 7, and 8, and then prepared their dichloro derivatives. After repeated distillation of the latter under reduced pressure, the degrees of purity of the resulting three standards, determined gas chromatographically[2], were 99.0, 95.5, and 94.1 %, respectively.

Polyethylene glycol was chlorinated with thionyl chloride in pyridine[15]. To determine the amount of thionyl chloride (excess) needed to ensure a complete reaction, the chlorination of polyethylene glycol with $\bar{n} = 22.3$ was checked by IR spectroscopy, NMR spectroscopy, and TLC.

The chlorination was carried out as follows: the polyethylene glycol sample was dried for 1 h at 60° and 10 mm Hg, and 0.5 g of it were dissolved in 0.5 ml of warm anhydrous pyridine in a 10-ml test-tube. The solution was cooled to 30°, and 0.2 ml of thionyl chloride were added dropwise over a period of 1/2 h, with continuous agitation. A further 0.4-ml portion of thionyl chloride was then added quickly, and the reaction mixture was heated for 3 h at 80 ± 2°. The mixture was first extracted with at least eight 10-ml portions of diethyl ether, then taken up in 5–10 ml of water, and the resulting aqueous solution was extracted with two 5-ml portions of ether. The ether extracts were combined and distilled first at atmospheric pressure and then at 10 mm Hg for 1 h at 60°. This gave the crude dichloro derivative, which was used without further purification.

The plates were prepared by appling Silica Gel G 20–40 μ (Merck) to optical glass supports (20 × 2.5 cm) by means of a spreader with a slit fixed at 200 μ. The silica gel was first made into an aqueous slurry as recommended by the manufacturers. At the wavelength at which the optical density was determined, the absorbance of the resulting plates was about 2.0. To eliminate any bubbles from the silica gel layer, the plates were activated by raising the temperature to 180 ± 5° over the course of 1 h, and by keeping them at this temperature for 1/2 h.

The sample was made into a 2 % w/v solution in chloroform, and this solution was applied to the plate with a 10-μl Hamilton microsyringe, so as to form a spot not bigger than 3 mm in diameter. The eluents were chromatographic solvents RS (Carlo Erba, Milan). The plates were developed to a distance of 17.2 cm by the ascending

technique in cylindrical Desaga tanks (20 × 5 cm) having a vapour-saturated atmosphere. The spots were visualised in similar tanks, which were placed in a horizontal position, and whose atmosphere was saturated with iodine vapour by placing a few iodine crystals at the bottom. The best contrast of the spots was obtained by first drying the plates in air for 1/2 h, and then exposing them to the iodine vapour at 20° for at least 1/2 h. When the spots had been visualised the plate was immediately covered by another glass plate of the same size, and the edges were sealed with adhesive tape to minimise the losses of iodine by evaporation.

The plates were then subjected to photometric evaluation with the aid of a Chromoscan Joyce Loebl apparatus, set for measuring the transmission. The plates were scanned along the direction of the migration. The mesaurements were made at

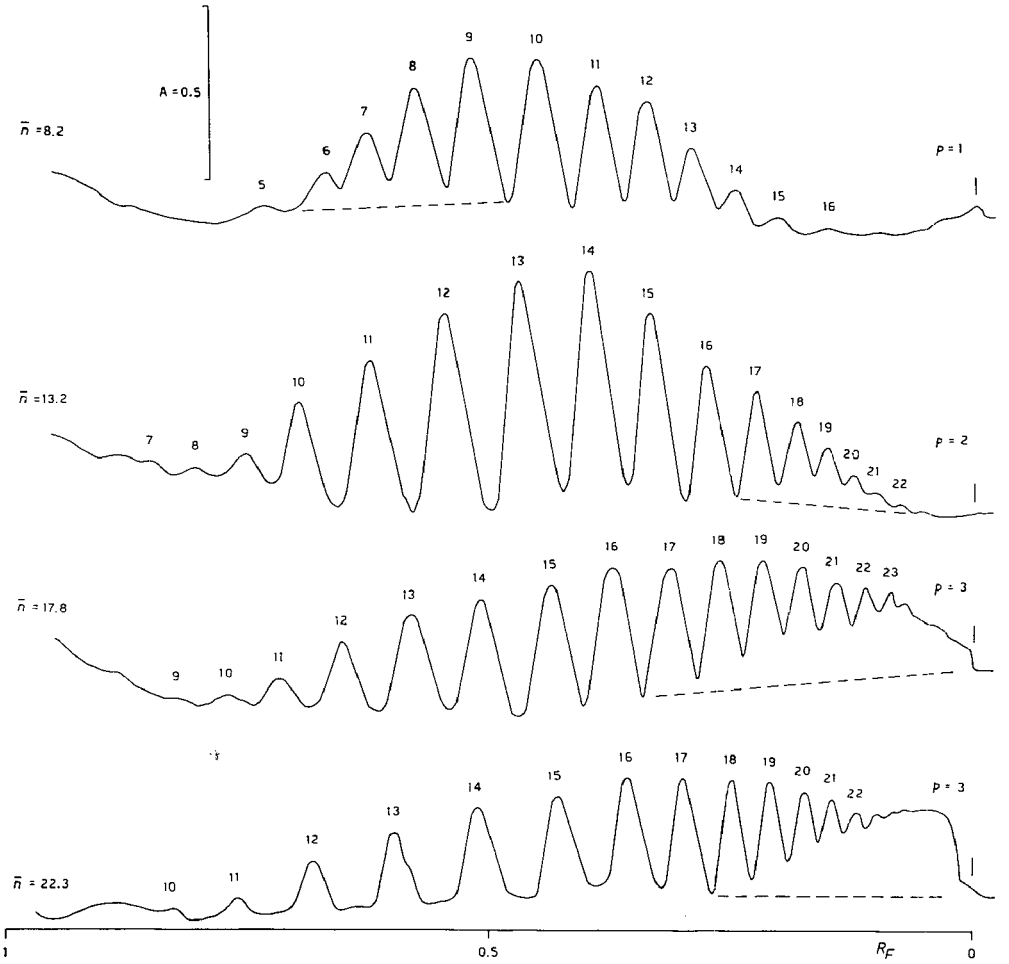


Fig. 1. Thin-layer chromatograms of the dichloro derivatives of polyethylene glycols, eluted at 20° with a 90:10 mixture of 2-butanone and water (upper curve), and with an 87.0:9.7:1.4:1.9 v/v nixture of 2-butanone, water, chloroform, and methanol (ratio before mixing) (other curves). Amount of sample applied to the plates: 15 μg (upper curve), and 20 μg (other curves). n is the degree of polymerisation of the parent polyethylene glycol.

a wavelength of 490 nm (green filter) and at slits of 5, 6, 7 × 1 mm. The response of the recorder was linearised for absorbances in the range of 0–1, and hence it was possible to determine the total absorbance of a peak from the area, which was found planimetrically.

The spectrophotometric determinations were carried out with a Perkin-Elmer Infracord apparatus, and the NMR spectra were obtained by means of a Jeol C-60 HL apparatus.

RESULTS AND DISCUSSION

Experiments with the three standards have shown that the total absorbance corresponding to the peaks varies linearly with the amount of solute placed on the sample (at least in the range 1–10 nmole). Furthermore, the mean square deviation of the gradient of the calibration curve is of the same order of magnitude as the error involved in the use of the microsyringe[16]. The linearity also holds for spots eluted over different migration distances, but the gradient varies from plate to plate, because the visualising conditions cannot be fixed in a fully reproducible manner. It has also been found that, when the three standards are eluted on the same plate, the recovery varies linearly with the value of n (the recovery is the ratio between the integrated absorbance, expressed in area units, and the amount of the sample in nmoles).

The chromatograms in Fig. 1 were obtained by eluting the sample up to three times with the same eluent and to the same distance. Although further repetition of the elution – we tried up to five elutions – slightly improves the resolution, the photometric homogeneity of the plates tends to deteriorate progressively after the third elution.

The polyethylene glycol samples with $\bar{n} = 8.2$ and 13.2 are seen to have been almost fully resolved. Even for some incompletely separated head and tail members, the separation factor is such as to permit their resolution by graphical means.

As regards polyethylene glycol samples with higher $\bar{n}$ values, three elutions give

TABLE I

R_F VALUES OBTAINED AFTER SINGLE, DOUBLE, AND TRIPLE ELUTION WITH THE SAME ELUENT AND TO THE SAME DISTANCE AT 20°.

p is the number of elutions. The sample was the dichloro derivative of polyethylene glycol with $\bar{n} = 22.3$. (n is the degree of polymerisation of the parent polyethylene glycol.) The eluent was an 87.0:9.7:1.9:1.4 v/v mixture of 2-butanone, water, methanol, and chloroform (ratio before mixing).

n	$(R_F)_p$				
	$p = 1$	$p = 2$		$p = 3$	
	Obs.	*Obs.*	*Calc.*	*Obs.*	*Calc.*
10	0.46	0.70	0.69	0.82	0.84
11	0.39	0.64	0.63	0.76	0.77
12	0.33	0.56	0.55	0.70	0.70
13	0.27	0.47	0.47	0.62	0.61
14	0.23	0.40	0.41	0.53	0.54
15	0.19	0.32	0.34	0.46	0.47
16	0.15	0.26	0.28	0.37	0.38
17	0.13	0.22	0.24	0.31	0.34
18	0.11	0.18	0.21	0.25	0.29

a practically complete separation of the pair with $n = 17$ and $n = 18$, and qualitative identification is possible up to $n = 22$–23.

The $(R_F)_p$ values observed after p successive elutions are in good agreement with the values calculated as follows from $(R_F)_1$, obtained after the first elution:

$$(R_F)_p = 1 - [1 - (R_F)_1]^p$$

Table I shows the observed and the calculated $(R_F)_p$ values for successive elutions of the dichloro derivative of the polyethylene glycol sample with $\bar{n} = 22.3$.

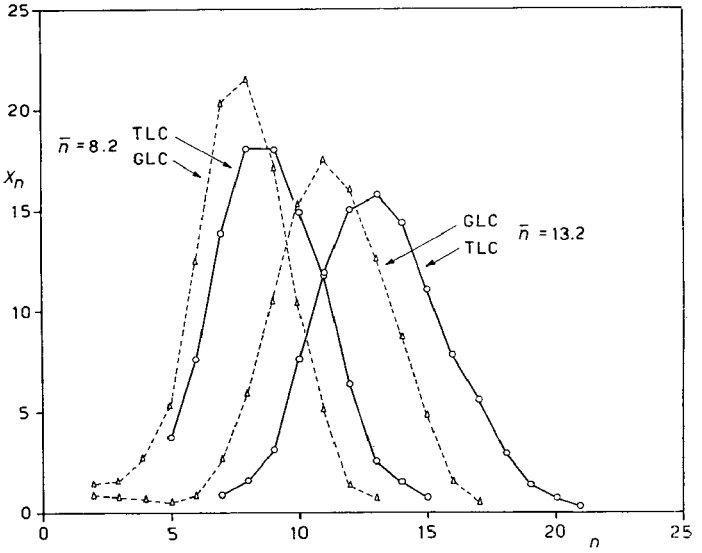


Fig. 2. Degree of polymerisation distribution as determined by TLC (full-lines) and by GLC (broken-lines) for the dichloro derivatives of polyethylene glycols $[Cl(CH_2CH_2O)_{n-1}CH_2CH_2Cl]$. n is the degree of polymerisation of the parent polyethylene glycol; $\bar{n}$ is its mean degree of polymerisation; and x_n is the average molar fraction in % (*cf.* Table II).

In the analysis of polyethylene glycol samples with $\bar{n} = 8.2$ and 13.2, there is a systematic discrepancy between the molecular-weight distribution found by TLC and that found[2] by GLC (*cf.* Table II and Fig. 2). This discrepancy increases with the mean molecular weight of the sample, as can also be seen by comparing the numerical average molecular weights. One reason for this lies in the fact that, for compounds with $n > 14$, the gas chromatographic analysis of the trimethylsilyl derivatives gives values that drop below the correct figures more and more as n increases. The other reason is that, in thin-layer chromatography, the affinity to iodine and the stability of the colour are lower for components with low n values. The influence of these two factors is clearly seen from the data in Table III, which shows the molar fractions as obtained by TLC and GLC for adjacent oligomer pairs (chosen for similar distributions so as to minimise the accidental error). The best agreement is found for the pair with $n = 12$ and 13. The reproducibility of the thin-layer chromatographic data is about 5–8 %, expressed as the mean square deviation from the average molar fractions.

Although the preparation of the derivatives is laborious, the present method scores over GLC by giving more accurate results for the molecular-weight distribution of polyethylene glycols with $n > 14$–15. The resolution of the dichloro derivatives is

TABLE II

DEGREE OF POLYMERISATION DISTRIBUTION OF POLYETHYLENE GLYCOL, AS FOUND BY TLC AND GLC

$\bar{n}$ (calc.) is the numerical-average degree of polymerisation, calculated from the distribution; $\bar{x}_n$ is the average molar fraction (%); s_n is the attendant mean square deviation found in five determinations; and n is the degree of polymerisation of the parent polyethylene glycol.

n	$\bar{n} = 8.2$		$\bar{n} = 13.2$	
	TLC (dichloro deriv.) $\bar{x}_n \pm s_n$	*GLC (TMS deriv.)* x_n	*TLC (dichloro deriv.)* $\bar{x}_n \pm s_n$	*GLC (TMS deriv.)* x_n
2	—	1.4	—	0.9
3	—	1.5	—	0.7
4	—	2.8	—	0.6
5	3.8 ± 1.3	5.3	—	0.5
6	7.5 ± 3.1	12.5	—	0.9
7	14.0 ± 2.3	20.2	0.9 ± 0.3	2.7
8	18.1 ± 2.9	21.6	1.5 ± 0.2	6.0
9	18.1 ± 1.9	17.1	3.1 ± 0.7	10.5
10	15.0 ± 1.9	10.4	7.8 ± 1.2	15.3
11	11.8 ± 2.0	5.2	11.9 ± 1.1	17.6
12	6.7 ± 1.7	1.3	15.1 ± 1.4	16.0
13	2.6 ± 0.4	0.7	15.8 ± 0.6	12.5
14	1.7 ± 0.8	—	14.4 ± 0.8	8.8
15	0.7 ± 0.2	—	11.0 ± 1.2	4.9
16	traces	—	7.8 ± 1.3	1.6
17	—	—	5.5 ± 0.6	0.5
18	—	—	2.8 ± 0.4	—
19	—	—	1.4 ± 0.3	—
20	—	—	0.7 ± 0.2	—
21	—	—	0.3 ± 0.2	—
22	—	—	traces	—
$\bar{n}$ (calc.)	8.98	7.74	13.24	11.08

TABLE III

DISTRIBUTION OF ADJACENT OLIGOMER PAIRS, AS FOUND BY TLC AND GLC

$\bar{x}_n$ is the average molar fraction (%); s_n is the attendant mean square deviation found in three determinations: and n is the degree of polymerisation of the parent polyethylene glycol.

Starting PEG	n	*TLC (dichloro deriv.)* $\bar{x}_n \pm s_n$	*GLC (TMS deriv.)* x_n
$\bar{n} = 8.2$	7	43.0 ± 6.8	48.3
	8	57.1 ± 7.2	51.7
$\bar{n} = 13.2$	12	50.5 ± 4.8	52.4
	13	49.5 ± 5.2	47.6
	16	58.8 ± 7.6	74.5
	17	41.8 ± 8.1	25.5

no longer quantitative beyond about $n = 20$, but this limit may be raised with other derivatives, prepared by substituting the OH groups with non-polar groups, and particularly with hydrophobic groups. Work in this direction is being carried out in our Department.

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CHARACTERIZATION OF AN ADDITIONAL ANTHOCYANIN PIGMENT IN EXTRACTS OF STRAWBERRIES, *FRAGARIA**,**

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SUMMARY

Investigation of the anthocyanin pigments of forty lots of strawberries representing thirteen selections and five varieties revealed the presence of a third pigment in most samples not previously identified. The pigment is not a chromatographic artifact as it is unchanged upon rechromatography. It is composed of pelargonidin and glucose in a 1:1 molar ratio. During acid hydrolysis the pigment readily forms pelargonidin-3-glucoside as an intermediate before pelargonidin formation. Infrared and ultraviolet-visible spectral properties eliminate the possibility of acylation with organic acids. Radiotracer experiments eliminate the possibility of incorporation of methanol as an ether or acetal into the pigment. On the basis of spectral data, chromatographic properties, and hydrolysis experiments, it is suggested that the pigment is either the furanose form or the alpha anomer of pelargonidin-3-glucoside.

INTRODUCTION

The main anthocyanin pigment in strawberries was identified as pelargonidin-3-glucoside (pgd-3-glu) by ROBINSON AND ROBINSON[1]. LUKTON *et al.*[2] identified a second minor pigment to be cyanidin-3-glucoside (cyd-3-glu). In assaying the relative amounts of these two pigments and the total anthocyanin pigment of thirteen different selections and five varieties of strawberries[3] (forty samples), we observed the presence of a third band in most analyses. The quantity of this pigment was as much as 5 % of the total anthocyanin content. This publication reports its characterization.

EXPERIMENTAL

Isolation of pigments

Strawberries supplied by the OSU Horticulture Department were washed,

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*** Author is holder of a research fellowship from the Royal Norwegian Council for Scientific and Industrial Research and on temporary leave from The Research Committee for Preservation of Agricultural Food Products, Ullevålsveien 72, Oslo 4, Norway.

individually quick frozen at $-35°$, and stored in polyethylene bags at $-26°$. Pigments were isolated from 500-g samples by adsorption on insoluble polyvinylpyrrolidone (PVP) as described by WROLSTAD AND PUTNAM[4].

Chromatography

Whatman No. 3 paper was used for preparative chromatography. Descending chromatography was used for all paper chromatography excepting sugar determinations which utilized Whatman No. 1 paper in an ascending system. All runs were carried out in the dark at $21 \pm 1°$.

Cellulose thin-layer plates of 0.25 mm thickness were developed in an ascending manner under the same conditions.

The following solvent systems were used:

(1) glacial acetic acid–water–conc. HCl (15:82:3) (AWHCl);
(2) less dense phase of *n*-butanol–glacial acetic acid–water (4:1:5) (BAW 415); equilibrated for three days;
(3) conc. HCl–water (3:97) (1 % HCl);
(4) *n*-butanol–pyridine–water (6:3:1) (BPW 631);
(5) glacial acetic acid–water (2:98) (2 % HAC);
(6) ethyl acetate–*n*-propanol–water (5:3:2) (EPrW 532);
(7) ethyl acetate–pyridine–water (2:1:2) (EPW 212).

Chromogenic spray reagents

Partridges reagent used for visualization of sugars was formulated as described by ALBACH *et al.*[5].

Organic acids were detected using Bromocresol Green spray reagent[6] and phenolics with tetrazotized benzidine[7].

Spectroscopy

UV-visible spectra were determined with a Beckman DK-1 spectrophotometer. AWHCl-developed chromatograms were air-dried and bands were extracted with spectro-grade MeOH. After determining spectra, solutions were concentrated and their purity assayed by TLC.

Methanol pigment solutions were concentrated in the tip of prepressed KBr "wick-sticks"* by ascending development at 4°. Concentration time was shortened by drying wick-sticks in a vacuum oven (25°, 25 mm pressure) between repeated MeOH developments. The tip containing concentrated pigment was dried, ground in an agate mortar and pestle, and pressed. IR spectra were run on KBr–pigment pellets using a Beckman IR 5 double beam spectrophotometer fitted with a beam condensor. Purity of material was checked by MeOH extraction of the pellet and subsequent chromatography.

Partial acid hydrolysis

Less than 1-mg samples of purified pigments were hydrolyzed in 2 ml of 1 *N* HCl at 97° using the procedure of LYNN AND LUH[8]. Aliquots were withdrawn at 5-min intervals, spotted on cellulose TLC plates, and developed in AWHCl.

* Harshaw Chemical Co., Division of Kewanee Oil Co., Cleveland, Ohio, U.S.A.

Pigment:aglycone ratio

The molar ratio of anthocyanin pigment and sugars yielded after complete hydrolysis were determined using a modified procedure of CHANDLER AND HARPER[9]. The molar quantity of anthocyanin was assayed spectrophotometrically on an aliquot of the pigment solution using the extinction coefficient of pgd-3-glu ($E = 36,600$, 510 nm). From 0.1–1 μmole of pigment was hydrolyzed (1 N HCl solution at 100° for 60 min in the dark under N_2). The molar quantity of sugars in the hydrolysate was assayed using anthrone reagent[10].

Distribution number

Purified pigment (0.2 mg) in absolute MeOH was evaporated to dryness and dissolved in 15 ml 0.01 N HCl in n-butanol. The concentration of pigment was determined by measuring the absorbance of an aliquot diluted with the same solvent. Five milliliters of the concentrated solution were shaken with 5 ml of 0.01 N HCl in water for 2 min and the absorbance of the organic layer determined. The distribution number is expressed as the per cent pigment remaining in the butanolic phase.

Measurement of radioactivity

A sample of fruit (ORE-US 2931, 2.29 g) was extracted with $^{14}CH_3OH$ having an activity of 0.120 μCi/ml (1.05×10^7 d.p.m./mole). A total of 30 ml was used in the extraction process. The extract was concentrated to 0.5 ml and stored for 10 days at $-5°$. Quantities of 1 μl were spotted on TLC plates, free MeOH was allowed to evaporate and the plates were developed in AWHCl. The developed plates were dried and scanned using a Packard Model 7200 Radiochromatogram Scanner. Efficiency was determined by scanning a plate spotted with 21,078 d.p.m. of [1-^{14}C]tyrosine and found to be 19.1 %. Peak areas were calculated by triangulation.

RESULTS AND DISCUSSION

A third pigment could be resolved from pgd-3-glu and cyd-3-glu when anthocyanin extracts of *Fragaria* were chromatographed in AWHCl on paper or cellulose thin-layer. It had a higher R_F value than pgd-3-glu in this system and in 1 % HCl but could not be resolved using BAW 415 (Table I). There appeared to be a varietal relationship in that some selections consistently contained substantial amounts of this pigment (band 3) and in others it was absent or barely detectable. Quantities of band 3 tended to increase with storage at $-5°$. No band 3 was detectable in a sample of *Fragaria ananassa* Duch. cv. "Northwest" when chromatographed immediately after isolation; however, after two weeks subsequent storage at $-5°$ the same sample contained band 3.

Two-dimensional thin-layer chromatography (TLC) using AWHCl in both directions resulted in only three spots, which lay along a 45° angle, indicating that band 3 was not a chromatographic artifact[11]. Band 3, extracted from preparative paper chromatograms with 0.01 % HCl in methanol (MeOH), had the same R_F value as pgd-3-glu when concentrated and rechromatographed. When extracted with absolute MeOH, however, band 3 could be isolated in pure form, retaining its original R_F value.

The UV-visible spectra of band 3 and pgd-3-glu are shown in Fig. 1. The $A_{440}/A_{vis\ max}$ value indicates that the 5 position is free[12]. The visible absorption maxi-

TABLE I

CHROMATOGRAPHIC INVESTIGATION OF PIGMENTS AND THEIR HYDROLYSIS PRODUCTS

Compound	R_F *values*				*Color (Partridges reagent)*
	AWHCl	*1% HCl*	*BAW 415*	*BPW 631*	
Band 1 (cyd-3-glu)	0.35	0.12	0.34		
Band 2 (pgd-3-glu)	0.52	0.22	0.48		
Band 3	0.68	0.38	0.48		
Pelargonidin	0.18	0.06	1.00		
Band 2 hydrolysis pigment					
original	0.51				
aglycone	0.19		1.00		
Band 3 hydrolysis pigments					
original	0.67				
intermediate	0.49				
aglycone	0.18		1.00		
Band 2 sugars				0.23/0.31	olive/red-brown
Band 2 sugars (repurified)				0.23	olive
Band 3 sugars				0.24/0.31	olive/red-brown
Band 3 sugar (repurified)				0.24	olive
Authentic sugar standards					
glucose				0.24	olive
galactose				0.21	brown
arabinose				0.31	red-brown
xylose				0.38	red-brown
rhamnose				0.49	olive

mum eliminates the possibility of substitution in the 7 position[13,14]. The absence of absorption peaks in the 300–330 nm range eliminates the possibility of acylation with cinnamic acids[15]. The intensity differences in the UV range were similar to those reported by SUN AND FRANCIS[16] for cyanidin-3-arabinoside and cyanidin-7-arabinoside; however they also reported a difference in the visible maximum of 3 nm. As the spectra in our study were not determined on crystalline compounds we question whether the intensity differences in the UV region are meaningful.

A compound having the same R_F value as pgd-3-glu was formed as an intermediate in the partial hydrolysis of band 3 to pelargonidin (pgd), the hydrolysis of

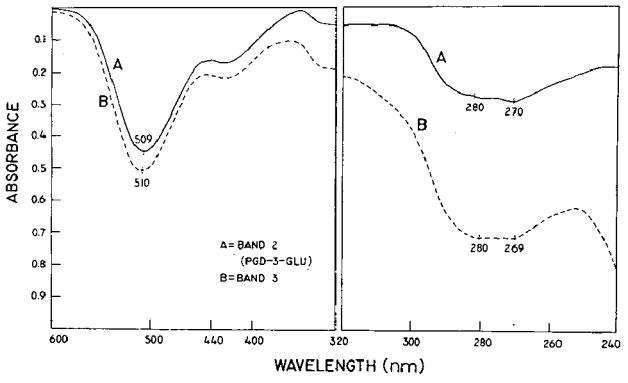


Fig. 1. UV-visible spectra of band 2 (pgd-3-glu) and band 3. Solvent: Spectro-grade MeOH.

band 3 to pgd-3-glu being complete after 5 min. After the developing solvent had evaporated from the thin-layer chromatogram of the band 3 hydrolysis samples, the plate was sprayed with tetrazotized benzidine; there was no indication of phenolic compounds (other than pelargonidin) being liberated with hydrolysis. BOCKIAN *et al.*[17] suggested a minor pigment of *Cabernet sauvignon* grapes with similar chromatographic properties to be a complex of malic acid and malvin. An ether extract of the band 3 hydrolysate was chromatographed in AWHCl along with malic, benzoic, ascorbic, *p*-hydroxybenzoic, caffeic, ferulic, and *p*-coumaric acids. The developed plate was sprayed with Bromocresol Green visualization reagent; no organic acids were detected in the band 3 ether extract.

The IR spectra of pgd-3-glu and band 3 are shown in Fig. 2. The absence of an absorption band at 1725–1750 cm^{-1} eliminates the possibility of esterification with an organic acid. Differences in the O–H stretching region (3100–3500 cm^{-1}) are due to the presence of MeOH, the "wick-stick" developing solvent. Band 3 shows additional bands at 794 and 1143 cm^{-1}, which are due to contamination with free arabinose, an artifact arising from preparative paper chromatography. Thus no obvious differences between the two spectra exist.

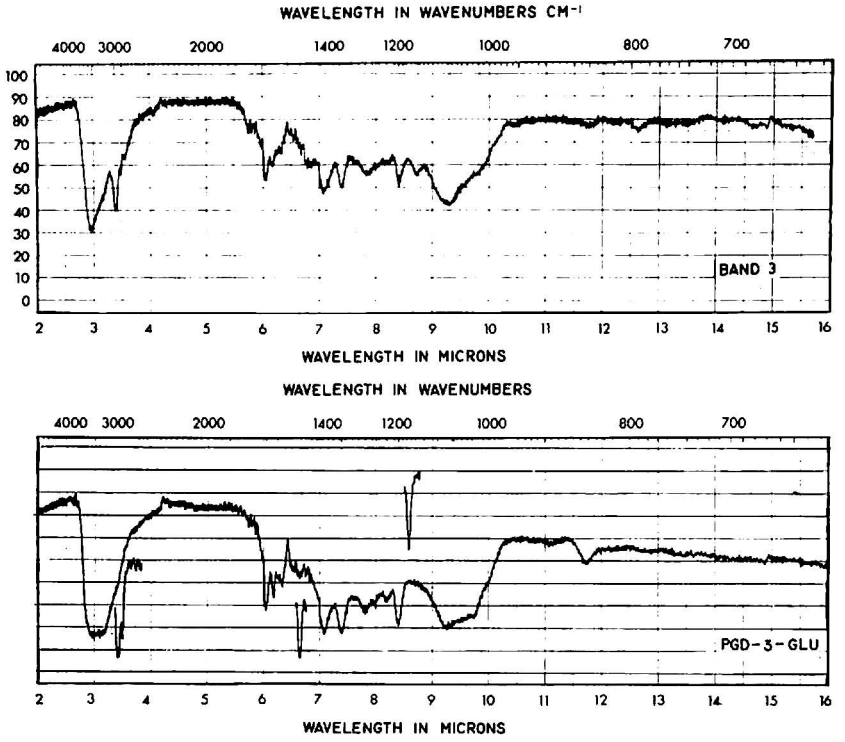


Fig. 2. IR spectra of band 3 and pgd-3-glu (KBr pellet).

After removal of acid from the hydrolysate by extracting with 10% di-*n*-octylmethylamine in chloroform, the sugars were chromatographed. Only glucose was detected in hydrolysates of pgd-3-glu and band 3. In a second experiment, however,

chromatography of the sugar fraction obtained from the sugar:pigment molar ratio determination showed substantial quantities of pentose sugar in band 3 hydrolysate and trace amounts in the pgd-3-glu sample. The sugar: pigment molar ratio and the partition behavior between butanol and water (Table II) showed that a large quantity of sugar was present in band 3. Subsequent investigation showed the pentose sugar was an artifact arising from development of Whatman No. 3 paper in AWHCl. The contaminating sugar could be removed by rechromatographing the isolated bands in 2 % acetic acid. The sugar:pigment molar ratios and the partition values (Table II) of band 3 thus purified showed it to be a monoglycoside. Chromatography of the sugar fraction of this hydrolysate showed only glucose present. MATHEW[18], in studying the anthocyanidins formed from leucoanthocyanins by treatment with alcoholic HCl,

TABLE II

SUGAR:PIGMENT MOLAR RATIO AND DISTRIBUTION NUMBERS OF PIGMENTS

	Sugar:pigment molar ratio	*Distribution number (% pigment remaining in butanolic phase)*
Pgd-3-glu	1.18, 1.31	50, 47
Pgd-3-glu (repurified)	0.97	
Band 3	4.0	39
Band 3 (repurified)	0.82	45, 49

reported that additional pigments were formed which were ethers of the anthocyanidin and the alcohol. The facile hydrolysis of band 3 would not be characteristic of ethers. An acetal of MeOH and pgd-3-glu (Fig. 3) could conceivably be formed, and its hydrolysis rate would be anticipated to be in accord with band 3. Anthocyanins were isolated from ORE-US 2931, a selection which consistently contained substantial quantities of band 3, using $^{14}CH_3OH$ as the extracting solvent. Radiochromatogram scanning of an AWHCl-developed thin-layer chromatogram of the anthocyanins showed slight incorporation of $^{14}CH_3OH$ at the solvent front. A typical experiment had 600 d.p.m. at the front. The background across the plate was 200 d.p.m. Regions of the plate containing pgd-3-glu, cyd-3-glu, and band 3 were indistinguishable from

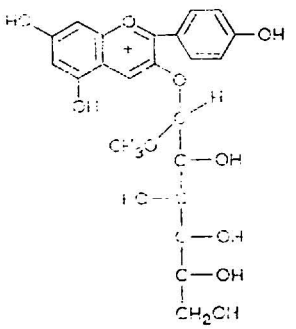


Fig. 3. Pelargonidin-3-β-methylglucose.

the background. Incorporation of $^{14}CH_3OH$ into band 3 in a 1:1 molar ratio would have resulted in a count of 750 d.p.m. It was concluded that there was no incorporation of $^{14}CH_3OH$ into the anthocyanins.

Evidence that the transformation of band 3 to pgd-3-glu was reversible was shown in our preparation of pgd. Pure pgd-3-glu was hydrolyzed in 1 *N* HCl. After the reaction was approximately 50 % complete, the pigments were chromatographed. The solution contained not only pgd-3-glu and pgd, but also band 3.

We are confident that band 3 is composed of pgd and glucose in a 1:1 molar ratio and that it does not contain MeOH, organic acids, or additional phenolics. The existence of different glycosidic forms is an interesting possibility. Several workers (ISHERWOOD AND JERMYN[19], AVIGAD AND BAUER[20] and GEE[21]) have established that α- and β-anomers and pyranose and furanose isomers of glycosides can be separated by paper and thin-layer chromatographic techniques. Furanoside linkages are considerably more labile than pyranosides, furanose compounds having hydrolysis rates in 0.01 *N* HCl 50–200 times greater than the corresponding pyranosides[22]. Hydrolysis rates of α- and β-anomers differ, which anomer having the faster rate being dependent on the sugar derivative[23].

We were interested in learning whether band 3 was an artifact of isolation or storage. Chromatography of an amyl alcohol extract of juice obtained by pressing fresh fruit (ORE-US 2786, *Fragaria ananassa* Duch. cv. "Tioga") through cheesecloth showed the presence of band 3. This would indicate that cold storage is not necessary for formation of band 3 and it is highly unlikely that its formation depended on the extremely mild isolation procedure.

Numerous workers have described minor pigments in anthocyanin extracts whose chromatographic properties are similar to our observations. ALBACH *et al.*[5] reported that a minor band (band 1) having a high R_F value in BAW 612 was interconvertible with band 2, (*p*-coumaric acid derivatives of the 3-monoglucosides of malvidin, peonidin, petunidin, and delphinidin) and speculated that band 1 was the quinoidal anhydro base and band 2 the oxonium salt form separated because of a sharp pH transition on the developed chromatogram. FRANCIS AND HARBORNE[24] observed that in chromatography of huckleberry extracts, band 2 (98 % petanin and 2 % negretein) would separate into two bands which in subsequent chromatography would recombine. The separation was not a resolution of the two pigments but rather two bands each containing both pigments; they attributed its cause to the acid-front phenomenon described by ALBACH *et al.*[5]. HETMANSKI AND NYBOM[25], in studying the anthocyanin pigments of rhubarb, reported the pigments were particularly labile, forming two intermediate pigments in their destruction which had higher R_F values in the solvent systems used. VON ELBE AND SCHALLER[26] isolated pigments not naturally occurring in Montmorency cherries and attributed their formation to ion-exchange resins and HCl. Cyanidin, one of the artifact pigments, and increasing quantities of naturally occurring cyanidin-3-glucoside, were formed from hydrolysis of cyanidin-3-gentiobioside and cyanidin-3-rhamnoglucoside. An additional pigment, however, was a cyanidin-3-diglucoside whose complete identity was not proposed but could not have arisen by partial hydrolysis.

The chromatographic characteristics and facile transformations of the above pigments are similar to what we have observed with band 3. Further study of these pigments should, in our opinion, consider the possibility of different glycosidic forms.

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GELIONOPHORETISCHE TRENNUNG UND CHARAKTERISIERUNG GEMISCHTER CYANO–THIOCYANATOCHROMATE(III)

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SUMMARY

Separation and characterisation of mixed cyano–thiocyanatochromates(III) by gel ionophoresis

The mixed ligand complexes $[Cr(SCN)_x(CN)_{6-x}]^{3-}$ are separated by an electrophoretic apparatus consisting of a separating tube. The composition of the complexes is confirmed by elementary analysis and absorption spectra.

EINLEITUNG

Die papierionophoretische Untersuchung der Reaktion von $K_3[Cr(SCN)_6]$ mit KCN in Acetonitril, die zu Gemischen sämtlicher Gemischtligandkomplexe $[Cr(SCN)_x(CN)_{6-x}]^{3-}$ ($x = 0, 1, \ldots, 6$) führt, wurde bereits beschrieben[1]. Die in Acetonitril leichtlöslichen Komplexe mit $x = 2, \ldots, 6$ wurden als Komplexgruppe *a*, die drei in Acetonitril schwerlöslichen Komplexe ($x = 0, 1$ sowie das nicht zur Reihe gehörende Komplexion X^{n-}) als Komplexgruppe *b* bezeichnet. Einen ersten Beleg für die Zusammensetzung dieser Komplexe stellten die ermittelten R_B-Werte dar.

In der vorliegenden Arbeit werden die gemischten Cyano–Thiocyanatochromate (III) in der neu entwickelten Trennschlauchapparatur[2] getrennt. Als Hydrolyse ausschliessendes Trennmedium für die Komplexgruppe *a* dient ein Acetonitril–Alkohol-Gel, für Komplexgruppe *b* ein Formamid-Gel. Die Charakterisierung der einzelnen Komplexe erfolgt durch Elementaranalysen und UV-Spektren.

CHEMIKALIEN UND GERÄTE

Chemikalien

Acetylcellulose F 900 der Fa. Bayer/Dormagen, Acetonitril p.a. (0.3 % Wasser) der Fa. Schuchardt, alle anderen Substanzen pro-analysi-Präparate der Fa. Merck. Über die Darstellung der im Handel nicht erhältlichen Komplexsalze $K_3[Cr(CN)_6]$ und $K_3[Cr(SCN)_6]$ wurde bereits berichtet[3].

Messgeräte

Absorptionsspektralphotometer PM Q4 der Fa. Carl Zeiss (photometrische CrO_4^{2-}-Bestimmung), Absorptionsspektralphotometer DK 2A der Fa. Beckman (Aufnahme der Spektren).

Trennschlauchapparatur[2]

Sie enthält vier Trenneinheiten. Als Kühlaggregat dient der Ultrakryomat K DWN der Fa. Messgeräte Lauda, als Spannungsquelle das Gerät SX 2001 der Fa. Knott.

DARSTELLUNG DER GEMISCHTLIGANDKOMPLEXE

In einer vorhergehenden Veröffentlichung[1] sind die günstigsten Reaktionsbedingungen zur Darstellung des Gemisches sämtlicher Gemischtligandkomplexe angegeben. Durch gezielte Wahl der Reaktionsbedingungen können jedoch einzelne Komplexe der Komplexgruppe *a* im Überschuss dargestellt werden (Tabelle I). Sie sind auf den entsprechenden Pherogrammen durch starke Zonen gekennzeichnet.

TABELLE I

GEZIELTE REAKTIONSFÜHRUNG INNERHALB DER KOMPLEXGRUPPE *a*

Reaktions-temperatur (°C)	*Reaktions-zeit* (*h*)	CN^- : $[Cr(SCN)_6]^{3-}$	*Komplex im Überschuss*
81	2.5	1 : 1	$[Cr(SCN)_5(CN)]^{3-}$
68	3	3 : 1	$[Cr(SCN)_4(CN)_2]^{3-}$
73	3	3 : 1	$[Cr(SCN)_3(CN)_3]^{3-}$
81	3	4 : 1	$[Cr(SCN)_2(CN)_4]^{3-}$

Es werden jeweils 5 mMol $K_3[Cr(SCN)_6]$ mit KCN (maximal bis 20 mMol) in 10 ml Acetonitril umgesetzt.

Die Komplexe der Gruppe *b* entstehen unter diesen Bedingungen nur in untergeordneten Mengen. Der Niederschlag, der bei diesen relativ langen Reaktionszeiten bräunlich ist, wird verworfen.

Zur Darstellung der Komplexe der Komplexgruppe *b* wird die Umsetzung unter folgenden Bedingungen durchgeführt: Temperatur 81°; CN^- : $[Cr(SCN)_6]^{3-}$ = 10 : 1; Reaktionszeit 1 h. Die kurze Reaktionszeit von 1 h garantiert das Vorliegen der drei Komplexe nebeneinander ohne störende Polymerprodukte.

TRENNUNG DER KOMPLEXGRUPPE *a*

Die Auftrennung der Komplexionen der Komplexgruppe *a* ist auf zwei Arten möglich: (1) Ionophorese der wandernden Grenzflächen unter Verwendung eines Acetonitril-Gels und (2) Kombination von Zonenionophorese und Ionophorese der wandernden Grenzflächen unter Verwendung eines Acetonitril–Alkohol-Gels.

(1) *Ionophorese der wandernden Grenzflächen unter Verwendung eines Acetonitril-Gels.* In Acetonitril findet keine Solvolyse der Komplexe statt[1]. Als schnellwanderndes Anion wird das Thiocyanation, als langsamwanderndes Anion das Benzoation benutzt. Das gemeinsame Gegenion aller Anionen (SCN^-, Komplexionen, Benzoation) ist $[N(CH_3)_4]^+$ (Lit. 4). Die Tetramethylammoniumsalze werden aus den bei der Darstellung anfallenden Kaliumsalzen der Komplexe durch Ionenaustausch an Amberlyst 15 in Acetonitril gewonnen. Im Trennschlauch ordnen sich die Komplexionen mit fallendem Thiocyanat-Gehalt hintereinander (Fig. 1).

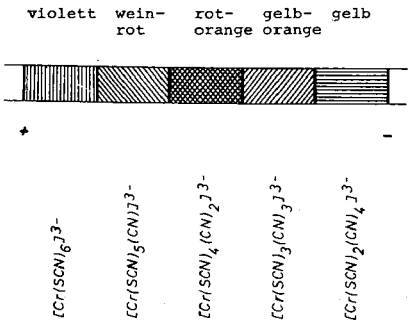


Fig. 1. Ionophorese der wandernden Grenzflächen der Komplexgruppe *a* in Acetonitril.

Der Durchsatz an Komplexionen in der Trennschlauchapparatur ist durch die geringe Löslichkeit des Tetramethylammoniumbenzoates und der cyanidreichen Komplexsalze in Acetonitril begrenzt. Eine präparative Trennung nach diesem Verfahren erfordert grossen Zeitaufwand. Vorteilhaft ist deshalb die primäre Anreicherung einzelner Komplexe durch gezielte Darstellung und eine Vortrennung nach dem Prinzip der Zonenionophorese.

(2) *Kombination von Zonenionophorese und Ionophorese der wandernden Grenzflächen unter Verwendung eines Acetonitril–Alkohol-Gels.* In einem Acetonitril–Alkohol-Gel können die bei der Darstellung anfallenden Gemische der Kaliumsalze der Komplexe direkt getrennt werden. Hier findet bei 0° selbst nach 100 h keine Solvolyse statt. Auf den Ionopherogrammen sind nur die fünf Zonen der Komplexgruppe *a* zu sehen.

Als langsamwanderndes Anion wird das Acetation eingesetzt. Kaliumacetat, Kaliumthiocyanat sowie die Kaliumsalze der Komplexe sind in dem Lösungsmittelgemisch genügend löslich, so dass grössere Komplexmengen getrennt werden.

Herstellung des Acetonitril–Alkohol-Gels

50 ml Acetonitril und 50 ml Alkohol werden mit 13.5 g Celluloseacetat (17 Gew.-%) und der entsprechenden Menge an Elektrolyt 1 h bei 60–65° unter Rückfluss gerührt. Bei Raumtemperatur ist dieses Gel dünnflüssig (Einfüllen in die Elektrodentröge und Schläuche), bei 0° wird es steif.

Durchführung der Trennung

Die Trennbedingungen lauten wie folgt:

Leitelektrolyt für Zonenionophorese	0.5 *M* KSCN
Schneller Elektrolyt für Ionophorese der wandernden Grenzflächen	0.5 *M* KSCN
Trennlösung	0.12 *M* Komplexe in K^+-Form
Langsamer Elektrolyt	0.4 *M* CH_3COOK
Spannung	0.7 kV
Stromstärke	4 mA pro Schlauch
Trennzeit	10 h
Kühltemperatur	−4°
Schlauchlänge	maximal 1 m

Überschreitet die angelegte Spannung 700 V, so ergeben sich höhere Stromstärken als 4 mA pro Schlauch. Durch die Kühlsole wird dann nicht genug Joulesche Wärme abgeführt und es beginnt an der Verbindung Kathodentrog–Schlauch nach kurzer Zeit zu brennen.

Zonenionophorese. Zur Vortrennung durch Zonenionophorese enthält die gesamte Trenneinheit (Schlauch und Elektrodentröge) die gelierte KSCN-Lösung. Die bei der gezielten Darstellung vorliegenden 10 ml Komplexgemisch werden mit absolutem Alkohol auf 20 ml aufgefüllt. Von dieser Lösung werden 1.5 ml in ein Schlauchstück von 20 cm Länge eingesaugt. Dies wird mittels zweier Glasrohre (3 mm Innendurchmesser) direkt vor den Kathodentrog in die Trenneinheit eingesetzt. Um selbst bei einer langen Trennstrecke eine Feldstärke von 7 V/cm nicht zu unterschreiten, wird wie folgt vorgegangen:

Ist die vorderste Komplexzone bis kurz vor den Anodentrog (etwa 3–5 cm) gewandert, wird der Trennschlauch hinter der letzten, langsamsten Komplexzone durchschnitten und dieses Ende mit einem frisch gefüllten Kathodentrog verbunden. Anschliessend wird das vordere Ende des Schlauches kurz vor der ersten Komplexzone durchschnitten. Hier schliesst man einen neu gefüllten Anodentrog mit Schlauch (maximal 1 m) an. Als Verbindungsstück dient ein kurzes Glasrohr.

Führt man diese Arbeitsoperation zweimal aus, können Trennstrecken bis zu 3 m bewältigt werden. Weiterhin wird die elektroosmotische Wanderung des durch anodische Oxydation von SCN^- entstehenden Schwefels in Richtung Kathode durch das Auswechseln des Anodentroges samt Schlauch jedesmal unterbunden. Dadurch entfällt die Verunreinigung der vordersten Komplexzone mit Schwefel.

Ionophorese der wandernden Grenzflächen. Die nach 30 h Zonenionophorese ausgebildete Zone des im Überschuss vorhandenen Komplexes wird aus dem Trennschlauch geschnitten und zur Reinigung von KSCN einer Ionophorese der wandernden Grenzflächen unterworfen.

Hierzu wird das eine Ende dieses Schlauchstückes direkt mit einem neuen Kathodentrog (jetzt CH_3COOK), das andere Ende mittels eines Glasrohres mit einem frisch gefüllten Anodenschlauch und Anodentrog (KSCN) verbunden. Nach weiteren 10–15 h Trennzeit ist alles KSCN aus der farbigen Komplexzone in Richtung Anode gewandert.

TRENNUNG DER KOMPLEXGRUPPE *b*

In Formamid als Lösungsmittel ist die Solvolyse der Komplexionen der Komplexgruppe *b* gering[1].

Herstellung des Formamid-Gels

100 ml Formamid werden mit 2.8 g Celluloseacetat (2.5 Gew.-%) und der entsprechenden Menge an schnellem bzw. langsamem Elektrolyten auf 140° erhitzt. Nach 14 min ist das fertige Gel durchsichtig klar. Beim Abkühlen auf Raumtemperatur wird es starr und kann so gelagert werden. Zum Einfüllen in Elektrodentröge und Schläuche muss man es auf 90° erwärmen.

Durchführung der Trennung

Die Trennbedingungen lauten wie folgt:

Schneller Elektrolyt	0.66 *M* KBr
Trennlösung	0.2 *M* Komplexe in K^+-Form
Langsamer Elektrolyt	0.66 *M* CH_3COOK
Spannung	1.5 kV
Stromstärke	5 mA pro Schlauch
Trennzeit	50 h
Kühltemperatur	$-4°$
Schlauchlänge	1.5 m

Es werden 360 mg Komplexgemisch, das sind bezogen auf $K_3[Cr(SCN)(CN)_5]$ rund 1 mMol, in 5 ml Formamid gelöst. Dabei bleibt der grösste Teil des $K_3[Cr(CN)_6]$ ungelöst. Nach Abzentrifugieren dieses schwerlöslichen Rückstandes wird die Lösung in die Trennschlauchapparatur gefüllt.

Die vorderste hellgelbe Zone enthält $[Cr(CN)_6]^{3-}$. Sie ist sehr schlecht zu erkennen. Erstens ist infolge der Schwerlöslichkeit des $K_3[Cr(CN)_6]$ in Formamid nur eine geringe Menge im Trennschlauch enthalten, zweitens ist der molare Extinktionskoeffizient ε im sichtbaren Bereich klein.

Die Auftrennung des gelben Komplexions $[Cr(SCN)(CN)_5]^{3-}$ gegen das ebenfalls gelbe Komplexion X^{n-} ist optisch nicht wahrnehmbar. Deshalb wird $K_3[Cr(SCN)_6]$ zur Trennlösung gegeben. Das violette $[Cr(SCN)_6]^{3-}$ lagert sich zwischen den langsameren Komplex X^{n-} und den schnelleren Komplex $[Cr(SCN)(CN)_5]^{3-}$ (Fig. 2).

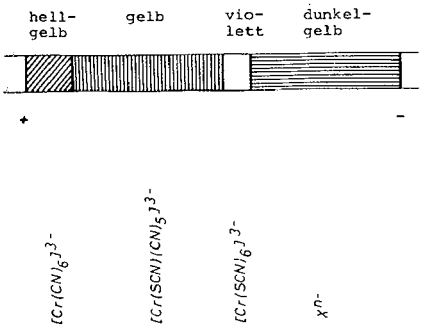


Fig. 2. Trennung der Komplexgruppe *b*.

Die Umkehr der Beweglichkeiten der Komplexionen in Acetonitril–Alkohol gegenüber denen in Wasser bzw. Formamid ist wahrscheinlich auf Primärdissoziationseffekte zurückzuführen[2].

ELUTION DER KOMPLEXE AUS DEN GELEN

Bei Versetzen der Gele mit Wasser flockt Celluloseacetat aus. Die wasserlöslichen Kaliumsalze der Komplexionen können somit leicht abgetrennt werden. Die Elution mit dem entsprechenden Gelpartner (Acetonitril–Alkohol bzw. Formamid) empfiehlt sich nicht, da zuviel Celluloseacetat gelöst wird. Im UV-Spektrum sind dann die charge-transfer-Banden der Komplexe durch die $n \rightarrow \pi^*$-Banden der freien Elektronenpaare von N und O des Celluloseacetats überlagert. Eine Trocknung der gelierten Zonen erhöht die Elutionszeit mit Wasser auf 1 h, so dass weitgehende Hydrolyse stattfindet.

Bewährt hat sich folgende Methode: Die vereinigten identischen Zonen aus vier Trennschläuchen der Ionophorese der wandernden Grenzflächen werden schnell mit 10–15 ml Wasser von maximal 5° verrieben. Das Gemisch lässt man sofort durch ein Faltenfilter in ein 50 ml-Kölbchen laufen und füllt bis zur Marke mit Wasser auf. Diese Stammlösungen der einzelnen Komplexe werden für die folgenden Untersuchungen eingesetzt.

CHARAKTERISIERUNG DER GEMISCHTLIGANDKOMPLEXE

Die papierionophoretische Reinheitskontrolle der oben angegebenen Stammlösungen ergibt jeweils nur eine Zone.

Elementaranalysen

Photometrische CrO_4^{2-} Bestimmung[5,6]. 5 ml der jeweiligen Stammlösung mit einer Konzentration von etwa 1 mMol Cr/l werden mit 20 ml 10^{-1} *M* NaOH auf 60–70° erhitzt. Anschliessend wird dreimal im Abstand von 15 min 3 ml 30%-iges H_2O_2 zugetropft. Hierbei entsteht quantitativ CrO_4^{2-}. Nach Verkochen des überschüssigen H_2O_2 wird auf 22° abgekühlt, die Lösung in einem 50 ml-Messkolben aufgefüllt und ihre Extinktion bestimmt.

Argentometrische CN^-- und SCN^--Bestimmung[3]. Zur Bestimmung gelangen 5 ml der jeweiligen Stammlösung. Die Mittelwerte aus fünf Bestimmungen je Komplex zeigt Tabelle II.

TABELLE II

ZUSAMMENFASSUNG DER ANALYSENERGEBNISSE

Komplex	*Farbe*	*Atomverhältnis*		
		Cr	*SCN*	*CN*
$[Cr(SCN)_5(CN)]^{3-}$	weinrot	1	4.91	0.98
$[Cr(SCN)_4(CN)_2]^{3-}$	rotorange	1	3.95	1.99
$[Cr(SCN)_3(CN)_3]^{3-}$	gelborange	1	2.97	2.97
$[Cr(SCN)_2(CN)_4]^{3-}$	gelb	1	1.94	3.97
$[Cr(SCN)(CN)_5]^{3-}$	goldgelb	1	0.97	4.96
X^{n-}	dunkelgelb	1	3.96	0.96

Spektren im sichtbaren und UV-Bereich

Die Konzentrationen für den sichtbaren Bereich betragen 10^{-3} Mol/l, die für

den UV-Bereich 10^{-5}–10^{-4} Mol/l. Für den sichtbaren Bereich werden Glasküvetten der Dicke 1 cm, für den UV-Bereich Quarzküvetten der Dicke 1 mm benutzt. Eine mögliche Hydrolyse wird wegen der geringen Verweilzeit in den Küvetten nicht berücksichtigt.

Fig. 3–5 zeigen die Spektren sämtlicher untersuchter Komplexe im Bereich 15000–50000 cm^{-1}.

Langwellige d–d Banden. Bei den Komplexionen $[Cr(CN)_6]^{3-}$ und $[Cr(SCN)_6]^{3-}$ sind lediglich die beiden ersten Laporte-verbotenen, spinerlaubten d–d Übergänge zu sehen: $^4A_{2g}(F) \rightarrow {}^4T_{2g}(F)$ als Bande I und $^4A_{2g}(F) \rightarrow {}^4T_{1g}(F)$ als Bande II. Die dritte dieser d–d Banden, die dem Übergang $^4A_{2g}(F) \rightarrow {}^4T_{1g}(P)$ entspricht, wird wie allgemein bei Cr(III) Komplexen durch die breite Laporte-erlaubte charge-transfer-Bande im nahen UV verdeckt[7].

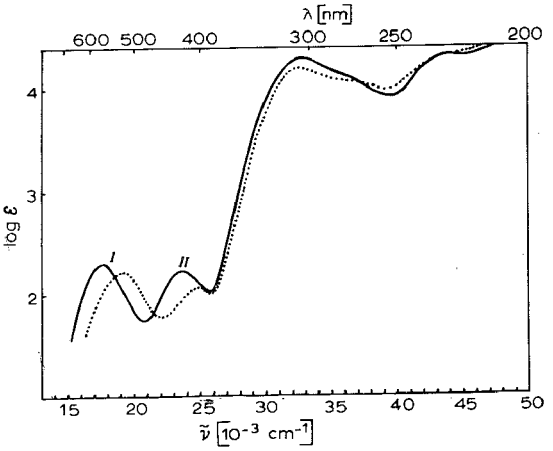


Fig. 3. Absorptionsspektren der Komplexionen $[Cr(SCN)_6]^{3-}$ (———) und $[Cr(SCN)_5(CN)]^{3-}$ (· · · · · ·).

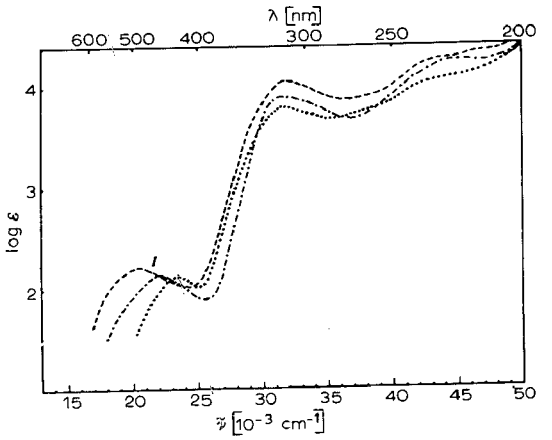


Fig. 4. Absorptionsspektren der Komplexionen $[Cr(SCN)_4(CN)_2]^{3-}$ (– – – –), $(Cr(SCN)_3(CN)_3]^{3-}$ (–·–·–) und $[Cr(SCN)_2(CN)_4]^{3-}$ (· · · · · ·).

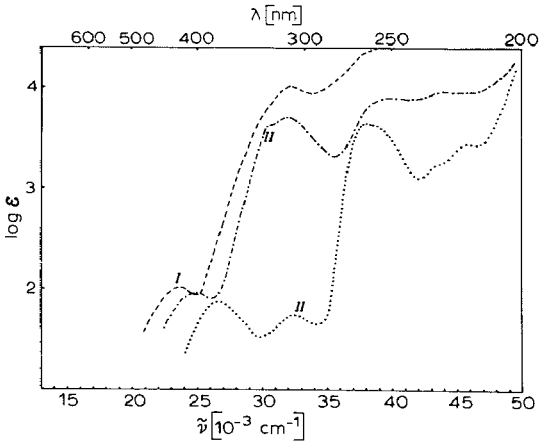


Fig. 5. Absorptionsspektren der Komplexionen X^{n-} (– – – –), $[Cr(SCN)(CN)_5]^{3-}$ (–·–·–·–) und $[Cr(CN)_6]^{3-}$ (·····).

Die Bande II wird ausserdem nur bei den Komplexen $[Cr(SCN)_5(CN)]^{3-}$ und $[Cr(SCN)(CN)_5]^{3-}$ als Schulter beobachtet. Wegen der spektrochemischen Verschiebung in Richtung kürzerer Wellenlängen durch sukzessiven Ersatz einer SCN- gegen eine CN-Gruppe kommt auch diese Bande in das Gebiet der starken charge-transfer-Bande Cr → NCS zu liegen, so dass sie bei den übrigen Gemischtligandkomplexen nicht mehr aufgelöst werden kann.

Die Aufspaltung des $^4T_{2g}$-Termes des oktaedrischen Ligandenfeldes in die C_{4v}, C_{2v}, D_{4h} bzw. C_{3v}-Komponenten bei tetragonaler bzw. trigonaler Ligandenfeldsymmetrie kann nicht festgestellt werden, obwohl SCN und CN in der spektrochemischen Reihe ziemlich weit auseinanderstehen. In guter Näherung kann also oktaedrische Symmetrie O_h des Ligandenfeldes für sämtliche hier betrachteten Gemischtligandkomplexe angenommen werden. In diesem speziellen Fall gilt die Regel der mittleren Umgebung[8].

$$Dq_{[Cr(SCN)_x(CN)_{6-x}]^{3-}} = \frac{x}{6} \cdot Dq_{[Cr(SCN)_6]^{3-}} + \frac{6-x}{6} \cdot Dq_{[Cr(CN)_6]^{3-}}$$

Die nach dieser Formel berechneten Bandenlagen sind zusammen mit den experimentellen Bandenlagen des Überganges $^4A_{2g}(F) \rightarrow {}^4T_{2g}(F)$ mit $\bar{v} = 10\ Dq$ in Tabelle III aufgeführt. Die Übereinstimmung ist gut.

TABELLE III

BERECHNETE UND EXPERIMENTELLE BANDENLAGE DER d–d-BANDE I

Komplex	$\bar{v}_{ber.}$	$\lambda_{ber.}$	$\bar{v}_{exp.}$	$\lambda_{exp.}$
$[Cr(SCN)_6]^{3-}$	—	—	17 730	564
$[Cr(SCN)_5(CN)]^{3-}$	19 231	520	19 200	521
$[Cr(SCN)_4(CN)_2]^{3-}$	20 736	482	20 500	488
$[Cr(SCN)_3(CN)_3]^{3-}$	22 233	450	22 000	454
$[Cr(SCN)_2(CN)_4]^{3-}$	23 734	421	23 500	425
$[Cr(SCN)(CN)_5]^{3-}$	25 235	396	24 800	402
$[Cr(CN)_6]^{3-}$	—	—	26 738	374

Ligandenbande Cr → NCS. Die Cr → NCS-charge-transfer-Bande der gemischten Thiocyanato–Ammin-Chrom(III)-Komplexionen, die zwischen 300 und 318 nm liegt, gibt die Zahl der SCN-Gruppen je Cr an[9]. Lg ε wächst linear mit jeder neu hinzukommenden SCN-Gruppe.

Auch für die hier behandelten gemischten Cyano–Thiocyanatochromate(III) erhält man diese lineare Beziehung zwischen lg ε bei 305 bis 318 nm und der Zahl der SCN-Gruppen im Komplex (Fig. 6).

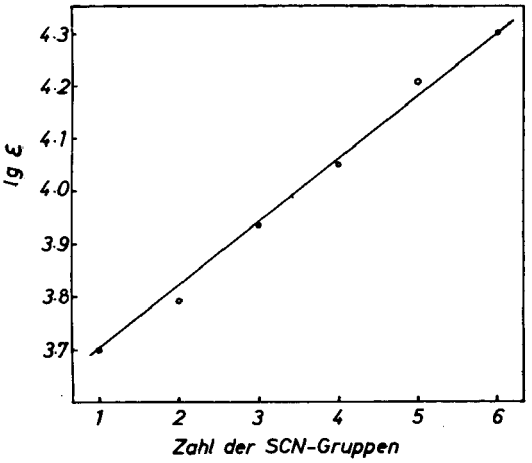


Fig. 6. Lg ε als Funktion der Zahl der SCN-Gruppen.

Die Zahl der SCN-Gruppen pro Cr-Atom des Komplexes X^{n-} lässt sich mit $\lg \varepsilon = 4.03$ zu 3.7 angeben.

Allgemein können bei Gemischtligandkomplexen des Ligandenverhältnisses 4:2, 3:3 und 2:4 Stereoisomere vorliegen. Bei der Umsetzung von $K_3[Cr(SCN)_6]$ mit KCN sind auf Grund der *trans*-Reihe von TSCHERNAJEW[10] die *trans*-Isomeren zu erwarten. Die experimentellen Daten geben jedoch keinen Hinweis für das ausschliessliche Vorliegen der *cis*- oder *trans*-Form.

Komplexzusammensetzung

Sowohl die Elementaranalysen als auch die Elektronenbandenspektren bestätigen die auf Grund der R_B-Werte[1] der Hochspannungspapierionophorese vermutete Zusammensetzung der gemischten Cyano–Thiocyanatochromate(III).

Der mit X^{n-} bezeichnete, gelbe, in Acetonitril schwerlösliche Komplex könnte als $[Cr_2(SCN)_8(CN)_2]^{4-}$ formuliert werden. Darauf weist einerseits sein gleichzeitiges Auftreten mit $[Cr(SCN)_5(CN)]^{3-}$ bei der Darstellung des Komplexgemisches[1] als auch die Elementaranalyse hin. Diese Formulierung wird weiterhin durch den molaren Extinktionskoeffizienten der Cr → NCS-charge-transfer-Bande bei 310 nm gestützt. Entsprechend der Zahl von vier SCN-Gruppen pro Cr-Atom (experimentell 3.7 SCN-Gruppen) beträgt die molare Extinktion an dieser Stelle $\varepsilon = 10700$.

ZUSAMMENFASSUNG

Die Trennung der Gemischtligandkomplexe $[Cr(SCN)_x(CN)_{6-x}]^{3-}$ $(x = 0, 1, \ldots, 6)$ wird mit Hilfe einer früher beschriebenen Trennschlauchapparatur vorgenommen. Beweise für die Zusammensetzung der Komplexionen erbringen Elementaranalysen und Absorptionsspektren.

DANK

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Notes

CHROM. 4780

Eine Fehlermöglichkeit bei der Kopfraumanalyse

Die Kopfraum-(head space)-Analyse ist, besonders in der Aromastoff-Forschung, eine wichtige Technik. Sie dient dazu, die flüchtigen Stoffe im Gasraum über einer kondensierten Phase zu ermitteln oder zu bestimmen. Im einfachsten Fall wird die kondensierte Phase (Flüssigkeit oder Festkörper) in ein Gefäss gegeben, welches mit einer Gummimembran verschlossen ist. Mit einer gasdichten Spritze können dann Gasproben entnommen und in einen Gaschromatographen eingespritzt werden.

Bei Versuchen mit Festkörpern und reinen Aromastoffen beobachteten wir unerklärliche Abnahmen der Aromastoffgehalte. Wir spritzten deshalb in leere Ampullenflaschen nach Schmitt (Fa. B. Braun, Melsungen, 80 ml Rauminhalt) 0.2–10.0 μl flüssiges Aceton ein und bestimmten nach dem Verdampfen die Acetongehalte im Gasraum der Flaschen. Sie nahmen zunächst stark, später weniger ab, und zwar stärker bei grösseren Konzentrationen (Fig. 1). Der Entnahmefehler, welcher durch die Volumenvergrösserung bei der Entnahme und durch mehrmalige Entnahme aus derselben Flasche entstand, wurde durch Multiplizieren der gaschromatographisch ermittelten Werte mit Korrekturfaktoren

$$k_n = \left(\frac{y + z}{y}\right)^n$$

eliminiert. Dabei bedeuten:

y = Gasraum in der Flasche (ml),

z = Entnahmevolumen + Totvolumen der Spritze, soweit es sich bei der Entnahme ausserhalb der Gummimembran befindet (ml),

n = Zahl der Entnahmen.

Das allmähliche Abfallen der Kurven in der Fig. 1 deutet auf eine relativ langsame Sorption, und zwar an den Gummistopfen, hin. Es wurde deshalb die relative Sorption der Dämpfe verschiedener Aromastoffe studiert. Stopfen, wie sie bei den Ampullenflaschen verwendet werden, wurden gesättigten Dämpfen ausgesetzt und gewogen. Fig. 2 zeigt die Ergebnisse bei jeweils fabrikneuen, 1.4 g schweren Gummistopfen, Fig. 3 diejenige bei Siliconstopfen. Letztere sorbierten Wasser praktisch nicht. Lipophile Aromastoffe hingegen werden allgemein besonders gut sorbiert. Amylacetat, zu 0.2 μl im Gemisch mit anderen Aromastoffen in eine Ampullenflasche eingespritzt, konnte nach 1 Std. nicht mehr nachgewiesen werden.

Die Prüfung der Temperaturabhängigkeit bei Aceton ergab, dass sowohl unter Zimmertemperatur (−17°, +7°) als auch darüber (+35°) der Acetongehalt stärker abnahm als bei +23°. Es erscheint möglich, dass dies auf die grössere Sprödigkeit des Gummis bei tiefer und auf die Erleichterung des Eindringens von Aceton in Gummi bei hoher Temperatur zurückzuführen ist.

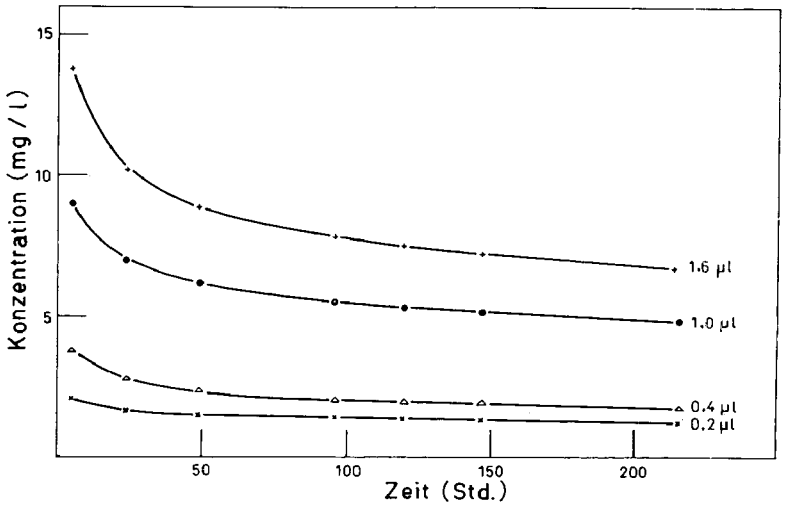


Fig. 1. Acetongehalt in Ampullenflaschen nach dem Einspritzen von 0.2–1.6 µl (+ 23°).

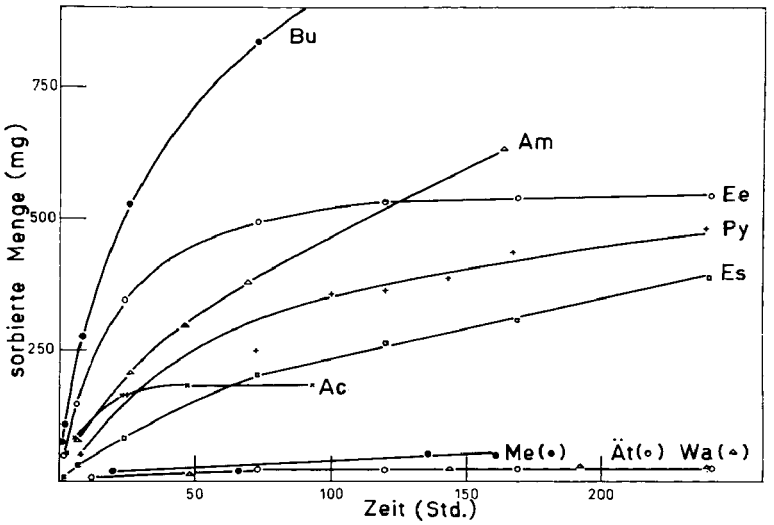


Fig. 2. Sorption von Dämpfen an Gummistopfen (+ 23°). Bu = *n*-Butylamin; Am = Ameisensäure; Ee = Äthylacetat; Py = Pyridin; Es = Essigsäure; Ac = Aceton; Me = Methanol; Ät = Äthanol; Wa = Wasser.

Auch bei verschiedenen Gummistopfen anderer Herkunft wurden ähnliche Sorptionen beobachtet. Es gelang nicht, diese durch Tempern, Aufkleben von Aluminium- oder Teflonfolie so zu beseitigen, dass mehrmalige fehlerfreie Entnahmen aus einem Gefäss möglich waren. Wesentlich geringer war die Sorption an versilberten Stopfen, ihre Reproduzierbarkeit an verschiedenen Stopfen dann aber schlecht. Da in Versuchsanordnungen mit Glashähnen anstelle von Gummistopfen nach mehreren Stunden noch grössere Verluste an Aceton auftraten, wurden für Versuche mit Fest-

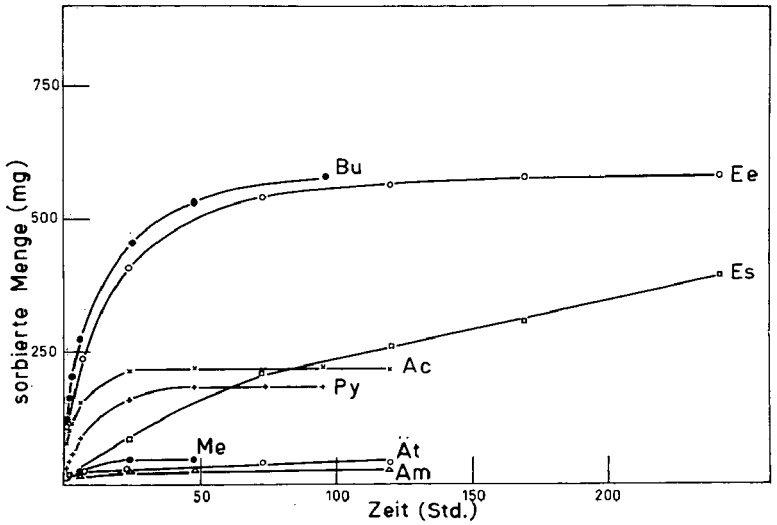


Fig. 3. Sorption von Dämpfen an Siliconstopfen. Bezeichnungen wie bei Fig. 2.

körpern zwar Gummistopfen verwendet, die Fehler aber mit Hilfe der Blindwerte durch eine relativ umständliche Berechnung korrigiert. Dabei erwies es sich als günstig, dass die in Fig. 1 gezeigten Kurven im doppelt logarithmisch geteilten Netz Geraden sind.

Wurde der Kopfraum über Flüssigkeiten analysiert, so wurden keine Abnahmen der Aromastoffgehalte im Gasraum beobachtet, wahrscheinlich deshalb, weil die Aromastoffe aus der Flüssigkeit schnell genug nachgeliefert wurden.

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CHROM. 4802

Artefacts in amino acid analysis

Ninhydrin-positive products of carbohydrate hydrolysis

Amino acids analysed using standard procedures for amino acid analysers[1,2] often can be identified solely on the basis of the time of their elution from the resin column. This criterion has met general acceptance for the common amino acids in protein hydrolysates, but is not satisfactory for many components of physiological fluids because of the presence of a number of artefacts and unknown compounds.

Many of the hydrolysates analysed in this laboratory contain polysaccharide and lignin derivatives. A number of ninhydrin-positive substances appeared between cysteic and aspartic acids at the beginning of analyses. This paper describes experiments which indicate that some of the peaks were artefacts derived from 'caramelisation' of carbohydrates during hydrolysis.

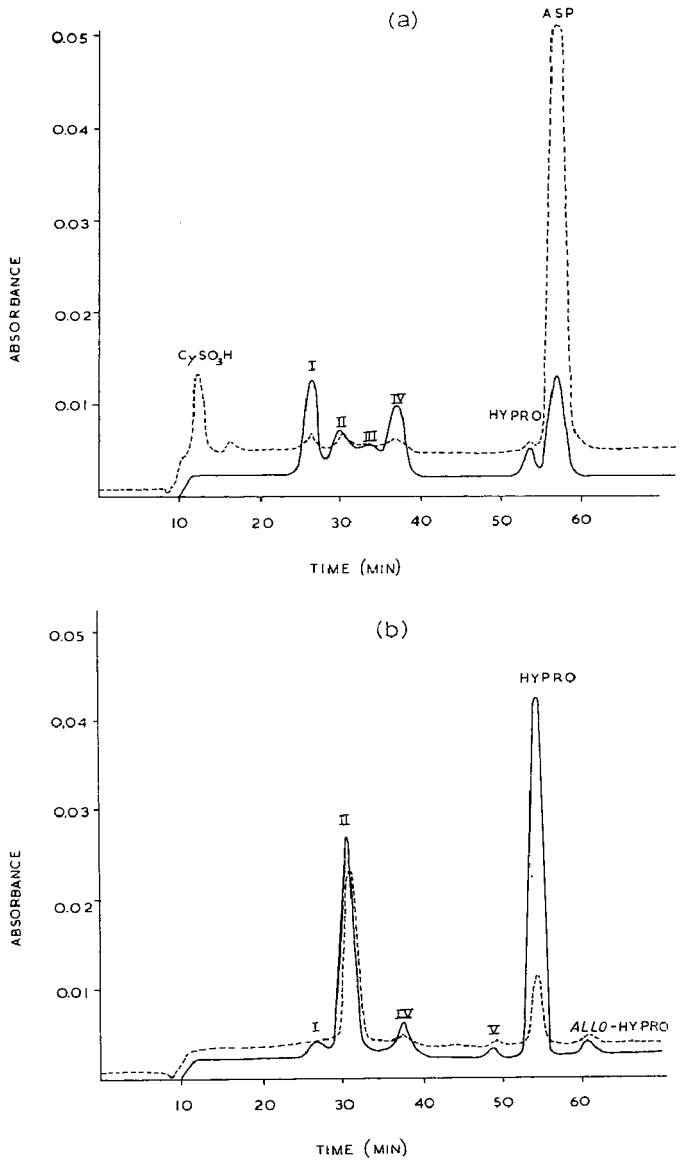


Fig. 1. Analysis of hydrolysates containing hydroxyproline and carbohydrates. (a) Part of the amino acid analysis of hydrolysed seed coats. (b) Complete analysis of a standard mixture of hypro and sucrose (see text). Peaks I, II, IV and V are derived from carbohydrate hydrolysis. ——, Absorbance at 440 nm; - - - - -, absorbance at 570 nm.

Materials and methods

Dried pea (*Pisum sativum*, L.) seed coats, which contain polysaccharide, lignin and protein[3], were hydrolysed in 6 *N* HCl at 105° for 20 h *in vacuo*. Test solutions containing 2 μmole 4-hydroxyproline (hypro) and 0.2 μmole sucrose either together or separately were hydrolysed in 0.2 ml 6 *N* HCl under the same conditions. The hydrolysates were made to a final volume of 5 ml with citrate buffer pH 2.2 (ref. 4), and 1.0 ml was analysed using a Beckman 120C amino acid analyser by the recommended 4-h procedure with expanded recorder range card.

Results and discussion

Fig. 1a shows the first part of the elution pattern obtained during amino acid analyses of hydrolysed pea seed coat material. Peaks I–IV were characterised by their high absorbance at 440 nm relative to that at 570 nm. Peak II was further characterised as having almost equal absorbance at the two wavelengths.

Fig. 1b shows the elution pattern obtained by amino acid analysis of an hydrolysate containing hypro and sucrose. Peaks I, II and IV correspond exactly with those in Fig. 1a; peak III is absent and there is an additional peak (V). The absorbance pattern of peak II was the same in both natural and synthetic hydrolysates.

None of the peaks I–V were present in hydrolysates of hypro. There were no peaks in the positions of hypro or *allo*-hypro when sucrose hydrolysates were analysed. All hydrolysates of seed coat or sucrose appeared pale yellow before analysis but there was no absorbance at 440 nm without reaction with ninhydrin. It is likely that the yellow colour was due to 'caramelised' products of carbohydrate hydrolysis. No attempt has been made to characterise these artefacts further. Their existence serves to emphasise the need for care in identification of ninhydrin-positive components, particularly in view of the fact that the elution positions of 3-hydroxyproline and 3,4-dihydroxyproline[5] are in this part of the chromatogram.

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CHROM. 4783

Simultaneous gas chromatographic determination of 2,4-D and dicamba in human blood and urine

The compounds 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methoxy-3,6-dichlorobenzoic acid (dicamba) are herbicides commonly used to control a variety of broadleaf plants. An acute poisoning case was recently investigated by the Hawaii Community Study on Pesticides in which the victim intentionally ingested a formulation containing 2,4-D and dicamba. The occurrence of this attempted suicide created a need for a rapid and sensitive method for detecting these compounds in small amounts of human blood and urine.

Several methods for detecting 2,4-D residues in a variety of substrates were considered for the analysis of 2,4-D and dicamba in human blood and urine[1-3]. While generally sensitive, these methods were neither rapid nor applicable to the relatively small samples available.

The Hawaii Community Study on Pesticides uses the method of BEVENUE *et al.*[4] to determine pentachlorophenol (PCP) residues in human blood. In an evaluation done by this laboratory[5], the procedure was shown to be rapid, accurate, sensitive and reproducible. Because 2,4-D and dicamba display chemical characteristics similar to those of PCP, these compounds are also detected by the procedure.

This note describes the application of the method of BEVENUE *et al.*[4] to the simultaneous determination of 2,4-D and dicamba in human blood and urine.

Experimental

Solvents and reagents. The following solvents and reagents were used: benzene, hexane, isooctane (Mallinckrodt Nanograde Reagents), used as received; 0.1 *N* H_2SO_4, benzene extracted; 2,4-dichlorophenoxyacetic acid; 2-methoxy-3,6-dichlorobenzoic acid; N-methyl-N'-nitro-N-nitrosoguanidine, supplied by Aldrich Chemical Co., Milwaukee, Wisc.

The diazomethane solution[6] was prepared as follows: hexane (25 ml) and 2 ml of 20% aqueous NaOH were combined in a 50-ml erlenmeyer flask and 0.5 g of N-methyl-N'-nitro-N-nitrosoguanidine was added in small increments. When the reaction was complete, the hexane layer was decanted into a small glass bottle equipped with a Teflon-lined screw cap. The diazomethane solution was kept in a freezer for up to one week. Because diazomethane is a skin irritant and a carcinogen, its preparation and use was confined to a well-ventilated hood.

Standard solutions. Stock solutions of 2,4-D and dicamba in benzene were prepared, each containing 1 mg of compound per ml of solvent. In separate 100-ml volumetric flasks 1.0 ml of each stock solution was treated with 0.5 ml of diazomethane solution and allowed to stand for 15 min. The solutions were then diluted to 100 ml with isooctane, thus giving solutions which contained 10 μg/ml of each compound as its methyl ester. From these solutions, working standards were prepared in appropriate concentrations.

Apparatus. Gas chromatographic determinations were made on a MicroTek MT 220 gas chromatograph equipped with a ^3H electron capture detector and a 0.32 cm I.D. × 180 cm borosilicate glass column packed with a mixture of 4%

SE-30 and 6% QF-1 on Chromosorb W (AW-DMCS), high-performance grade, 80–100 mesh.

The instrument conditions were: inlet temperature 220°, detector temperature 208°, column temperature 190°; nitrogen flow rate 85 ml/min.

The 25-ml evaporative concentrator tube was Kontes No. 570050-2526, the evaporative concentrator column Kontes No. 569251-0324.

Extraction. One milliliter of blood serum or 5 ml of urine were combined with 20 ml of 0.1 *N* H_2SO_4 and 12 ml of benzene in a 125-ml glass-stoppered erlenmeyer flask equipped with a 1-in. Teflon-coated magnetic stirring bar and mixed for 20 min at 50° on a heated stirplate. After cooling, the contents of the flask were transferred to a 35-ml centrifuge tube and centrifuged for 15 min to effect phase separation. The benzene (top) layer was transferred to a 25-ml evaporative concentrator tube, and the aqueous phase was re-extracted three times with 5-ml portions of benzene. With each wash the mixture was shaken vigorously for 1 min, and the phases were separated by centrifugation. The benzene extracts were combined in the 25-ml evaporative concentrator tube, a boiling chip was added, an evaporative concentrator column was attached, and the combined extract was concentrated to 2 ml in a water-bath at 100°.

Methylation. Diazomethane solution (0.2 ml) was added to the concentrated extract, and the mixture was allowed to stand for 15 min. Excess diazomethane was then removed by gently bubbling dry nitrogen through the extract until the yellow color disappeared, and the volume was adjusted to 10.0 ml with isooctane.

Gas chromatography. The methylated extract was diluted as necessary, and 2 to 8 μl were injected into the gas chromatograph. Following each extract injection, an amount of standard which gave a response of similar peak height ($\pm$ 10%) was injected.

Results and discussion

The 2,4-D and dicamba levels in the poisoning case samples are given in Table I. The results of a recovery study to determine the applicability of the procedure to the analysis of human blood and urine for 2,4-D and dicamba residues are given in Table II. The samples were fortified, prior to extraction, with 2,4-D and dicamba acids at levels which compared generally with the wide range of concentrations found in the poisoning case samples.

In all cases responses to 2,4-D and dicamba (as their methyl esters) were quantitated by comparison with standard responses in terms of peak height. By comparing responses with peak heights differing by 10% or less, quantitation problems associated with lack of linearity and changing sensitivity, sometimes encountered with electron capture detectors, were minimized.

The limits of detectability in 1 ml of blood serum were 0.03 p.p.m. for dicamba and 0.05 p.p.m. for 2,4-D. In 5 ml of urine the limits of detectability were 0.01 and 0.02 p.p.m. for dicamba and 2,4-D, respectively.

Under the conditions given, dicamba and 2,4-D methyl esters had retention times of 1.2 and 1.9 min, respectively; PCP methyl ether had a retention time of 2.2 min. Because the three compounds are well separated on the gas chromatographic column, the procedure is useful for detecting any or all of the compounds in the same sample.

TABLE I

2,4-D AND DICAMBA LEVELS IN POISONING CASE SAMPLES

Sampling	*Blood serum*		*Urine*					*2,4-D (p. p. m.)*		*Dicamba (p. p. m.)*	
	Date[a]	*Time*	*Date*[a]	*Time*	*to Date*[a]	*Time*	*Vol. (ml)*	*Serum*	*Urine*	*Serum*	*Urine*
1	10/14	7:00 a.m.	10/14	11:00 a.m.	10/14	3:00 p.m.	740	1006	145.2	46.4	215.0
2	10/14	3:00 p.m.	10/14	3:00 p.m.	10/14	11:00 p.m.	375	1031	274.0	22.3	280.3
3	No sample taken		10/14	11:00 p.m.	10/15	9:00 a.m.	1230	—	151.5	—	47.5
4	10/15	9:00 a.m.	10/15	9:00 a.m.	10/15	4:00 p.m.	—	911.9	762.0	8.52	12.8
5	10/15	4:00 p.m.	10/15	4:00 p.m.	10/15	11:00 p.m.	775	931.2	599.8	8.12	1.95
6	10/15	11:15 p.m.	10/15	11:00 p.m.	10/16	7:45 a.m.	1170	736.4	784.3	6.99	4.22
7	10/16	7:50 a.m.	10/16	7:45 a.m.	10/16	4:15 p.m.	720	648.6	408.9	4.47	1.08
8	10/16	4:15 p.m.	10/16	4:15 p.m.	10/16	11:00 p.m.	835	461.0	1333	3.66	0.95
9	10/16	11:00 p.m.	10/16	11:00 p.m.	10/17	9:00 a.m.	740	383.1	1900	2.54	1.35
10	10/17	9:00 a.m.	10/17	9:00 a.m.	10/17	5:00 p.m.	310	364.3	1442	1.97	1.06
11	10/17	5:00 p.m.	10/17	5:00 p.m.	10/18	10:00 a.m.	1640	72.4	409.6	1.30	0.80
12	10/18	10:00 a.m.	10/18	10:00 a.m.	10/18	4:30 p.m.	910	5.23	77.9	0.47	0.54
13	10/18	4:30 p.m.	10/18	4:30 p.m.	10/19	9:45 a.m.	1400	1.69	8.60	0.23	0.40
14	10/19	9:45 a.m.	10/19	9:45 a.m.	10/19	3:00 p.m.	450	1.78	3.88	0.15	0.25
15	No sample taken		10/19	3:00 p.m.	10/20	9:00 a.m.	2150	—	4.51	—	0.12
16	10/20	9:00 a.m.	10/20	9:00 a.m.	10/21	8:30 a.m.	2420	1.26	2.90	0.15	0.06
17	10/21	8:30 a.m.	10/21	8:30 a.m.	10/22	9:30 a.m.	3710	0.38	1.58	0.15	<0.01
18	10/22	9:30 a.m.	10/22	9:30 a.m.	10/23	8:00 a.m.	2600	0.16	1.74	0.03	<0.01
19	10/23	7:45 a.m.	10/23	8:00 a.m.	10/24	8:45 a.m.	4465	0.30	0.54	0.07	<0.01
20	10/24	8:45 a.m.	10/24	8:45 a.m.	10/25	1:00 p.m.	2960	0.88	0.71	0.06	0.03
21	10/25	1:00 p.m.	10/25	1:00 p.m.	10/27	10:45 a.m.	3800	0.38	0.25	<0.03	0.04
22	10/27	10:45 a.m.	10/27	10:45 a.m.	10/29	8:00 a.m.	2110	0.28	0.42	<0.03	<0.01
23	No sample taken		10/29	8:00 a.m.	10/31	1:00 p.m.	3030	—	1.15	—	<0.01
24	10/31	1:00 p.m.	10/31	1:30 p.m.	11/3	7:30 a.m.	1360	0.33	1.13	<0.03	<0.01
25	11/7	9:00 a.m.	11/7	6:00 a.m.	11/8	6:00 a.m.	1300	0.56	0.84	<0.03	<0.01

[a] 1969

TABLE II

RECOVERY OF 2,4-D AND DICAMBA ADDED TO HUMAN BLOOD SERUM AND URINE

Sample[a]	*Fortification level (p. p. m)*		*Found (p. p. m.)*		*% recovery*	
	Dicamba	*2,4-D*	*Dicamba*[b]	*2,4-D*[b]	*Dicamba*	*2,4-D*
Urine	—	—	< 0.01[c]	< 0.02[c]	—	—
	65.0	685	68.8	779	106	114
	13.0	137	12.5	132	96	96
	1.30	13.7	1.15	11.6	88	85
	0.13	1.37	0.11	1.35	85	98
	0.013	0.137	0.01	0.15	77	109
	0.006	0.068	< 0.01[c]	0.07	—	103
Blood serum	—	—	< 0.03[c]	< 0.05[c]	—	—
	65.0	685	61.0	636	94	93
	6.50	68.5	5.91	68.9	91	101
	0.65	6.85	0.55	5.96	85	87
	0.065	0.685	0.07	0.60	108	88
	0.006	0.068	< 0.03[c]	0.06	—	88

[a] Each sample a single analysis.
[b] Detected as their methyl esters.
[c] Limit of detectability.

No interfering responses were encountered in any of the samples analyzed; however, the use of the diazomethane solution in amounts greater than those indicated in the procedure can lead to interferences due to impurities in the reagent.

Using this procedure, an experienced analyst can process approximately sixteen samples per day.

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Fluorescent visualization of trioses on silica gel thin-layer chromatograms

The present paper describes a method for the detection of glyceraldehyde and dihydroxyacetone on silica gel thin-layer chromatograms. When the trioses are heated with mineral acid, they are readily converted to pyruvaldehyde[1], and the latter reacts with chromotropic acid in sulfuric acid to form a soluble yellow condensation product which on irradiation at 380–510 mμ gives an intense green fluorescence[2]. So far, no report has appeared describing the application of the above reaction to the visualization of the trioses on chromatograms.

Experimental

Preparation of chromatoplates. 30 g of 'Kieselgel G nach Stahl' (E. Merck, A.G., Darmstadt, G.F.R.) was mixed with 60 ml of 0.1 *M* boric acid. The slurry obtained was applied to 20 × 20 cm glass plates in the usual manner (0.25 mm layer). These plates were allowed to stand overnight at room temperature and then heated for 1 h at 100–105°.

Solvent-development of chromatograms. 1 μl of a sample solution containing appropriate quantities of the trioses (preferably 1 μg per μl each) was applied to the chromatoplate with a microsyringe. The development was carried out by the one-dimensional ascending technique with a developing solvent of *n*-butanol–ethyl acetate–isopropanol–acetic acid–water (35:100:60:35:30) (ref. 3). Pre-equilibration of the plate in the developing tank (23 × 23 × 5 cm) was continued for more than 30 min. At the end of a 10-cm solvent run, the plate was dried by means of a fairly strong current of air, provided by a fan, for about 1 h at room temperature. Then, the odor of the solvent was very faint, if detectable at all.

Detection of the spots. After drying, the plate was sprayed with a slight excess of the following reagent solution: 0.5 g of chromotropic acid disodium salt dissolved in 100 ml of a 1:10 (v/v) mixture of sulfuric acid and water. The quantity of the reagent solution required to give satisfactory results was 0.04–0.05 ml per sq. cm. The plate was then heated in an oven at 100–102° for 10 min. To obtain good results it is advisable to raise the temperature of the oven up to 100° within 10 min after the plate has been placed in the oven; heating over 102° must be avoided. The finished chromatogram was submitted to UV irradiation in the dark to detect the trioses. In the present experiments, a 'Super-Light, Model LS-DI' (Osawa Shigaisen Kogyo Kenkyusho, Ltd., Tokyo, Japan) was used as a UV light source. With this instrument, it is possible to irradiate the chromatogram at 254 mμ or at 365 mμ. For reasons stated below, irradiation at 254 mμ was adopted. The irradiation revealed the trioses as spots showing a brilliant yellow fluorescence tinged with green on a violet background.

To obtain the most satisfactory results, it was required to complete the whole process described above, except for the preparation of the plates, within as short a time as possible because of the instability of the trioses and other sugars on the chromatoplates.

Results and discussion

When a mixture of an aqueous solution of a triose and a 1% solution of chromo-

tropic acid in 50 % sulfuric acid was heated in a test tube placed in boiling water[4], the resulting solution was brownish-yellow in transmitted light and exhibited a green fluorescence. By using a fluorescence spectrophotometer (Model MPF-2A, Hitachi, Ltd., Tokyo, Japan) it was found that the fluorescence was most intense when the solution was irradiated at 385–412 mμ. When the reaction was carried out on the chromatoplates as described above and the treated chromatograms were irradiated at 254 or 365 mμ with the above-mentioned instrument (Super-Light), however, it was observed that the fluorescence of the spots was yellow rather than green in color and that the irradiation at 254 mμ was more effective for the present purpose than at 365 mμ. The cause of disagreement between the results obtained in the above two cases has not been determined yet.

TABLE I

R_F VALUES (× 100) ON 0.1 *M* BORIC ACID-IMPREGNATED SILICA GEL

Solvent system: *n*-butanol–ethyl acetate–isopropanol–acetic acid– water (35:100:60:35:30). Detecting reagent[a]: naphthoresorcinol–sulfuric acid solution[3].

	R_F
Glycolaldehyde	44
DL-Glyceraldehyde	72
Dihydroxyacetone	57
D-Erythrose	45
L-Arabinose	42
D-Xylose	53
D-Lyxose	49
L-Rhamnose	60
D-Glucose	42
D-Mannose	44
D-Galactose	35
D-Fructose	36
L-Sorbose	34

[a] The fluorescence method, as described in the text, is fairly specific for trioses, and the detection of sugars other than the trioses is not very effective. For this reason, a universal detection reagent was used.

Experiments which were made using 13 monosaccharides listed in Table I as test samples (1 μl of each sample solution was chromatographed as described above), revealed that the limits of detection of the trioses were 0.10–0.12 μg per μl, respectively and that quantities of each less than 2.5 μg/μl of glucose, mannose, galactose, fructose and sorbose and 1.2 μg/μl of glycolaldehyde, erythrose, arabinose, xylose, lyxose and rhamnose gave no fluorescence. From these results, including the R_F values obtained (Table I), it seems likely that the present method can be used effectively for detecting the trioses in complicated monosaccharide mixtures.

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Thin-layer electrophoresis of indole derivatives

The chromatography of indole derivatives has been a subject of much interest for some time. A number of articles dealing with the separation of indoles of plant origin and animal origin have appeared in the last few years. Thin-layer chromatography (TLC) especially has proved to be a very useful technique[1–4]. The most recent development has been the use of "gradient TLC" as proposed by STAHL AND DUMONT[5]. Thin-layer electrophoresis (TLE) on the other hand has not been widely applied. However, that TLE can be of great value is well documented by some recent reports on the separation of polynuclear aza heterocyclic compounds[6] and in the fractionation of plant extracts[7]. Also STAHL[8] has added an entirely new chapter on TLE in his recent book on TLC, which suggests that TLE can be of value in the separation of different categories of compounds. In view of lack of knowledge on the use of TLE for the separation of indoles, it was thought to be worthwhile to study the behaviour of some physiologically important indole derivatives by means of electrophoresis and a preliminary survey of commercially available indoles using TLE was made.

Materials

Apparatus. A Shandon electrophoresis chamber was used for TLE separations in this study. Thin-layer plates were also prepared using a Shandon applicator and TLC kit.

Standards. Indole-3-acetic acid (IAA), DL-3-indole lactic acid (ILA), indole acrylic acid (IAcA), indole acetonitrile (IAN), L-tryptophane (TP), DL-5-hydroxytryptophane (5-HTP), DL-5-fluorotryptophane (5-FTP), tryptamine hydrochloride (T), and 5-hydroxytryptamine creatinine sulphate (5-HT) were purchased from Nutritional Biochemicals Corporation. The chemicals 3-indole glyoxylic acid (IGA) and 5-hydroxyindole-3-acetic acid (5-HIAA) were purchased from Sigma Chemicals Company: *γ*-(indole-3)-*n*-butyric acid (IBA) was obtained from Eastman Kodak Company. All other chemicals used were purchased from Fischer Scientific Company and used without further purification.

Buffers. Borate, pH 10.0: A quantity of 7.5 g of boric acid and 3.75 g sodium hydroxide were dissolved in distilled water and made into a solution of 2.5 l in volume.

Borate, pH 12.4: An amount of 3.9 g boric acid and 4 g sodium hydroxide were dissolved in distilled water and made 1 l in volume.

Acetate: Sodium acetate solution (0.1 *N*) was adjusted to the desired pH by adding a normal sodium hydroxide solution.

Formic acid–glacial acetic acid, pH 2.0: Amounts of 78 ml of formic acid (85–90 %) and 148 ml of glacial acetic acid were mixed and diluted with distilled water to make 2.5 l.

Pyridine–glacial acetic acid, pH 6.1: Pyridine (100 ml) and 5 ml of glacial acetic acid were mixed and diluted with distilled water to make 2.5 l.

Methods

Thin-layer plates of 0.5 mm thickness were prepared (STAHL[8]). Silica Gel G (Merck) was used as the sorbent. The plates were heated at 100° for 30 min before

use. The standard indole compounds were dissolved in ethanol or water to give a concentration (of the compound) of 1 mg/ml. A quantity of 2–5 μg of each indole was spotted on to the plate for electrophoresis or chromatography. The TLC was performed in conventional glass tanks. The indoles were located by spraying the plates with a 1 % solution of *p*-dimethylaminocinnamaldehyde in concentrated HCl (w/v). The reagent was always prepared just before use and diluted with ethanol in 1:4 proportion (v/v) before spraying.

For TLE the 20 × 20 cm plate was spotted with the standard indole compounds and the plate was sprayed very gently with the buffer solution. It is extremely important that excess of buffer on the plate be avoided because it may result in the displacement of the original spot samples besides destroying the uniformity of the gel layer. The cathode (−) and anode (+) edges of the plate were connected with the buffer solution in the electrophoresis chamber by means of paper strips wetted with buffer. To avoid condensation, cold water was run in the jacket around the electrophoresis chamber. This was absolutely essential if the separation time was over 60 min and the voltage in use was high. Cold water flow also keeps the temperature of the buffer from rising very high which could otherwise result in the concentration of the buffer solution and thus a change in pH. Immediately after the required time, plates were taken out of the chamber and dried in a fumehood. If TLC were to be performed, the dried plate was put in a glass tank having an appropriate solvent. Otherwise the plate was immediately sprayed with the chromogenic reagent, dried, and then heated at 100° for 5–10 min to develop the spots; heating was not always necessary.

Results and discussion

The electrophoretic mobilities of the test substances under various combinations of current strength and buffer solutions are reported in Tables I and II. Electrophoretic mobility is expressed in arbitrary units 10^{-6} $g^{\frac{1}{2}}$ $cm^{-1\frac{1}{2}}$ by calculating from the following formula[9]:

$$U = dl/te$$

where

U is the mobility in 10^{-6} $g^{\frac{1}{2}}$ $cm^{-1\frac{1}{2}}$
l is the length of electrophorogram in cm
d is the distance travelled by the test substance in cm
t is the time in h
e is the current in V.

The movement of the compounds towards the cathode or the anode is expressed by the use of a + or − sign before the figures for electrophoretic mobility in Tables I and II.

The movement of neutral and basic indoles was very rapid in all the buffer solutions used and under all the voltages applied. However, a high voltage (500 V) acting over a small time (1 h) gave the best results. Both T and 5-HT moved very rapidly; at lower pH T moved faster than 5-HT. This behaviour was reversed when borate buffer of pH 10.0 or 12.4 was used. Similarly IAN showed a faster migration rate at higher pH and high voltage. The best separation of TP and 5-HTP took place only with borate buffer (pH 12.4) at 500 V (Fig. 1). For the separation of a mixture of nine common and important indole derivatives a system employing acetate buffer

TABLE I

ELECTROPHORETIC MOBILITES OF INDOLES AT DIFFERENT VOLTAGES[a]

A: acetate buffer, pH 5.2; current, 110 V; time, 5 h. B: pyridine–acetic acid, pH 6.1; current, 440 V; time, 1,5 h. C: acetate buffer, pH 6.0; current, 220 V; time, 3 h. D: acetate buffer, pH 6.2; current, 220 V; time, 3 h. E: pyridine–acetic acid, pH 6.1; current, 220 V; time, 3 h. F: borate buffer, pH 10.0; current, 220 V; time, 3 h.

Indoles	*Electrophoretic systems*					
	A	*B*	*C*	*D*	*E*	*F*
IAA	+0.10	+0.10	+0.07	+0.07	+0.15	+0.08
IAN	−0.05	−0.04	−0.05	−0.05	−0.03	−0.12
IBA	+0.06	+0.05	+0.03	+0.04	+0.12	+0.04
TP	−0.05	−0.01	−0.07	−0.07	−0.05	−0.11
T	−0.24	−0.20	−0.20	−0.23	−0.19	−0.21
5-HT	−0.29	−0.17	−0.23	−0.25	−0.20	−0.24
5-HTP		−0.02	−0.08	−0.07	−0.07	−0.12

[a] The electrophoretic mobility is expressed in arbitrary units; for details see text.

TABLE II

ELECTROPHORETIC MOBILITY OF INDOLES AT 500 V[a]

A: formic acid-acetic acid, pH 2.0. B: pyridine-acetic acid, pH 6.1. C: borate, pH 10.0. D: borate, pH 12.4. Time, 1 h.

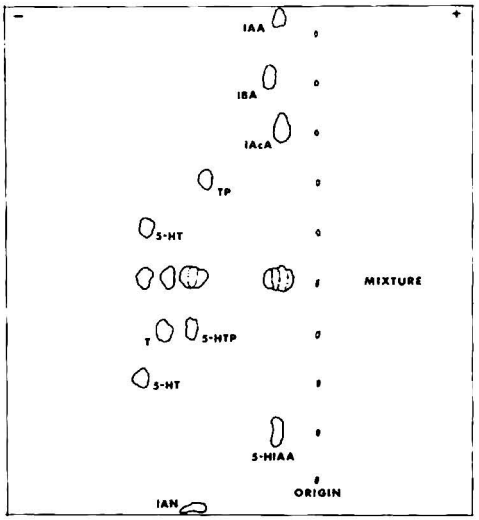
Indoles	*Buffer systems*			
	A	*B*	*C*	*D*
IAA	−0.04	+0.11	+0.04	−0.06
IAN	−0.05	−0.10	−0.16	−0.19
IBA	−0.06	+0.09	+0.02	−0.08
IAcA	−0.04	+0.08	+0.03	−0.06
5-HIAA	−0.06	+0.10	+0.01	−0.07
ILA		+0.06		
IGA		+0.12		
TP	−0.13	−0.09	−0.15	−0.18
5-FTP		−0.06		
5-HTP	−0.12	−0.08	−0.16	−0.20
T	−0.24	−0.25	−0.22	−0.24
5-HT	−0.19	−0.28	−0.28	−0.28

[a] Electrophoretic mobility is expressed in arbitrary units; for details see text.

(pH 6.0) was found to be the most suitable (Fig. 2). The time required for separation was 3 h because the movement of IAA and IBA was very slow.

The separation of indolic acids was not very clear in any of the buffer solutions and current strength described for basic or neutral compounds. The electrophoretic mobility did not give sufficient data to warrant identification, except for the colour of the spots produced by the chromogenic reagent. Only pyridine–acetic acid buffer (pH 6.1) was found useful for the separation of IAA, IBA, IAcA and 5-HIAA at a voltage of 500 V. Both IAA and IBA could also be separated easily by the use of acetate buffer (pH 6.0) and a 220 V current. Borate buffer of any strength was not useful for separating acidic indole compounds.

During the later stages of this work, a combination of the electrophoretic and the chromatographic technique proved to be the best procedure for separating acidic



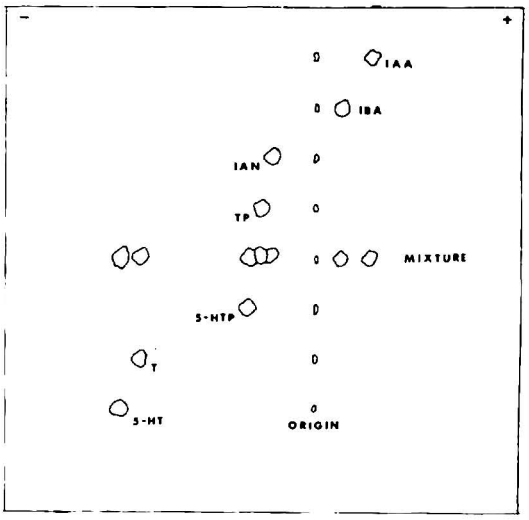


Fig. 1. Thin-layer electropherogram of indole derivatives in borate buffer, pH 12.4. Time, 1 h.

Fig. 2. Thin-layer electropherogram of indole derivatives in acetate buffer, pH 6.0. Time required to separate the components of the mixture was 3 h.

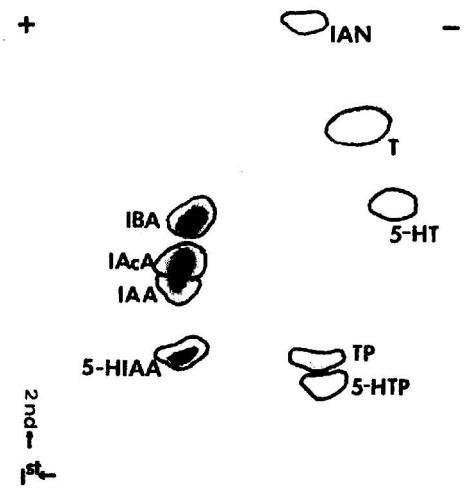


Fig. 3. A mixture of nine indoles was subjected to electrophoresis in the 1st direction using borate buffer pH 12.4; time, 1 h. Chromatography was performed in the 2nd direction using isopranol–ammonia–water (8:1:1) as the solvent system.

indoles. A mixture of nine indoles was subject to electrophoresis using borate buffer (pH 12.4) and a current of 500 V. After drying the plate, it was chromatographed in a second direction using isopropanol–ammonia–water (8:1:1, v/v) as the solvent (Fig. 3). The separation of all the components of the mixture was quite good and easily reproducible. Therefore, for acidic indoles a combined electrophoresis and chromato-

graphic technique is much more useful, while for basic and neutral indoles either technique could be used without much problem.

It is hoped that this TLE procedure may be of help to researchers in the field of biogenic amines (especially 5-HT) besides being of some value for those working on the metabolism of TP or IAA, where often it is necessary to separate a mixture of indolic substances. It would be advisable to extend the TLE procedure to a study of more compounds; moreover, a study of the use of different adsorbents could yield valuable results.

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Electrophoresis of human salivary secretions at acid pH

Electrophoretic methods have been extensively used in investigations concerning the protein composition of human salivary secretions. Thus, paper[1,2], polyacrylamide[3,4], cellulose acetate[5], paper curtain[6,7], immunoelectrophoresis[8], and more recently iso-electric focusing[9] have been shown to be valuable tools in the separation and identification of proteins from parotid and submandibular, as well as mixed saliva. An excellent review on the subject has been published[10]. Polyacrylamide electrophoresis has also been utilized in an attempt to determine abnormal protein patterns in certain pathological conditions such as cystic fibrosis[11] and osteoporosis[12]. All of the above-mentioned studies have been carried out at alkaline pH, although in one case[3], an attempt to separate the cathodal components of submaxillary saliva was made by reversing the electrodes, a theoretically objectionable procedure. Thus, although the behavior of human salivary proteins in electrophoresis above pH 7 has been studied extensively, their behavior under acid conditions has not yet been investigated and is therefore poorly understood. This report describes the use of acidic, 6 *M* urea polyacrylamide gels for the separation of parotid and mixed human saliva.

Materials and methods

Human salivary secretions. Human parotid fluid was collected from healthy volunteers using a modified stainless-steel Carlson Crittenden vacuum cup[13] at a flow rate of 1.00 ml min with a mean protein concentration of 2.00 mg ml. Mixed human saliva was obtained from healthy laboratory personnel in a cold room (2°) by expectoration into a vessel containing thymol crystals in order to inhibit bacterial growth. All samples were stored at 2° and processed no later than 2 h after collection. The saliva was then cleared by centrifugation at 48,200 × *g* for 30 min and the supernatant fluid lyophilized and stored at −40° until used.

Sample preparation. Samples were prepared by dissolving a given amount of lyophilized saliva (50–100 mg) in 1.0 ml of 0.1 *M* Tris–citric acid buffer, pH 3.8 containing urea in 4 *M* concentration; insoluble proteins were then removed by centrifugation (27,000 × *g* 30 min, 2°) and the supernatant fluid utilized for the electrophoretic analysis. It was found necessary to use only freshly dissolved samples as further precipitation occurred, especially with parotid fluid, during prolonged storage (20 h or more).

Electrophoresis. Electrophoresis in acidic, 6 *M* urea polyacrylamide gels was carried out as previously described[14], but modified in order to obtain the following experimental conditions: voltage, 250 V (constant); current, 100 mA; pre-run, 4.0 h; electrolyte, 0.37 *M* glycine–citric acid buffer, pH 2.0; load, 25–75 μl (approximately 0.90 mg protein); separations were carried out for 5–6 h at room temperature but coolant (4°) was circulated through the cooling plates at all times. In all instances a 14.0% Cyanogum-41 gel was used.

The gels were stained for 24 h with Amido Schwarz and destained electrophoretically (40 min) with a mixture of methanol–acetic acid–water (5:1:5).

Results and discussion

Excellent resolution of human mixed (20 zones)and parotid (17 zones) saliva can be achieved under the present conditions of electrophoresis (Fig. 1); furthermore, there is no problem in identifying the origin of each particular secretion, mainly because parotid and whole saliva differ drastically not only in the number but also in the concentrations of the basic components migrating faster than lysozyme.

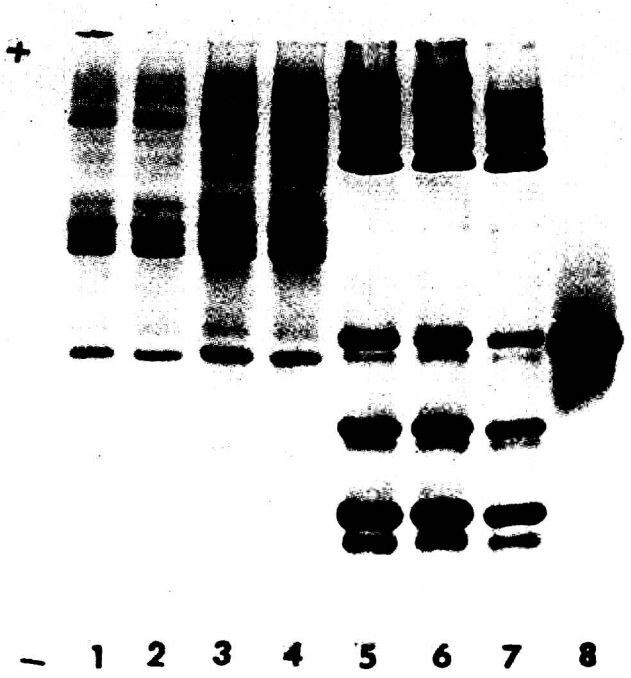


Fig. 1. Polyacrylamide electrophoresis of human salivary secretions at acid pH in the presence of 6 *M* urea. Channels 1 and 3 duplicate samples (25 and 50 μl) of whole human saliva from a 28-year-old normal male; channels 2 and 4 (25 and 50 μl) of whole saliva from a 26-year-old normal female. Channels 5, 6 and 7 parotid fluid from three different normal individuals. Channel 8, lysozyme marker.

It is of great interest to note that the lysozyme zone (see arrow, Fig. 2) can also be used to differentiate mixed from parotid saliva. Two zones, differing in concentration, are seen to migrate at the level of hen's egg white lysozyme. In mixed saliva, the band appearing in greater concentration has a mobility identical to lysozyme, while in parotid fluid the same zone has a slightly lower mobility. The reverse is true for the less concentrated protein band. At this time no explanation can be given for this phenomenon, but it is likely that these bands represent two distinct salivary lysozymes. MILLIN AND SMITH[15] reported the existence of three chromatographically

distinct amylases in human saliva. We have obtained identical results using parotid lysozyme isolated by the method of BALEKJIAN *et al.*[16] as a standard in lieu of hen's egg white lysozyme. With the exception of lysozyme[16] little is known about the basic proteins in human salivary secretion. It is of interest to note, in this regard, the presence of several very basic proteins in both parotid and mixed saliva which are seen to migrate at faster rates than lysozyme (see Fig. 1). These basic proteins constitute less than 1% (0.86) of the total protein in parotid fluid and are characterized[17] by having isoelectric points above pH 11.0 (range, 11.0–12.6) and very small molecular weights (range, 3256–8500) and have been partially purified by adsorption chromatography on highly cross-linked polyacrylamide gel[18], dialysis and ion-exchange chromatography on carboxymethyl cellulose. The human parotid basic proteins are significant from an evolutionary standpoint since similar proteins have been isolated from the venom gland secretion of several species of North American Crotalid snakes[19].

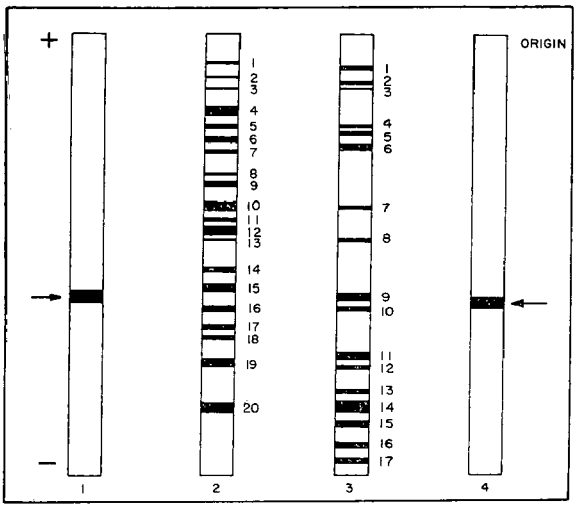


Fig. 2. Schematic representation for the electrophoretic separation of human salivary secretions under the conditions described in the text. Channels 1 and 4 represent the lysozyme marker (arrows). Channel 2, location of 20 protein zones in whole saliva, five of which (15–20) lose most of their intensity upon destaining. Channel 3, location of 17 protein zones in parotid fluid, two of which (16 and 17) are lost during extensive destaining. The bands with lysozyme-like mobility referred to in the text correspond to 14 and 15 in whole saliva and 9 and 10 in parotid fluid. Bands 13–17 in parotid fluid (channel 3) constitute the small-molecular-weight basic proteins described in the text.

Since some of the weaker components are lost when transcription of the gel pattern to the photograph is made, a schematic representation of the electrophoretic separation is shown in Fig. 2. In summary, as shown in Figs. 1 and 2, polyacrylamide electrophoresis in acidic 6 *M* urea can become a very useful tool for the study of the basic proteins and polypeptides in human salivary gland secretions.

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Elution peak positions of linear phosphates in gradient elution chromatography with an anion-exchange resin

Gradient elution chromatography has been used with good results for the separation of a series of similar ionic species such as linear phosphates[1-4] with an ion exchanger. An apparatus suitable for gradient elution has a constant-volume mixing bottle which delivers an eluent whose concentration follows the equation

$$C = C_r - (C_r - C_0) \cdot \exp(-V/V_m) \quad (1)$$

where C denotes the concentration of the eluent when V ml of the eluent has flowed from the mixing bottle, C_0 the initial concentration of the eluent in the mixing bottle, C_r the concentration of the eluent in a reservoir, and V_m the constant volume of the eluent in the mixing bottle.

In previous papers[5,6] some equations have been proposed for calculating where an elution peak will appear in such a gradient elution. The purpose of this paper is to compare the elution peak positions of linear phosphates obtained experimentally with those predicted from the equations.

The abbreviated notations, P_1, P_2, P_3, etc. are used in this paper for ortho-, di-, triphosphate, etc.

Experimental

Resin. An anion-exchange resin, Dowex 1-X4 (100–200 mesh), in the chloride form, was dried at 60° in a vacuum desiccator for 20 h. The conversion factor between the resin bed volume and resin gravity was found to be 3.3 ml/g.

Reagents. The phosphates employed in this investigation were as follows: potassium dihydrogen orthophosphate, KH_2PO_4, tetrasodium diphosphate decahydrate, $Na_4P_2O_7 \cdot 10H_2O$, pentasodium triphosphate hexahydrate, $Na_5P_3O_{10} \cdot 6H_2O$ and glassy sodium polyphosphate with an average degree of polymerization, $\bar{p}$ of 10. Potassium chloride solutions adjusted to pH 9.3 with an ammonia–ammonium chloride buffer were used as eluents. The concentration of ammonium ion in the eluents was 0.01 M.

Gradient elution. The apparatus shown in Fig. 1 was devised for the separation of linear phosphates. Reservoir 2 was inserted between reservoir 1 and the mixing bottle in order to keep the liquid level constant. A small hole, G in Fig. 1, plays the role of regulator for the liquid level. For the purpose of reference a typical chromatogram for a series of linear phosphates is illustrated in Fig. 2.

Although a borate buffer was used for the elution shown in Fig. 2, an ammonia–ammonium chloride buffer was employed in the present case in order to simplify the system of ionic species in an eluent.

C_0 and C_r were chosen as 0.200 and 0.500 M, respectively. For these values of C_0 and C_r, the parameter α is 0.600.

$$\alpha = (C_r - C_0)/C_r \quad (2)$$

As was mentioned in the previous paper[6], when a value of α is simple such as 0.500, 0.600 and 0.700 the calculation of the elution peak positions can be easily

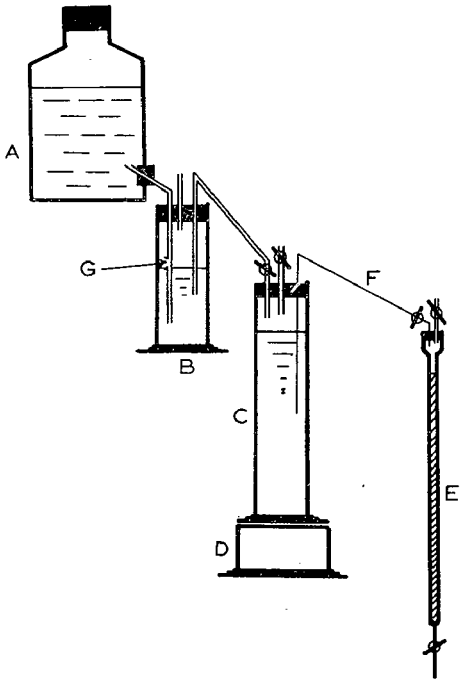


Fig. 1. Apparatus for gradient elution. A = reservoir 1, 2000-ml bottle; B = reservoir 2, 250-ml measuring cylinder; C = mixing bottle, 1000-ml measuring cylinder; D = magnetic stirrer; E = column; F = Teflon tube, I.D. 1 mm; G = a small hole.

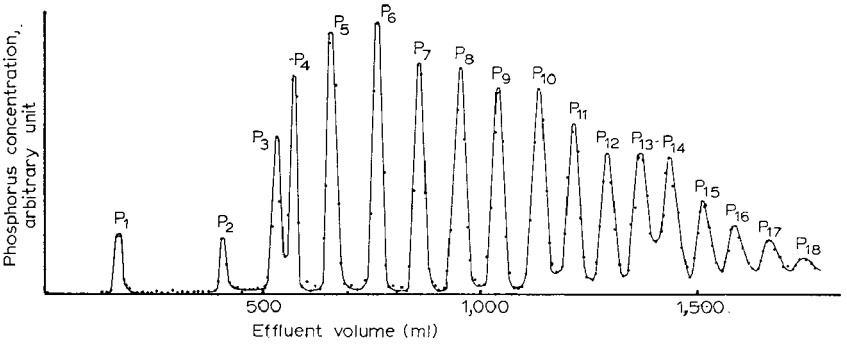


Fig. 2. Elution curve of linear phosphates. Sample: a mixture of orthophosphate, diphosphate and glassy sodium polyphosphate with a $\bar{p}$ of 10. Total amount of the phosphates loaded: 18 mg P. Resin: Dowex 1-X4 (100–200 mesh), Cl^- form. Bed size: 1.2 × 69 cm. Eluent: KCl solution, pH 8.0, buffered with borate. $C_0 = 0.2\ M$; $C_r = 0.45\ M$; $V_m = 1000$ ml. Flow rate: 0.3 ml/min. Temperature: room temp.

carried out. V_m was kept constant at 1,000 ml. The bed volume of the resin, S, was 87.8 ml, and the interstitial volume of the resin bed was 41.4 ml. A phosphate mixture was eluted into 8.7 ml fractions at a flow rate of 0.56 ml/min at 21 ± 1°. Phosphate concentrations in the fractions were determined colorimetrically. Chloride concentrations in the effluent were determined titrimetrically.

Determination of distribution ratios. Potassium dihydrogen orthophosphate, tetrasodium diphosphate decahydrate and pentasodium triphosphate hexahydrate were used as the linear phosphates with degrees of polymerization, p of 1, 2 and 3. Linear phosphates with p 4 to 9 were obtained from a glassy sodium polyphosphate with a $\bar{p}$ of 10 by means of the gradient elution chromatography described above.

Dry resin (0.5–1.0 g) and 15 or 20 ml of a 0.1–0.5 M potassium chloride solution adjusted to pH 9.3 with an ammonia–ammonium chloride buffer were allowed to stand in a stoppered erlenmeyer flask overnight. 5 ml of phosphate solution (0.1–0.2 mg as P) was then added to the flask, the mixture was shaken mechanically for about 1 h at 21 ± 1°. The phosphorus content in the solution or in the resin was determined colorimetrically. The distribution ratio, D, is calculated as follows

$$D = \frac{\text{mmoles of phosphorus per ml resin phase}}{\text{mmoles of phosphorus per ml solution}} \quad (3)$$

Results and discussion

A mixture of potassium dihydrogen orthophosphate, tetrasodium diphosphate and glassy sodium polyphosphate with $\bar{p}$ of 10 was loaded onto a resin bed and separated by the method described above. The elution curve thus obtained is shown in Fig. 3. It is evident that the linear phosphates with p 4 to 9 were almost completely separated from each other. These isolated linear phosphates were immediately employed for the measurement of their distribution ratios.

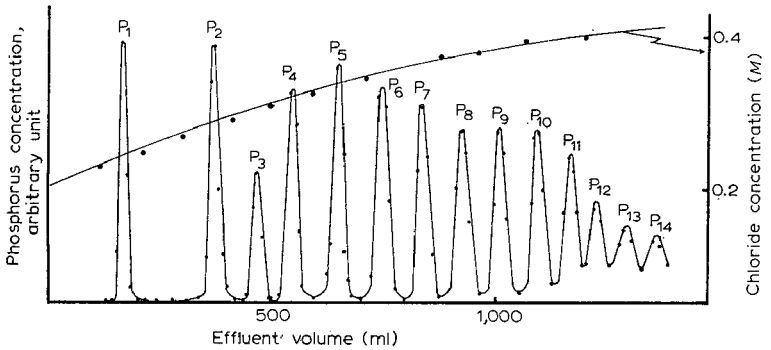


Fig. 3. Elution curve of linear phosphates. Sample: a mixture of orthophosphate, diphosphate and glassy sodium polyphosphate with a $\bar{p}$ of 10. Total amount of the phosphates loaded: 6 mg P. Resin: Dowex 1-X4 (100–200 mesh), Cl^- form. Bed size: 1.3 × 66.2 cm. Eluent: KCl solution, pH 9.3 buffered with NH_3 and NH_4Cl. C_0 = 0.200 M; C_r = 0.500 M; V_m = 1000 ml. Flow rate: 0.56 ml/min. Temperature: 21 ± 1°. Concentration of chloride ion: observed •, calculated —.

In the separate chromatographic run, it was demonstrated that the elution peaks of tetrameta- and trimetaphosphate appear at 1122 and 1383 ml of the effluent, respectively. Since a glassy sodium polyphosphate contains, in general, small amounts of trimeta- and tetrametaphosphate, the fractions containing the linear phosphates with p 10 to 14 may be contaminated with tri- and tetrametaphosphate.

It was verified experimentally that there is a linear relationship between log D and log C for each member of the linear phosphates with p 1 to 9.

$$\log D \propto -n \log C \quad (4)$$

As examples, the distribution ratios of the linear phosphates with p of 2, 5 and 9 at various concentrations of chloride ion are shown in Fig. 4. n in eqn. 4 can be obtained as the slope from such a diagram.

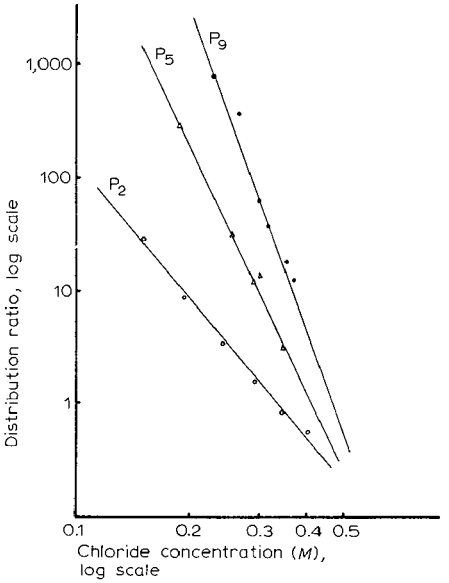


Fig. 4. Distribution ratios of P_2, P_5 and P_9 at various concentrations of chloride ion.

According to the method described in the previous paper[6], the elution peak position, $V_{max.}$, can be calculated from the following equation

$$y = x - f_n(\alpha) + f_n(\alpha e^{-x}) \quad (5)$$

where $y = D_r S/V_m$ and $x = V_{max.}/V_m \cdot D_r$ is the distribution ratio at C_r and S is the volume of the resin bed. The relationships between x and y when $\alpha = 0.6$ and $n = 0$–6

TABLE I

CALCULATED AND OBSERVED VALUES OF V_{max}

Phosphate species	n	D_r	y	$V_{max.}$		
				Calculated (ml)	*Observed (ml)*	*Error (%)*
P_1	2.8	0.092	0.008	151	165	+9.3
P_2	4.1	0.22	0.019	371	374	+0.8
P_3	5.2	0.21	0.018	481	470	−2.3
P_4	5.9	0.19	0.017	566	548	−3.2
P_5	6.9	0.22	0.019	711	653	−8.2
P_6	7.5	0.22	0.019	776	748	−3.6
P_7	8.5	0.25	0.022	914	844	−7.9
P_8	9.5	0.24	0.021	986	931	−5.6
P_9	10.2	0.26	0.023	1078	1018	−5.6

are given graphically in the previous paper[6]. When n is larger than 6 the relationship between x and y can be calculated in a similar way.

Since the values of α, n, D_r, S and V_m are known, one can obtain a value of V_{max}. The values of $V_{max.}$ should be corrected by adding the interstitial volume of the resin bed. The results obtained are summarized in Table I. The observed values are in rough accordance with the calculated values. It can be said that elution peak positions of linear phosphates in gradient elution chromatography with an anion-exchange resin are predicted by the method previously described.

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News

New Apparatus

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CHROM. 4774

CONTRIBUTION TO THE THEORY OF THE RETENTION INDEX SYSTEM

II. ERRORS ON THE DETERMINATION OF THE RETENTION INDICES: THE ERDEY EQUATION

IN MEMORIAM PROF. L. ERDEY*

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SUMMARY

The increasing importance of the retention index system has stimulated the authors to investigate some of the problems in the field. On the basis of retention indices as defined by KOVÁTS and general methods of error calculation, the error of the determination of the retention indices under conditions of classical gas chromatography (T, F, p_i and j are all constants), and the effects of various factors on the error are discussed. An equation is presented for the calculation of the error. This equation was named after ERDEY. The results of the calculations support practical experience in many cases.

INTRODUCTION

The increasing importance of the retention index system has been pointed out in previous papers[1,2], as well as its role in qualitative analysis and other fields of chemistry. In the present paper one of the most important questions of the application of retention indices in qualitative analysis, namely the accuracy of the determination of retention indices, is discussed. Although this problem has already been successfully studied by several workers, no relationship has been deduced as yet to the calculation of the error of the index determination. Experience has shown that the error inherent in index determination first of all depends on the carbon number of the reference hydrocarbons. This, however, has not given any information on the size of errors. Realizing the importance of the calculation of error of index determinations and its dependence on various factors the authors have made extensive studies in this field.

* This is one of the last works of Prof. L. ERDEY of the Institute for General and Analytical Chemistry, as he died on February 21st, 1970. The equation giving the error of the determination of the retention indices (eqn. 9) has been named after ERDEY.

THEORY

In one of his recent papers KOVÁTS defined the retention index as follows[3]:

$$I^{\text{stationary phase}}_{\text{substance}}(T) = 100\left[\frac{\log t_R'(x) - \log t_R'(z)}{\log t_R'(z+1) - \log t_R'(z)} + z\right] \quad (1)$$

where:

I = retention index under the conditions of classical gas chromatography (T, F, p_i and j are all constants) (the index unit)
T = temperature of column (°K)
F = flow rate of carrier gas (ml/min)
p_i = inlet pressure of carrier gas (kp/cm²)
j = compressibility factor according to James–Martin
$t_R' = t_R - t_M$ = reduced retention time (min)
t_R = retention time (min)
t_M = time of passage of the inert substance through the column (min)
x = symbol for the unknown substance
z and $z+1$ = symbols for normal paraffins with carbon number z and $z+1$, respectively
z = carbon number

As is familiar, the error of an equation describing a given process can be obtained by summing up the corresponding partial derivatives. The error of the determination of the index (E_I) can be given in a general form as follows:

$$E_I = \left[\left|\frac{\partial I}{\partial \bar{t}_R(x)}\right| \cdot \Delta t_R(x) + \left|\frac{\partial I}{\partial \bar{t}_R(z)}\right| \cdot \Delta t_R(z) + \left|\frac{\partial I}{\partial \bar{t}_R(z+1)}\right| \cdot \Delta t_R(z+1) + \left|\frac{\partial I}{\partial \bar{t}_M}\right| \cdot \Delta t_M\right] \cdot 100 \quad (2)$$

where:

E_I = total error of the index determination (index unit) under the conditions of classical gas chromatography

$$\Delta t_R(i) = \frac{|t_{R,1}(i) - \bar{t}_R(i)| + |t_{R,2}(i) - \bar{t}_R(i)| + \ldots\ldots + |t_{R,s}(i) - \bar{t}_R(i)|}{n} \quad (3)$$

$$\bar{t}_R(i) = \frac{t_{R,1} + t_{R,2} + \ldots\ldots + t_{R,s}}{n} \quad (4)$$

$\bar{t}_R(i)$ = average retention time (min)
i = symbol for x, z and $z+1$
s = serial number
n = number of parallel measurements; in this case $n = 11$.

Forming the corresponding partial derivatives of eqn. (2) the following relationships were obtained:

$$\left|\frac{\partial I}{\partial \bar{t}_R(x)}\right| \cdot \Delta t_R(x) = \left|\frac{1}{(\bar{t}_R(x) - \bar{t}_M) \cdot \log\left(\frac{\bar{t}_R(z+1) - \bar{t}_M}{\bar{t}_R(z) - \bar{t}_M}\right) \cdot 2.30258}\right| \cdot \Delta t_R(x) \quad (5)$$

$$\left|\frac{\partial I}{\partial \bar{t}_R(z)}\right| \cdot \Delta t_R(z) = \left|\frac{\log\left(\frac{\bar{t}_R(z+1) - \bar{t}_M}{\bar{t}_R(x) - \bar{t}_M}\right)}{(\bar{t}_M - \bar{t}_R(z)) \cdot \log^2\left(\frac{\bar{t}_R(z+1) - \bar{t}_M}{\bar{t}_R(z) - \bar{t}_M}\right) \cdot 2.30258}\right| \cdot \Delta t_R(z) \quad (6)$$

$$\left|\frac{\partial I}{\partial \bar{t}_R(z+1)}\right| \cdot \Delta t_R(z+1) = \left| \frac{\log\left(\frac{\bar{t}_R(x)-\bar{t}_M}{\bar{t}_R(z)-\bar{t}_M}\right)}{(\bar{t}_M-\bar{t}_R(z+1))\,\log^2\left(\frac{\bar{t}_R(z+1)-\bar{t}_M}{\bar{t}_R(z)-\bar{t}_M}\right)\cdot 2.30258} \right| \cdot \Delta t_R(z+1) \quad (7)$$

$$\left|\frac{\partial I}{\partial \bar{t}_M}\right| \cdot \Delta t_M =$$

$$= \left| \frac{\left(\frac{\bar{t}_R(x)-\bar{t}_R(z)}{(\bar{t}_R(x)-\bar{t}_M)\cdot(\bar{t}_R(z)-\bar{t}_M)}\right)\cdot\log\left(\frac{\bar{t}_R(z+1)-\bar{t}_M}{\bar{t}_R(z)-\bar{t}_M}\right) - \frac{\bar{t}_R(z+1)-\bar{t}_R(z)}{(\bar{t}_R(z)-\bar{t}_M)\cdot(\bar{t}_R(z+1)-\bar{t}_M)}\cdot\log\frac{\bar{t}_R(x)-\bar{t}_M}{\bar{t}_R(z)-\bar{t}_M}}{\log^2\left(\frac{\bar{t}_R(z+1)-\bar{t}_M}{\bar{t}_R(z)-\bar{t}_M}\right)\cdot 2.30258} \right| \cdot \Delta t_M \quad (8)$$

Eqns. 5–8 not only permit the exact calculation of the error of the index determination but also the study of the effect of various factors on the error.

Then, substituting the terms from eqns. 5–8 into eqn. 2:

$$E_I = \left\{ \left| \frac{1}{(\bar{t}_R(x)-\bar{t}_M)\cdot\log\left(\frac{\bar{t}_R(z+1)-\bar{t}_M}{\bar{t}_R(z)-\bar{t}_M}\right)\cdot 2.30258} \right| \cdot \Delta t_R(x) + \left| \frac{\log\left(\frac{\bar{t}_R(z+1)-\bar{t}_M}{\bar{t}_R(x)-\bar{t}_M}\right)}{(\bar{t}_M-\bar{t}_R(z))\cdot\log^2\left(\frac{\bar{t}_R(z+1)-\bar{t}_M}{\bar{t}_R(z)-\bar{t}_M}\right)\cdot 2.30258} \right| \cdot \Delta t_R(z) + \right.$$

$$+ \left| \frac{\log\left(\frac{\bar{t}_R(x)-\bar{t}_M}{\bar{t}_R(z)-\bar{t}_M}\right)}{(\bar{t}_M-\bar{t}_R(z+1))\,\log^2\left(\frac{\bar{t}_R(z+1)-\bar{t}_M}{\bar{t}_R(z)-\bar{t}_M}\right)\cdot 2.30258} \right| \cdot \Delta t_R(z+1) +$$

$$\left. + \left| \frac{\left(\frac{\bar{t}_R(x)-\bar{t}_R(z)}{(\bar{t}_R(x)-\bar{t}_M)\cdot(\bar{t}_R(z)-\bar{t}_M)}\right)\cdot\log\left(\frac{\bar{t}_R(z+1)-\bar{t}_M}{\bar{t}_R(z)-\bar{t}_M}\right) - \frac{\bar{t}_R(z+1)-\bar{t}_R(z)}{(\bar{t}_R(z)-\bar{t}_M)\cdot(\bar{t}_R(z+1)-\bar{t}_M)}\cdot\log\left(\frac{\bar{t}_R(x)-\bar{t}_M}{\bar{t}_R(z)-\bar{t}_M}\right)}{\log^2\left(\frac{\bar{t}_R(z+1)-\bar{t}_M}{\bar{t}_R(z)-\bar{t}_M}\right)\cdot 2.30258} \right| \cdot \Delta\bar{t}_M \right\} \cdot 100 \quad (9)$$

This equation giving the error of the determination of the retention indices (eqn. 9) was named after ERDEY.

EXPERIMENTAL

Extensive experimental studies were made, by means of Carlo Erba Fractovap Model C, D, GD, GV and GI-452 gas chromatographs. As an example, calculations made with data obtained under the conditions given in Table I will be presented.

Experiments were repeated under the same conditions by a moderately skilled technician, to estimate the standard deviation. The number of replicate measurements was reduced to 5, since in practice this is more usual than the eleven used in the course of our investigations. The analysis of the errors of the determinations of the index and comparison of the results have shown that the skill of the gas chromatograph operator and the number of measurements only influences the value of $\Delta\, t(i)$, sometimes quite remarkably, but the reproducibility of the retention

TABLE I

GAS CHROMATOGRAPHIC PARAMETERS USED IN DETERMINING THE DATA FOR CALCULATION

Gas chromatograph: Carlo Erba Fractovap Model D
Detector: FID
Sensitivity: 16 × 100
Sample introduction: 1.0 μl gas by means of a Hamilton syringe, and 1.5 ml gas by semi-automatic introduction, respectively
Column: aluminium spiral 3.0 m length and 2.0 mm I.D.
Packing: 10.0 wt% squalane on Chromosorb W (60–80 mesh)
Carrier gas: nitrogen
Inlet pressure of carrier gas: 1.86 kp/cm^2
Auxiliary gases: hydrogen, inlet pressure: 1.27 kp/cm^2; oxygen, inlet pressure: 1.74 kp/cm^2
Temperature of column: 70.0 ± 0.1°
Temperature of evaporator: 210.0 ± 1.0°
Recorder: Speedomax G; 2.5 mV; 1.0 sec
Chart speed: 5.08 cm/min

times, *i.e.* stability of the operation of the gas chromatograph, is the most important factor in determining the error.

From practical considerations, three model compounds were chosen: ethene, which produced a peak between those of methane and ethane; cyclobutane, eluted between *n*-C_4 and *n*-C_5; and styrene, the gas chromatographic peak of which appeared between those of *n*-C_8 and *n*-C_9.

The time to pass through the chromatograph was calculated by mathematical

TABLE II

RETENTION DATA DETERMINED UNDER CONDITIONS GIVEN IN TABLE I AND USED FOR THE ERROR CALCULATIONS

Component	*Average retention time (sec)*	*Value of* $\Delta t_R(i)$ *(sec)*
Methane	28.3	0.26
Ethene	29.1	0.26
Ethane	29.9	0.28
n-Butane	43.0	0.45
Cyclobutane	53.0	0.55
n-Pentane	67.8	0.68
n-Octane	637.8	3.20
Styrene	1070.0	5.40
n-Nonane	1520.7	7.60
t_M	26.4	0.25

means on the basis of data published earlier[4]. Retention data used for the calculations are summarized in Table II.

As an example, the calculations concerning styrene will be presented here. The fraction of error of the determination of the retention index due to the error of the determination of t_M can be calculated by means of eqn. 8:

$$\left|\frac{\partial I}{\partial \bar{t}_M}\right| \cdot \Delta \bar{t}_M =$$

$$= \left| \frac{\frac{1070.0-637.8}{(1070.0-26.4)\cdot(637.8-26.4)} \log\left(\frac{1520.7-26.4}{637.8-26.4}\right) - \frac{1520.7-637.8}{(637.8-26.4)\cdot(1520.7-26.4)} \cdot \log\left(\frac{1070.0-26.4}{637.8-26.4}\right)}{\log^2\left(\frac{1520.7-26.4}{637.8-26.4}\right)\cdot 2.30258} \right| \cdot 0.25 =$$

$$= \left| \frac{\frac{432.2}{1043.6\cdot 611.4}\cdot 0.38811 - \frac{882.9}{611.4\cdot 1494.3}\cdot 0.23198}{0.15063\cdot 2.30258} \right| \cdot 0.25 = \left| \frac{0.00387\cdot 0.25}{0.3469} \right| = 0.00003 \text{ index units} \quad (10)$$

The fraction of error due to the error in the determination of the retention time of the hydrocarbon preceding the component in question can be calculated by means of eqn. 6:

$$\left|\frac{\partial I}{\partial \bar{t}_{R(z)}}\right| \cdot \Delta t_{R(z)} = \left| \frac{\log\left(\frac{1520.7-26.4}{1070.0-26.4}\right)}{(26.4-637.8)\cdot \log^2\left(\frac{1520.7-26.4}{637.8-26.4}\right)\cdot 2.30258} \right| \cdot 3.2 = \left| \frac{\log\left(\frac{1494.3}{1043.6}\right)}{(-611.4)\cdot \log^2(2.44406)\cdot 2.30258} \right| \cdot 3.2 =$$

$$= \left| \frac{0.15588\cdot 3.2}{(-611.4)\cdot 0.3469} \right| = \left| \frac{0.4988}{212.09} \right| = 0.24\cdot 10^{-2} \text{ index units} \quad (11)$$

The fraction of error due to the error of the determination of the retention data of the hydrocarbon, in this case *n*-nonane, following the component in question can be calculated according to eqn. 7:

$$\left|\frac{\partial I}{\partial \bar{t}_{R(z+1)}}\right| \cdot \Delta t_{R(z+1)} = \left| \frac{\log\left(\frac{1070.0-26.4}{637.8-26.4}\right)}{(26.4-1520.7)\cdot \log^2\left(\frac{1520.7-26.4}{637.8-26.4}\right)\cdot 2.30258} \right| \cdot 7.6 =$$

$$= \left| \frac{\log\frac{1043.6}{611.4}}{(-1494.3)\cdot \log^2(2.44406)\cdot 2.30258} \right| \cdot 7.6 = \left| \frac{0.23198\cdot 7.6}{(-518.37)} \right| = 0.34\cdot 10^{-2} \text{ index units} \quad (12)$$

Finally, the error contribution due to the inaccuracy of the determination of the retention time of the component in question is as follows, according to eqn. 5:

$$\left|\frac{\partial I}{\partial \bar{t}_{R(x)}}\right| \cdot \Delta t_{R(x)} = \left| \frac{1}{(1070.0-26.4)\cdot \log\left(\frac{1520.7-26.4}{637.8-26.4}\right)\cdot 2.30258} \right| \cdot 5.4 =$$

$$= \left| \frac{5.4}{1043.6\cdot 0.38811\cdot 2.30258} \right| = \left| \frac{5.4}{932.79} \right| = 0.58\cdot 10^{-2} \text{ index units} \quad (13)$$

The total error is the sum of the error contributions:

$$E_I \begin{matrix}\text{squalane}\\ \text{styrene}\end{matrix} (70.0°C) = 0.24 + 0.34 + 0.58 + 0.00 = 1.2 \text{ index units}$$

TABLE III

CONTRIBUTIONS TO THE ERROR OBTAINED UNDER THE CONDITIONS GIVEN IN TABLE I

Component	*Error (index unit) caused by*				*Total error (index units)*
	$t_R(x)$	$t_R(z)$	$t_R(z+1)$	t_M	
Ethene	15.8	9.5	7.5	0.7	33.5
Cyclobutane	2.3	1.4	0.9	0.1	4.7
Styrene	0.6	0.2	0.3	0.0	1.1

The partial errors of the determination of the indices are summarized for the model compounds studied in Table III.

DISCUSSION

The deviation of the average retention index, calculated from a number of replicate measurements, from the correct index value does not give information about the reproducibility and accuracy of the index determination, since relatively high positive and negative deviations of single index values from the correct one may compensate each other. For this reason the error was studied on the basis of partial derivatives.

In this way our earlier experience could be supported by experimental results and conclusions drawn from them. The practical fact that the error of the determination of retention indices of substances with retention times similar to t_M is in most cases high is already known, as is also the fact that the error due to the inaccuracy of t_M determinations is practically equal to zero for components appearing in the chromatogram later; this could be proved by the calculations.

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CHROM. 4756

EFFECTS OF THE COLUMN LENGTH ON THE HETP IN GAS CHROMATOGRAPHY

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SUMMARY

The effects of varying the length of a gas chromatography column operated under laminar flow conditions on the course of the HETP *versus* flow velocity curve have been discussed in terms of the mean absolute column pressure. Systems have been considered with both high and low liquid load packings, operated at either a constant outlet and variable inlet pressures or *vice versa*. An account has been given of the effects of varying the absolute mean column pressure at a constant column length.

The application of Van Deemter's equation[1,2] in gas chromatography presents certain problems stemming from the compressibility of the mobile phase. A rational approach to these problems was the concept of a local HETP[3–5], which led to the formulation of equations expressing the apparent HETP in the original Van Deemter terms corrected for the compressibility effects. Though it can be inferred from a number of fundamental studies[3,6–12] and has also been stated explicitly by some authors[5,13–16], it is pertinent to point out that the variables occurring in the HETP–carrier gas velocity dependence are interdependent, each having a specific influence on the shape and position of the respective curve. For instance, although the effect of a mere pressure drop across the column has been found insignificant[5,13,14], the changes of the absolute column pressure incident to the pressure drop changes may essentially influence the course of the HETP–carrier gas velocity curve. The same obviously holds for the absolute column pressure changes brought about by the changes in the column length, other permeability parameters, and either the column outlet or inlet pressures. However, the above effects are rather small under current conditions and may be accurately perceived only upon very careful measurements. The results of such measurements have shown[17,18] that the problem is still open.

The aim of the present paper is to show that simple concepts based on appreciating the effects of the mean absolute column pressure may render a consistent view on the interrelations between the course of an HETP–carrier gas velocity curve and the column length as well as the other permeability characteristics, taking into account

the degree of the stationary phase load and the method of accomplishing the carrier gas flow. A case of streamlined carrier gas flow will be considered.

Owing to the secondary influence of the pressure gradient alone on the HETP as well as with regard to other approximations further introduced, the corrections for the decompression effects[3-5] will be neglected in this treatment. A chromatographic zone having passed under the actual conditions through the column will be looked upon as if it had passed under the average carrier gas velocity ($\bar{u}$) and the mean absolute pressure ($\bar{P}$) in the column. Since $\bar{P}\bar{D}_m = P'D'_m$ where $\bar{D}_m$ and D'_m are the solute diffusion coefficients in the mobile phase at the mean pressure in the column and at a unit pressure (P'), respectively, the apparent HETP ($\bar{H}$) can be immediately expressed by

$$\bar{H} = A + B_s/\bar{u} + B_m/\bar{P}\bar{u} + C_m\bar{P}\bar{u} + C_s\bar{u} \qquad (1)$$

where the constants A–C_s should be independent of pressure. Besides the usual terms, a term $B_s/\bar{u}$ has been introduced in the equation to allow for longitudinal diffusion in the stationary phase[19]; this term may become comparable with $B_m/\bar{P}\bar{u}$ at higher absolute column pressures. This holds for the mean (time average) carrier gas velocity $\bar{u} = u_o(P_o/\bar{P}) = u_o j$ where u_o and P_o are the velocity and pressure at the column outlet and j is the well-known James–Martin correction factor. Apart from the term $B_s/\bar{u}$, eqn. 1 is one of the most used forms of expressing the Van Deemter equation in gas chromatography[11,16].

As the dependences of the diffusion coefficient and carrier gas velocity are the same, the contributions of the B_m and C_m terms to the HETP are constant throughout the given column (*cf.* refs. 3, 14). However, this invariability only applies to a position in the column under given conditions. In fact, it is just the above terms that render the $\bar{H}$ generally dependent on the absolute column pressure, regardless whether the $\bar{H}$ has been expressed as a function of $\bar{u}$ or u_o.

It can be derived from Darcy's law that the mean column pressure is related to the average velocity and the other quantities involved by

$$\bar{u} = (K/\eta L)\,(P_i^2 - P_o^2)/2\bar{P} \qquad (2)$$

where K, η, and L are the column permeability constant, carrier gas dynamic viscosity, and column length, P_i and P_o stand for the column inlet and outlet pressures, respectively. The pressure term can be expressed in the form $(P_i + P_o)\,(P_i - P_o)/2\bar{P}$ where, at moderate pressure drops, $(P_i + P_o)/2\bar{P}$ is close to unity, and the $\bar{P}$ can be expressed approximately by either $\bar{P} \simeq P_o + (P_i - P_o)/2$ or $\bar{P} \simeq P_i - (P_i - P_o)/2$. Thus, one can write

$$\bar{u} \simeq (K/\eta L)\,(P_i - P_o) \simeq (2K/\eta L)\,(\bar{P} - P_o) = (2K/\eta L)\,(P_i - \bar{P}) \qquad (3)$$

which yields, upon rearranging,

$$\bar{P} \simeq P_o + \eta L\bar{u}/2K = P_i - \eta L\bar{u}/2K \qquad (4)$$

The combination of eqns. 1 and 4 renders an illustrative picture of the effects of varying the column length on the separation efficiency at either a constant outlet pressure and variable inlet pressures or *vice versa*. Thus, one can obtain by substituting for $\bar{P}$ in eqn. 1 from eqn. 4 either

$$\bar{H} = A + \frac{B_s}{\bar{u}} + \frac{B_m}{\bar{u}P_o + (\eta L/2K)\bar{u}^2} + C_m P_o \bar{u} + C_m(\eta L/2K)\bar{u}^2 + C_s\bar{u} \qquad (5)$$

or

$$\bar{H} = A + \frac{B_s}{\bar{u}} + \frac{B_m}{\bar{u}P_i - (\eta L/2K)\bar{u}^2} + C_m P_i \bar{u} - C_m(\eta L/2K)\bar{u}^2 + C_s\bar{u} \qquad (6)$$

It is interesting to note the presence of the terms with $\bar{u}^2$ in eqns. 5 and 6. This justifies HALÁSZ's presumption that the Van Deemter equation in its usual form should be regarded as "no more than a short McLaurin series"[16]; the terms with $\bar{u}^2$ correspond with those suggested by PAPENDICK[20]. It is worth emphasizing that the above terms may acquire either positive or negative signs.

With regard to eqn. 1, it can be derived for the optimum $\bar{u}$ ($\bar{u}_{opt}$) and the corresponding $\bar{H}$ ($\bar{H}_{opt}$) that

$$\bar{u}_{opt} = \{[(B_m/\bar{P}_{opt}) + B_s]/(C_m\bar{P}_{opt} + C_s)\}^{\frac{1}{2}} \qquad (7)$$

and

$$\bar{H}_{opt} = A + 2\{[(B_m/\bar{P}_{opt}) + B_s](C_m\bar{P}_{opt} + C_s)\}^{\frac{1}{2}} \qquad (8)$$

However, $\bar{P}_{opt}$ is also a function of $\bar{u}_{opt}$ (*cf.* eqn. 4) so that eqns. 7 and 8 are not very illustrative; the explicit expression of $\bar{u}_{opt}$ and $\bar{H}_{opt}$ implies the solving of biquadratic equations, which is tedious and yields cumbrous relations. Therefore, in conformity with the procedures used by other authors[13,15,16], we shall consider only two limiting cases characterized by either $(B_m/\bar{P}) \gg B_s$ and $C_m\bar{P} \gg C_s$ or $(B_m/\bar{P}) \gg B_s$ and $C_s \gg C_m\bar{P}$, so that eqns. 7 and 8 will be reduced to either

$$\bar{u}_{opt} = (1/\bar{P}_{opt})(B_m/C_m)^{\frac{1}{2}} \qquad (9)$$

and

$$\bar{H}_{opt} = A + 2(B_mC_m)^{\frac{1}{2}} \qquad (10)$$

or

$$\bar{u}_{opt} = [(1/\bar{P}_{opt})(B_m/C_s)]^{\frac{1}{2}} \qquad (11)$$

and

$$\bar{H}_{opt} = A + 2(B_mC_s/\bar{P}_{opt})^{\frac{1}{2}} \qquad (12)$$

respectively. It is possible to assess from eqns. 9–12 the direction and limits in which $\bar{u}_{opt}$ and $\bar{H}_{opt}$ will vary upon changing the column length. Thus, in the work at a constant outlet pressure, both $\bar{u}_{opt}$ and $\bar{H}_{opt}$ will decrease on increasing the column length. As for the $\bar{u}_{opt}$, the extent of the decrease rises with decreasing importance of the C_s term, whereas the decrease of the $\bar{H}_{opt}$ slackens under the same circumstances; if $C_s \ll C_m\bar{P}$, the $\bar{H}_{opt}$ tends to be independent of the column length. In the case of work at a constant inlet pressure and variable outlet pressure, the situation is converse as to the direction of the shifts, but the same as to their extent. Combining eqns. 9 and 11 with the appropriate form of eqn. 4 and solving the resulting equations, one obtains

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$$\bar{u}_{opt} = (K/\eta L)\ \{[P_o^2 + (2\eta L/K)\ (B_m/C_m)^{\frac{1}{2}}]^{\frac{1}{2}} - P_o\} \tag{13}$$

and

$$\bar{u}_{opt} = (K/\eta L)\ \{P_i - [P_i^2 - (2\eta L/K)\ (B_m/C_m)^{\frac{1}{2}}]^{\frac{1}{2}}\} \tag{14}$$

for the case described by eqn. 9 and for a constant outlet pressure and a constant inlet pressure, respectively. The situation corresponding analogously to eqn. 11 is characterized by

$$\bar{u}_{opt} = -a + [b - a^3 + (b^2 - 2ba^3)^{\frac{1}{2}}]^{\frac{1}{3}} + [b - a^3 - (b^2 - 2ba^3)^{\frac{1}{2}}]^{\frac{1}{3}} \tag{15}$$

where

where $a = (2P_o/3)\ (K/\eta L)$ and $b = (B_m/C_s)\ (K/\eta L)$, and

$$\bar{u}_{opt} = a + [a^3 - b + (b^2 - 2ba^3)^{\frac{1}{2}}]^{\frac{1}{3}} + [a^3 - b - (b^2 - 2ba^3)^{\frac{1}{2}}]^{\frac{1}{3}} \tag{16}$$

where $a = (2P_i/3)\ (K/\eta L)$ and b is the same as in eqn. 15.

It can be inferred from eqns. 5 and 6 that the column length also essentially influences the slope of the ascending branch of the $\bar{H}$ *vs.* $\bar{u}$ curve. In the case of higher velocities (*cf.* eqn. 1) it holds that

$$\frac{d\bar{H}}{d\bar{u}} = \frac{d(C_m\bar{P} + C_s)}{d\bar{u}} = C_s + C_m\left(\frac{d\bar{P}}{d\bar{u}}\,\bar{u} + \bar{P}\right) \tag{17}$$

which, on combining with eqn. 4 results in

$$\frac{d\bar{H}}{d\bar{u}} = C_s + C_m\left(\frac{\eta L}{K}\,\bar{u} + P_o\right) \tag{18}$$

and

$$\frac{d\bar{H}}{d\bar{u}} = C_s + C_m\left(P_i - \frac{\eta L}{K}\,\bar{u}\right) \tag{19}$$

It is evident from the above relations that the column elongation will generally enhance the dependence of the slope on the carrier gas velocity and, consequently, the curvature of the slope. Thus, when working at a constant outlet pressure, the $\bar{H}$ *vs.* $\bar{u}$ curve measured on a longer column will display a steeper ascending branch, which leads to higher $\bar{H}$ values on longer columns at a higher velocity. However, in the work at a constant inlet pressure, the column elongation will cause a more gentle slope of the ascending branch, as the velocity-dependent component of the slope is negative. This obviously results in lower $\bar{H}$ values measured on longer columns at a higher velocity. The negative contribution of the term $C_m\eta L\bar{u}/K$ causes the ascending part of the $\bar{H}$ *vs.* $\bar{u}$ curve to become concave at higher velocities when measured at a constant inlet pressure.

It readily occurs that what has been said about the effects of the column length actually applies to the whole expression $\eta L/K$, which substantially extends the applicability of the above relations. The possibility of assessing the effects of varying parametrically the outlet pressure at a constant inlet pressure and *vice versa* represents

another important feature. Hence, the question what effect will the column elongation have on the apparent HETP is quite indefinite without quoting the conditions. The results may be summarized as follows:

(1) Column operated at a constant outlet pressure. An increase of the parameter $\eta L/K$ results in a decrease in both the $\bar{u}_{opt}$ and $\bar{H}_{opt}$ when employing a high liquid load packing, and in a more pronounced decrease of the $\bar{u}_{opt}$ and no change of the $\bar{H}_{opt}$ in the case of a low liquid load packing. The ascending branch of an $\bar{H}$ *vs.* $\bar{u}$ curve grows steeper on increasing $\eta L/K$, which renders higher $\bar{H}$ values at a given velocity in the respective region; the branch is always convex, provided the C_s and C_m are really constants, and the increase of the slope upon raising the $\eta L/K$ is given by the product $C_m\bar{u}$. The consequences of varying the column outlet pressure as a parameter are similar to those incidental to varying the $\eta L/K$; the increase of the slope upon increasing the P_o is given by the C_m itself.

(2) Column operated at a constant inlet pressure. The situation is opposite in all aspects mentioned in the preceding case, the functions of $C_m\bar{u}$ and C_m in connection with increasing the $\eta L/K$ and decreasing the P_i, respectively, being negative in this case. In the region of higher velocities, the ascending part of the curve becomes concave.

If one increases the inlet pressure while decreasing the outlet pressure so as to keep the $\bar{P}$ constant, one should obtain an ideal Van Deemter curve, with its shape and position practically independent of varying the $\eta L/K$ and with the ascending branch approaching a straight line ($d\bar{P}/d\bar{u} = 0$, eqn. 17) at high velocities.

It may be concluded that the minimum attainable HETP in a system operated at a constant outlet pressure may be either lowered or unchanged upon elongating the column, provided no sources of extra-column zone broadening have been introduced by coupling the columns. In the constant inlet pressure operation, the minimum HETP either rises or remains practically unchanged upon elongating the column. A number of excellent examples for both the constant outlet pressure operation[9–11] and inlet pressure operation[3,21] as well as for measurement at a constant mean column pressure[8] may be found in the literature to corroborate the consistency of the above account.

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A GAS–LIQUID CHROMATOGRAPHIC EXAMINATION OF STILBENE DERIVATIVES

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SUMMARY

Trimethylsilyl ether derivatives of twenty hydroxystilbenes were separated by gas–liquid chromatography on Apiezon-L, OV-1, OV-17, and SE-54. Relative retention times were highly dependent upon the degree of hydroxylation and methoxylation, the positions of these groups and on *cis–trans* isomerism. OV-1 was the best liquid phase for separating compounds with different numbers of trimethylsilyl ether groups while OV-17 was best for separating on the basis of different numbers of methoxyl groups. The method holds promise for analysis of stilbene derivatives in plant extracts.

INTRODUCTION

Stilbenes are an important group of natural products found in wood, bark and leaves of a variety of forest trees[1]. These compounds are noted for their fungi-toxic properties[2,3], their inhibition of wood delignification by acid sulphite pulping methods[4] and discoloration of wood[5]. Pinosylvin and pinosylvin monomethyl ether were found in the heartwood but not sapwood of all pine species investigated[6,7], and pinosylvin dimethyl ether was present in the bark of *P. banksiana*[8]. 3,5,4′-Trihydroxy-3′-methoxystilbene 3-β-D-glucoside[9] and glucosides of 3,5,3′,4′-tetrahydroxystilbene[10] were isolated from spruce bark. HATHWAY[11] found 3,5,4′-trihydroxystilbene and its 3-β-D-glucoside in the heartwoods of a group of *Eucalyptus* species but not in *E. wandoo* sapwood[12]. Both 3,5,2′,4′-tetrahydroxystilbene[13] and 3,5,3′,4′,5′-pentahydroxystilbene[14] were found in wood. The glucosides of 3,5,3′,4′-tetrahydroxy-, of 3,5,3′-trihydroxy-4′-methoxy- and of 3,5,4′-trihydroxystilbene were isolated from the leaves of *E. sideroxylon*[15–17]. The 4,4′-dihydroxy-3,3′-dimethoxystilbene was formed during bisulphite pulping of *P. radiata* and caused a pink coloration of the pulp[18].

HILLIS AND ISHIKURA[19] recently surveyed the paper and thin-layer chromatographic behaviour and spectral properties of stilbenes. Gas–liquid chromatography of trimethylsilyl ether (TMS) derivatives of plant polyphenols showed GLC to be a very useful and sensitive adjunct to PC and TLC in the analysis of flavonoids[20] and lignans[21]. Analysis of stilbenes by GLC would appear to hold similar promise but

little work has been reported to date. SATO *et al.*[22] studied GLC of TMS-pinosylvin and TMS-pinosylvin monomethyl ether on DC-430 liquid phase but did not separate these two compounds[22]. However, they did separate the stilbenes from the flavonoids, TMS-pinocembrin and TMS-pinobanksin as well as the lower molecular weight phenols usually present in plant extracts. SATO AND VON RUDLOFF[23] were able to separate TMS-pinosylvin from TMS-pinosylvin monomethyl ether using a 15% SE-52 liquid phase. SINSHEIMER AND SMITH[24] used GLC for the quantitative analysis of 2-hydroxy-, 4-hydroxy-, 4,4'-dihydroxy- and 4-hydroxy-3-methoxystilbenes as TMS derivatives on SE-52. This paper reports a survey of the separations obtained with twenty stilbenes on four different liquid phases.

EXPERIMENTAL

Gas chromatography

All analyses were carried out on a Beckman GC-4 gas chromatograph with a flame ionisation detector using glass columns (2 m × 3 mm I.D.). The nitrogen carrier gas flow rate was 50 ml/min while hydrogen and air flow rates were 30 and 275 ml/min, respectively. All liquid phases were coated on acid-washed, silylated, 80–100 mesh Chromosorb W* using the filtration method. The liquid phases studied were: (1) 2% Apiezon-L* (hydrocarbon), (2) 1% OV-1* (methyl-silicone gum), (3) 2% OV-17* (methyl-phenyl-silicone gum), and (4) 2% SE-54* (methyl-phenyl-vinyl-silicone gum). Light loading of liquid phase was required for analysis of stilbene glucosides. Oven temperatures varied depending on the group of stilbenes to be separated and on the liquid phase but the detector and injection port temperatures were controlled to at least 25° above oven temperature.

Silylation of stilbenes

The silylating reagent contained 1 ml of N,O-bis(trimethylsilyl) acetamide (BSA)**, 2 ml of hexamethyldisilazane (HMDS)** and 1 ml of trimethylchlorosilane (TMCS)** in 10 ml of dry pyridine. Mixtures of pure stilbenes (0.2–0.5 mg each) were dissolved in 1 ml of the silylating reagent in 1-ml glass vials with silicone gum septum stoppers. Aglucones were reacted for 10 min at room temperature while glucosides were heated to 105° for 5 min and injected within 10–15 min after heating. As a further precaution against moisture, vials were stored in a desiccator over P_2O_5.

Preparation of cis-stilbenes

cis-Stilbenes were prepared by irradiating ethanol solutions of the pure *trans*-isomers with UV light at a wave length of 254 nm. The isomerisation was followed by changes in the UV spectra. After the samples had been irradiated for 24 h, they were divided, a portion silylated and analysed, and a larger amount was applied to Whatman No. 3 paper. The paper chromatograms were developed with 6% acetic acid and the band containing the *cis*-isomer was extracted with diethyl ether. The

* 80–100 mesh Chromosorb W was obtained from Varian Pty. Ltd., Australia, and acid washed and silylated in our laboratory. Apiezon-L was from Assoc. Elect. Ind. Ltd., Great Britain. OV-1, OV-17 and SE-54 were all obtained from Varian Pty. Ltd., Australia.

** BSA was obtained in 1-ml glass ampoules from Pierce Chem. Co., U.S.A. HMDS and TMCS were obtained from Varian Pty. Ltd., Australia.

ether solution was washed twice with distilled water and dried over sodium sulphate before the evaporated extract was dried under high vacuum at room temperature. A sample of this material was silylated and analysed by GLC.

Preparation of wood extracts

Chips from the heartwood of a *Pinus radiata* tree grown near Penola, South Australia, were freeze dried, ground to pass a 40-mesh screen on a Wiley mill and the ground wood soaked in 40–60° petrol for two days. The wood was air dried and then soaked in ethanol–acetone (50 : 50) at room temperature for three days. The filtrate was evaporated under high vacuum and analysed by TLC and after silylation by GLC.

RESULTS AND DISCUSSION

Silylation conditions

The TMS-ether derivatives of stilbene aglucones had sharp individual peaks after a reaction period of 10 min at room temperature when silylated with either HMDS–TMCS in pyridine (2 : 1 : 10) or BSA–HMDS–TMCS in pyridine (1 : 2 : 1 : 10). Additional peaks were sometimes observed after 2–3 h of storage of sample vials over P_2O_5 in a desiccator.

Silylation of the glucosides was more difficult than of the aglucones. Attempts to use HMDS and TMCS at increasing concentrations in pyridine continued to give low yields. Addition of BSA and 5 min of heating at about 100° were necessary to increase yields significantly. More care in the exclusion of moisture from the TMS-stilbene glucosides appeared to be necessary than with the corresponding aglucones, since additional peaks were observed to develop more rapidly. Injection of the solution within 10–15 min after heating gave best results. Solutions were injected directly into the gas chromatograph without prior separation of the ammonium chloride precipitate and no difficulties arising from this procedure were evident. Attempts to use dimethylformamide instead of pyridine gave lower yields and additional peaks.

Relative retention times of TMS-stilbene derivatives

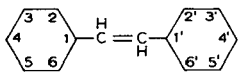
The relative retention times of the TMS-stilbene aglucones were determined with respect to TMS-4,4′-dihydroxystilbene on four different liquid phases and are summarised in Table I. 4,4′-Dihydroxystilbene was chosen as an internal standard because it does not occur in nature, was readily obtainable, and had a central retention time. The relative retention times for the glucoside derivatives were calculated with respect to TMS-3-methoxy-4′-O-β-D-glucosylstilbene. This compound has not been found in plant extracts.

Isomers

On the liquid phase OV-1, the relative retention times of the *cis*-stilbenes were approximately 0.38 those of the corresponding *trans*-isomers. There was only a small change in the relative retention times of these geometrical isomers with increasing degree of hydroxylation (Table II). The *cis*-isomers are frequently observed in paper chromatograms of plant extracts, *e.g.* ref. 11. BATTERSBY AND GREENOCK[25] among

TABLE I

RELATIVE RETENTION TIMES OF STILBENE AND TMS-STILBENE DERIVATIVES



Stilbene	*Liquid phase and oven temperature*			
	Apiezon-L 215°	*OV-1* 180°	*OV-17* 195°	*SE-54* 215°
Aglucones[a]				
cis-Stilbene	—	—	—	0.033
trans-Stilbene	—	—	—	0.062
4-Hydroxy-	0.34	0.22	0.27	0.30
Pinosylvin monomethyl ether (3-hydroxy-5-methoxy-)	0.51	0.44	0.62	0.36
4-Hydroxy-3-methoxy-	0.51	0.43	0.57	—
Pterostilbene (4′-hydroxy-3,5-dimethoxy-)	1.62	1.43	2.36	0.94
4-Hydroxy-4′,3-dimethoxy-	1.25	1.19	1.87	—
Pinosylvin (3,5-dihydroxy-)	0.53	0.55	0.58	0.40
4,4′-Dihydroxy-	1.00	1.00	1.00	1.00
4,4′-Dihydroxy-3,3′-dimethoxy-	2.58	2.96	4.26	1.89
Resveratrol (3,5,4′-trihydroxy-)	1.84	2.35	2.13	1.30
3,5,3′,4′-Tetrahydroxy-	2.97	4.58	3.58	2.38
3,5,2′,4′-Tetrahydroxy-	2.39	4.05	3.58	—
	Apiezon-L 275°	*OV-1* 225°	*OV-17* 240°	*SE-54* 250°
Glucosides[b]				
3-Methoxy-4-O-β-D-glucosyl-	1.00	1.00	1.00	1.00
Pinosylvin monomethyl ether glucoside (5-methoxy-3-O-β-D-glucosyl-)	—	0.92	—	0.92
4′,3-Dimethoxy-4-O-β-D-glucosyl-	2.00	1.10	2.58	—
Piceid (5,4′-dihydroxy-3-O-β-D-glucosyl-)	1.79	2.62	1.77	2.11
Rhapontin (5,3′-dihydroxy-4′-methoxy 3-O-β-D-glucosyl-)	2.19	3.30	2.50	2.62
Astringin (5,3′,4′-trihydroxy-3-O-β-D-glucosyl-)	2.93	3.83	2.34	2.87

[a] Aglucone relative retention time with respect to 4,4′-dihydroxystilbene.
[b] Glucoside relative retention time with respect to 3-methoxy-4-O-β-D-glucosylstilbene.

many others have observed the facile photo-chemical isomerisation of *trans*- to *cis*-stilbenes. Consequently, care must be taken in this respect when preparing samples and interpreting chromatograms.

There was often a large difference in retention time for various structural isomers (Table III). Compounds that had TMS-ether or methoxyl groups on both rings of the molecule showed longer retention times than compounds with the same number of the same groups on one ring. TMS-4,4′-dihydroxystilbene had a much longer retention time than TMS-3,5-dihydroxystilbene on all liquid phases. On Apiezon-L, TMS-3,5,3′,4′-tetrahydroxystilbene had a significantly longer retention time than TMS-3,5,2′,4′-tetrahydroxystilbene. There was little separation of TMS-3-hydroxy-5-methoxy- from TMS-4-hydroxy-3-methoxystilbene on all liquid phases.

TABLE II

RELATIVE RETENTION TIMES OF STEREOISOMERS OF STILBENES

Stilbene	*Relative retention time using liquid phase OV-1*
cis-Stilbene	0.36
trans-Stilbene	1.00
cis-4-Hydroxy-[a]	0.38
trans-4-Hydroxy-[a]	1,00
cis-4,4′-Dihydroxy-[a]	0.40
trans-4,4′-Dihydroxy-[a]	1.00

[a] TMS derivatives.

OV-17 gave the best separation of this pair with a separation factor of 1.09. These compounds are more easily separated by TLC[19] but since 4-hydroxy-3-methoxystilbene has not been isolated from plants, the GLC method is suitable for phytochemical studies. There was a large separation of TMS-4′-hydroxy-3,5-dimethoxystilbene from TMS-4-hydroxy-4′,3-dimethoxystilbene on all liquid phases. Despite the large increase in molecular size and weight of the only pair of isomeric glucosides studied, the separation of the pair was similar to that obtained with the aglucone pair (Table III).

Effect of the number of TMS-ether groups

Although there were exceptions, an increase of one TMS-ether group resulted in about twice the relative retention time (Fig. 1). The methyl-silicone gum OV-1 liquid phase gave the largest separation of compounds with different numbers of TMS-ether groups. Another methyl-silicone gum (SE-30) also gave the largest separation of flavonoids with different numbers of TMS-ether groups[21]. OV-17 and Apiezon-L gave somewhat less separation, while SE-54 gave the least separation

TABLE III

RELATIVE RETENTION TIMES OF TMS DERIVATIVES OF STRUCTURAL ISOMERS OF STILBENES

Stilbene isomers	*Liquid phase*		
	Apiezon-L	*OV-1*	*OV-17*
3,5-Dihydroxy-	0.53	0.55	0.58
4,4′-Dihydroxy-	1.00	1.00	1.00
3-Hydroxy-5-methoxy-	1.00	1.02	1.09
4-Hydroxy-3-methoxy-	1.00	1.00	1.00
4′-Hydroxy-3,5-dimethoxy-	1.30	1.20	1.26
4-Hydroxy-4′,3-dimethoxy-	1.00	1.00	1.00
3,5,3′,4′-Tetrahydroxy-	1.24	1.13	1.00
3,5,2′,4′-Tetrahydroxy-	1.00	1.00	1.00
3-Methoxy-4-O-β-D-glucosyl-	—	1.10	—
5-Methoxy-3-O-β-D-glucosyl-	—	1.00	—

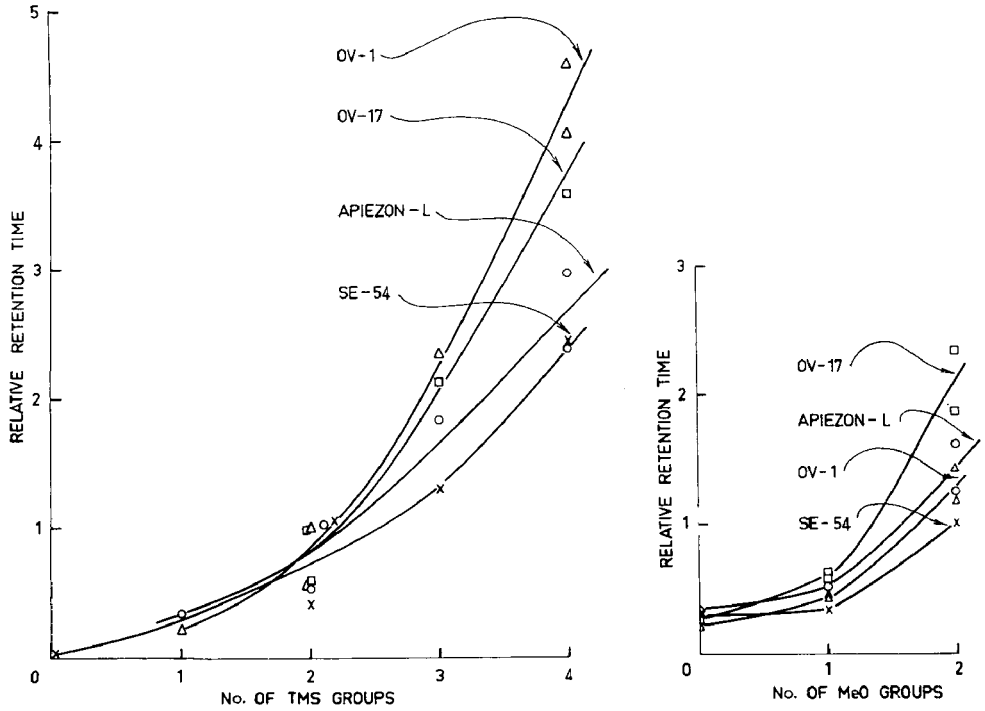


Fig. 1. Influence of the number of TMS-ether groups on the relative retention times of stilbene derivatives chromatographed on four liquid phases. □, OV-17; △, OV-1; ○, Apiezon-L; ×, SE-54.

Fig. 2. Influence of the number of methoxyl groups on the relative retention times of stilbene derivatives chromatographed on four liquid phases. □, OV-17; △, OV-1; ○, Apiezon-L; ×, SE-54.

studied. Addition of the first two TMS-ether groups increased retention times less than addition of the third and fourth.

TMS derivatives of the stilbenes on OV-1 showed some peak tailing, as did TMS-flavonoids on SE-30 (ref. 21). However, the previous work with flavonoids indicated that peak symmetry could be improved by temperature programming with a temperature increase of about 1°/min[21]. The other liquid phases did not show a significant amount of tailing of compounds with large numbers of TMS-ether groups.

Effect of number of methoxyl groups

The increase in retention time resulting from an increase in the number of methoxyl groups was greater than or equal to that caused by increasing an equal number of TMS-ether groups (Fig. 2). Increasing numbers of methoxyl groups caused an exponential increase in retention time on all liquid phases. OV-17 gave the best separation of compounds with different numbers of methoxyl groups. There was little difference between Apiezon-L and OV-1 in this respect, while the SE-54 again gave the least separation based on the number of methoxyl groups. The first

based on the number of TMS-ether groups. The increase in relative retention time with increasing number of TMS-ether groups was exponential on all liquid phases. The second methoxyl increased retention times significantly more, to a minimum of 2.5 times for SE-54 and 3.5 times for OV-17.

Glucosides

The TMS-stilbene glucosides usually gave sharp individual peaks on Apiezon-L, OV-17, and SE-54 liquid phases. Peak tailing was small but significant on the OV-1 liquid phase and this occurred also with the aglucones that had a large number of TMS-ether groups. It was apparent that OV-1 gave the best separation of compounds with different numbers of TMS-ether groups and that OV-17 gave the best separation based on the number of methoxyl groups. Increasing the number of methoxyl groups from one to two increased the retention time to 2.5 times on the OV-17 liquid phase. The retention time of TMS-5,4'-dihydroxy-3-O-β-D-glucosylstilbene increased to 1.5 times its original value with an additional TMS-ether group at the 3' position when chromatographed on the OV-1 liquid phase. The OV-1 liquid phase was not effective in the separation of TMS-4',3-dimethoxy-4-O-β-D-glucosylstilbene from TMS-3-methoxy-4-O-β-D-glucosyl- or TMS-5-methoxy-3-O-β-D-glucosylstilbenes. However, the former two stilbene glucosides are not natural products. TMS-3-methoxy-

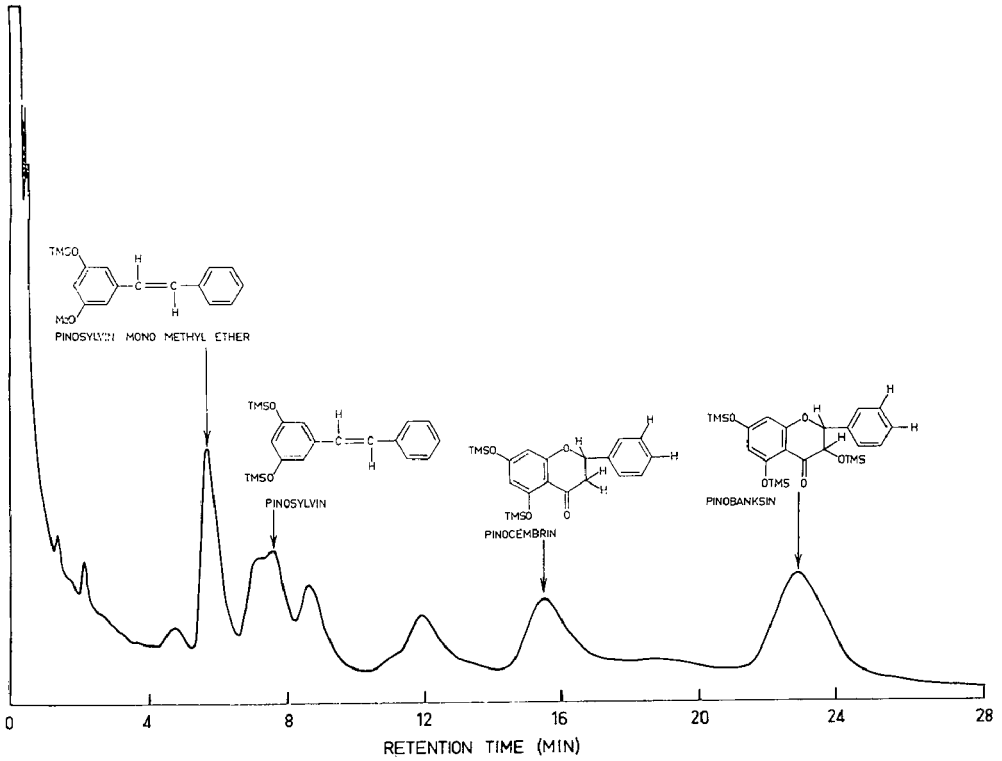


Fig. 3. Gas–liquid chromatogram of the silylated products of the ethanol–acetone soluble components of *Pinus radiata* heartwood. Chromatographic conditions: oven temperature, 150°; injection port and detector temperatures 200°; carrier gas flow 50 ml/min; column, 2 m × 3 mm I.D. glass packed with 2% OV-1 on 80–100 mesh DMCS-treated Chromosorb W.

methoxyl on a monohydroxystilbene increased the relative retention time to a minimum of 1.2 times on SE-54 and to a maximum of 2 times for OV-1 and OV-17. 4-O-β-D-glucosylstilbene was a useful internal standard; it was close to but adequately separated from TMS-5-methoxy-3-O-β-D-glucosylstilbene.

Application to pine heartwood

Compounds previously found in *Pinus radiata* heartwood include pinosylvin, pinosylvin monomethyl ether, pinocembrin and pinobanksin[6,7]. Both TLC and GLC of the wood sample we have studied indicated significant quantities of additional compounds. Unfortunately one unknown compound has a relative retention time very similar to that of TMS-pinosylvin on an OV-1 liquid phase (Fig. 3). A better separation was obtained by lowering the oven temperature to 140°.

CONCLUSIONS

Both stilbene glucosides and their aglucones were successfully silylated and separated by GLC. The relative retention times obtained for various stilbene derivatives were highly dependent upon the degree of hydroxylation and methoxylation. OV-1 was the best liquid phase for separating on the basis of number of hydroxyls while OV-17 was best for separating compounds with differing numbers of methoxyls. Relative retention times were also highly dependent upon the geometry of the compound both with respect to *cis*- and *trans*-isomers and the position of substituent groups. While analyses of natural extracts may be complicated by peaks from unidentified compounds, this problem can probably be overcome. GLC of silylated plant extracts appears to hold considerable promise as an analytical tool.

ACKNOWLEDGEMENTS

We are grateful to Profs. M. HASEGAWA, T. KONDO, T. J. KING, B. LINDBERG, T. R. SESHADRI, J. W. CLARK-LEWIS, Drs. F. H. YORSTEN, E. WALCZAK and W. WIENIAWSKI for providing some of the stilbenes.

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CHROM. 4769

ANALYTICAL CHEMICAL STUDIES ON STEROIDS

XXXVII. GAS CHROMATOGRAPHY OF 2,3-OXYGENATED ESTRATRIENES

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SUMMARY

Gas chromatography of the 2,3-oxygenated estratrienes was carried out employing 3% SE-30 as a stationary phase and their "steroid number (SN)" values were determined according to the proposed definition. The SN contributions of various oxygen functions at C-2 or C-3 were estimated by means of estra-1,3,5(10)-triene as a reference. The contribution of two functional groups on ring A and D was found to be in agreement with the summation of the values characteristic of each substituent. It was also demonstrated that the additivity rule is applicable to the 2,3-oxygenated estratrienes, when the SN contributions of the vicinal functional groups on ring A are regarded as a set.

INTRODUCTION

Several articles dealing with the correlation between the structures of steroids and their retention times in gas chromatography have already been reported. In 1962, VANDENHEUVEL AND HORNING introduced the concept of "steroid number (SN)", which is of use for characterization of the steroids in gas chromatography[1]. The SN contributions of representative functional groups have been determined with the neutral steroids, but not fully with the aromatic steroids. In connection with the biochemical problems concerning catechol estrogens it appeared to be of interest to us to examine the effect of various substituents on the SN with respect to the estratriene nucleus. The present paper describes the gas chromatographic behavior of the estratrienes having an oxygen function at C-2 and/or C-3.

EXPERIMENTAL

Materials

All the estratriene derivatives employed in this work were synthesized by established methods in this laboratory[2,3].

Preparation of derivatives

Trimethylsilyl (TMS) derivatives were prepared by treatment with hexamethyldisilazane and trimethylchlorosilane in pyridine in the manner described by SWEELEY *et al.*[4]. After evaporation of the solvent the residue was extracted with *n*-hexane, centrifuged and the supernatant was used. Trifluoroacetates (TFA) were prepared with trifluoroacetic anhydride and pyridine in tetrahydrofuran according to the procedure of VANDENHEUVEL *et al.*[5]. After evaporation of the solvent the residue was dissolved in tetrahydrofuran and injected into the gas chromatograph.

Gas chromatography

The apparatus used for this work was a Shimadzu Model GC-1C gas chromatograph equipped with a hydrogen flame ionization detector and a U-shaped stainless-steel column (1.875 m × 3 mm I.D.). The column was packed with 3% SE-30 on a support of Chromosorb W (60–80 mesh). The detector and flash heater were kept at 250°, while the column was at 218°. Nitrogen was used as the carrier gas at a flow rate of 55 ml/min. The relative retention time of each compound was measured using cholestane as a reference compound. According to the definition proposed by VANDENHEUVEL AND HORNING a plot of log relative retention time against steroid number was made, whereby the values of androstane and cholestane were taken as 19 and 27, respectively.

RESULTS AND DISCUSSION

Gas chromatography was performed with 62 estratriene derivatives and their relative retention times (RRT) and SN were determined. As listed in Table I SN

TABLE I

RELATIVE RETENTION TIMES AND STEROID NUMBERS OF MONOSUBSTITUTED ESTRATIENES

Compound functional group	*RRT*	*SN*	*SN contribution of functional group*
Estra-1,3,5(10)-triene	0.13	19.0	
17-Oxo	0.23	21.3	2.3
16-Oxo	0.22	21.2	2.2
17β-OH	0.24	21.4	2.4
16β-OH	0.23	21.3	2.3
17β-OAc	0.32	22.6	3.6
17β-OTMS	0.28	22.0	3.0
17β-OTFA	0.18	20.4	1.4
3-OH	0.28	22.0	3.0
2-OH	0.28	22.0	3.0
3-OCH_3	0.25	21.6	2.6
2-OCH_3	0.23	21.3	2.3
3-OAc	0.38	23.2	4.2
2-OAc	0.34	22.8	3.8
3-OTMS	0.31	22.4	3.4
2-OTMS	0.28	22.0	3.0
3-OTFA	0.19	20.5	1.5
2-OTFA	0.17	20.1	1.1
Cholestane	1.00 (14.0 min)		
Androstane	0.13 (1.8 min)		

TABLE II

RELATIVE RETENTION TIMES AND STEROID NUMBERS OF DISUBSTITUTED ESTRATRIENES

Compound functional group	*RRT*	*SN*		*ΔSN observed*
		Expected[a]	*Observed*	
17-Oxo				
3-OH	0.51	24.3	24.4	0
2-OH	0.51	24.3	24.4	
3-OAc	0.65	25.5	25.3	0.3
2-OAc	0.60	25.1	25.0	
3-OTMS	0.55	24.7	24.7	0.5
2-OTMS	0.48	24.3	24.2	
3-OTFA	0.33	22.8	22.7	0.4
2-OTFA	0.30	22.4	22.3	
17β-OH				
3-OH	0.55	24.4	24.7	0
2-OH	0.55	24.4	24.7	
3-OCH_3	0.47	24.0	24.1	0.3
2-OCH_3	0.44	23.7	23.8	
17β-OAc				
3-OH	0.74	25.6	25.8	0
2-OH	0.74	25.6	25.8	
3-OCH_3	0.65	25.2	25.3	0.3
2-OCH_3	0.60	24.9	25.0	
3-OAc	0.99	26.8	27.0	0.3
2-OAc	0.93	26.4	26.7	
3-OTMS	0.81	26.0	26.2	0.4
2-OTMS	0.73	25.6	25.8	
3-OTFA	0.46	24.1	24.0	0.3
2-OTFA	0.43	23.7	23.7	
17β-OTMS				
3-OCH_3	0.57	24.6	24.8	0.2
2-OCH_3	0.54	24.3	24.6	
3-OTMS	0.72	25.4	25.7	0.5
2-OTMS	0.63	25.0	25.2	
17β-OTFA				
3-OCH_3	0.36	23.0	23.0	0.2
2-OCH_3	0.34	22.7	22.8	
3-OTFA	0.28	21.9	22.0	0.3
2-OTFA	0.26	21.5	21.7	
Cholestane	1.00 (14.0 min)			
Androstane	0.13 (1.8 min)			

[a] The values used in these calculations were obtained from Table I.

contributions of the functional groups at C-17 or C-16 were estimated with the monosubstituted compounds by subtracting the value of estra-1,3,5(10)-triene, respectively. Some of these functional group values are in good accordance with those which have already been reported for the saturated steroids[1]. This finding is of particular interest in suggesting that a structural alteration far removed from the site of the functional group exerts no significant influence and the angular distortion due to the distant part of a molecule may be of subtle nature. In addition, the SN contribution of an aromatic ring A was found to be 1.0.

The SN contributions of the oxygen functions such as hydroxyl, methoxyl, acetoxyl, trimethylsilyloxyl and trifluoroacetoxyl groups at C-2 or C-3 were deter-

mined in a similar fashion employing estra-1,3,5(10)-triene as a reference. Table I also contains SN and contribution values of these functional groups. With respect to the free hydroxylic compounds no difference can be seen between the two positional isomers. However, it seems likely that a more distinct resolution could be attained, when a sufficiently bulky group is introduced to accentuate the existing difference. It is also apparent from Table II that the contribution values of the two functional groups on ring A and D are in agreement with the summation of the values characteristic of each substituent. The additive relationship will be of great use for characterization of these oxygenated steroids, which include the important estrogen metabolites. The constancy of SN difference (Δ SN) between a pair of positional isomers is evidently observed irrespective of the nature of the C-17 substituent.

With the 2,3-dioxygenated compounds the experimental SN values are not necessarily identical with the summation of values related to the steroid skeleton and the functional groups (see Table III). BROOKS *et al.* examined the effect of the functional groups on the gas chromatographic behavior of the estratrienes, including several 2,3-oxygenated derivatives, and in consequence observed that the adjacent functional groups modify the retention effects of each other[6]. The marked deviation from the expected value may be ascribable to the intramolecular interaction of the vicinal substituents on ring A. So far as examined, however, the SN contribution of both substituents on ring A appears to be almost constant. This finding implies that the SN contribution of the adjacent functional groups can be regarded as a set, and

TABLE III

RELATIVE RETENTION TIMES AND STEROID NUMBERS OF 2,3-OXYGENATED ESTRATRIENES

Compound functional group			*RRT*	*SN*		*SN contribution of functional groups at C-2 and C-3*
C-2	*C-3*	*C-17*		*Expected*[a]	*Observed*	
OH	OCH_3	β-OH	0.79	27.0	26.1	4.7
		Oxo	0.75	26.9	25.9	4.6
OCH_3	OH	β-OH	0.74	26.7	25.8	4.4
		Oxo	0.70	26.6	25.6	4.3
OCH_3	OTMS	β-OTMS	1.00	27.7	27.0	5.0
		Oxo	0.79	27.0	26.1	4.8
OTMS	OCH_3	β-OTMS	0.98	27.6	26.9	4.9
		Oxo	0.75	26.9	25.9	4.6
OTFA	OCH_3	β-OTFA	0.44	24.1	23.8	3.4
		Oxo	0.50	25.0	24.3	3.0
OCH_3	OTFA	β-OTFA	0.43	24.2	23.7	3.3
		Oxo	0.50	25.1	24.3	3.0
OCH_3	OAc	β-OAc	1.44	29.1	28.4	5.8
OAc	OCH_3	β-OAc	1.48	29.0	28.5	5.9
Cholestane			1.00 (14.0 min)			
Androstane			0.13 (1.8 min)			

[a] The values used in these calculations were obtained from Table I.

that the additivity rule is also applicable for the 2,3-oxygenated estratrienes.

It is hoped that the present results may help to identify metabolites derived from the modified steroids as well as the naturally occurring estrogens.

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CHROM. 4805

GAS–LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY OF DEUTERIUM-CONTAINING AMINO ACIDS AS THEIR TRIMETHYLSILYL DERIVATIVES

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SUMMARY

The gas–liquid chromatography and mass spectrometry of specifically labeled deuterium-containing amino acids as their trimethylsilyl derivatives has been investigated. In many cases the retention time of the deuterated compound was measurably shorter than that of its protium analog. The small retention time differences, to a first approximation, were proportional to the number of deuterium atoms. Under our experimental conditions separation of mixtures (bifurcated peak) was observed within only two pairs of compounds—leucine and leucine-D_{10}, and isoleucine and isoleucine-D_{10}. Fractionation of the protium and deuterium forms within a single gas–liquid chromatography peak has been demonstrated by combined gas–liquid chromatography–multiscan mass spectrometry. Comparison of the mass spectra of normal amino acid trimethylsilyl derivatives and their deuterium-containing analogs has facilitated the elucidation of the mass spectral fragmentation patterns observed for these compounds.

INTRODUCTION

We have recently reported on the gas–liquid chromatography–mass spectrometry (GLC–MS) of the trimethylsilyl (TMSi) derivatives of carbon-13 enriched (15% randomly labeled) amino acids[1,**]. The presence of the isotopic labels facilitated the assignment of structure to mass spectral fragmentation products. While no evidence was found for the GLC separation or fractionation of the normal and corresponding carbon-13 enriched amino acids, there are reports in the literature on GLC separation of a variety of deuterated compounds from their protium analogs. The methyl esters of perdeutero fatty acids have been shown to possess shorter retention times than the corresponding normal esters[4], and isotope fractionation of protium and deuterium

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** BAKER *et al.*[2] and TEETER[3] have reported on the MS of TMSi derivatives of several amino acids.

forms of sugars (as TMSi ethers) during GLC has also been reported[5,6]. More recently, WALLER *et al.*[7] observed the GLC separation of TMSi and TMSi-D_9 derivatives of α-D-glucose prepared from normal and deuterated reagents, respectively; for a penta-ether, this results in a molecular weight difference of 45 amu, but the deuterium-containing derivative exhibits the shorter retention time. Other workers have also observed the effect of isotope substitution upon GLC behavior (see refs. 1 and 4–7). The availability of a considerable number of isotopically pure deuterated amino acids (obtained from algae grown in deuterium oxide (99%) by hydrolysis[8] and separated by ion-exchange chromatography[9]) has enabled us to investigate the effect of deuterium content upon the GLC behavior of the TMSi derivatives of these important natural products. Further, mass spectrometry of these specifically labeled compounds has allowed an instructive comparison with our earlier comments[1]. We now wish to present the results of our study on the GLC and MS of deuterated amino acids.

EXPERIMENTAL

GLC retention behavior studies were carried out with a Barber-Colman Model 5000 instrument (hydrogen flame ionization detection). The column was a 12 ft. × 4 mm glass W-tube; 2% F-60 (DC-560) coated over 1.5% SE-30 on 80–100 mesh acid-washed and silanized[10] Gas-Chrom P; 20 p.s.i. Four different temperatures were employed for the isothermal separations because of the widely different retention times of the amino acid derivatives: 90° (alanine, glycine), 105° (valine, proline, leucine, isoleucine, serine, threonine), 150° (methionine, aspartic acid, phenylalanine, glutamic acid), 180° (tyrosine, lysine); the amino acids are listed in order of increasing retention time. Unless indicated otherwise, all carbon-bound hydrogen is replaced by deuterium in the deuterated amino acids. Partial exchange during the isolation procedure resulted in incomplete deuteration with aspartic and glutamic acids[9]. Methionine-α,β-D_3 was prepared synthetically by MARKLEY[11]* following the method of MARTIN AND MORLINO[12]. The TMSi derivatives were prepared as in ref. 1.

The LKB Model 9000 instrument, fitted with a 6 ft. × 3.5 mm I.D. spiral glass column packed with 3% OV-1 on acid-washed and silanized[10] Gas-Chrom P, was employed for combined GLC–MS experiments. Spectra were obtained with the previously reported instrument conditions[1].

RESULTS AND DISCUSSION

The retention times of the deuterium-containing amino acid TMSi derivatives are less than those of their protium analogs. To a first approximation, the retention time difference was proportional to the number of deuterium atoms. For example, the separation factor (retention time normal acid/retention time deuterium-containing acid) for the pair glycine and glycine-D_2 is < 1.01, and that for phenylalanine and phenylalanine-D_8 is 1.02 (see Table I). In addition to a measurable difference in retention times between the members of most pairs of amino acid derivatives when chromatographed individually, in a number of cases GLC of a mixture of the two compounds resulted in a peak broadening effect. This is reflected by a peak width at half-height for the mixed peak measurably larger than that for either component run

* We are grateful to Dr. MARKLEY for this sample.

TABLE I

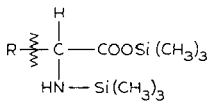
EFFECT OF DEUTERIUM CONTENT UPON GLC BEHAVIOR OF AMINO ACIDS AS TMSi DERIVATIVES

Compound	*Separation factor*	*Peak width (cm) at half height*[a]	
		Individually	*Mixture*
Gly	<1.01	0.28	0.29
Gly-D_2		0.27	
Ala	1.02	0.25	0.30
Ala-D_4		0.23	
Val	1.02	0.22	0.31
Val-D_8		0.22	
Phe	1.02	0.24	0.32
Phe-D_8		0.25	
Leu	1.04	0.29	0.59[b]
Leu-D_{10}		0.28	
Ile	1.04	0.31	0.68[b]
Ile-D_{10}		0.30	

[a] These values are ± 0.01 cm.
[b] Partial separation is observed on the chromatogram.

separately (see Table I), and can also be discerned in the more favorable instances by simple visual inspection. For example, the difference in GLC retention behavior between the TMSi derivatives of valine and valine-D_8 is reflected by a separation factor of 1.02, but the peak resulting from chromatography of a mixture shows only a slight broadening, and no indication of splitting. In only two instances (leucine and leucine-D_{10}, and isoleucine and isoleucine-D_{10}) was separation (peak bifurcation) clearly visible (see Fig. 1). Those compounds which differ the greatest in deuterium content exhibit the greatest separation. The greater volatility of the labeled compounds is in complete agreement with the observations of other workers[4-7].

In our previous paper[1] we described four pathways of fragmentation which amino acid TMSi derivatives undergo during MS, and suggested that they are (a) loss of a methyl group from the molecular ion M (M-15)*; (b) loss of methyl and CO with retention of the silyloxy group (M-43); (c) loss of carbotrimethylsilyloxy (M-117)*; (d) loss of the amino acid side chain group R, *i.e.*,



to yield a fragment of *m/e* 218 (previously reported by PIERCE[13]). Other signals at *m/e* 73, $[Si(CH_3)_3]^+$, and 147, $[(CH_3)_2Si{=}O{-}Si(CH_3)_3]^+$, are commonly observed with O-TMSi compounds. Concerning fragmentation (a) there can be little doubt with those amino acids which do not possess a side chain methyl group (*e.g.*, proline, phenylalanine) that the eliminated methyl comes from a TMSi group. For methyl group-containing amino acids such as valine and leucine, however, the methyl could

* Also observed by BAKER *et al.*[2].

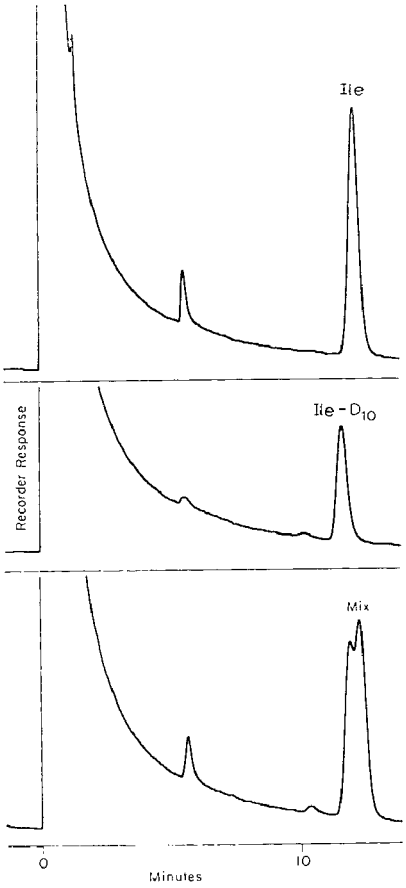


Fig. 1. GLC analysis of the TMSi derivatives of isoleucine (top panel), isoleucine-D_{10} (middle panel) and a mixture of these two compounds (bottom panel).

conceivably be lost from either a TMSi group or the amino acid moiety. Mass spectrometry of the carbon-13 enriched amino acids proved to be very helpful with this problem, and simple calculations based on the carbon-13 content of the fragments (reflected in the (S+1) isotope peak) indicated that a TMSi group is the source of the expelled methyl group[1]. Deuterium labeling has been employed extensively in MS to deduce fragmentation modes and the structure of fragment ions, as has been exemplified by the recent reports of CAPELLA AND ZORZUT[14] and DIEKMAN *et al.*[15]. That our conclusion concerning the source of the lost methyl group was correct can be seen in Table II, where one observes (M-15), rather than (M-18), fragments for the TMSi derivatives of the methyl group-containing deuterated amino acids. Within each pair of these compounds the difference in *m/e* values for the fragment ions resulting from loss of a methyl group corresponds exactly to the difference in molecular weight between the two derivatives. Loss of methyl and CO (b), and loss of carbotrimethylsilyloxy (c), are seen not to involve deuterium-containing portions of the amino acids. Finally, the identity of the 218 fragment, observed for many of the amino acids, is also con-

TABLE II

MASS SPECTRAL FRAGMENTS FOR NORMAL AND DEUTERATED AMINO ACIDS AS TMSi DERIVATIVES

Amino acid	*M*[a] *or M-15*[b]	*M-43*[c]	*M-117*[d]	*M-R*[e]
Gly	204	176	102	—
Gly-D_2	206	178	104	—
Ala	218	190	116	218[f]
Ala-D_4	222	194	120	219
Pro	244	216	142	—
Pro-D_7	251	223	149	—
Val	246	218[f]	144	218[f]
Val-D_8	254	226	152	219
Leu, Ile	260	232	158	218
Leu-D_{10}, Ile-D_{10}	270	242	168	219
Met	293*, 278	250	176	218
Met-D_3	296*, 281	253	179	219
Phe	294	266	192	218
Phe-D_8	302	274	200	219
Asp	349*, 334	306	232	218
Asp-$D_{1,2,3}$	350*, 335	307	233	219
	351*, 336	308	234	
	352*, 337	309	235	
Tyr	382	354	280	218
Tyr-D_7	389	361	287	219

[a] M indicated by asterisk.
[b] Loss of methyl.
[c] Loss of methyl + CO.
[d] Loss of carbotrimethylsilyloxy.
[e] Loss of amino acid side chain.
[f] See p. 410.

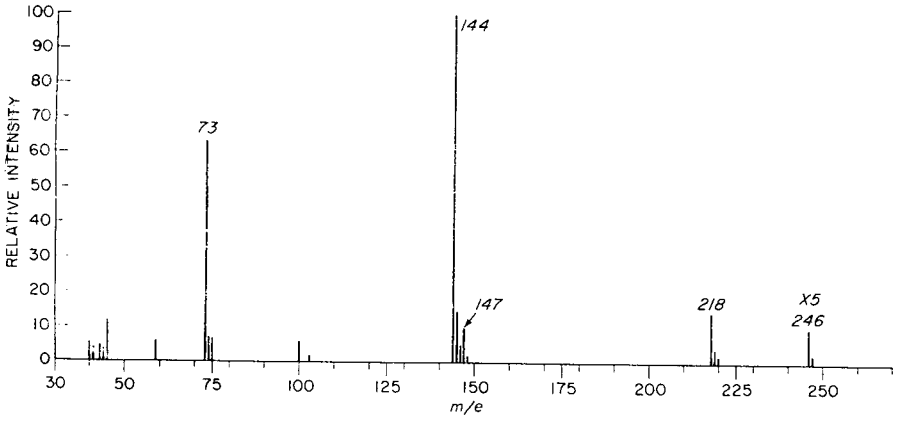


Fig. 2. Mass spectrum of the TMSi derivative of valine.

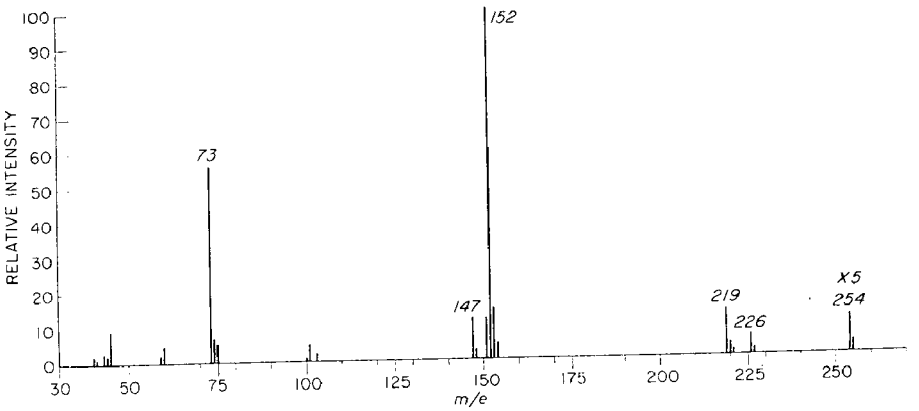


Fig. 3. Mass spectrum of the TMSi derivative of valine-D_8.

firmed, since it is replaced by a fragment 1 amu greater for the deuterated analogs (see Table II).

The mass spectrum of the TMSi derivative of valine is presented in Fig. 2, and contains fragments as described above, *e.g.*, (M-15) (*m/e* 246), and (M-COOSi$(CH_3)_3$) (*m/e* 144). A signal is also noted, as expected, at *m/e* 218. This fragment could arise either by loss of methyl and CO (b), or by loss of the isopropyl side chain (d); both result in loss of 43 amu. Data obtained from the carbon-13 enriched amino acid led to the suggestion that the main pathway was the latter[1]. As is clear from the mass spectrum of the TMSi derivative of valine-D_8 (Fig. 3), this is indeed the main pathway, for there is a strong signal at *m/e* 219 (M-50). The alternative pathway, loss of methyl and CO, is not insignificant, however, for a fragment of *m/e* 226 (M-43) is readily apparent. It was also deduced from work with carbon-13 enriched alanine that its *m/e* 218 fragment is formed by loss of methyl from a TMSi group (a), and not the amino acid methyl (d)[1]. Examination of the mass spectrum of the TMSi derivative of alanine-D_4 has shown this to be essentially correct; however, in addition to the expected *m/e* 222 signal, one also observes a much less intense (1/7) signal at *m/e* 219, indicating a minor contribution of pathway (d).

A number of methods are frequently employed to determine peak homogeneity in GLC, *viz.* increase in column efficiency (*e.g.*, use of a longer column)[5], use of both non-selective and selective stationary phases[10,16], and exposure of the sample to derivative formation conditions[10]. The homogeneity of a peak may also be examined by taking a mass spectrum, either in direct combination with a GLC column[10,17–19] or by conventional means following collection[20,21]. If the peak is multi-component in nature, and if the components exhibit significantly different mass spectra, the non-homogeneity will be apparent from visual inspection of the composite spectrum. If the co-eluted compounds undergo partial fractionation but not separation, collection of several fractions during elution of the peak followed by MS of each "cut" may indicate the multi-component nature of the peak[20]. The peak may be scanned successively during combined GLC–MS, and the spectra will be seen to change with time across the peak. With this latter approach, one is using MS as a highly selective detection system, and, in a sense, the combination of techniques possesses greater

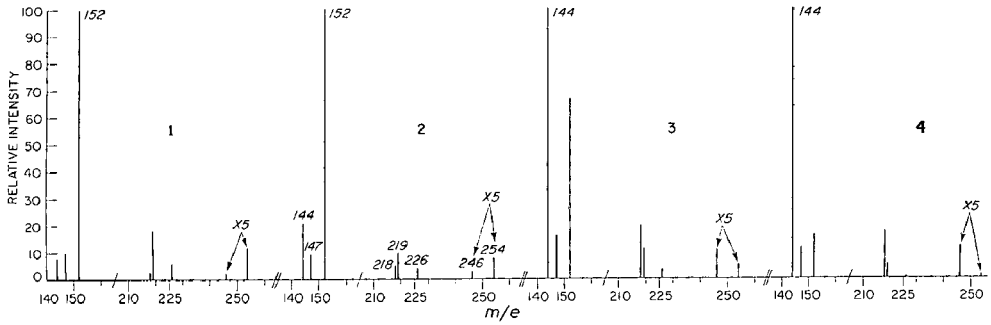


Fig. 4. Partial mass spectra (separated by double slash marks) obtained for four successive scans across the GLC peak resulting from chromatography of a mixture of the TMSi derivatives of valine-D_8 and valine. Spectra 1 and 2 were produced by scanning the ascending portion of the GLC peak, and spectra 3 and 4 result from scans on the descending side of the peak. The *m/e* values for all the signals are given in spectrum 2—152, 219, 226 and 254 arise from valine-D_8; 144, 218 and 246 are the corresponding signals for valine.

GLC resolution than the same column equipped with a normal mass detector (*e.g.*, hydrogen flame detector). SWEELEY *et al.*[6] were able to demonstrate isotope fractionation of the TMSi derivatives of α-D-glucose and α-D-glucose-D_7 on a short (2 m) GLC column by this means. We have utilized this multiple scanning MS approach to detect unresolved normal and deuterated amino acids in GLC effluents (Fig. 4). The symmetrical peak resulting from the chromatography of an approximately 1:1 mixture of valine and valine-D_8 (TMSi derivatives) was subjected to four successive scans, two (1 and 2) on the ascending side, and two (3 and 4) on the descending side. The partial (*m/e* 140–260) mass spectra from the four scans are presented in Fig. 4. Panel 1 (scan 1) corresponds favorably to the mass spectrum of valine-D_8 (Fig. 3), with only relatively small signals at *m/e* 144, 218 and 246, indicating that the fraction of the peak scan 1 represents is mainly valine-D_8. The proportion of normal valine is seen to have increased by the time of scan 2. The composition of the peak at scan 3 has changed radically—the signals for the valine fragments are of greater intensity than the corresponding signals from valine-D_8 (*m/e* 152, 219 and 226, and 254), and the spectrum resulting from scan 4 is essentially that of the TMSi derivative of valine (Fig. 2). The number of deuterium atoms necessary for the occurrence of such GLC fractionation is not large; using the multi-scan GLC–MS technique we have demonstrated this phenomenon within pairs of amino acids which exhibit separation factors of 1.01 or less (*e.g.*, glycine and glycine-D_2).

The use of deuterium-labeled drugs as an aid in the mass spectrometric identification of metabolites has been discussed by BOYD AND BULLOCK[22]; the recognition of fragment ions arising from drug-related compounds is facilitated by the presence of the elevated isotope peaks. MCCLOSKEY *et al.*[23] have discussed the value of deuterium-labeled trimethylsilyl derivatives (*i.e.*, TMSi-D_9) in the structure determination of unknown molecules. Combined GLC–MS techniques are finding increasing use in drug metabolism studies[24–31], and the possibility of encountering the GLC isotope fractionation of deuterium-labeled and non-labeled drug or metabolite is very real. Indeed, it could serve as an additional means for distinguishing drug-related substances.

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A NEW TECHNIQUE OF PROTEIN HYDROLYSIS AND ANALYSIS OF THE AMINO ACIDS ON AN AUTOANALYZER

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SUMMARY

A new technique of protein hydrolysis is presented which has been shown to be more efficient than the classical HCl hydrolysis.

The new method consists in hydrolysis with crystallized oxalic acid with some added HCl in a sealed tube. The temperature of hydrolysis seems to be more critical than the time. After hydrolysis the frozen mass is dissolved, filtered, suitably diluted and chromatographed directly without neutralization. No damage to the columns was observed after fifty runs.

Only serine and threonine appear to suffer slight losses due to destruction and the arginine content was lowered somewhat. All the other amino acids show a better recovery.

INTRODUCTION

The hydrolysis of a protein or a polypeptide plays an important role in the determination of its amino acid content. As pointed out by LIGHT AND SMITH[1], this step is one of the weakest links in the entire chain of events leading to a complete analysis of a protein or polypeptide.

Until the present time there has not been a single method described which is able to hydrolyze completely the peptide link without the destruction of one or other of the amino acids. In addition, all the recommended processes for hydrolysis are tedious and time consuming.

So far the refinement of any method necessarily leads to a decrease in yields or to the formation of artifacts (see the excellent review by LIGHT AND SMITH).

Since the introduction of the automatic amino acid analyzer, the micro scale method of MOORE AND STEIN[2] is now employed routinely with or without some modification. This process is considered very good despite the fact that tryptophan is completely destroyed, cystine is reduced to a very low level or completely decomposed, and the threonine and serine levels are lowered to a certain extent.

In order to find a more effective hydrolyzing agent and a less time consuming method we have tried other substances capable of breaking the peptide bond. Some of them have already been mentioned in the literature, *e.g.* monochloroacetic acid

(LIEBEN AND TANDLER[3]) and crystallized oxalic acid (FEIGL[4] and IACHAN *et al.*[5]). Other acidic substances with low melting points (below 100°) have also been used. With the exception of oxalic acid and trichloroacetic acid, no good results were obtained. The method of hydrolysis using oxalic acid as described by IACHAN *et al.*[5] showed more promise, if improved or modified.

EXPERIMENTAL

Both in preliminary and in subsequent experiments, partially purified crystalline bovine albumin was used. In addition, the new technique was tried out on several other crystalline proteins, defatted cocoa beans, and biological fluids.

Acid hydrolysis with 6 *N* HCl was carried out in sealed tubes previously evacuated, using from 1 mg up to 20 mg of material and 5 ml of acid. Hydrolysis conditions were: temperature from 115 to 130° and a hydrolysis time of 16 to 24 h.

After the hydrolysis the tubes were opened, the liquid was diluted, filtered through hard filter paper, and desiccated under low pressure over sodium hydroxide and phosphorus pentoxide, as generally recommended. The residue was redissolved in buffer pH 2.2, filtered again, conveniently diluted and chromatographed in Model 120C amino acid analyzer (Spinco Division of Beckman Instruments, Inc.) using Custom Research Resin type PA-28 and PA-35 for both columns, and a standard programmed operation.

When solid hydrolyzing agents were used they were weighed and one half was introduced into the tube, followed by the protein to be analyzed and finally the rest of the hydrolyzing agent. Some water was added when necessary. The tubes were evacuated and sealed.

All the sealed tubes were of heavy walled borosilicate glass, measuring approximately 18 mm in diameter and 80 mm long; the neck was constructed in order to facilitate the evacuation and sealing operation.

The method finally adopted may be summarized as follows:

Reagents

Crystallized oxalic acid (1.5 g); 6 *N* HCl (0.5 ml).

Protein material

From 1 to 20 mg was used for high protein content. (With defatted cocoa bean, 25% protein, up to 100 mg has been used with good results.)

Procedure

Half of the oxalic acid is placed inside the tube, then the protein, followed by the remaining oxalic acid, and finally, the HCl solution. The tube is evacuated (a few minutes) and sealed, as usual. The sealed tube is immersed in a can containing mineral oil, transferred to an oven regulated at 130° and left overnight (16 h). After cooling and freezing, the tubes are opened and the frozen mass dissolved in water and filtered through a very fine filter paper. The filtrate is suitably diluted and is ready for analysis in the amino acid analyzer.

All the experiments with the new hydrolyzing agents were compared with

those with 6 *N* HCl as described in the experimental part, and chromatographed in a standard operation.

The preliminary hydrolysis with crystallized oxalic acid was made essentially according to IACHAN *et al.*[5], either in a sealed or open tube using 1.5 g of oxalic acid, a temperature of 120° and from 1 mg up to 20 mg of protein.

For the monochloroacetic, trichloroacetic and formic (99%) acid experiments 1.5 g of acid was used and 20 mg of protein. To provide the necessary water for the hydrolysis 0.5 ml of distilled water was added. The temperature was the same as for oxalic acid.

It is very important to note that all the new hydrolyzing agents develop an internal pressure, despite the prior evacuation of the tube. The gas formed contains H_2S (from cystine?). It is important to call attention to the necessity of freezing the ampoule before opening in order to avoid a possible explosion.

RESULTS AND DISCUSSION

The preliminary results obtained are summarized in Tables I and II. Only oxalic and trichloroacetic acids showed some promise with respect to further examination.

TABLE I

HYDROLYSIS WITH NEW HYDROLYSIS AGENTS

Temperature 115°. Time of hydrolysis 24 h, sealed tube. 1.5 g of monochloroacetic, trichloroacetic and formic acids were used and 0.5 ml water.

Reagent	*Moles amino acid/ 100 000 g protein*
HCl 6 *N*	587.8
Monochloroacetic acid	354.7
Trichloroacetic acid	476.4
Formic acid	165.6
Oxalic acid	538.6
Monochloroacetic acid	—no cystine, no valine, no methionine, low serine
Trichloroacetic acid	—no cystine, no methionine, serine preserved
Formic acid	—very low hydrolysis
Oxalic acid	—see Table II

IACHAN *et al.*[5], when describing this method of "dry hydrolysis" with crystallized oxalic acid, claimed a very good recovery of the amino acids. They emphasized the fact that amino acids ordinarily destroyed in the normal acid hydrolysis (such as tryptophan, cystine and serine) were preserved. These authors measured the degree of hydrolysis by the "Sörensen and ninhydrin reactions, and paper electrophoresis". No chromatographic analysis was made.

Our first attempt at using this method, as described by its authors, in an automatic amino acid analyzer, the hydrolysis experiments being made both in a sealed tube and in an open tube, showed low results when compared with normal acid hydrolysis, cystine and tryptophan being completely destroyed. In the open tube

TABLE II

COMPARISON OF HYDROLYSIS WITH HYDROCHLORIC AND OXALIC ACIDS

Moles amino acid/100 000 g protein. Temperature 115°; time of hydrolysis 24 h; crystallized oxalic acid without any addition. Average of two hydrolyses and two chromatographic runs of each.

Amino acids	*6 N HCl*	*Oxalic acid*	
		Sealed tube	*Open tube*
Lysine	78.7	75.3	70.5
Histidine	12.6	12.6	4.3
Arginine	26.3	21.9	15.3
Aspartic acid	57.0	62.8	38.0
Threonine	39.9	26.4	5.2
Serine	23.6	8.8	0.0
Glutamic acid	79.5	80.3	34.8
Proline	31.9	30.3	29.4
Glycine	18.7	18.6	16.6
Alanine	61.3	56.6	67.4
Cystine	0.0	0.0	5.3
Valine	33.4	27.4	29.8
Methionine	3.6	3.3	2.9
Isoleucine	12.9	11.5	8.4
Leucine	63.5	57.7	51.9
Tyrosine	17.6	19.2	15.2
Phenylalanine	31.2	25.9	25.6
Total	591.7	538.6	420.6

results were much lower and serine was also destroyed. However cystine was partially preserved. See Table II.

Furthermore it was observed that neutralization with calcium carbonate lowered the yields of many amino acids. Two hydrolysis experiments were made with the same material, one neutralized with calcium carbonate and diluted with buffer, pH 2.2, and the other without neutralization. Both solutions were chromatographed in the Beckman analyzer. The results obtained showed a decrease of about 15% in the yields of almost all the amino acids for the neutralized solution, showing that calcium oxalate or the excess of calcium carbonate adsorbed some of the amino acids.

As the direct chromatography of the acid hydrolyzate worked so well in the above experiment we were encouraged to repeat this technique, as it could represent a considerable time saving for the overall operation. All the subsequent experiments with oxalic acid were made without neutralization despite its high initial pH. The HCl hydrolyzate was always dried under vacuum and over sodium hydroxide and phosphorus pentoxide. At least fifty runs were made under these conditions without any trouble with the columns.

Apparently addition of water does not decrease the rate of hydrolysis. In experiments not included in this paper the addition of up to 2 ml of water to 1.5 g of crystallized oxalic acid did not alter the rate of hydrolysis. This is very useful for possible analyses of biological fluids.

In order to improve the rate of hydrolysis a higher temperature was tried. A temperature of 130° for 24 h showed the best results, as recorded in Table III.

TABLE III

HYDROLYSIS AT 130° FOR 24 h

Moles amino acid/100 000 g protein; average of two hydrolyses and two chromatographic runs; crystalline oxalic acid without any addition.

Amino acids	*6 N HCl*	*Crystallized oxalic acid*
Lysine	80.4	96.0
Histidine	13.4	12.8
Arginine	27.4	25.4
Aspartic acid	59.2	70.1
Threonine	38.5	28.4
Serine	20.9	7.0
Glutamic acid	84.9	91.1
Proline	32.8	43.3
Glycine	19.1	21.7
Alanine	60.1	68.0
Valine	35.4	37.3
Methionine	3.6	4.1
Isoleucine	13.3	15.2
Leucine	65.5	70.8
Tyrosine	18.3	23.6
Phenylalanine	31.6	35.4
Total	604.3	650.2

Under these conditions a relative decrease in threonine and serine content was observed (*cf.* Table III).

It was also investigated whether the addition of some HCl together with the oxalic acid could improve the hydrolysis rate.

TABLE IV

COMPARISON OF HYDROLYSES AT 130° FOR 16 h

Moles amino acid/100 000 g protein; average of two hydrolyses and two chromatographic runs of each.

Amino acids	*Oxalic acid plus*				*6 N HCl*	
	0.5 ml H_2O	*1 ml H_2O*	*0.25 ml 6 N HCl*	*0.5 ml 6 N HCl*	*16 h at 130°*	*24 h at 115°*
Lysine	78.4	79.1	83.5	93.1	80.4	75.5
Histidine	8.5	11.1	11.7	14.2	13.4	12.1
Arginine	19.1	22.2	21.2	20.3	27.4	27.3
Aspartic acid	61.3	61.0	64.2	64.0	59.2	55.3
Threonine	24.8	26.6	24.3	34.6	38.5	33.6
Serine	8.3	11.1	8.6	14.6	20.9	17.1
Glutamic acid	71.7	70.1	79.3	99.2	84.9	83.8
Proline	35.1	32.0	34.1	36.6	32.8	32.9
Glycine	20.4	20.5	17.9	18.5	19.1	15.8
Alanine	67.7	65.0	65.0	67.2	60.1	66.1
Valine	33.0	32.6	33.8	37.5	35.4	35.8
Methionine	3.8	4.1	3.3	3.5	3.6	3.3
Isoleucine	13.4	13.5	14.5	16.6	13.3	14.9
Leucine	65.6	66.5	66.8	69.0	65.4	66.5
Tyrosine	21.0	20.7	20.5	19.8	18.3	18.3
Phenylalanine	29.7	29.3	29.7	34.0	31.6	30.3
Total	561.7	565.4	578.4	642.7	604.3	588.6

As the temperature of 130° showed better results all the subsequent programmed experiments were made with only 16 h hydrolysis. The results obtained are summarized in Table IV.

The addition of 0.5 ml of HCl to 1.5 g of crystallized oxalic acid proved to be the most effective hydrolyzing agent. In general all the amino acids show higher yields except arginine, threonine and serine which show a relative decrease. Lysine and glutamic acid showed much higher figures. Aspartic acid is also somewhat increased in yield. It is interesting to note that the addition of HCl apparently affords some protection to threonine and serine.

In view of the results obtained we tried to use the new method for the hydrolysis of different materials such as crystalline proteins, defatted cocoa beans and biological fluids.

The hydrolysis of the cocoa bean showed better results than with the normal HCl hydrolysis. Apparently less humin is formed and a larger amount of solid residue is observed probably because inert materials such as fiber or polysaccharides are less hydrolyzed.

It is also interesting that the temperature is more critical than the duration of hydrolysis.

CONCLUSIONS

The hydrolysis of protein material with crystallized oxalic acid together with some added HCl has been shown to be more efficient than the classical hydrolysis method with HCl alone. Only serine and threonine appear to be destroyed to a certain extent, and the arginine content is lowered somewhat. All the other amino acids show a better recovery.

The neutralization step of the hydrolyzate, which is a tedious and time-consuming operation, may be omitted without any danger to the columns of the automatic amino acid analyzer or to the precision of the analysis.

Also, solutions may be analyzed without previous concentration or need only be partially concentrated, provided that the proportion of the remaining water does not exceed very much that recommended, and that concentrated HCl is added to the proportion recommended.

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CHROM. 4764

GEL PERMEATION CHROMATOGRAPHY OF THE LINEAR MONOMER AND OLIGOMERS IN POLYAMIDES

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SUMMARY

The linear monomers and oligomers in nylon 6, nylon 66, and nylon 12 were separated and determined by gel permeation chromatography using Sephadex LH-20 as the gel and 0.05 *N* HCl–methanol as eluant. The water–ethanol extractables of the polyamides were reacted with 2,4-dinitrofluorobenzene and the reaction solution was chromatographed. The effluent was channeled into a flow cell and the absorbance of the DNP-derivatives of the linear monomer and oligomers separated was measured continuously by a spectrophotometer in the 370 to 450 mμ range. The cyclic monomers and oligomers did not interfere with the separation and determination of the linear monomer and oligomers. Higher oligomers, up to the octamer of nylon 6, the tetramer of nylon 66, and the hexamer of nylon 12, were separated and determined. As little as 0.01 μmole of the DNP-derivatives has been determined. A linear relationship was obtained between the elution volume and the logarithm of the molecular weight of monomer and oligomers from which that of the dinitrophenyl group had been subtracted.

INTRODUCTION

In order to study the kinetics of polymerization of caprolactam or polycondensation of the bifunctional compound such as diamine-dicarboxylic acid salt or ω-amino acid, it is necessary to know the linear monomer and oligomer content in the equilibrium product, as well as cyclic monomer and oligomer content. The linear monomer and oligomers are formed by the hydrolysis of the amide group of the corresponding cyclic monomer and oligomers or of the polyamide, and by the amide-interchange reaction of the polyamide. They remain in the final polymerization product in low concentrations. In addition, ε-aminocaproic acid (linear monomer of nylon 6) is used, in some cases, instead of water as the initiator for the polymerization of caprolactam to nylon 6.

Paper chromatography has been widely used in the separation of ε-aminocaproic acid and the linear oligomers of nylon 6 (refs. 1–4). HERMANS and co-workers[1] separated the aminocaproic acid from the alcohol extractables of nylon 6 on a paper chromatogram by use of a suitable eluant, and evaluated the spot visually against known aminocaproic acid standards after developing it with ninhydrin. The concentration of aminocaproic acid in the equilibrium polymer ranged from 0.0008 to 0.005 mmole per g of polymer[5]. The linear monomer, dimer and the higher oligomers were separated by paper chromatography (PC) with a solvent mixture of *sec.*-butanol–20% aq. ammonia (80 : 20) and the concentration of each component on the paper chromatogram was determined colorimetricaly by extracting the component from the paper into water and by developing the color of the component with sodium hypochlorite solution[2]. The components of the initial phase products of the polymerization of ε-caprolactam were examined and separated into linear monomer to pentamer fractions with isobutanol–acetic acid–water (4 : 1 : 1) on the paper chromatogram[3]. Thin-layer chromatography (TLC) was also possible[6]. The linear monomer and oligomers of nylon 66 have been separated in a similar manner by PC[7,8].

The methods described above are complicated, time-consuming and give poor reproducibility for the purpose of quantitative analysis of each component. ROBINSON[9] has reported a polarographic procedure for the analysis of aminocaproic acid in nylon 6. The aminocaproic acid was reacted with formaldehyde to form the Schiff base and polarographic reduction of the Schiff base was carried out in an alkaline medium. This procedure has very good possibilities for the analysis of the linear monomers of other types of polyamide, but in the case cited caprolactam and oligomers included in the water extractables depressed the wave for the Schiff base. Furthermore, the linear dimer and higher oligomers could not be detected by ROBINSON's procedure.

Gel permeation chromatography has enabled us to separate the cyclic monomers and oligomers in nylon 6 and nylon 66 and each component was determined continuously by UV spectrophotometry[10]. The linear monomer and oligomers could not be detected in these experiments because of their low concentration in the polymers and low molar absorption coefficient at 210 mμ.

Since its introduction in 1945 by SANGER[11], 1-fluoro-2,4-dinitrobenzene (DNFB) has frequently been employed as an analytical reagent in studies involving proteins, peptides, amino acids, and amines. Various techniques for identifying the 2,4-dinitrophenyl (DNP)-derivatives have been reported[12]. KESNER and co-workers[13] have separated ether- and water-soluble DNP-derivatives of amino acids, peptides and amines by means of silica gel column chromatography with a gradient elution system. The absorbance of the effluent was measured at 340 mμ. SATAKE and co-workers[14] have reported the automatic analysis of amino acids and peptides based on dinitrophenylation with DNFB which was used for the colorimetry instead of ninhydrin[14]. The DNFB reaction has been applied to the determination of amine end groups in nylon 6, by reacting nylon fiber heterogeneously[15] or homogeneously[16] with DNFB.

The method described in this paper gives quantitative results for the linear monomers and oligomers in nylon 6, nylon 66, and nylon 12. The linear monomers and oligomers are converted into the DNP-derivatives with DNFB and their separation

accomplished by means of gel permeation chromatography. The gels used in this study were Sephadex LH-20 (Pharmacia) which was used with organic solvents and Bio-Beads S-X2 (Bio-Rad Lab.) made of cross-linked polystyrene. The effluent was passed through a flow cell from which the absorbance was continuously recorded. The previous isolation of the cyclic monomer and oligomers from the extractables was not necessary.

EXPERIMENTAL

Materials

The gels used were Sephadex LH-20 (particle size 25–100 μ, Lot. No. 1152) and Bio-Beads S-X2 (particle size 200–400 mesh, control No. 6604). The solvent regains were determined in our laboratory. The eluant was a 0.05 N hydrochloric acid solution in methanol for LH-20 and a 0.05 N hydrochloric acid solution in methanol–benzene (1 : 1) for Bio-Beads. DNFB and ε-aminocaproic acid (ACA) (linear monomer of nylon 6) were commercial products of reagent grade. A sodium borate–sodium phosphate buffer solution[14] was prepared as follows: 0.1 M sodium borate solution was mixed with 0.8 M mono-sodium dihydrogen phosphate solution until the pH of the mixture was near 8.5. The linear dimer, trimer and tetramer of nylon 6 were synthesized according to VAN DER WANT's method[17,18]. The higher linear oligomers of nylon 6, the linear monomer and oligomers of nylon 66 and nylon 12 were obtained by extracting them from the corresponding polymers.

Packing

Packing of the gels in the columns was carried out according to the method previously reported[10]. Columns and fittings were also identical.

Dinitrophenylation

A 10-mg portion of ACA was weighed into a 30-ml round-bottomed flask and 700 mg of DNFB (50-fold molal excess), 12 ml of ethanol, 1 ml of the buffer solution, and 2 ml of water were added. The flask was attached to a water-cooled condenser and the solution was refluxed on a water bath for 1 h. After cooling, the solution was transferred to a 25-ml volumetric flask and made up to volume with 0.05 N HCl–methanol. DNP-oligomers of nylon 6 (dimer to tetramer) were prepared in the same manner, adding 50-fold molal excess of DNFB-reagent.

Both linear and cyclic monomers and oligomers were extracted from 10 g of the corresponding polymers with 85% ethanol and the extract solution was evaporated nearly to dryness. The dried extract was transferred to a 30-ml round-bottomed flask and 500 mg of DNFB, 6 ml of ethanol, 0.5 ml of the buffer solution and 0.5 ml of water were added. After the reaction, the mixture was transferred to a 10-ml volumetric flask and diluted to volume.

Elution

A 1-ml portion of the solution of the DNP-derivatives was placed on the column. A constant flow rate was kept at 30 ml/h for the Sephadex columns and 20 ml/h for the Bio-Beads column. Blue Dextran 2000 (Pharmacia), methylated with dimethylsulphate[19], was also applied in order to determine the void volume (V_0)

of the columns. The other conditions and procedures were as described in a previous report[10].

Detection

The effluent was delivered to a flow cell adapted to the Hitachi spectrophotometer Model 124 equipped with an absorbance recorder. The absorbance of the linear monomer and oligomer dinitrophenylates in the effluent was recorded continuously. The wavelength was selected in the range of 370 mμ and 450 mμ according to their concentration and elution position.

Calibration

Calibration curves were plotted from prepared solutions of DNP-ACA. The solutions were prepared so that the concentration of DNP-ACA varied within the range expected. Injection of the solutions was made in duplicate at each wavelength. The calibration curves were prepared by plotting the peak area (unit: absorbance × ml) *vs.* the concentration of DNP-ACA (mmole/l) for each wavelength.

RESULTS AND DISCUSSION

The reaction rate of DNFB with amine groups in ethanol depends upon the water content and the pH. The rate increases with increasing water content up to about 15% by weight[16], and also increases ten-fold on increasing the pH value by 1 unit[14]. The dinitrophenylation procedure described under EXPERIMENTAL was developed in the light of these considerations. Under the prescribed conditions, the reaction was complete in 1 h. If the refluxing was stopped in 30 min, the conversion rate was not satisfactory—only about 70%.

The UV and visible absorption spectra of DNP-ACA and the reagent blanks (dinitrophenol, dinitrophenetole, and unreacted DNFB) in 0.05 *N* HCl–methanol are shown in Fig. 1. The maximum molar absorption coefficient (ε) of DNP-ACA in

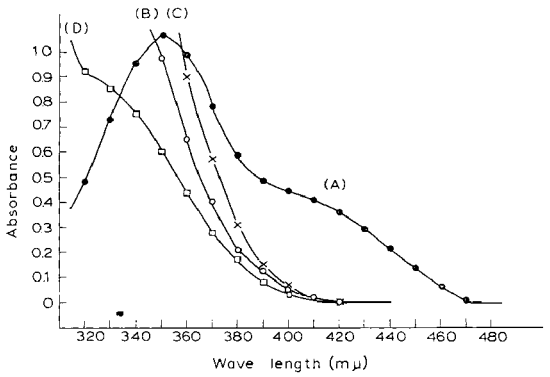


Fig. 1. Spectra of DNP-ACA and reagent blanks. (A) 22.5 mg (0.076 mequiv.) of DNP-ACA in 1250 ml of 0.05 *N* HCl–methanol. (B) 700 mg of DNFB, 12 ml ethanol, and 200 mg $NaHCO_3$ were added and refluxed for 1 h and then diluted to 1250 ml with 0.05 *N* HCl–methanol. (C) 700 mg of DNFB, 12 ml of water, and 1 ml of the buffer solution were added and refluxed for 1 h and then diluted to 1250 ml with 0.05 *N* HCl–methanol. (D) 600 mg of DNFB in 1250 ml of 0.05 *N* HCl–methanol. Cell length: 1 cm; reference: 0.05 *N* HCl–methanol.

TABLE I

THE MOLAR ABSORPTION COEFFICIENTS OF DNP-ACA

Wavelength (mμ)	*M.A.C.*[a] *(l cm^{-1} moles^{-1})*
360	16 050
370	12 700
380	9 530
390	7 870
400	7 220
410	6 660
420	5 890
430	4 740
440	3 950
450	2 210

[a] Measured in 0.05 *N* HCl–methanol.

0.05 *N* HCl–methanol was measured as 17 400 at 351 mμ. This wavelength could be employed for measurement of DNP-derivatives, if no blank materials elute near the DNP-derivatives. The molar absorption coefficients of DNP-ACA in 0.05 *N* HCl–methanol were also measured in the range of 360 and 450 mμ and listed in Table I. The absorbance of the reagent blanks decreases on the acid side, and in the range 420–450 mμ the effect of the reagent blanks is negligible. A 0.05 *N* HCl–methanol solution was thus employed as an eluant. Provided that the elution peaks of DNP-derivatives and the reagent blanks do not overlap, the behavior of the reagent blanks deserves little consideration. In the chromatography as described later, the influence of the reagent blanks must be considered. The choice of 410 mμ (Fig. 3) is a compromise between the sensitivity and magnitude of the reagent blanks. The molar absorption coefficients of DNP-oligomers were considered to be almost identical to that of DNP-ACA.

The column constants showing the column performance for Sephadex LH-20 and Bio-Beads S-X2 are listed in Table II. The elution volume, 143.5 ml, on the

TABLE II

COLUMN CONSTANTS

	Column		
	LB	*LC*	*S*
Type of gel	LH-20	LH-20	S-X2
Column head; cm	91	142	90
Dry gel weight, a; g	41.7	65.1	65
Total volume of gel bed, V_t; ml	174	271	172
Void volume, V_0; ml	72.5	113	50
Inner volume, V_i; ml	71	111	62
Volume of gel phase, V_x; ml	101.5	158	122
Volume of gel matrix, V_g; ml	30.5	47	60
Solvent regain, S_r; ml/g of dry gel	1.7	1.7	0.95
Bed volume per g dry gel; ml	4.2	4.2	2.6

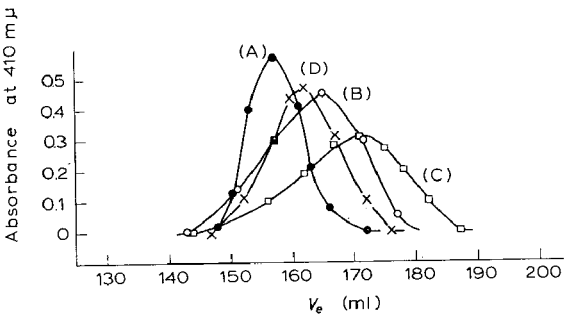


Fig. 2. Elution behavior of DNP-ACA in several solutions on the LB column. Sample volume injected: 1 ml. (A) A 15 mg portion of DNP-ACA was dissolved in 25 ml of ethanol. (B) A 9.8 mg portion of ACA and 1 ml of the buffer solution were refluxed with DNFB in ethanol and diluted to 25 ml with ethanol. (C) A 14.9 mg portion of DNP-ACA and 10 ml of the buffer solution were mixed and diluted to 25 ml with ethanol. (D) A 10.1 mg portion of ACA and 1 ml of ammonia (28%) were refluxed with DNFB in ethanol and diluted to 25 ml with ethanol.

LB column represents a K_d value of unity. If a solute has a higher K_d value than unity, it implies that adsorption (or something else to retard the elution) is taking place between the solute and the gel matrix. The elution behavior of DNP-ACA in the presence of electrolyte on the LB column is shown in Fig. 2. The elution volume of DNP-ACA is between 157 and 171 ml and the K_d value is higher than unity. An electrolyte, such as sodium borate (buffer solution), sodium bicarbonate, retards the elution of DNP-ACA by 8 to 14 ml and in addition broadens the peak. The neutralization of the alkali with hydrochloric acid was not efficacious. The use of ammonia instead of alkali was effective in eliminating the influence of the electrolyte. Without use of alkali, buffer solution, or ammonia the conversion rate of ACA to DNP-ACA was only about 77%, even after refluxing for 1 h. The effect of the electrolyte will be discussed separately[20].

The elution curves of the linear monomer and oligomer of nylon 6, nylon 66, and nylon 12 on the LH-20 column are shown in Fig. 3. Peaks representing unreacted DNFB, dinitrophenol, and dinitrophenetole appear before and after the DNP-ACA (Fig. 3A) and are not shown. Fig. 3 demonstrates good resolution in the fractionation of a mixture of the linear monomer and oligomers. The higher oligomers (up to the octamer of nylon 6, the tetramer of nylon 66, and hexamer of nylon 12) were separated and determined by use of the LC column (LH-20, gel bed 142 cm). The elution volumes of the linear monomer and oligomers on each gel column are presented in Table III. The elution volume of DNP-ACA is large in comparison with the value of $V_0 + V_i$ in Table II. This result shows that the elution of the DNP-derivatives was retarded by adsorption or some other mechanism. A linear relationship can be seen (Fig. 4) between the elution volume and logarithm of the molecular weight of the raw monomer and oligomers minus that of the dinitrophenyl group. The relationship between elution volume and molecular weight of DNP-derivatives will be discussed in detail in a separate paper[20].

The linear monomer and oligomers of nylon 66 are generally classified into three types from their end groups as follows: diamine type (which has two amine groups in both ends), dicarboxylic acid type (two carboxylic acids), and amino acid type (an amine group and a carboxylic acid group). In Fig. 4, the plot of the

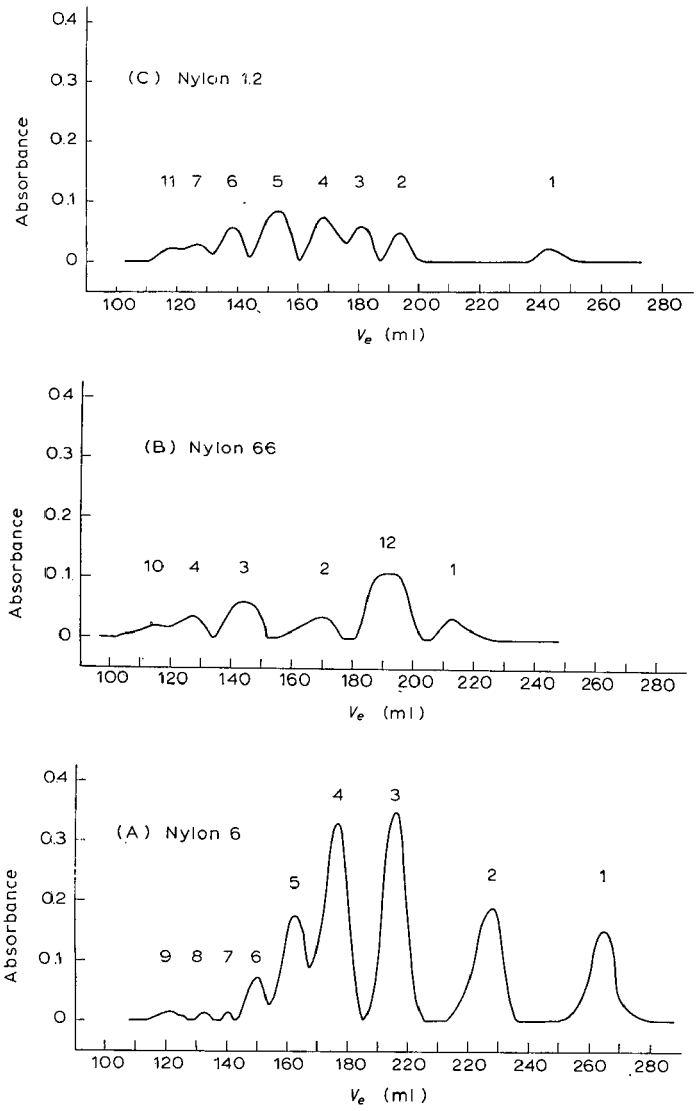


Fig. 3. Elution chromatograms of the DNP-derivatives of linear monomer and oligomers in polyamides on the LC column (LH-20). (A) Absorbances were measured at 370 mμ for oligomers and 450 mμ for the monomer. (B) Absorbances were measured at 410 mμ. (C) Absorbances were measured at 410 mμ for oligomers and 450 mμ for the monomer. 1 = monomer; 2 = dimer; 3 = trimer; 4 = tetramer; 5 = pentamer; 6 = hexamer; 7 = heptamer; 8 = octamer; 9 = nonamer and higher; 10 = pentamer and higher; 11 = octamer and higher; 12 = diamine type monomer of nylon 66.

elution volumes of the monomer and oligomers of nylon 66 fitted the positions for the molecular weight of the amino acid type and a linear relationship was obtained. From this result it was concluded that the linear monomer and oligomers of nylon 66 are of the amino acid type. A solute which eluted between the monomer and the dimer

TABLE III

THE ELUTION VOLUMES (V_e) (ml) OF THE DNP-DERIVATIVES OF LINEAR MONOMER AND OLIGOMERS

Oligomer	*Nylon 6*				*Nylon 66*			*Nylon 12*		
	Column			*Mol. wt.*	*Column*		*Mol.*[a] *wt.*	*Column*		*Mol. wt.*
	LB	*LC*	*S*		*LB*	*LC*		*LB*	*LC*	
Monomer	165	264	114	131	124	212	244	153	242	215
Dimer	130	227	102	244	102	170	470	116	193	410
Trimer	115	195	64	357	88	143	696	102	180	605
Tetramer	103	176	59	470	78	127	922	91	167	800
Pentamer	95	162		583				83	153	995
Hexamer	88	150		696				78	137	1190
Heptamer	82	140		809					127	1385
Octamer	72	132		922						
Higher	~75	114 ~128								

[a] The molecular weights of the linear monomer and oligomers of nylon 66 were calculated by assuming that they have both amino and carboxyl groups as end groups.

of nylon 66 (in Fig. 3B 113 ml on Column LB and 193 ml on Column LC) is looked upon as the linear monomer of the diamine type (molecular weight 342).

Peak areas were calculated from the elution chromatograms of the dinitrophenylated extractables of each polyamide, and the concentration (mmole/l) of each DNP-derivative was determined from the calibration curves. The content of each monomer or individual oligomer in the polymer was also calculated on a weight percentage basis from the molecular weight of corresponding monomer or oligomer and the weight of the polyamide from which the monomer and oligomers were extracted. The results are shown in Table IV. Consistent results were obtained with known mixtures of the monomer and oligomers, provided that the conditions for

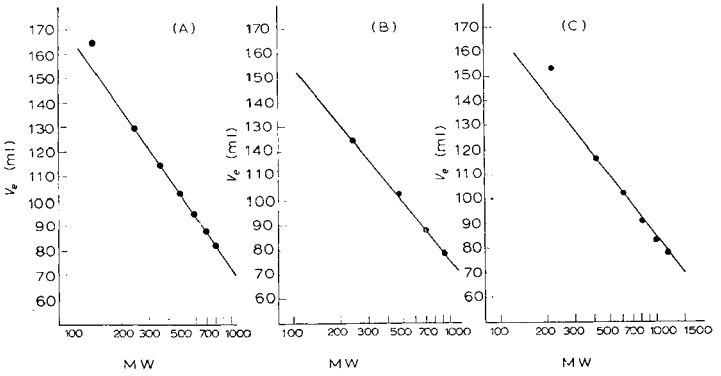


Fig. 4. Correlations between elution volume and molecular weight of the linear monomer and oligomers in polyamides on the LB column (LH-20). (A) nylon 6; (B) nylon 66; (C) nylon 12. Molecular weight of dinitrophenyl group is not included.

TABLE IV

PER CENT OF LINEAR MONOMER AND OLIGOMERS IN THE POLYAMIDES

1×10^{-2} wt.% *vs.* polymer.

	Nylon 6	*Nylon 66*	*Nylon 12*
Monomer	3.6	0.9	1.0
Dimer	2.0	2.5	0.9
Trimer	4.8	5.2	1.5
Tetramer	4.2	3.4	5.4
Pentamer	1.5		9.8
Hexamer	0.9		4.0
Heptamer	0.1		2.0
Octamer	0.1		
Diamino monomer		2.2	
Sum	17.2	14.2	24.6

dinitrophenylation and for the separation of the DNP-derivatives were held constant. An identifiable peak at 410 mμ is obtained with as little as 0.01 μmole of the DNP-derivatives. The amount of the higher oligomers (hexamer to octamer) is less than that of the lower oligomers. According to Flory's theory, the content of higher oligomers would be expected to increase with increasing the degree of polymerization. From the results in Table IV it would appear that the higher oligomers are barely extracted from the polymer. The content of the linear monomer in nylon 6 and nylon 12 is much more than that of the linear dimer. This fact gives some clue as to the mechanism of the polymerization; the oligomers greater than the dimer are considered to be a part of the polymer; on the other hand, the monomer acts as a catalyst for the polymerization and will be an intermediate to the polymer in the polymerization process.

Dinitrophenol and dinitrophenetole are formed by reaction of DNFB with water and ethyl alcohol in the dinitrophenylation process as by-products and they are included in the reaction products in addition to unreacted DNFB. The molar absorption coefficients of dinitrophenol and unreacted DNFB decrease with decreasing pH value and thus methanol acidified with HCl was employed as an eluant. The elution volumes of the various constituents on the LC column are as follows: 257 ml for unreacted DNFB, 278 ml for dinitrophenol, and 320 ml for dinitrophenetole. These reagent blanks interfere, therefore, with the absorption measurement of the DNP-ACA which elutes between unreacted DNFB and dinitrophenol. The interference can be eliminated by the measurement at higher wavelengths such as 420 $\sim$ 450 mμ and if necessary the addition of ammonia after the dinitrophenylation of the mixture of monomer and oligomers could be considered when the dinitrophenol and unreacted DNFB would be converted to dinitroaniline. The elution order of these reagent blanks is in contrast to their molecular weights. According to NILSSON[21], the estrogenic isoflavones in red clover are effectively separated on Sephadex G-25 in 0.1 *M* ammonium hydroxide and daidzein, genistein, formonetin, and biochanin A are eluted in this order. Daidzein where a hydrogen atom is substituted for the hydroxyl group in genistein elutes earlier than genistein, and biochanin A containing a methoxy group in place of the hydroxyl group in genistein elutes later than genistein. His result is therefore consistent with ours.

In the course of the chromatography of the solution of reagent blanks, unknown solutes were measured before the peak of unreacted DNFB and after the peak of dinitrophenol by the measurement of absorption at 370 mμ. The wavelength of 410 mμ was therefore selected for the measurements after due consideration of the sensitivity and the magnitude of the reagent blanks. The wavelength range of 440–450 mμ was used, if necessary, to reduce the absorption which appeared near the peak concerned due to the reagent blanks when the absorbance of the DNP-monomer of nylon 6 and nylon 12 was measured.

The swelling of Bio-Beads S-X2 with methanol–benzene (1 : 1) solvent was so incomplete that separation above the DNP-trimer of nylon 6 was incomplete.

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CHROM. 4784

PARTITION CHROMATOGRAPHY OF SUGARS ON ION-EXCHANGE RESINS

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SUMMARY

In separations of monosaccharides in aqueous ethanol on ion-exchange resins in their lithium and sulfate forms, the sugars are eluted, with few exceptions, in the order of an increased number of hydroxyl groups. With oligomers there exists a straight-line relationship between the logarithm of the distribution coefficient and the number of monomeric units. Higher saccharides of various types exhibit large individual differences.

INTRODUCTION

Partition chromatography on ion-exchange resins in aqueous ethanol is a valuable tool in the separation of various mono- and oligosaccharides. Within the range of ethanol concentration applied, the ratio water:ethanol is higher inside the resin than in the external solution. This explains why sugars are retained in the resin phase. In addition, interacting forces between the solutes and the counterions of the resin influence the uptake[1].

The method is of practical importance in analyzing monosaccharides in the hydrolyzate of plant materials[2]. The purpose of this work is to study the elution behavior of mono- and oligosaccharides not previously examined. An attempt has been made to correlate the structure of the sugars with their elution behavior.

MATERIALS

Talose was prepared by reduction of talono-1,4-lactone with 5% sodium amalgam[3]. The product was purified and isolated by partition chromatography on an anion exchanger in the sulfate form. Commercial samples of erythrose from various suppliers were found to contain a complex mixture of sugars. Erythrose, as well as threose, was therefore prepared by lead tetraacetate degradation of glucose and galactose, respectively[4]. The products were isolated as above, and the identity of the tetroses was verified by gas chromatography–mass spectrometry of the trimethylsilyl ethers of the corresponding alditols.

Cellotriose was prepared by phosphoric acid hydrolysis of cotton[5], whereas cellotetraose, cellopentaose and cellohexaose were gifts from Dr. J. K. HAMILTON

(Shelton, Wash.). Samples of 5,6-di-O-isopropylidene gulose and methyl-6-deoxy-α-D-glucoside, kindly supplied by Drs. W. MEYER ZU RECKENDORF (Münster) and T. E. TIMELL (Syracuse, N.Y.), were used for the preparation of the corresponding monosaccharides. Nystose, 1-kestose, planteose and stachyose were gifts from Dr. W. W. BINKLEY (New York), 1,6-anhydro-β-D-glucofuranose and 1,6-anhydro-β-D-glucopyranose from Dr. J. H. SLONEKER (Peoria, Ill.), allose, altrose, 3-ribo-hexulose, mannobiose, xylobiose from Dr. O. THEANDER (Stockholm) and 2,3-di-O-methyl-6-deoxyallose from Dr. MICHAEL H. B. HAYES (Birmingham).

EXPERIMENTAL

The equipment used in the chromatographic separation was the same as described previously[6]. Two kinds of resins were used: one cation exchanger in the lithium form (Dowex 50W-X8) and one strongly basic anion exchanger in the sulfate form (T5C). The particle sizes referred to below were determined in 92.4% ethanol for the H^+ and Cl^- forms, respectively.

Unless otherwise mentioned, the experiments were carried out at 75° to avoid decomposition of the saccharides. The eluate was analyzed automatically[6] with the orcinol method[7], using 3 g of orcinol per liter of 60% sulfuric acid as reagent solution. The reagent pump was made of Hastalloy C except for the cylinder and piston which were of glass and Teflon, respectively, and the bullets which were of ruby. The volume distribution coefficients (D_v) were calculated from the peak elution volumes as usual[8].

RESULTS AND DISCUSSION

Most monosaccharides studied were conveniently separated in 86–90% ethanol on an anion exchanger in the sulfate form. The separation of some monosaccharides not studied previously is illustrated in Fig. 1.

All aldoses studied in earlier work were eluted as expected, in the order of

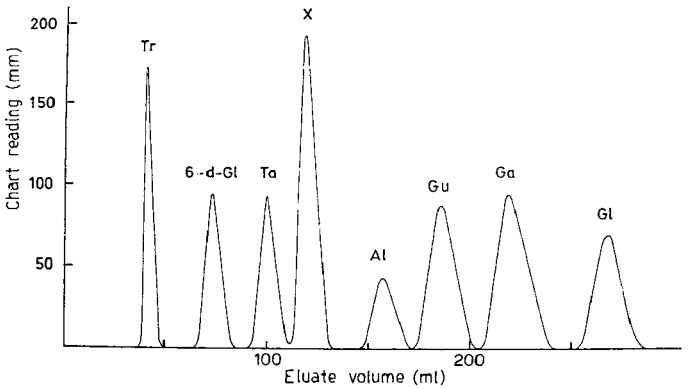


Fig. 1. Partition chromatography in 88% (w/w) ethanol. Resin bed: 4 × 550 mm, T5C, SO_4^{2-}, 14–17 μ. Flow rate: 2.2 ml·cm^{-2}·min^{-1}. Tr = threose, 500 μg; 6-d-Gl = 6-deoxyglucose, 250 μg; Ta = talose, 744 μg; X = xylose, 400 μg; Al = allose, 300 μg; Gu = gulose, 850 μg; Ga = galactose, 500 μg; Gl = glucose, 1000 μg.

TABLE 1

VOLUME DISTRIBUTION COEFFICIENTS OF SOME MONOSACCHARIDES AND DISACCHARIDES AT 75°

Saccharides	*SO_4^{2-} resin (14–17 μ) 88% ethanol*	*Li^+ resin (17–21 μ) 92.4% ethanol*	*Li^+ resin (17–21 μ) 96% ethanol*
Erythrose	3.1	1.9	2.3
Threose	3.8	1.4	1.8
Arabinose	10.1	3.5	4.2
Lyxose	8.9	2.7	3.2
Ribose	6.6		4.6
Xylose	12.5	2.7	3.2
Allose	16.6	6.0	8.3
Altrose	16.6	4.2	5.6
Galactose	23.4		7.6
Glucose	28.1	4.8	6.0
Gulose	20.4	4.9	6.3
Mannose	16.4		5.9
Talose	10.7	5.8	8.3
Fructose	13.5		6.8
3-Ribohexulose	9.8	4.7	6.6
6-Deoxy-D-glucose	7.3	0.9	1.1
Rhamnose	4.8	1.4	1.6
1,6-Anhydro-β-D-glucofuranose	6.3	1.4	1.6
1,6-Anhydro-β-D-glucopyranose	3.8	2.3	2.9
2,3-Di-O-methyl-6-deoxyallose (mycinose)	0.53	0.4	0.4
Mannobiose	41.6		
Xylobiose	35.8	5.4	7.3

increasing number of hydroxyl groups. The distribution coefficients of some monosaccharides, most of which have not been studied previously, are listed in Table I. The positions of erythrose and threose, as well as those of allose and gulose, are in agreement with this rule, whereas talose constitutes an exception and appears before xylose. Another exception is 3-ribohexulose which was eluted at a position close to that of arabinose. Interestingly, it was eluted much earlier than fructose and other hexuloses studied previously. Allose could not be separated from altrose under these conditions (Table I). As expected 6-deoxyglucose exhibited a low D_v value which, however, was much larger than that of 6-deoxymannose. The two isomeric forms of 1,6-anhydro-glucose appeared well separated much earlier than glucose.

As found in previous work, a few monosaccharides which could not be separated on the sulfate resin were eluted as discrete bands from a column filled with cation-exchange resin in the lithium form, whereas some species which were well-separated on the sulfate column overlapped seriously on the lithium column. With few exceptions the correlation between number of hydroxyl groups and elution order was found to hold true for this system as well. Among the species listed in Table I no exceptions were observed. Interestingly, talose was eluted later than the other hexoses with the exception of allose. Likewise a reversed order of elution occurred with other isomers, *e.g.* with the tetroses and with the two forms of 1,6-anhydroglucose. These results confirm that not only are the differences in solubility in aqueous ethanol of the species to be separated of importance, but also interacting forces with the counterions inside the resin.

TABLE II

VOLUME DISTRIBUTION COEFFICIENTS OF SOME MONOSACCHARIDES AND OLIGOSACCHARIDES AT 75°

	SO_4^{2-} resin (14–17 μ) 70% ethanol	Li^+ resin (17–24 μ) 85% ethanol
Monosaccharides		
Xylose	2.3	1.9
Glucose	3.1	3.0
Disaccharides		
Cellobiose	4.4	4.9
Isomaltose	6.5	8.6
Mannobiose	2.9	7.8
Trehalose	6.8	8.2
Xylobiose	3.0	2.0
Trisaccharides		
Cellotriose	5.8	8.7
Gentianose	9.6	12.3
1-Kestose	8.7	16.2
Maltotriose	6.6	7.7
Planteose	9.1	19.3
Higher saccharides		
Cellotetraose	7.9	15.4
Nystose	14.9	27.8
Stachyose	18.5	64.7
Cellopentaose	10.9	27.4
Cellohexaose	15.2	44.4

From a practical point of view, it is important that allose and altrose are well separated on the lithium resin. In practice most separations of mixtures of monosaccharides can be carried out either on a sulfate or on a lithium resin, but it is seen that with some very complex mixtures it is necessary to apply both types of resin.

At the temperature used, 75°, no interfering decomposition was recorded. Experiments carried out with erythrose showed, however, that an appreciable decomposition occurred at higher temperatures. At 90° about 65% of the added erythrose was lost during the run.

At an ethanol concentration of 88%, the disaccharides appear much later on a sulfate resin than do the monosaccharides. As shown in Table I xylobiose and mannobiose were eluted after the hexoses and in the order predicted from the rule discussed above. At 70% ethanol the distribution coefficients of xylobiose and mannobiose were very similar, however, and both were slightly lower than that of glucose (Table II). This reversal can be ascribed to the competition between the factors mentioned in the introduction which contribute to an accumulation of the sugars in the resin phase and factors which tend to exclude the sugars from the resin. The importance of the latter increases with a lowered ethanol concentration and in pure water the exclusion effect increases with an increased partial molar volume[9]. Evidently, an ethanol concentration of 70% is too low for separations of complicated mixtures in which mono- and disaccharides are involved. This ethanol solution was, however, suitable for a complete separation of the oligosaccharides in the glucose–cellohexaose series.

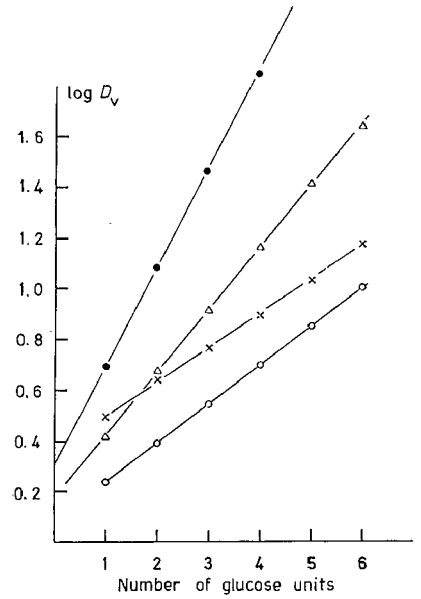


Fig. 2. Relationship between log D_v and the number of glucose units in the oligosaccharides. ×, Sulfate resin (14–17 μ) in 70% ethanol. Lithium resin (17–21 μ) at various ethanol concentrations: ○, 80%; △, 85%; ●, 92.4%.

Paper chromatographic studies[10] have shown that MARTIN'S rule[11] which predicts a straight-line relationship between log $R_F/(1 - R_F)$ and the chain length holds true for several types of oligosaccharides. It could be expected therefore that a plot of the logarithm of the D_V value against the number of hexose units would result in a straight line. As can be seen from Fig. 2, this holds true for the glucose–cellohexaose series, both for the runs on the sulfate and the lithium columns. On both columns the oligomers appeared well-separated in the order of increasing

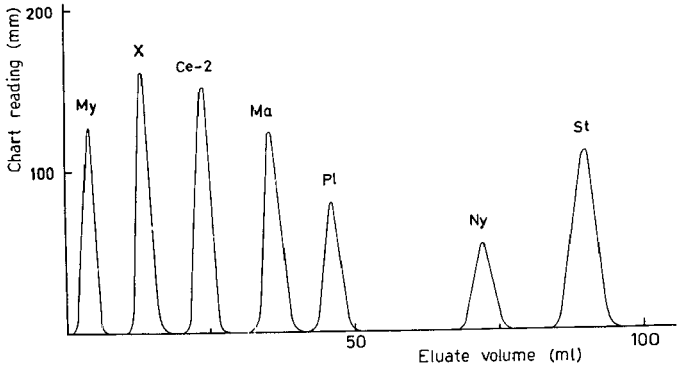


Fig. 3. Partition chromatography in 70% (w/w) ethanol. Resin bed: 4 × 550 mm, T5C, SO_4^{2-}, 14–17 μ. Flow rate: 1.9 ml·cm^{-2}·min^{-1}. My = mycinose, 88 μg; X = xylose, 50 μg; Ce-2 = cellobiose, 112 μg; Ma = maltotriose, 100 μg; Pl = planteose, 82 μg; Ny = nystose, 96 μg; St = stachyose, 200 μg.

molecular weight. Despite the fact that the swelling of the resin is much lower in ethanol than in water, no interfering screening effect[8] was observed. The results show that the method is extremely useful in separations of oligomers.

Chromatograms illustrating the application of sulfate resins and lithium resins in separations of complex mixtures including higher saccharides of various types are given in Figs. 3 and 4. As can be seen from these chromatograms, as well as from the results given in the table, some species are more easily separated on a sulfate column, whereas with others a lithium column is preferred. On both types of resins the elution curves were virtually symmetric. It is seen that the structure of the individual disaccharides and higher saccharides has a great influence upon the retention volume. As an example, it can be mentioned that isomaltose is held much

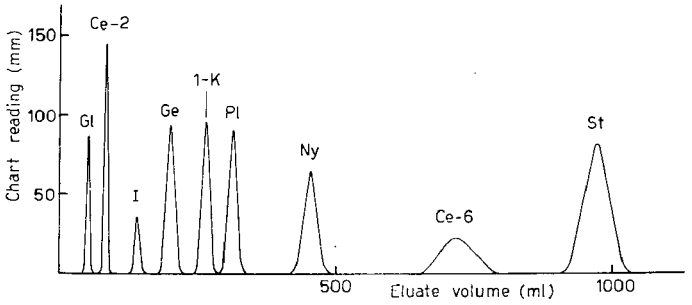


Fig. 4. Partition chromatography in 85% (w/w) ethanol. Resin bed: 4 × 1050 mm, Dowex 50W-X8, Li^+, 17–24 μ. Flow rate: 4.4 $ml \cdot cm^{-2} \cdot min^{-1}$. Gl = glucose, 20 μg; Ce-2 = cellobiose, 56 μg; I = isomaltose, 480 μg; Ge = gentianose, 80 μg; 1-K = 1-kestose, 135 μg; Pl = planteose, 82 μg; Ny = nystose, 160 μg; Ce-6 = cellohexaose, 180 μg; St = stachyose, 300 μg.

more strongly than cellobiose, both on the sulfate column and on the lithium column. Another striking example is the tetrasaccharide stachyose which is held more strongly on both columns than cellohexaose.

In agreement with the results published with various monosaccharides the method was found to be well suited to quantitative analyses. It is extremely important that the analyzing system is kept clean and that no gas bubbles are permitted to enter the system. In addition care must be exercised to avoid leakage in the pumps. When these precautions were taken, the deviations in the peak areas in successive runs were less than $\pm$ 2% from the mean.

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IDENTIFICATION AND QUANTITATIVE ANALYSIS OF *p*-HYDROXYBENZOIC ACID AND ITS ESTERS USING REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The *p*-hydroxybenzoic acid and its methyl, ethyl, propyl, butyl and benzyl esters are separated on thin layers of silanized silica gel using borate buffer (pH 11) as mobile phase, adding a given volume of organic solvent as required. The separation of the various compounds is complete and an accurate quantitative determination of the *p*-hydroxybenzoic acid and its esters is possible using a simple technique of elution and spectrophotometric determination.

INTRODUCTION

The increasing use of *p*-hydroxybenzoic acid esters as food and drug preservatives has led to the development of many analytical techniques for identifying and assaying these compounds. In recent years, thin-layer chromatography (TLC) in particular has often been used with good results[1–20].

In this report we shall briefly describe a reversed-phase TLC method based on the use of silanized silica gel as stationary phase. Coupled with a simple elution and spectrophotometric determination technique, it has proved highly effective in separating and determining *p*-hydroxybenzoic acid and its principal esters.

EXPERIMENTAL

Preparation of the chromatographic plates

About 30 g of HF_{254} (Merck) silanized silica gel were suspended in 60 ml of a (2 : 1) water–methanol mixture by shaking vigorously for a few minutes in order to obtain a homogeneous suspension. The latter was then distributed on the plates using a Desaga spreader so as to obtain a uniform layer of 0.25 mm thickness. The plates were dried in air for about 3 h and stored over silica gel in a desiccator. They should not be used for at least 24 h after preparation.

Preparation of the chromatography solutions

Methanol solutions of pure commercial products (Merck, BDH, Carlo Erba)

were used as reference solutions for *p*-hydroxybenzoic acid and its esters. The methods described in a previous report [21] were used to extract the preservatives from pharmaceutical specialities and to prepare the sample solutions.

Application of the solutions to the plates

To identify the substances, the sample and reference solutions were applied as spots in parallel on the same plate using a micropipette and taking care to pipette no more than 5 μl each time, and to deposit from 2 to 10 μg of each preservative on the plate. For the quantitative determination, the solutions to be analyzed were applied in bands on 5 × 20 cm plates, pipetting from 0.025 to 0.1 ml of solution so that from 20 to 100 μg of each substance are applied to each plate. The solutions were applied 2 cm from the starting edge leaving a margin of approx. 0.5 cm on both sides of the plate.

Chromatographic development

Chromatographic development was carried out using borate buffer, pH 11 (502 ml of 0.2 *M* H_3BO_3 solution diluted to 1000 ml with 0.2 *M* NaOH solution) as mobile phase, adding a given volume of organic solvent as required. The following buffer–solvent combinations have proved most suitable for separation of the various preservatives: buffer–methyl alcohol (90 : 10), buffer–ethylcellosolve (90 : 10), buffer–ethyl acetate (saturated solution at 25°), buffer–ethyl ether (saturated solution at 15°), buffer–tetrahydrofuran (95 : 5), buffer–dioxane (90 : 10).

The plates are developed in glass chromatography chambers lined with filter paper. Before putting the plates in them, the chromatographic chambers were saturated with the solvent vapours for about 1 h. The plates are allowed to develop until the solvent front has risen about 14 cm from the point where the substance was deposited (90 to 120 min). When double development is desired, the plate is dried at room temperature for about 1 h and then returned to the chromatographic chamber. Chromatographic development is performed at about 25° except when the mixture contains ethyl ether in which case the temperature of the chromatographic chamber should be 15°.

Detection of the compounds

After the plates have been developed they are dried at room temperature. The spots or bands of the various compounds were visualized under a 250-mμ UV light source.

Quantitative analysis

After detection, the band of each substance is isolated by cutting away the sides of the layer of adsorbent with a spatula, and then performing the quantitative determination by removing the silica gel and extracting it with methyl alcohol. A silica gel collection method using a vacuum described by several authors[22–25] has proved useful for this purpose. We have adopted the simple device shown in Fig. 1 which permits the easy collection of the silica gel and quick elution of the substances under vacuum. The device consists basically of a small glass funnel inserted, with a special plastic (polyvinylchloride) fitting, into a Pyrex glass volumetric flask (Fig. 1A). Near the lower end of the funnel stem is a perforated sintered glass filter plate with

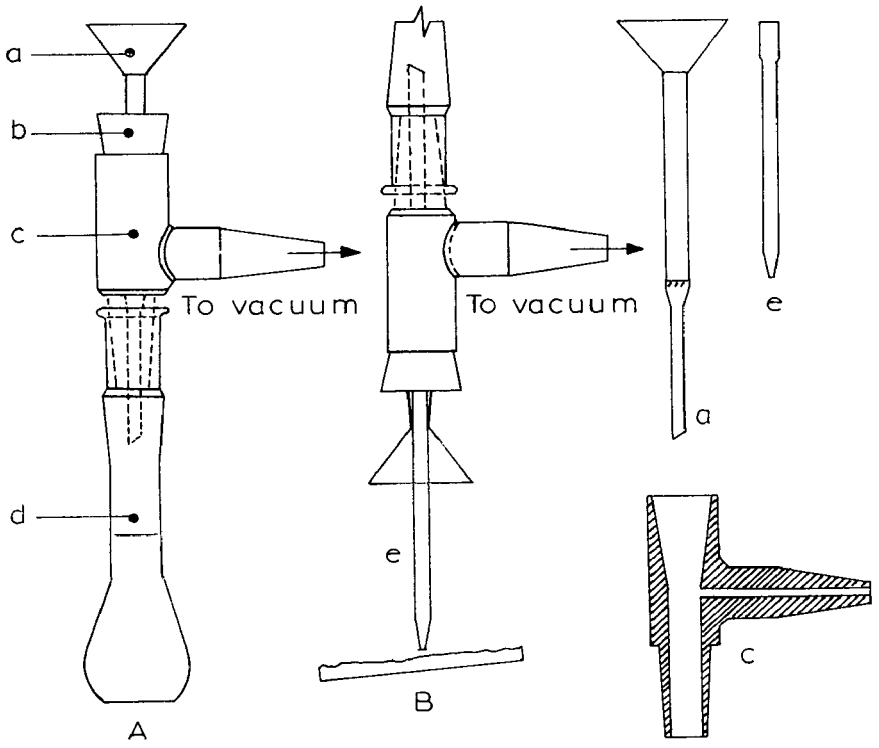


Fig. 1. (1:2 scale). A = system for elution of substances; B = system for suction of silica gel. a = Pyrex glass funnel with perforated sintered glass filter plate, b = rubber stopper, c = polyvinylchloride fitting with side inlet for vacuum, d = volumetric flask, e = glass tube with tygon ring.

gauze or fiber glass pressed onto it; the system can be connected to the vacuum pump by means of the side attachment on the plastic fitting. The silica gel is collected by inserting a small glass tube with a pointed end in the funnel using a tygon ring. The device is turned upside down, and the silica gel is sucked in by applying a low vacuum (Fig. 1B). When the silica gel has been collected, a few tenths of ml of methyl alcohol are sucked through to wash the inlet tube (taking care that no solvent emerges from the bottom end of the funnel stem), and the device is quickly uprighted. The vacuum is broken, the inlet tube is removed, 1 or 2 ml of methyl alcohol are pipetted into the funnel, the vacuum is again applied, and the solvent is collected in the volumetric flask. Methyl alcohol is added a couple more times until elution of the substance to be determined is complete (generally after 4–5 ml of methyl alcohol have passed through the funnel, elution is complete).

The eluent in the volumetric flask is diluted to volume with methyl alcohol and divided into two portions. One was acidified and the other was made alkaline by adding 2% of 1 *N* HCl and 1 *N* NaOH, respectively. Light absorption is measured at 297 mμ using the alkaline solution as test solution and the acidified solution as blank following the differential spectrophotometric method described in our previous report[21].

TABLE I

R_F VALUES OBTAINED ON HF_{254} SILANIZED SILICA GEL FOR THE VARIOUS COMPOUNDS STUDIED

Compound	R_F *values*[a]					
	Mixture A pH 11 buffer–methyl alcohol (90:10)	*Mixture B pH 11 buffer–ethyl cellosolve (90:10)*	*Mixture C pH 11 buffer saturated with ethyl acetate at 25°*	*Mixture D pH 11 buffer saturated with ethyl ether at 15°*	*Mixture E pH 11 buffer–tetra-hydrofuran (95:5)*	*Mixture F pH 11 buffer–dioxane (90:10)*
Benzyl-*p*-hydroxybenzoate	0.04	0.08	0.03	0.02	0.03	0.08
Butyl-*p*-hydroxybenzoate	0.06	0.11	0.06	0.05	0.08	0.13
Propyl-*p*-hydroxybenzoate	0.15	0.22	0.14	0.15	0.14	0.20
Ethyl-*p*-hydroxybenzoate	0.23	0.32	0.25	0.25	0.24	0.28
Methyl-*p*-hydroxybenzoate	0.30	0.38	0.34	0.35	0.33	0.35
p-Hydroxybenzoic acid	0.60	0.70	0.69	0.75	0.57	0.59

[a] Each R_F is the mean value of six separate chromatographic runs.

RESULTS AND DISCUSSION

Table I shows the R_F values obtained on developing the chromatograms with the various buffer–solvent mixtures.

Of the mobile phases studied, mixtures C, D, E, and F gave the best separation of the various compounds, especially of benzyl- and butyl-*p*-hydroxybenzoates. However, mixtures C and D are rather difficult to use because the solvents are volatile and the solutions are saturated; good chromatographic development and reproducibility of data are closely controlled by temperature stability in the chromatographic chamber. Mixtures E and F are more practical, and we normally use them for the analysis of preservatives in pharmaceutical preparations.

In quantitative zone chromatography, the separation may not be good enough

TABLE II

R_F' VALUES CALCULATED AFTER SECOND DEVELOPMENT ON HF_{254} SILANIZED SILICA GEL

Compound	R_F' *value*[a]	
	Mixture E pH 11 buffer–tetrahydrofuran (95:5)	*Mixture F pH 11 buffer–dioxane (90:10)*
Benzyl-*p*-hydroxybenzoate	0.06	0.17
Butyl-*p*-hydroxybenzoate	0.14	0.25
Propyl-*p*-hydroxybenzoate	0.24	0.38
Ethyl-*p*-hydroxybenzoate	0.36	0.49
Methyl-*p*-hydroxybenzoate	0.47	0.58
p-Hydroxybenzoic acid	0.68	0.75

[a] Each R_F' is the mean value of six separate chromatograms.

for elution and quantitative determination of the components of the mixture when the amounts of some of the compounds to be separated are much larger than others. In this case, the mobility of the substances and the chromatographic separation can be greatly enhanced by performing a second development with the same solvent mixture, especially when low R_F paraseptics are involved.

Table II shows the R_F' values obtained with mixtures E and F after a second development. In this case the R_F' values represent the proportion between the distance of the spots of substance from the point of application after the second development and the distance of the solvent front from the point of application (the solvent run was the same in the first and second development).

The quantitative analysis of the eluents by differential spectrophotometry eliminates background absorption due to the extraction of the silica gel with methanol. The amount of the test compounds may be calculated from the spectrophotometric results using a calibration straight line plotted from results of chromatographic analyses performed on solutions containing known amounts of preservatives.

Greater precision in quantitative analyses may be obtained by using the internal standard method. In this case a known amount of a compound with a different R_F from those of the test compounds is added to the test solution before chromatography (since the six preservatives discussed here are not generally used together in the same pharmaceutical speciality, one which is known to be absent in the test sample may be used as an internal standard). After chromatographic development and spectrophotometric determination of all the compounds, it is

TABLE III

QUANTITATIVE CHROMATOGRAPHIC ANALYSIS OF METHANOL SOLUTIONS CONTAINING DIFFERENT CONCENTRATIONS OF METHYL AND PROPYL-*p*-HYDROXYBENZOATE

Chromatography was performed on 5 × 20 cm H_{254} silanized silica gel plates using a pH 11 buffer–dioxane mixture (90:10) as mobile phase; 0.05 ml of sample solution was pipetted as a band on the plates. Method A = determinations performed using the calibration straight line. Method B = determinations performed by adding butyl-*p*-hydroxybenzoate as internal standard.

Solution No.	*Theoretical (mg/ml)*	*Found*[a]			
		Method A		*Method B*	
		mg/ml	*% difference*	*mg/ml*	*% difference*
(a) Methyl-p-hydroxybenzoate					
1	0.424	0.426	+0.5	0.424	0.0
2	0.824	0.807	−2.1	0.836	+1.5
3	1.227	1.196	−2.5	1.260	+2.7
4	1.595	1.552	−2.7	1.585	−0.6
5	2.006	1.992	−0.7	2.046	+2.0
(b) Propyl-p-hydroxybenzoate					
1	2.008	2.045	+1.8	2.053	+2.2
2	1.621	1.580	−2.5	1.613	−0.5
3	1.206	1.164	−3.5	1.211	+0.4
4	0.804	0.781	−2.9	0.807	+0.4
5	0.419	0.420	+0.2	0.416	−0.7

[a] Mean of three chromatographic determinations performed under the same conditions.

easy to calculate the amount of each single compound in the test solution using the formula:

$$\frac{P \cdot Ec \cdot F}{Est} = \text{mg of compound contained in the sample solution}$$

where P is the amount of internal standard added, expressed in mg; Ec and Est are the differential absorption of the eluents of the test compound and of the compound used as internal standard respectively; F is a correction factor calculated from the ratio between the differential absorptions of equal amounts of the internal standard and test compounds (experimentally; the F factor is determined by repeated quantitative chromatographic analyses of mixtures containing known amounts of the two compounds).

Tables III (a) and (b) show the results of a quantitative chromatographic determination performed on solutions with different concentrations of methyl-*p*-hydroxybenzoate and propyl-*p*-hydroxybenzoate using the calibration straight line method and the internal standard method.

The data given in Table III show that the quantitative analysis, especially using the internal standard method, is quite accurate; the internal standard method is also more precise than the calibration straight line method because the quantitative determination is not affected by pipetting errors which may occur when the solutions are applied to the chromatographic plates. A repeatibility test consisting of 15 chromatographic determinations on a methyl- and propyl-*p*-hydroxybenzoate mixture showed that the coefficient of variation for both paraseptics is about 3% for the calibrated straight line method and about 1.5% for the internal standard method.

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CHROM. 4787

ERGOT ALKALOIDS

XXXVI*. SEPARATION OF DIASTEREOMERIC (+)-1-HYDROXY-2-BUTYLAMIDES OF D- AND L-LYSERGIC AND D- AND L-ISOLYSERGIC ACIDS BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

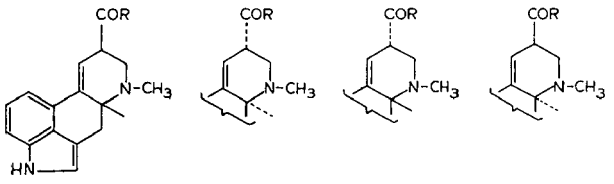
A procedure for the separation of diastereomeric (+)-1-hydroxy-2-butylamides of D- and L-lysergic and D- and L-isolysergic acids by thin-layer chromatography has been developed. By using aqueous solutions of imidazole as one of the components of the solvent system, greater differences were found in the R_F values of the substances studied than was the case without imidazole.

INTRODUCTION

The object of this study was to find conditions giving a good separation of diastereomeric (+)-1-hydroxy-2-butylamides of D- and L-lysergic and D- and L-isolysergic acids (I–IV) on thin layers of silica gel and alumina, and to attempt to use the solubilization effect of imidazole for the purpose. The idea of using imidazole originated after we had found experimentally, in the course of our preparatory work, that imidazole could solubilize methylergobasine (I) and methylergobasinine (III) in aqueous medium.

MACEK AND VANĚČEK[1] have studied the separation of substances I–IV by paper chromatography. However, the differences in the R_F values of the amides I and II were too slight. Various authors have used thin-layer chromatography for the separation of amides of lysergic acid (see refs. 2–7). For instance, GENEST AND FARMILO[4,5] have separated the diethylamide of lysergic acid alongside of heroin and other narcotics. GENEST[5] determined the diethylamide of lysergic acid so isolated

* Part XXXV: *Collection Czech. Chem. Commun.*, 34 (1969) 2819.



I II III IV

I – IV R = NH CH(CH_2OH) CH_2CH_3-(+)

by a densitometric method. BIANCHINE *et al.*[3] used this procedure for the separation of methysergid, *i.e.* (+)-1-hydroxy-2-butylamide of 1-methyl-D-lysergic acid, and methergin (I) in a pure form and after extraction from biological tissues and fluids. HOFMANN AND TSCHERTER[6,7] determined the amides of lysergic and isolysergic acids, chamoclavine, elymoclavine and lysergol in *Rives corymbosa* and *Ipomosea tricolor* Cava by thin-layer chromatography.

EXPERIMENTAL

Chemicals and reagents

The amides I–IV used were prepared in our laboratories[8]. All the substances were chromatographically pure. Chemicals were of reagent grade quality. Imidazole pur. was manufactured by Messrs. Koch-Light. Chromatographic separation was carried out on Silica Gel G Merck layers with the addition of 2% of a fluorescence indicator (UV 254 nm). Aluminium oxide, Lachema, Czechoslovak Standard 685131, pH 8.6, grain size 0.075 mm, was also used, activity approx. III (azobenzene R_F 0.70; *p*-methoxyazobenzene 0.42; Sudan Yellow 0.20; Sudan Red 0.06 and *p*-aminoazobenzene 0.03, in carbon tetrachloride).

Apparatus

The usual type of equipment for chromatographic separation[9,10] was used. Substances isolated were detected by means of a Uvis-lamp (Desaga) at wavelengths of 254 and 365 nm.

Counter-current distribution was studied in a device consisting of six ground-glass separators on a common axis[11]. A Unicam IR-700 spectrophotometer was used.

Method

After preparation, the layers of Silica Gel G were dried for 24 h at room temperature. Prior to use the layers were heated at 100–110° for 30 min in an oven.

Layers of aluminium oxide were activated and adjusted in the usual way[10]. Separated substances and their mixtures were spotted as a 0.05% solution in 60% ethanol in amounts of 10 μ on the chromatographic plates (dimensions 10 × 24 cm). Chromatographic separation was performed in chambers which were saturated for 30 min with the solvent system used in the case of silica gel, while the saturation time was 120 min when loose aluminium oxide was used.

The partition coefficient K was determined by shaking 4 ml of a solution of

amide I or amide III in ether–ethanol (95 : 5) (concentration 1 mg of base in 1 ml of the solution) with 4 ml of water, or with 4 ml of a 0.01, 0.1, 1 or 2 *M* aqueous solution of imidazole. The concentration of the amides was determined spectrophotometrically at 315 nm; $K = C_1/C_2$ (C_1 = concentration of amides in organic phase, C_2 = concentration of amides in aqueous phase).

RESULTS AND DISCUSSION

In experiments described above the solubilization effect of the imidazole upon amides I and III was verified by the determination of their partition coefficient K in the systems ether–ethanol (95 : 5) / water and ether–ethanol (95 : 5) / aqueous solution of imidazole. In the first system, K for amide I was 0.94 and K for amide III was 7.51, in the second system, using 1 *M* and 2 *M* imidazole, substantially lower values were found as a result of solubilization ($K = 0.2$ for amide I and $K = 1.26$ for amide III with 2 *M* imidazole); the solubilization effect of 0.01 *M* and 0.1 *M* imidazole was only slight and the partition coefficients were nearly the same as when water was used in the first system.

The results of our experiments for the separation of amides I–IV on thin layers of silica gel and alumina, in various solvent systems, and also the effect of imidazole on the separation of these amides are summarized in Table I.

As was the case in paper chromatography, the R_F values of amides III and IV

TABLE I

SEPARATION OF DIASTEREOMERIC (+)-1-HYDROXY-2-BUTYLAMIDES I–IV BY THIN-LAYER CHROMATOGRAPHY

Number of solvent system	*Solvent system*	R_F *values*							
		Silica gel				*Aluminum oxide*			
		I	*II*	*III*	*IV*	*I*	*II*	*III*	*IV*
1	Ether–ethanol (95:5)	0.07	0.07	0.07	0.07	0.60	0.60	0.75	0.75
2	Ether–ethanol–water (90:5:5)	0.35	0.15	0.47	0.68				
3	Ether–ethanol–0.1 *M* imidazole (90:5:5)	0.28	0.08	0.35	0.58				
4	Ether–ethanol–0.75 *M* imidazole (90:5:5)	0.30	0.13	0.54	0.69				
5	Ether–ethanol–1 *M* imidazole (90:5:5)	0.48	0.35	0.65	0.75	0.76	0.76	0.76	0.76
6	Chloroform–acetone (50:50)	0.20	0.20	0.25	0.25	0.47	0.47	0.75	0.75
7	Chloroform–acetone–water (50:45:5)	0.12	0.07	0.27	0.35				
8	Chloroform–acetone–0.1 *M* imidazole (50:45:5)	0.22	0.15	0.51	0.75	0.57	0.57	0.75	0.75
9	Chloroform–acetone–1 *M* imidazole (50:45:5)	0.27	0.07	0.31	0.47	0.50	0.32	0.62	0.70
10	Chloroform–ethanol (90:10)	0.72	0.60	0.80	0.80	0.87	0.87	0.93	0.93
11	Chloroform–ethanol–water (90:5:5)	0.30	0.27	0.43	0.50				
12	Chloroform–ethanol–0.1 *M* imidazole (90:5:5)	0.42	0.35	0.62	0.82	0.55	0.47	0.78	0.80
13	Chloroform–ethanol–1 *M* imidazole (90:5:5)	0.55	0.50	0.65	0.80	0.68	0.84	0.92	0.96

TABLE II

VISCOSITY AND pH DATA OF IMIDAZOLE AQUEOUS SOLUTIONS

Molarity of imidazole solution	*Viscosity**		*pH*
	Absolute	*Kinematic in cSt*	
0.1	1.1323	1.1323	9.65
0.2	1.1575	1.1575	9.55
0.5	1.1862	1.1840	9.40
0.75	1.2831	1.2778	9.30
1.0	1.3334	1.3250	9.2

* Wt. of ball used: 4.9789 g; specific weight at 20°: 2.409 g; ball constant: 0.009987.

were higher on thin layers as compared with amides I and II. Solvent systems including water (No. 2, 7, 11) and analogous systems using aqueous solutions of imidazole (systems No. 3, 4, 5, 8, 9, 12, 13) were shown to be useful for the separation of all four stereoisomeric amides I–IV. The effect of imidazole upon the separation of the compounds under study was followed for system No. 2 by using 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 0.8 and 1.0 *M* aqueous solutions of imidazole instead of water. By using the system ether–ethanol–0.75 *M* imidazole (No. 4) the difference between the R_F values of substances II and III was greater than that in system No. 2. Comparison of the solvent systems chloroform–acetone–water (No. 7) and chloroform–acetone–0.1 *M* imidazole (No. 8) shows the favorable effect of imidazole on the separation of compounds III and IV. The presence of imidazole in the solvent systems made it possible, in contrast to systems without imidazole, to obtain compact and clearly outlined spots of I–IV. The separation of the substances studied did not directly depend on the basicity of the used system (see Table II).

The favorable effect of imidazole on the separation of the amides I–IV, up to a certain concentration of the added solubilizer, can be explained by formation of intermolecular bonds between this component of the solvent system with the butanolamides present. A concentration of 0.75 *M* imidazole was found experimentally to be the most favorable for the separation. In concentrations above this limit, the differences in the R_F values of the substances I–IV separated were decreased and the separation coefficients also increased. Thus, in excessively concentrated solutions the solubilizing effect decreases and hence the separation of these substances on thin layers is worse. This phenomenon is in accordance with the data of HECKER[13], GENEST[5] and KÜTTEL[14].

When the solvent systems are modified as in this study, not only does a change occur in the R_F values on thin-layer chromatography of the substances studied at higher concentrations of added imidazole, but a considerable increase of viscosity was found experimentally (Table II), which is in agreement with BÜCHI[15], who studied this problem by measuring the surface tension of the solubilizers.

Attempts to interpret the data obtained by IR spectroscopy and NMR measurements with a view to proving a direct or indirect interaction of imidazole with the diasteroisomeric amides I–IV has not met with any success so far. The solution of this problem will be the subject of further study.

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CHROM. 4761

DENSITOMETRIC DETERMINATION OF BILE PIGMENTS ON THIN-LAYER CHROMATOGRAMS

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SUMMARY

Densitometric quantitation of bile pigments directly on the scored TLC silica gel and polyamide plates is described. Calibration curves are given for mesobiliviolin, stercobilin, mesobilirubin and also for dimethyl esters of stercobilin and biliverdin.

INTRODUCTION

The direct quantitation of compounds separated on thin-layer chromatographic (TLC) plates has the obvious advantage that in most cases the preliminary treatment required is minimal. The application of this method has already been quite extensive, including amino acids[1,2], fatty acids[3], proteins[4], pharmaceuticals[5], lipids[6–9], steroids[10,11], and other substances[12–15]. These uses have recently been divided into several general chemical groups[12–15]. For present purposes, however, a division into colored and colorless compounds is important. The latter have to be sprayed with a visualizing agent which introduces an additional possible source of error. Colored compounds (350–700 nm) are visible on the TLC plates without any treatment, and the bile pigments in particular absorb in the visible range, with exception of their chromogens which are readily oxidized to colored pigments. Hence they can be quantitated by use of visible light, and in the present study we have been mainly concerned with measurement of transmitted light[12] or of visible fluorescence excited by an appropriate source of light[12,14,15].

Extensive theoretical studies have been carried out as to the use of transmitted[12,22], reflected[12,22] and fluorescent light[13–15,22] for direct quantitation of compounds separated on TLC plates. These have revealed several factors which influence and limit the precision of the method, *e.g.* in particular a precision in spotting, the spot size on origin and spot size after development, the amount of substance in the spot, R_F values, visualization and quality of the thin-layer material[16–19]. It is extremely difficult to predict the total effect of the above-mentioned factors from the semi-theoretical studies when the influence of only one factor at a time is investigated. Thus we have chosen a different, purely pragmatic approach to the problem by experimentally standardizing the chromatography and spectrodensitometry for direct quantitation of bile pigments on TLC plates. Using the disc integrator for calculating the optical densities and peak areas, the calibration curves

have been plotted for each pigment. The most precise ranges of concentration and the limitations of the method have been determined.

EXPERIMENTAL

Materials

Silica Gel G was obtained from E. Merck, A. G. (Darmstadt, G.F.R.) for preparation of TLC plates, as described[20]. Polyamide ITLC plates 15 × 15 cm, coated on both sides, were from Gallard Schlesinger Chemical Manufacturing Corp. Chloroform, ethanol, benzene, methanol, ammonia were all analytical grade reagents; water was deionized and distilled. The Schoeffel double-beam densitometer was equipped with a monochromator and disc integrator[22].

Methods

Plates were scored into bands 10 mm wide: silica gel plates were scored with the Schoeffel scoring device and polyamide plates manually with a scalpel. Every second band was spotted, the unspotted band serving as a reference[22].

The chromatography systems were described previously[20], great care being taken to achieve reproducibility of R_F values[12]. Activation and storage of silica gel plates were rigorously followed. In an air conditioned laboratory the temperature was maintained at 21 ± 1°, and the relative humidity varied from 43 to 61%. These variations did not influence the quality of separation on TLC plates if the plates were not stored more than 48 h after activation. Silica Gel G plates are much more sensitive to humidity than polyamide plates, and the latter could be stored indefinitely without any visible change.

Special care was taken in spotting and developing the plates. Spotting was done with a micropipet, and approx. 0.3 μl were delivered at a time, followed by drying with a stream of nitrogen after which the next portion of the solution was delivered. In this way spot size could be controlled and did not exceed 30 sq. mm. The original size had some influence on quantitation, but the important point was the application of the pigment with formation of spots of the same sizes.

Development for each system must be performed in precisely the same way. The solvent front should always be the same distance from the origin. Whether the chosen distance was 10 or 12 cm was less important and could be selected according to R_F values of the separating pigments (the smaller the R_F value, the greater the distance from origin to front). Once the distance was chosen, it had to be adhered to in preparation of calibrating curves and for later analyses.

Spot area measurements were done with the Desaga–Brinkmann standard labeling template. The $\lambda_{max.}$ of each pigment was found by repeated optical density measurements of the same spot on the TLC plate by manual change of wavelength. Preceding each series of measurements, the instrument was checked with a set of five red filters. These were cut in pieces 2, 6, 7.5, 10 and 26 mm, positioned on the glass plate, and their optical densities and integrator readings were made. At least 32 measurements for each of the five filters provided sufficient data to calculate the standard deviation for the densitometer–recorder–integrator system. Coefficients of variation were ± 3.4, ± 3.9, ± 0.9, ± 5.5 and ± 4.0%, correspondingly.

Before standard curves could be prepared, it was necessary to determine the

error connected with spotting and chromatography. This was achieved by repeated spotting of 3 μl of the same solution, followed by chromatography and densitometric measurements. When integrator readings varied no more than $\pm$ 7%, the method was assumed to be acceptable for standard curve preparation. Such factors as chromatography, tank location, position of the TLC plate during the saturation, and uniform conditions of saturation had to be examined carefully as they might relate to significant variations. Background scintillations were measured by running a clean TLC plate in the spectrodensitometer, thus establishing values for Silica Gel G and polyamide plates as well as certain chromatographic papers.

The general method for preparation of standard curves was as follows. The pigment of known concentration was spotted on a plate with several spots containing various amounts. Preliminary runs of widely varying amounts gave a good indication of the desired range. On this basis more exact amounts were selected, *i.e.* 0.01, 0.05, 0.1, 0.3, 0.6, 1.0, 1.3, 1.6 and 2 μg. The plates were developed, and optical density was measured in the spectrodensitometer with the peaks integrated. The plots were then made with a quantity of pigment on the abscissa and the integrator reading on the ordinate. These plots permitted definition of the upper and lower limits of accuracy of densitometry (maximal sensitivity within the area in which the Lambert–Beer law was obeyed) (see Fig. 1).

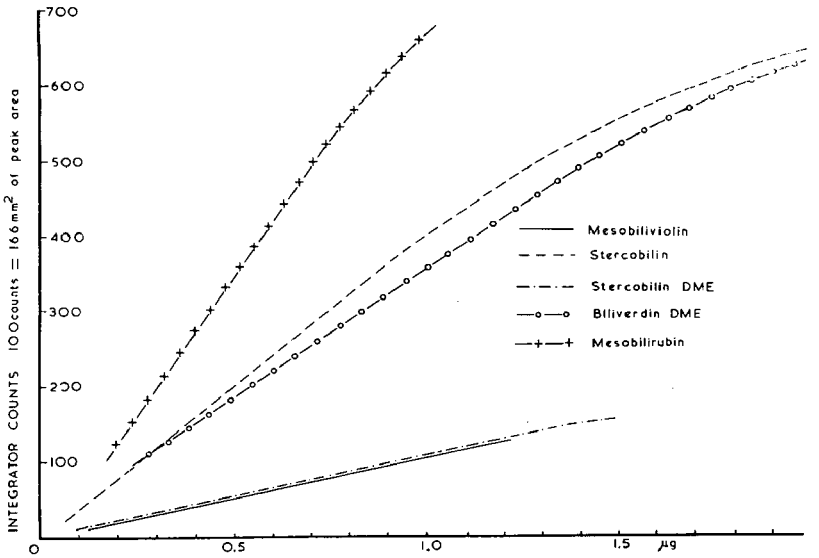


Fig. 1. Standard curves for bile pigment dimethyl esters on silica gel layers and standard curves for free bile pigments on polyamide. Chromatography systems as in ref. 20; calculated standard errors of estimate in Table II.

The influence of the R_F value on the results was determined by spotting the same amount of pigment at various distances from the bottom of the plate. The influence of spot size on the integrator reading was ascertained by spotting the same amount of pigment in varying dilution so that spots of various sizes were formed. The differences found were in agreement with published results[12].

TABLE I

ABSORPTION MAXIMA FOR BILE PIGMENTS ON TLC

Pigment	*Wavelength (nm)*	
	Free pigments on polyamide layer	*Pigment dimethyl esters on Silica Gel G*
Mesobilirubin	420	—
Mesobiliverdin	—	360
Stercobilin	480	480
Urobilin	480	480
Mesobiliviolin	580	—
Bilirubin	440	410
Biliverdin	370	320

RESULTS AND DISCUSSION

It is evident that the greatest sensitivity of this method is at the wavelength corresponding to the $\lambda_{max.}$ of each pigment, and these wavelengths were determined for each pigment on silica gel and polyamide thin-layer plates. Results are given in Table I.

Standard curves for bile pigment dimethyl esters on silica gel layers and standard curves for free bile pigments on polyamide are shown in Fig. 1. The sensitivity of direct quantitation on TLC is different for each bile pigment. Ranges of possible application (when integrator readings are directly proportional to pigment quantities, Beer's law and the ratios of integrator reading to μg pigment are constant)

TABLE II

STANDARD ERRORS AND RANGES FOR QUANTITATION OF BILE PIGMENTS

Pigment	*Standard error of estimate (ref. 23) (μg)*	*Range (μg)*	*Ratio* $\frac{\text{integrator counts}}{1\ \mu g\ \text{of pigment}}$
Mesobilirubin	± 0.015	0.02 → 0.7	831.9
Mesobiliviolin	± 0.021	0.1 → 0.7	104.7
Stercobilin	± 0.006	0.03 → 0.8	396.8
Biliver din DME[a]	± 0.041	0.082 → 1.2	362.3
Mesobilirubin DME	± 0.036	0.072 → 0.7	143.5
Stercobilin DME	± 0.055	0.11 → 1.0	90.9

[a] DME = Dimethyl ester.

are given in Table II. Examples of measurement of bile pigment mixtures at different wavelengths are shown in Fig. 2.

Absorption spectra of bile pigments are very dependent on media, and normally they are studied in solution. We have found that they differ when the pigment is dispersed on the absorbing medium of TLC plates. Since the maximum sensitivity

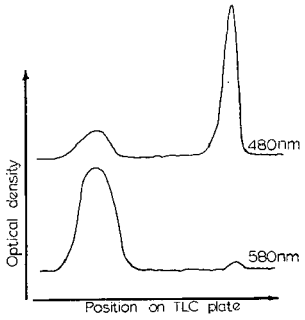


Fig. 2. Results of measurements of mesobiliviolin (left-hand peak) and stercobilin (right-hand peak) at 480 and 580 nm.

is at $\lambda_{max.}$ for a given pigment, the $\lambda_{max.}$ on the absorbing media was determined. The values are rounded up to 10 nm, corresponding to the smallest grating on the monochromator which permits duplication of the wavelength settings. True maxima are the recorded values $\pm$ 5 nm, but for purposes of quantitation this difference is insignificant.

Standard curves have different ratios of integrator reading to amount of pigment for each pigment. This is due to differences in the molar extinction coefficient, ε, and there was good correspondence between the ratio and ε as calculated for pigments in solution[21]. The greater the ratio, the greater the sensitivity of the densitometric method. The standard curves can be interpolated to 0; the lowest detectable quantity is considered to be an integrator value corresponding to doubled value for zero line variation of integrator reading or twice the standard error of estimate, whichever is greater.

The upper limits of standardization curves useful for quantitation are the points where the curve departs from the straight line, and those limits are given in Table II. The curves rise more rapidly at first and then level off as more pigment is added. This region might also be useful for quantitation; however there is no direct proportionality between integrator readings and the amount of pigment. All factors vital to reproducibility of the results are even more critical in this region. Leveling off of the curves is due to oversaturation with the pigment in the spot area[17], and then Beer's law is not obeyed (Fig. 1). If, however, too large an amount is applied for quantitation, simple dilution will permit a shift to a more favorable region on the calibration curve.

Dependence of calibration and quantitation on R_F values and spot size has been emphasized by others[12]. We have confirmed this and in addition have found an explanation for the dependence by means of using the scored TLC plates. Theoretically there is a maximal R_F value when no self-absorption and no scoring effect (error of quantitation due to scoring) are observed. The scoring effect changes the ratio of integrator counts to μg pigment and lowers the sensitivity. Scoring effect is related to a fraction of the pigment in a given spot on the very edge of the 10 mm band which is never placed in the light beam for measurement of absorption. The effect influences larger spots related to greater R_F values. It is closely related to the spot size on the origin, since the larger the spot, the sooner the scoring effect becomes noticeable—hence the necessity of rigorous attention to the conditions of spotting,

developing and measuring. When the pigment is spotted on the origin there is no scoring effect, since the whole spot is under the beam and then the oversaturation becomes prevalent. The straight part of the curve corresponds to the absence of both effects, scoring and oversaturation.

Mixtures of pigments are readily analyzed by means of the prepared calibration curves. Each pigment represented by a peak can be distinguished by density measurements at the appropriate wavelength (Table I). This technique can be used for quantitation of pigments separated on TLC. Also, it could be applied to qualitative identification of pigments by estimation of corresponding absorption maxima for each pigment. An example of this application limited to two pigments is shown in Fig. 2; however, it can be employed to multi-component mixtures.

Introduction of the densitometric method for direct determination of bile pigments on TLC necessitates some changes in the preliminary preparation of the solution. Previously it has been necessary to separate and partially purify pigments to be determined. With the present method a solution of a mixture of pigments can be subjected directly to TLC and densitometry.

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CHROM. 4800

SEPARATION AND IDENTIFICATION OF TETRYL AND RELATED COMPOUNDS BY TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A two-dimensional TLC method for the separation and identification of tetryl and related compounds is described. Production-grade tetryl contains N-methyl-N,2,4-trinitroaniline, picryl chloride, picric acid, picramide or N,2,4,6-tetranitroaniline, and N-methyl-2,4,6-trinitroaniline. Samples heated at 120° for 48 h contain picric acid and 2,4,6-trinitroanisole as the major decomposition products, with decreasing amounts of picramide and/or N,2,4,6-tetranitroaniline, N-methyl-2,4,6-trinitroaniline, 1,3,5-trinitrobenzene, and N-methyl-N,2,4-trinitroaniline.

INTRODUCTION

Thin-layer chromatography (TLC), as a method of separating organic compounds, enables the analyst to perform qualitative and quantitative analyses on substances present in minute quantities. With proper selection of substrate and solvent, separations of simple and complex mixtures, not possible by conventional techniques, can be achieved by TLC. A requirement to identify the impurities associated with N-methyl-N,2,4,6-tetranitroaniline (tetryl) prompted us to investigate the applicability of the technique to this problem.

Several studies on the thermal decomposition of tetryl have been reported in the literature, but only DUBOVITSKII *et al.*[1] attempted to identify the degradation products, using column chromatography and UV absorption for this purpose. The two-dimensional TLC method described below provides a convenient and simple method of separating and identifying the components of such mixtures.

REAGENTS AND EQUIPMENT

The applicator, chromatojar, Silica Gel G, and glass plates (200 by 200 mm) were purchased from Brinkmann Instruments, Inc.

All solvents were reagent grade. Petroleum ether with a boiling range of 30 to 60° was used.

Zinc metal dust, A.R. grade, was obtained from Mallinckrodt Chemical Works.

The spray reagent consisted of 0.25 g of *p*-diethylaminobenzaldehyde (*p*-DEAB) and 2.1 ml of concentrated HCl made up to 100 ml with absolute ethanol.

Sample aliquots were delivered with an Agla micrometer syringe.

Several of the compounds included in this study were not available commercially. The N-methyl-2,4-dinitroaniline and N-methyl-2,6-dinitroaniline were prepared by condensing methylamine with the corresponding chlorodinitrobenzene. The procedure of GLAZER *et al.* was used for the preparation of N-methyl-N,2,4-trinitroaniline[2]. The purity of these samples was established by TLC, chemical tests, melting point, and elemental analyses.

EXPERIMENTAL

Preparation of thin layer

A slurry consisting of 30 g of Silica Gel G and 0.5 g of zinc dust in 65 ml of distilled water was prepared and coated on five glass plates by pulling the applicator across them at a constant speed with a Bodine speed-reducer motor. The coated plates were air-dried for 15 min and activated at 110° for 2 h before use.

R_G measurement

Twenty-three compounds related to tetryl were assembled, and the R_G value for each was measured relative to 1,3-dinitrobenzene on an activated Silica Gel G–zinc plate using a variety of solvents. To locate the spots, *p*-DEAB–HCl was

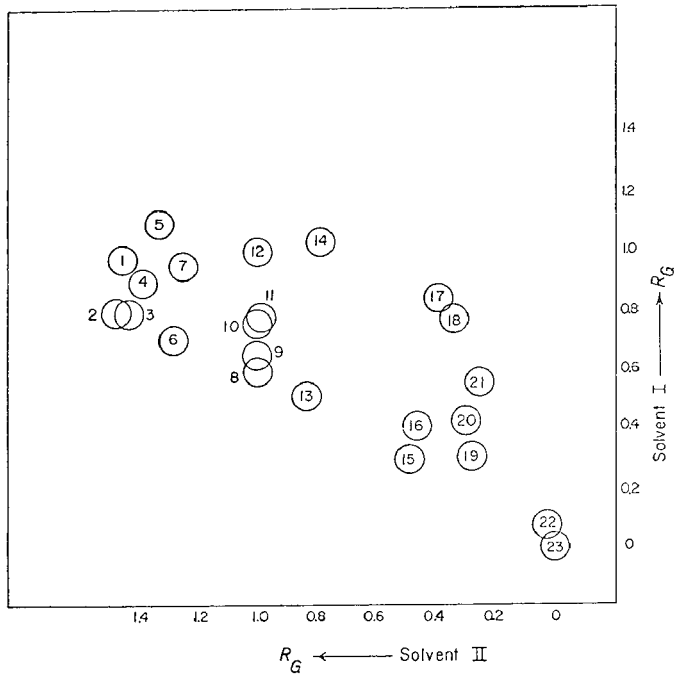


Fig. 1. R_G measurements of tetryl and related compounds. For identification of compounds, see Table 1.

TABLE I

IDENTIFICATION OF THE SPOTS IN FIGS. 1 AND 2 OF TETRYL AND RELATED COMPOUNDS

Abbreviations of colors: y = yellow, b = brown, o = orange, r = red and g = gray.

No.	*Compound*	*Self-indicator*	*Color with p-DEAB*
1	1-Chloro-2,4,6-trinitrobenzene (picryl chloride)	—	y-b
2	N,N-Dimethylaniline	—	y to g
3	1,3,5-Trinitrobenzene (TNB)	—	o
4	2,4,6-Trinitroanisole	+	y
5	1-Chloro-2,4-dinitrobenzene	—	y
6	N-Methylaniline	—	y
7	N-Methyl-2,6-dinitroaniline	+	y
8	2,4,6-Trinitroaniline (picramide)	+	o
9	N,2,4,6-Tetranitroaniline	—	b
10	N-Methyl-2,4,6-trinitroaniline	+	y-b
11	*o*-Nitroaniline	+	y
12	1,3-Dinitrobenzene (reference compound)	—	o
13	N-Methyl-N,2,4,6-tetranitroaniline (tetryl)	—	y-b
14	1-Chloro-2,6-dinitrobenzene	—	o
15	3,5-Dinitroaniline	+	y
16	*m*-Nitroaniline	—	y
17	2,4-Dinitroanisole	—	o
18	N-Methyl-2,4-dinitroaniline	+	y
19	2,4-Dinitroaniline	+	o
20	*p*-Nitroaniline	—	r
21	N-Methyl-N,2,4-trinitroaniline	—	y
22	2,4-Dinitrophenol	+	o
23	2,4,6-Trinitrophenol (picric acid)	—	b

used as the spray reagent. By cross-plotting the R_G values, chloroform (solvent I) and a 1 : 3 ethyl acetate–petroleum ether mixture (solvent II) were found to be the most effective solvent pair. Fig. 1 shows a plot of the data obtained with solvents I and II. The colors developed with the *p*-DEAB reagent and the selfindicating compounds are listed in Table I.

Procedure

A benzene solution containing 0.3–0.5 mg of sample is applied about 1 in. from the lower right-hand corner of an activated Silica Gel G–zinc plate. The sample is chromatographed in 200 ml of solvent I for 55 min and then exposed to the atmosphere for 10 min. In an equal volume of solvent II, the sample is rechromatographed at 90° to the direction of flow of solvent I for an additional 55 min. The visible spots are located, and the chromatogram is sprayed with *p*-DEAB–HCl reagent. With the exception of N,N-dimethylaniline, which requires 1 h for color development, the components react immediately to form brown, yellow, and red spots on the chromatogram.

RESULTS AND DISCUSSION

Fig. 1 indicates that 23 compounds can be partially or completely resolved for identification purposes. To verify this, a synthetic mixture consisting of all the

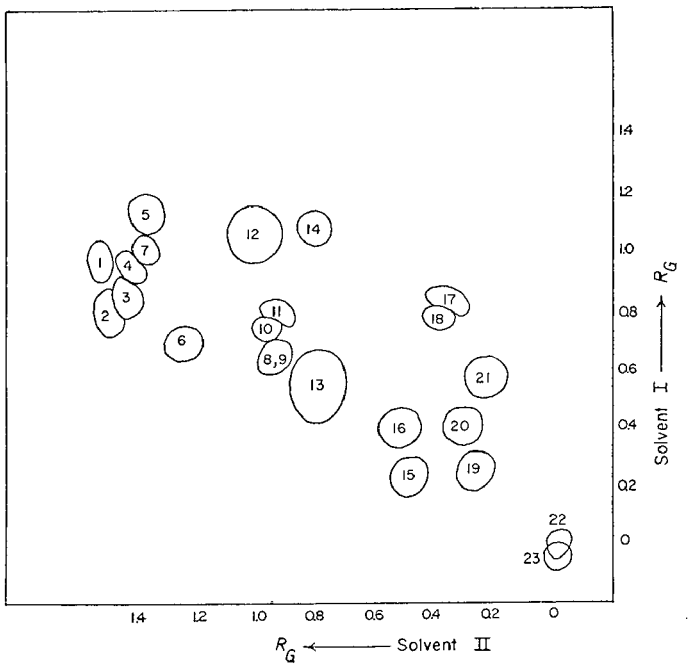


Fig. 2. Two-dimensional thin-layer chromatography of a synthetic mixture. For identification of compounds, see Table I.

TABLE II

IMPURITIES IN TWO TYPICAL LOTS OF PRODUCTION-GRADE BEFORE AND AFTER HEATING

Sample	*Impurities*
Tetryl A	N-Methyl-N,2,4-trinitroaniline > picric acid > picramide or N,2,4,6-tetranitroaniline = picryl chloride
Tetryl B	N-Methyl-N,2,4-trinitroaniline > picryl chloride > picric acid > N-methyl-2,4,6-trinitroaniline (trace)
Tetryl A (heated at 120°, 48 h)	Picric acid ≥ 2,4,6-trinitroanisole > N-methyl-2,4,6-trinitroaniline > picramide or N,2,4,6-tetranitroaniline > TNB (trace) N-methyl-N,2,4-trinitroaniline (trace)
Tetryl B (heated at 120°, 48 h)	Picric acid > 2,4,6-trinitroanisole > picramide or N,2,4,6-tetranitroaniline > TNB (trace) N-methyl-N,2,4-trinitroaniline (trace)

components was prepared and chromatographed. Fig. 2 is a tracing of the developed chromatogram.

In general, the relative position of each compound is similar to that shown in Fig. 1. N-Methyl-2,6-dinitroaniline is displaced further than predicted, while picramide and N,2,4,6-tetranitroaniline overlap completely, making identification impossible. Slight displacement of a compound is expected in a complex mixture of this sort, as the migration rate of each component is affected by the migration of other components in the sample.

The detection limit of each compound was determined after chromatographing the sample. With the exception of N-methylaniline and N,N-dimethylaniline, 0.5 to 1 μg of each compound can be readily detected with the *p*-DEAB reagent. N-Methylaniline and N,N-dimethylaniline require 4 and 8–10 μg, respectively.

Production-grade and heated tetryl samples were analyzed by this method. The impurities found are listed in Table II in the approximate order of decreasing concentration.

An unexpected impurity, N-methyl-N,2,4-trinitroaniline, was found in production-grade tetryl. Its identity was established by comparing the melting point and R_G value with those of an authentic sample.

With the heated tetryl samples, the 2,4,6-trinitroanisole, picric acid, and N-methyl-2,4,6-trinitroaniline listed in Table I were also reported by DUBOVITSKII, *et al.*; however, the picramide or N,2,4,6-tetranitroaniline, and the traces of TNB and N-methyl-N,2,4-trinitroaniline, were not reported by these authors.

ACKNOWLEDGEMENTS

The author thanks P. N. NEUMAN for the preparation of certain nitroaromatic compounds. This work was done under the auspices of the U. S. Atomic Energy Commission.

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CHROM. 4807

SEPARATION AND IDENTIFICATION OF BILIVERDIN ISOMERS AND ISOMER ANALYSIS OF PHYCOBILINS AND BILIRUBIN

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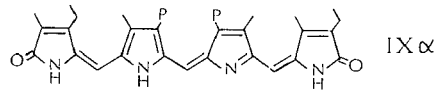
SUMMARY

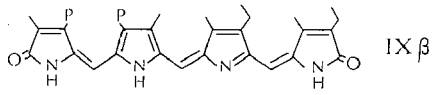
One-dimensional thin-layer chromatographic systems are described for the resolution of the IXα, IXβ, IXγ and IXδ isomers of protobiliverdin and mesobiliverdin, and each isomer is unambiguously identified. The separations may be applied on a preparative scale or as a micro-scale method for isomer analysis of biliverdin preparations or of bilins which can be converted thereto. Besides being very sensitive and reliable, the analytical method also yields much more information than degradative methods. Applied to some naturally occurring bilins, it shows that bilirubin from pig bile is about 99.6% IXα with traces of IXδ and of IXβ and no IXγ. Phycoerythrobilin and phycocyanobilin seem to be entirely IXα. Mesobiliverdin IXα, prepared from bilirubin by the usual alkaline reduction followed by dehydrogenation, contains two extra minor, seemingly isomeric mesobiliverdins. A new method of preparation, avoiding alkaline reduction, yields only true mesobiliverdin IXα.

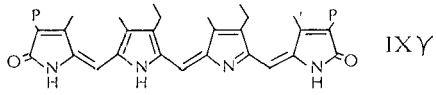
INTRODUCTION

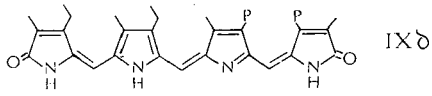
Biliverdins are derived formally from haems by oxidative removal of a methine bridge and loss of the iron atom. The asymmetric nature of haem IX results in the possibility of four different biliverdin isomers, each corresponding to the removal of a different methine bridge and named accordingly. Thus the IXα, IXβ, IXγ and IXδ isomers illustrated in scheme 1 correspond respectively with removal of the α, β, γ and δ bridges of mesohaem. Such isomerism is of importance in studying haem and bilin metabolism (*cf.* refs. 1 and 2).

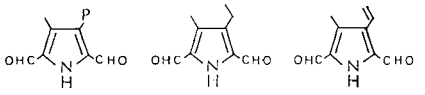
Until recently the only method available for investigating the isomeric nature of bilins was alkaline permanganate degradation[3,4], a destructive method requiring about 6–10 mg of material, an amount not readily available from most of the processes we have been studying. We have found that results obtained with smaller quantities of material are unreliable[5]. Moreover, only the pure IXα and IXγ isomers can be distinguished by this technique; the IXβ, IXδ, and all mixtures of isomers are indistinguishable. The recently introduced dichromate degradation technique of











Scheme 1. Structures of the mesobiliverdin isomers (IX α, β, γ, δ) and of pyrrole dialdehydes I, IIa, and IIb. In the protobiliverdins the ethyl groups are replaced by vinyl groups as in pyrrole dialdehyde IIb. P represents a methyl propionate side-chain in the esterified compounds (or propionic acid side-chain in the free pigments).

RÜDIGER[6–8] is far more sensitive and reliable, but it suffers from the same ambiguity with samples other than pure IXα or IXγ. Furthermore, degradative methods do not distinguish isomers of the IX series uniquely (from those of the III series for example).

NICOL AND MORELL[9] have recently described the combined use of mass spectrometry and NMR as a method of isomer analysis, but the technique is complex, requires relatively large quantities of material, and the results seem to be open to serious misinterpretation (see below).

RÜDIGER[6,7] has briefly described a method of isomer analysis applicable to deutero- and proto-biliverdins and based on thin-layer chromatographic separation of the isomers, but the IXβ and IXδ isomers were not distinguished and the system described for the protobiliverdins is a two-dimensional one, inconvenient for comparative or preparative work.

We describe here one-dimensional chromatographic methods which allow the complete resolution of the four isomers of both protobiliverdin IX and mesobiliverdin IX either on a preparative scale or as a microanalytical method, and we identify each isomer unequivocally. The method is applied here to the isomer analysis of biliary bilirubin and the phycobilin prosthetic groups of the algal biliproteins. We have also used the technique successfully in studying the specificity of coupled oxidation of haemproteins[1] and the specificity of biliverdin reductase[10].

MATERIALS AND METHODS

General

Crystalline protohaemin was obtained from Sigma. Mesohaemin was prepared by catalytic hydrogenation of protohaemin over palladium[11] followed by reinsertion of the iron atom into the mesoporphyrin formed[12,13]. Bilirubin IXα (twice crystallized) was obtained from Sigma. Its isomeric purity was checked by the pH 1.7 dichromate degradation technique of RÜDIGER[6,8]. Both the dichromate degradation technique and the chromic acid degradation (to imides) were performed by the "on the plate" technique and the pyrrole dialdehydes and imides were separated by TLC, detected and identified as described by RÜDIGER[6,8].

Handling, esterification and TLC of biliverdins

Biliverdins and other bilins are prone to oxidative and other modifications and throughout all procedures they were kept as far as possible under nitrogen and were stored at 2° in the dark. It is essential to ensure that all solvents used are completely free of peroxides.

Conversion to the dimethyl esters was achieved by dissolving the biliverdins in ice-cold 5% H_2SO_4 in methanol, the solutions being allowed to stand for 12–16 h at 0–4° under nitrogen. (Esterification of bilins at more elevated temperatures should be avoided as it may lead to formation of artifacts[7].) The mixtures were then diluted with four volumes of water and extracted with chloroform. The chloroform extracts were washed with distilled water until the washings were no longer acid (four washings with four volumes of water usually sufficed). The chloroform solutions were then evaporated to dryness under a stream of nitrogen and the pigment was dissolved in a drop of acetone for application to the thin-layer plates. (When the chloroform solutions were not washed sufficiently to remove all the acid, it was found that the pigment remained at the origin during subsequent chromatography. This could usually be overcome by drying the plate, spotting a little concentrated ammonia onto the biliverdin spot, drying off under a stream of nitrogen, and rechromatographing.)

Thin-layer chromatography was carried out on 20-cm plates coated with 0.25-mm layers of Silica Gel G (Merck) activated at 100° for at least 1 h. The chromatographic systems developed are sufficiently insensitive to deactivation of the silica gel to permit multiple development. It is necessary, however, during multiple development with those solvent systems containing acetic acid to ensure that the latter is well dried off in a stream of cool air between each development. The 13% gypsum present in the Silica Gel G (originally as a binder) appears to be essential for the separations reported here. Pre-coated silica gel plates containing other binders, or no binder, gave very poor results.

Conversion of proto- to mesobiliverdins

It was found that reduction of the vinyl side chains of protobiliverdins could be carried out specifically on a micro-scale by hydrogenation over palladium in formic acid. (Other methods of reduction reduced the central methine bridge preferentially or concomitantly, yielding bilirubins.) The protobiliverdin (*ca.* 20–50 μg as the dimethyl ester or the free diacid) was dissolved in 99–100% formic acid (1 ml)

in a stoppered test-tube. Palladium black (4 mg) was added, hydrogen was bubbled through and the tube (filled with hydrogen) was stoppered and shaken at 40° for 10 min (until the color changed from green to blue). Water (6 ml) was added and the pigment extracted into chloroform which was then washed with 10% sodium acetate (10 ml twice) followed by water (10 ml twice) and evaporated to dryness.

Biliverdin preparations

Standard protobiliverdin IXα dimethyl ester was prepared by dehydrogenation of bilirubin IXα by the ferric chloride–glacial acetic acid method[14] followed by esterification. Standard mesobiliverdin IXα dimethyl ester was prepared by catalytic hydrogenation of protobiliverdin IXα dimethyl ester as described above. Mesobiliverdin preparations were also prepared from bilirubin IXα by catalytic hydrogenation to mesobilirubin or amalgam-reduction to mesobilirubinogen in aqueous alkali[15] followed by ferric chloride dehydrogenation and esterification. The latter type of preparation is termed mesobiliverdin IXα type II and was found to be inhomogeneous (see RESULTS AND DISCUSSION). Synthetic mesobiliverdin IXβ dimethyl ester[16] was kindly supplied by Professors G. W. KENNER and A. H. JACKSON.

Coupled oxidation of protohaemin with ascorbate was carried out as described by LEVIN[17], and of mesohaemin by mixing 30 mg mesohaemin in 50 ml pyridine with 100 mg sodium ascorbate in 150 ml water and incubating under an atmosphere of oxygen at 60°, with further additions of ascorbate if necessary, until the solution turned green (30–60 min). This procedure was necessary as mesohaemin was much more resistant to coupled oxidation than was protohaemin. The resulting verdohaemochromes were extracted as described by LEVIN[17] and were converted to biliverdins and at the same time esterified by following the standard esterification procedure.

Conversion of some naturally occurring bilins for isomer analysis

Phycocyanobilin, the blue chromophore of phycocyanins, was partly released from the apoprotein and simultaneously isomerized to mesobiliverdin[18,19] by heating 40 mg of C-phycocyanin (isolated from *Nostoc muscorum*[20]) in 1 *N* KOH (4 ml) at 100° for 5 min. 11 *N* HCl (8 ml) was then added and the mixture was allowed to stand at 40–50° for 2 h to complete the hydrolytic release of the pigment from the protein. The mixture was diluted with water (32 ml) and extracted with chloroform (10 ml three times), into which practically all the pigment went. The combined chloroform extracts were washed with water (20 ml) and evaporated to dryness, and the pigment was esterified for chromatography in 5% methanolic H_2SO_4 as usual.

Phycoerythrobilin, the red chromophore of phycoerythrins, was also isomerized to mesobiliverdin by similar KOH treatment of R-phycoerythrin (isolated from *Ceramium rubrum*[20]). The pigment was similarly released from the apoprotein, and esterified. Prior to analysis it was submitted to preliminary TLC in carbon tetrachloride–ethyl acetate (2 : 1) to remove traces of artifact pigments formed by modifications of the vinyl group initially present in phycoerythrobilin before its isomerization.

Biliary bilirubin for isomer analysis was prepared according to the method of OSTROW *et al.*[21] but the fractionation procedures were cut to a minimum to avoid possible loss of isomers present in small amounts. All washings were re-extracted

and any bilirubin recovered was returned to the bulk of the bilirubin. Crystallization steps were omitted. The total crude bilirubin was then converted to biliverdin by refluxing with ferric chloride in glacial acetic acid[14] and the biliverdin was esterified for TLC in the usual manner. Certain non-bilin impurities came through the limited fractionation procedures and were removed by preparative chromatography on Silica Gel G in carbon tetrachloride–ethyl acetate (2 : 1)[19]. (This solvent moved protobiliverdins as a homogenous band ahead of the impurities, with no fractionation of the isomers.) The biliverdin band was scraped off, eluted into acetone, concentrated and used for isomer analysis.

RESULTS AND DISCUSSION

Separation of the isomers

Bilins are, in general, more easily chromatographed as the diesters than as the free diacids. The dimethyl esters of the biliverdins were therefore used for TLC throughout this investigation. Silica Gel G was found to be the most suitable adsorbent; as mentioned above, the calcium sulfate present as binder plays an important role in the separations reported here and cannot be omitted.

The esterified protobiliverdin and mesobiliverdin preparations, produced by coupled oxidation of pyridine haemochromes followed by treatment of the resulting verdohaemochromes with acid methanol, were used to search for suitable chromatographic systems, as it had been shown[6,22] that such a preparation of protobiliverdin is a mixture of isomers and it was anticipated that the same would be true of the mesobiliverdin preparation.

The mesobiliverdin preparation was indeed eventually separated into four blue components by multiple development in one dimension with either of the solvent systems A, B or C listed in Table I. Solvent A gave the most consistent results and was adopted for further studies although it requires about eight-fold development for full resolution (Fig. 1A, position 1). Sharper resolution was obtained by multiple development than by continuous (open ended) TLC. The two top spots were the most difficult to resolve with all solvent systems, and the composition given for solvent system A seems to be optimal, and rather critical, for this separation.

The corresponding protobiliverdin preparation was separated into four green components by triple development in one dimension in solvent system D (Fig. 1B, position 1) or by double development in solvent E (Table I). Solvent D was marginally

TABLE I

SOLVENT SYSTEMS DEVELOPED FOR TLC SEPARATION OF BILIVERDIN ISOMERS, AS THE DIMETHYL ESTERS, ON SILICA GEL G

For mesobiliverdins
(A) Carbon tetrachloride–ethyl acetate–cyclohexane (32:9:4)
(B) Carbon tetrachloride–ethyl methyl ketone (4:1)
(C) Toluene–carbon tetrachloride–ethyl methyl ketone (5:5:2)
For protobiliverdins
(D) *n*-Heptane–ethyl methyl ketone–glacial acetic acid (10:5:1)
(E) Toluene–ethyl methyl ketone–glacial acetic acid (10:5:0.5)

more reproducible, and was used thereafter. The slight "bearding" effect observable in Fig. 1 becomes progressively worse when the plates are overloaded, but at concentrations of biliverdin sufficient to give good visible spots the effect is not troublesome, and when bands rather than spots are applied only the ends of the bands are affected.

Identification of the isomers

The results establishing the identities of the separated isomers are summarized in Fig. 1. The separated spots were subjected to RÜDIGER's pH 1.7 dichromate degradation technique[6,8] as illustrated in Fig. 2. The pyrrole dialdehydes produced from each spot are indicated on the left of Fig. 1. As reported by RÜDIGER[6,8], only the two internal pyrrole rings of bilins yield dialdehydes; the outer rings yield maleimides. Therefore, the production of I as the only dialdehyde from the front mesobiliverdin spot (see Fig. 1A) identifies this as the IXα isomer, since this is the only isomer both of whose internal rings carry a propionate side-chain (see scheme 1). This isomer is also identified by its chromatographic identity with the authentic IXα preparation (Fig. 1A, position 2). The production of only dialdehyde IIa from the second spot identifies this as the IXγ isomer (see scheme 1). The remaining two spots yield both pyrrole dialdehydes I and IIa indicating that they are the IXβ and IXδ

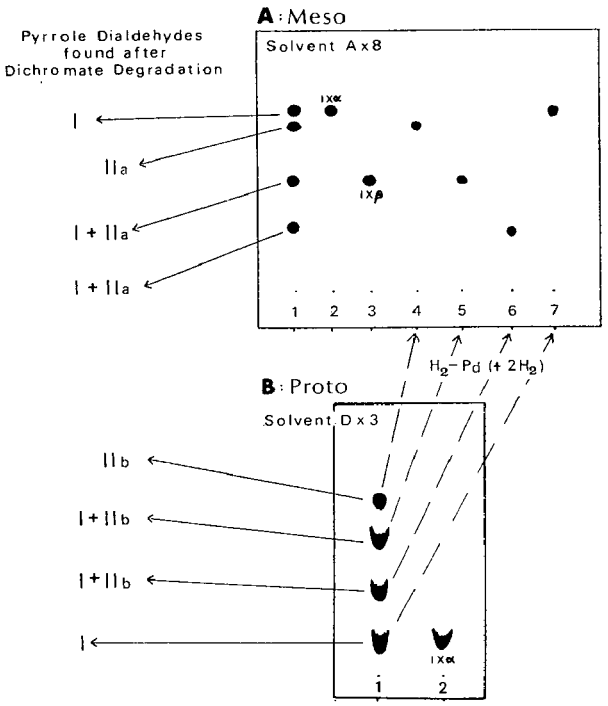


Fig. 1. Separation of (A) mesobiliverdins and (B) protobiliverdins as the dimethyl esters, and identification of the individual isomers (see text). For solvent systems see Table I; for structures of the pyrrole dialdehydes see Scheme 1. (1) Preparations obtained after coupled oxidation of haemins; (2) authentic biliverdin IXα dimethyl ester preparations (A,2 prepared by hydrogenation of B,2 in formic acid); (3) mesobiliverdin IXβ dimethyl ester; (4, 5, 6 and 7) separated isomers of B,1 hydrogenated to mesobiliverdin in formic acid.

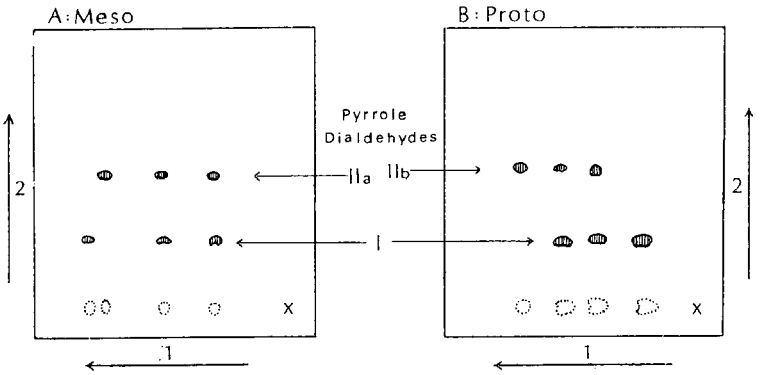


Fig. 2. Dichromate degradation of (A) separated meso- and (B) separated protobiliverdin isomers by the on-the-plate method of RÜDIGER[6,8]. The biliverdin ester mixtures were applied at ×, and, by chromatography in direction 1 as in Fig. 1, they were separated into the individual isomers (areas bounded by dotted lines). A 1% aqueous, pH 1.7 dichromate–acetic acid (2:1) mixture was carefully applied over each biliverdin spot and after 1 h the degradation products were separated by chromatography in direction 2 with carbon tetrachloride–ethyl acetate–cyclohexane (5:3:1). After evaporation of the solvent the pyrrole dialdehydes were visualized as red-brown spots (hatched areas) by spraying with dinitrophenylhydrazine[6,8]. The indicated identifications of the dialdehydes were confirmed by running standards (not shown) in direction 2.

isomers but without distinguishing between them. However, the synthetic mesobiliverdin IXβ dimethyl ester provided by Professors KENNER and JACKSON is chromatographically identical with the third mesobiliverdin spot (Fig. 1A, position 3; the chromatographic identity was confirmed by co-chromatography in solvents A, B and C). Thus the third spot is the IXβ isomer and the bottom one must then be the IXδ isomer.

Of the four protobiliverdin spots (Fig. 1B) the last spot corresponds with authentic protobiliverdin IXα dimethyl ester. Dichromate degradation (on the basis of the same reasoning as applied above to the mesobiliverdins) confirms the identity of the last spot as the IXα isomer and shows that the front spot is the IXγ and the middle two spots are the IXβ and IXδ isomers.

The catalytic hydrogenation procedure, described in MATERIALS AND METHODS, enabled the direct conversion of the separated protobiliverdin isomers (after elution from preparative-scale plates) to mesobiliverdins and thus allowed a correlation of the isomers of the two biliverdin series as illustrated in Fig. 1 (arrows from B to positions 4 to 7 of A). The second protobiliverdin spot (from the front) was thus identified unambiguously as the IXβ isomer and the third as the IXδ. The confirmatory cross-correlation of the IXα and IXγ isomers of the two series also adds further weight to the identifications.

The identities of the isomers are summarized in Figs. 3 and 4.

The separations may be carried out on a preparative scale on thick layers of Silica Gel G and, after elution from the silica gel with acetone, the isolated pigments may be converted to the free diacids by hydrolysis in 10 *N* HCl.

Isomer analysis

Micro-analysis of the isomer composition of a bilin preparation based on the

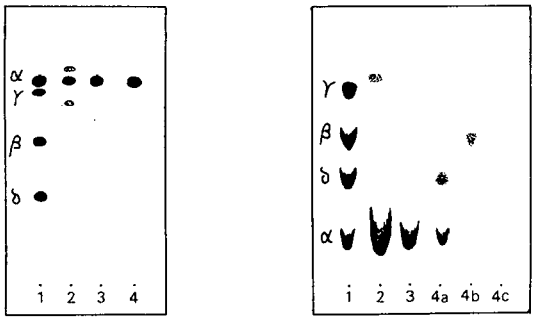


Fig. 3. Mesobiliverdin dimethyl ester preparations separated by eight-fold multiple development with solvent system A (carbon tetrachloride–ethyl acetate–cyclohexane, 32:9:4). (1) Standard preparation obtained after coupled oxidation of pyridine mesohaemochrome showing identities of isomers; (2) mesobiliverdin IXα, type II (see text); (3 and 4) mesobiliverdin prepared respectively from C-phycocyanin and from R-phycoerythrin.

Fig. 4. Protobiliverdin dimethyl ester preparations separated by three-fold multiple development with solvent system D (*n*-heptane–ethyl methyl ketone–glacial acetic acid, 10:5:1). (1) Standard preparation obtained after coupled oxidation of pyridine protohaemochrome, showing identities of the isomers; (2) preparation obtained from one post-mortem human bile sample overloaded to show trace of artifact biliverdin; (3) preparation from crude bilirubin from fresh pig bile; (4a, b and c) rechromatography of concentrated eluates from: (a) the IXδ region, (b) the IXβ region, and (c) the IXγ region, scraped from a number of plates heavily overloaded with bands of the preparation from crude pig bilirubin.

above TLC systems may be achieved if the bilin can be converted to proto- or mesobiliverdin. The methylated biliverdin is chromatographed alongside a mixed-isomer standard (prepared by coupled oxidation and esterification of the appropriate haemin) and the isomer(s) are thus identified. A rough indication of the relative proportions of the isomers may be obtained by visual inspection of the chromatograms. Reflectance densitometry using a red filter and calibrating with different quantities of the appropriate biliverdin may be used to obtain accurate figures. Alternatively, the sample is applied as a band and, after separation, the isomer zones are scraped off and extracted twice with acetone, the silica gel being removed completely by centrifugation. The extracts are made up to a fixed volume (1–2 ml) with acetone, the absorbancies read at $\lambda_{max.}$ and the relative proportions of the isomers calculated (assuming equal extinction coefficients for the four isomers). The latter method yields results which are reproducible to within about 5% except where a particular isomer is present in large excess over the others. About 50 μg of the biliverdin preparation is required for this method, while less than 5 μg is sufficient if a suitable reflectance densitometer is available.

Besides the green or blue biliverdin spots, faint red-purple and yellow spots due to artifact oxidation products (not shown in the figures) can usually be seen. As these do not absorb in the same spectral region as the biliverdins they do not interfere significantly with the estimations even where they coincide with a biliverdin spot.

NICOL AND MORELL[9] interpreted results obtained by a combination of mass spectrometry, NMR and alkaline permanganate degradation to indicate that the protobiliverdin produced by coupled oxidation of pyridine haemochrome with

ascorbate consisted of a single isomer—either the IXβ or IXδ. Since our results and those of RÜDIGER[6] indicate unequivocally that the product is a mixture of all four isomers, the validity of isomer analyses obtained by the methods of NICOL AND MORELL must therefore be open to doubt. However their deductions concerning the isomeric composition of phycoerythrobilin[9] are consistent with ours (see below).

Other methods of isomer analysis are dealt with in INTRODUCTION.

The phycobilins and bilirubin

The esterified mesobiliverdin prepared from both C-phycocyanin and R-phycoerythrin was found to consist entirely of the IXα isomer (Fig. 3 and Table II),

TABLE II

ISOMER COMPOSITION OF NATURALLY OCCURRING BILINS

Original bilin	*Converted to*	*% isomer composition*			
		IXα	*IXβ*	*IXγ*	*IXδ*
Phycocyanobilin	mesobiliverdin	100	0	0	0
Phycoerythrobilin	mesobiliverdin	100	0	0	0
Biliary bilirubin	protobiliverdin	*ca.* 99.6	*ca.* 0.1	0	*ca.* 0.3

no trace of any other isomer being detectable even when the plates were overloaded. Dichromate degradation confirmed this—only pyrrole dialdehyde I was obtained and no trace of IIa. Dichromate degradation of phycocyanobilin and phycoerythrobilin themselves (isolated as described in ref. 19) also yielded only pyrrole dialdehyde I. These results confirm that both these phycobilin prosthetic groups are entirely of the IXα configuration.

Protobiliverdin obtained by dehydrogenation of bilirubin from fresh pig bile at first seemed to consist entirely of the IXα isomer (Fig. 4, position 3), but when crude non-crystalline bilirubin (which had been submitted to a minimum of fractionation during its isolation) was used, and when the TLC plates were heavily overloaded with bands of the esterified biliverdin, a faint trace of some biliverdin in the IXδ position was detectable. The regions corresponding to the IXδ, IXβ and IXγ isomers were scraped off, eluted, concentrated and reapplied as spots on a new TLC plate as illustrated in Fig. 4 (positions 4a, b and c). In this way the occurrence of trace quantities showed up much better. The presence of a trace of the IXδ isomer was verified (still contaminated with some IXα owing to spreading on the overloaded preparative plates) and also an even smaller trace of the IXβ isomer. No biliverdin IXγ whatever could be detected. The proportion of the IXδ to total IXα was roughly estimated to be about 1 : 300 or 1 : 400, and of IXβ to IXδ about 1 : 3, giving the approximate figures listed in Table II. At this trace level it is not possible to give accurate figures and the risk of interference by trace quantities of artifacts is considerably increased. However it seems certain that bilirubin from pig bile consists of over 99.5% of the IXα isomer. PETRYKA[23], using the alkaline permanganate technique applied to large quantities of bilirubin (200 mg), previously obtained evidence that bilirubin from dog bile was not entirely the IXα isomer and that the

early labeled bilirubin fraction contained a higher proportion of other isomer(s). No indication of the nature or proportions of the other isomers could be obtained with that technique, however. Since early labeled bilirubin represents about 10% of the total bilirubin[24,25], the low level of non-IXα isomers which we find in the total bilirubin indicates that even the early labeled fraction must be at least 95% IXα.

Some unidentified biliverdin components

Some interesting biliverdin artifacts which we have encountered could cause confusion. Fig. 3 shows the pattern obtained with "type II" preparations of mesobiliverdin IXα dimethyl ester (see MATERIALS AND METHODS) prepared by the usual methods of preparing this substance—dehydrogenation of mesobilirubin or mesobilirubinogen obtained by alkaline reduction of bilirubin IXα. As illustrated in Fig. 3 (position 2), such preparations are resolved into two additional minor mesobiliverdin components which do not correspond with any of the isomers discussed above, besides a major spot corresponding with the single mesobiliverdin IXα spot observed in the other preparations (Figs. 1 and 3).

The three components were isolated on a preparative scale. Chromic acid degradation showed that all three had identical side-chain complements and they were also similar spectrally. The structural basis of the two minor components is therefore unclear and may possibly be due to some previously undetected type of isomerization, which presumably takes place during the alkaline reduction step, since the mesobiliverdin prepared from the same bilirubin, but avoiding such a step, does not contain the minor components (Fig. 1A, position 2). RÜDIGER and PAULMANN (unpublished results, quoted in ref. 7) have independently observed inhomogeneity in mesobiliverdin IXα prepared from mesobilirubinogen and have also suggested that an unknown type of isomerization may be responsible.

In the protobiliverdin series artifacts may arise readily by modifications of the labile vinyl side-chains, *e.g.* by methoxylation during esterification[7] or by hydroxylation or oxidation, but if the precautions outlined in MATERIALS AND METHODS are observed, such modifications are avoided. However, in one post-mortem sample of human bile, we observed a trace of a modified IXα derivative despite maximum precautions. This trace material could only be detected when the plates were overloaded (Fig. 4, position 2) and it ran just in front of the IXγ isomer, with which it might easily be confused. Sufficient material was available only for limited degradative studies and these indicated that it was a derivative of biliverdin IXα apparently with a modified (but not methoxylated) vinyl side-chain. It was not found in preparations from fresh biliary bilirubin and it may have been due to bacterial action.

ACKNOWLEDGEMENTS

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CHROM. 4796

ARTIFACTS IN ISOELECTRIC FOCUSING

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SUMMARY

Isoelectric focusing of an acidic protein from wool in acrylamide gels has shown that artifacts may occur possibly due to complexes of the protein with ampholines. The phenomenon has also been demonstrated for some basic proteins. Methods for demonstrating the presence of artifacts involve varying the ratio of ampholine to protein, re-running of discrete bands, or applying the protein sample at different positions on the gel.

INTRODUCTION

Although formation of complexes between proteins and ampholytes during isoelectric focusing is possible, it has been assumed that such a complex will have the same isoelectric value as the free protein[1]. During the course of experiments aiming to demonstrate micro heterogeneity in an alkylated wool protein by isoelectric focusing in acrylamide gels, serious variations in the number of zones obtained and their apparent isoelectric points were found according to the position of sample application on the gel. This raised the suspicion that the different zones observed might be due either to formation of a number of specific complexes with a single protein rather than to the separation of closely related proteins, or to the formation of multiple boundaries[2]. Experiments were therefore designed to investigate this behaviour more fully.

MATERIALS

The protein used in these experiments, component 8, is a major low-sulphur fraction prepared from reduced and S-carboxymethylated wool[3]. It is an acidic protein with a molecular weight of 45,000 and is homogeneous as determined by gel filtration on Sephadex G-200 and by starch gel electrophoresis in 8 *M* urea buffer[4]. However, recent evidence, both immunological[5] and chemical[6], indicates that the component is a mixture of closely related proteins.

Cyanogum was purchased from Kodak and ampholine from L.K.B. All other reagents were of analytical grade and were used without further purification with

the exception that urea solutions were deionised by passage through a mixed-bed ion-exchange resin and subsequently stored at 5°.

METHODS

Electrofocusing

The experiments were carried out in 5% acrylamide gels cast into trays 15 × 5 cm and 3 mm deep.

To make the acrylamide gel, the required amount of cyanogum was dissolved in 28 ml 8 *M* urea, and 2 ml of ampholine (3–10 range) and 0.04 ml of TMED* were added. Immediately before pouring the solution into the tray, 0.5 ml of 4% ammonium persulphate was added. The tray was then covered with a perspex sheet and left for 1 h.

Samples of protein (2% in 8 *M* urea) were applied on small pieces of filter paper pushed into slots cut into the gel.

Voltage was applied to the gel via two cellulose wicks dipping into 2.5% ethanolamine (cathode) and 2.5% phosphoric acid (anode). Focusing experiments were generally run at 150 V for 5 h, then at 200 V for a further 20 h.

For determination of the pH gradient along the gel, small discs were cut out with a cork borer and placed into 1 ml of water. The pH of the solution was measured after 3 h.

Staining of gels

Generally staining of acrylamide gels containing ampholine is very time consuming as the ampholytes must be first washed from the gel, and this may take up to several days. Two dyes, Light Green SF and Fast Green FCF, have been used[7] which do not form insoluble complexes with ampholine, but were considered inferior to Comassie Blue.

During the course of this work, a range of dyes was discovered which can be used directly to stain the gel, and which can be washed from the gel in a few hours. The dyes are characterised by being amphoteric, having generally two sulphonic acid groups and one or two charged nitrogens. In addition, the presence of bulky unsubstituted aromatic rings appears to favour the formation of insoluble complexes with ampholine.

The dyes investigated were Kiton Rhodamine B (C.I. 45100), Disulphine Blue VN (C.I. 42045), Fast Acid Blue B (C.I. 44035) and Coomassie Violet R (C.I. 42650). Although Kiton Rhodamine B is probably too weak to be used by itself, it is useful when mixed with other dyes (particulary Disulphide Blue VN and Fast Acid Blue B) as the contrast between the stained protein and the background is greatly enhanced. Although Fast Acid Blue B and Coomassie Violet R do form precipitates with ampholine, this only occurs in the staining liquid, and does not occur within the gel itself.

The dyes have satisfactory fastness properties provided that the pH of the staining or destaining solutions is not below 2.5. For this reason, trichloroacetic acid

* TMED = N,N,N′N′-tetramethylethylenediamine.

cannot be used, and satisfactory results were obtained by the use of aqueous 5% acetic acid both in the staining and destaining solution.

For staining the protein used in these experiments a mixture of 0.05% Fast Acid Blue B and 0.05% Coomassie Violet R in 5% acetic acid was used, followed by destaining in 5% acetic acid. After 4 or 5 h destaining, the background was sufficiently reduced for the gel to be photographed.

For prolonged storage of the gels it is recommended that they be kept in the original destaining solution and no attempt made to completely clear the gel. Even though the background is still somewhat coloured, the contrast obtained is very strong and remains so even after several weeks.

This dye mixture may not be satisfactory for all proteins, and some experimentation with different dyes may be necessary to obtain maximum sensitivity.

RESULTS

In the first experiment samples were applied to the gel at three positons corresponding to the alkaline, neutral and acid regions after the gradient had been

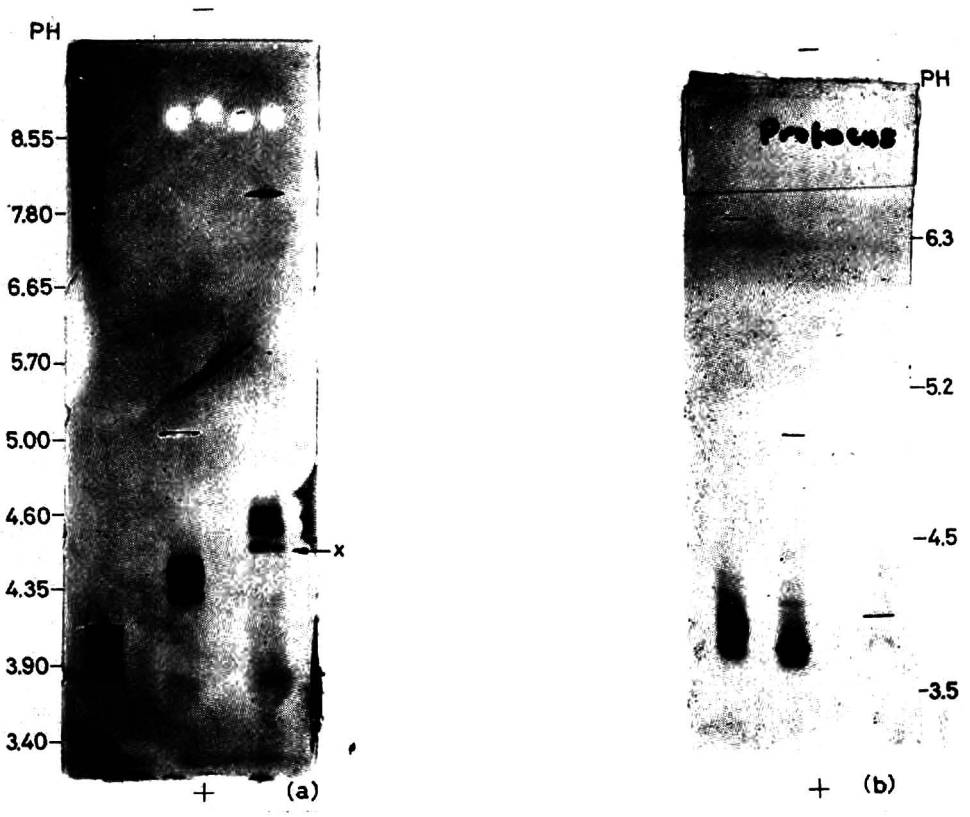


Fig. 1. Isoelectric focusing of a low-sulphur wool protein on acrylamide gel in the presence of 8 *M* urea. The gel contained 2 ml of 3–10 ampholine per 30 ml. (a) No prefocus; 25-h run. (b) 5 h prefocusing, then 20 h with protein; protein loaded at marked slot positions.

established. As can be seen from Fig. 1a the number of protein bands observed and their apparent isoelectric points vary according to the initial position on the gel.

If complex formation were responsible for this result, then it seemed reasonable that the differences seen were due to exposure of the protein to acidic and basic ampholines for different periods of time. Thus the sample loaded near the basic end would be exposed to more basic ampholines while the protein migrated through the establishing gradient than proteins loaded near the acidic end of the gel. If this were the case, then application of the protein after the gradient had been established should give different apparent isoelectric points. A gel was prepared in the normal manner and a potential of 150 V applied for 5 h after which time the current had dropped to a tenth of its initial value and the gradient was assumed to have been established. Samples of protein were then applied at the same three positions on the gel and electrofocusing continued for a further 20 h at 200 V.

The results of this experiment are seen in Fig. 1b and demonstrate that indeed prefocusing does affect the results. Because of the increased number of bands seen and the slightly higher apparent isoelectric points in protein exposed to more basic ampholines, it is likely that complexes are forming predominantly with basic

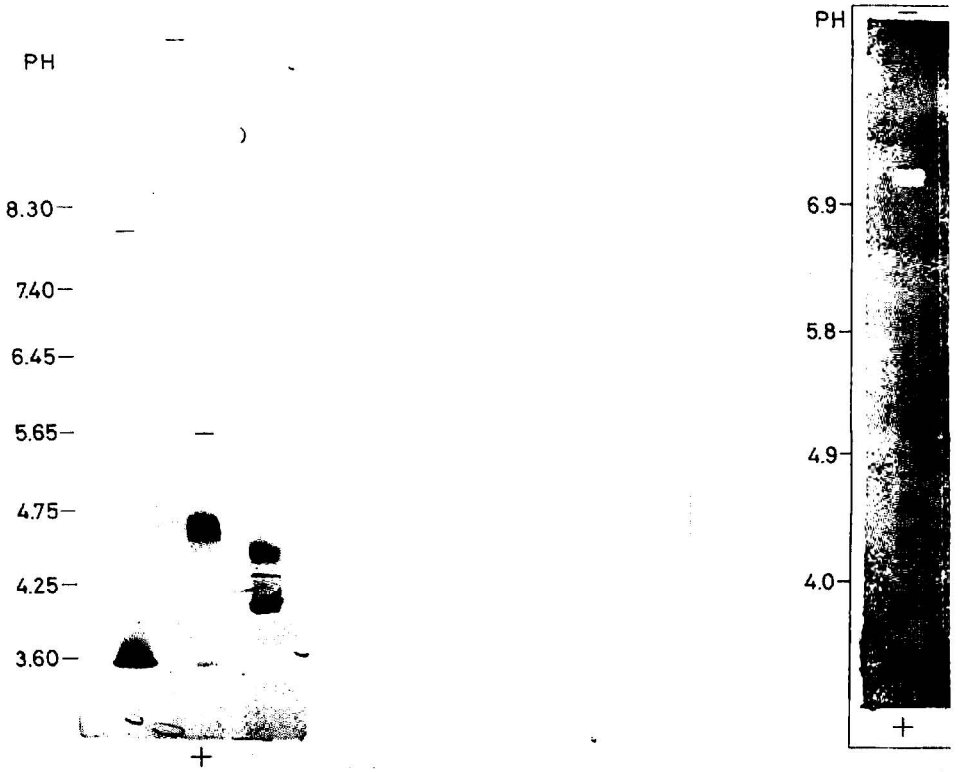


Fig. 2. Isoelectric focusing of wool protein. The gel contained 0.5 ml of 3–10 ampholine per 30 ml. No prefocus, focusing run for 25 h at 200 V.

Fig. 3. Repeated electrofocusing of band taken at point "X" in Fig. 1a. The gel contained 2 ml of 3–10 ampholine per 30 ml. No prefocus.

ampholines. An interesting observation here is that staining of the protein exposed only to acidic ampholines is very much weaker (Fig. 1b) than that exposed to basic ampholines, indicating that protein–ampholine complexes take up more of the acid dye than does the protein alone.

The last two experiments suggest that the results could be explained by complex formation. According to CANN[8], a characteristic of multiple protein zones caused by reversible buffer–protein interactions is that this may be modified by alteration in concentration of one of the constituents. In a demonstration of this phenomenon, the concentration of ampholine was decreased to 0.5 ml/30 ml gel, and samples of protein were run in three positions (no prefocusing). The results (Fig. 2) show that this modification causes marked alterations in the patterns of zones obtained.

Further evidence was obtained by re-electrofocusing one of the protein bands formed during the electrophoresis. To do this it was necessary to locate the protein in the gel without staining, and fortunately this could be done by simply soaking the gel in water to precipitate the protein. Thus the band corresponding to the one marked X in Fig. 1a was cut from the gel and inserted into a trough cut into the basic end of a fresh gel. Electrofocusing was then carried out in the usual manner, and, as can be seen in Fig. 3, multiple zones again reappear.

DISCUSSION

That artifacts may occur during electrofocusing experiments is amply demonstrated by the results presented. The artifacts may be due either to complex formation, or to the formation of multiple boundaries[2]. It should be noted that the positions of bands found are equilibrium positions and that alteration of the conditions of electrophoresis will alter this equilibrium.

As demonstrated for the acidic protein from wool it seems that basic ampholines are involved more than acidic ones and this is also supported by the fact that attempts to electrofocus the protein using a narrow-range ampholine (3–6) did not produce comparable results. In this case, no discrete bands were observed, but a broad, poorly staining smear extending from approx. pH 4.5 to 5.2 was seen.

Since these experiments were performed with only one acidic protein it is important to know if artifacts occur with other proteins. In a limited number of experiments with some basic proteins (myoglobin and haemoglobin) artifacts were not observed. However, in some experiments performed by Dr. J. R. YATES of this Division artifacts were observed in electrofocusing on acrylamide gels of some basic microbial proteases (pronase, alcalase, and serratia protease).

Thus it appears that artifacts may occur in the electrofocusing of both acidic and basic proteins, and this possibility should be considered when using the technique for any protein.

The use of acrylamide gels for these experiments greatly facilitates the observation of artifacts, and is a recommended preliminary before proceeding to larger-scale separations on columns.

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CHROM. 4781

A PAPER ELECTROPHORETIC STUDY OF ION PAIR FORMATION

V. PAPER ELECTROPHORESIS AND ANION-EXCHANGE PAPER CHROMATOGRAPHY OF INORGANIC ANIONS WITH VARIOUS ELECTROLYTES

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SUMMARY

Inorganic anions were studied by paper electrophoresis and ion-exchange paper chromatography employing electrolytes and eluents with various metal ions (Mg^{2+}, Al^{3+} and Cd^{2+}). It was found that the metal ion has a strong effect on the movement of anions in paper electrophoresis both by ion pair and complex formation In ion-exchange paper chromatography the effect is small. Data are given for twenty inorganic anions.

INTRODUCTION

Separations by paper electrophoresis are usually effected by choosing suitable conditions of pH or complexation (with borate for example) or by permitting the ions to move over large distances with high potentials. We have recently shown that there exists still another factor able to improve separations, namely ion pairing or outer-sphere complexing[1,2] and have shown that the separation of quarternary ammonium compounds can be improved by choosing suitable electrolytes and electrolyte concentrations[3].

In this paper we report a similar study for inorganic anions. Paper electrophoresis of inorganic anions has been effected in several alkaline electrolytes, and there is little difference in the separations obtained in *e.g.* NaOH or ammonium carbonate[4–7]. pH variations cannot be used very effectively for inorganic anions as many are unstable below neutrality. Thus improvement of separations can be obtained, as will be shown here, either by complex formation or by ion pair formation. We also thought it of interest to compare the effect of ion pairing in paper electrophoresis and ion-exchange chromatography. While the effect is considerable in the first and improves separations, it is not very marked in ion exchange.

EXPERIMENTAL

Paper electrophoresis

A Camag high-potential electrophoresis apparatus was used with Whatman

TABLE I

HIGH-VOLTAGE ELECTROPHORESIS OF INORGANIC ANIONS IN AMMONIUM NITRATE AS ELECTROLYTE

Whatman No. 1 paper; 1000 V for 30 min at 7–8°. The values are corrected for electroosmotic flow (movement of H_2O_2) and results of different sheets are compared by assuming the movement of ferricyanide (run on all sheets) constant. Chromatographic R_F values are given for each concentration to show where adsorption occurs, but no correction to the electrophoretic movement was made.

Anion	*1.0 M*		*0.5 M*		*0.1 M*	
	R_F	*mm*	R_F	*mm*	R_F	*mm*
F^-	1.0	73	1.0	63	1.0	57
Cl^-	1.0	114	1.0	96	1.0	89
Br^-	1.0	108	1.0	101	1.0	90
I^-	0.90	110	0.88	95	0.89	87
SCN^-	0.85	82	0.85	79	0.85	72
ClO_3^-	1.0	86	1.0	80	1.0	75
BrO_3^-	1.0	68	1.0	65	1.0	66
IO_3^-	1.0	41	1.0	38	1.0	39t
ClO_4^-	0.88	80	0.86	75	0.86	71
IO_4^-	0.90	44	0.87	39	0.90	38
ReO_4^-	0.84	57	0.80	52	0.83	53
$HCOO^-$	1.0	73	1.0	67	1.0	66
CH_3COO^-	1.0	50	1.0	46	1.0	42
Ox^{2-}	1.0	71	1.0	65	1.0	68
$Cr_2O_7^{2-}$	0.77	71	0.78	63	0.80	70
CrO_4^{2-}	0.77	75	0.78	68	0.80	65
$S_2O_3^{2-}$	1.0	82	1.0	78	1.0	77
$S_2O_8^{2-}$	1.0	87	1.0	76	1.0	74
SeO_3^{2-}	1.0	39	1.0	40	1.0	41
$Fe(CN)_6^{3-}$	1.0	91[a]	1.0	81[a]	1.0	78[a]
SO_4^{2-}	1.0	78	1.0	68	1.0	65

[a] Anion run on all sheets.
t = tailing.

No. 1 paper, water circulating at 7–8°, applying a potential of 1000 V for 30 min. During this time the current increased. However, we preferred such conditions to efficient cooling as they led to a fast separation. To compare the movement of ions from different electropherograms, we ran ferricyanide on all of them and recalculated the movement to the mean distance moved by ferricyanide (as indicated in the tables). For $Cd(NO_3)_2$ as electrolyte, perchlorate was used as standard instead of ferricyanide.

Adsorption on Whatman No. 1 paper was examined by chromatographing at about 5° by ascending development with the electrolyte. Electroosmotic movement was measured by observing the movement of H_2O_2, and the results shown in Tables I–V are corrected for electroosmotic flow. The movement of the anions in various concentrations of ammonium, magnesium and aluminium nitrates is shown in the tables. Some were insoluble under certain conditions and thus are not shown.

Generally there are two important changes in the movement of anions from NH_4^+ to Mg^{2+} and Al^{3+}. The divalent anions are slowed down as the charge of the metal ion increases while the halides move with about the same speed and there is a considerable difference in the separation of chlorate–bromate–iodate.

The greater tendency of oxyhalide anions to ion pairing is evident compared

TABLE II

HIGH-VOLTAGE ELECTROPHORESIS OF INORGANIC ANIONS IN MAGNESIUM NITRATE AS ELECTROLYTE

Conditions as in Table I.

Anion	*1.0 M*		*0.5 M*		*0.1 M*	
	R_F	*mm*	R_F	*mm*	R_F	*mm*
F^-	—	—	—	—	—	—
Cl^-	1.0	74	1.0	83	1.0	83
Br^-	0.90	77	1.0	86	0.92	85
I^-	0.82	75	0.86	84	0.83	83
SCN^-	0.83	56	0.82	73	0.82	66
ClO_3^-	1.0	64	1.0	72	1.0	68
BrO_3^-	1.0	44	1.0	51	1.0	56
IO_3^-	0.91	16	0.88	21t	0.92	25
ClO_4^-	0.90	67	1.0	76	0.86	72
IO_4^-	1.0–0.35	18–30	0.87t	22	0.89t	27
ReO_4^-	0.77	42	0.75	49	0.72	48
$HCOO^-$	1.0	21	1.0	37	1.0	47
CH_3COO^-	1.0	16	1.0	24	1.0	32
Ox^{2-}	—	—	—	—	—	—
$Cr_2O_7^{2-}$	0.70t	46–34	0.70	51	0.74	48t
CrO_4^{2-}	0.92t	33	0.90t	41	0.91t	46
$S_2O_3^{2-}$	1.0	38	1.0	45	1.0	49
$S_2O_8^{2-}$	1.0	62	1.0	69	1.0	67
SeO_3^{2-}	1.0	14	0.90	17	0.89	23
$Fe(CN)_6^{3-}$	1.0	41[a]	1.0	51[a]	1.0	55[a]
SO_4^{2-}	1.0	22	1.0	26	1.0	37

[a] Anion run on all sheets.
t = tailing.

TABLE III

HIGH-VOLTAGE ELECTROPHORESIS OF INORGANIC ANIONS IN ALUMINIUM NITRATE AS ELECTROLYTE

Conditions as in Table I.

Anion	*0.5 M*		*0.3 M*		*0.2 M*		*0.05 M*	
	R_F	*mm*	R_F	*mm*	R_F	*mm*	R_F	*mm*
F^-	—	—	—	—	—	—	—	—
Cl^-	1.0	70	1.0	78	1.0	77	1.0	82
Br^-	0.95	78	0.92	84	0.92	81	0.93	86
I^-	0.84	69	0.86	83	0.86	86	0.86	72
SCN^-	0.84	50	0.80	55	0.80	61	0.81	60
ClO_3^-	1.0	59	1.0	63	1.0	69	1.0	63
BrO_3^-	1.0	42	1.0	46	1.0	50	1.0	54
IO_3^-	1.0	9	0.90	11	0.88	14	0.85t	22t
ClO_4^-	0.88	64	0.85	67	0.83	73	0.88	70
IO_4^-	—	—	—	—	—	—	—	—
ReO_4^-	0.73	44	0.75	46	0.76	48	0.75	47
$HCOO^-$	—	—	—	—	—	—	—	—
CH_3COO^-	—	—	—	—	—	—	—	—
Ox^{2-}	—	—	—	—	—	—	—	—
$Cr_2O_7^{2-}$	0.75	44	0.80	46	0.82	49; 0.79	0.70	45; 38
CrO_4^{2-}	0.76	47	0.76	46	0.75	42; 0.76	0.68	46; 36
$S_2O_3^{2-}$	1.0	29	1.0	32	1.0	33	1.0	31
$S_2O_8^{2-}$	1.0	58	1.0	60	1.0	63	1.0	60
SeO_3^{2-}	1.0	+12	1.0	+4	0.91	0	0.84	0
$Fe(CN)_6^{3-}$	1.0	38[a]	1.0	41[a]	1.0	43[a]	1.0	37[a]
SO_4^{2-}	—	—	—	—	—	—	—	—

[a] Anion run on all sheets.
t = tailing.

TABLE IV

HIGH-VOLTAGE ELECTROPHORESIS OF INORGANIC ANIONS IN CADMIUM NITRATE

Perchlorate is used as reference anion for comparison of different sheets. Conditions as in Table I.

Anion	*1.0 M*		*0.5 M*		*0.1 M*	
	R_F	*mm*	R_F	*mm*	R_F	*mm*
F^-	1.0	15–52	1.0	23–60	1.0	63–38–0
Cl^-	0.92	+11	0.88	+ 7	0.92	8; +1
Br^-	0.89	+15	0.88	+ 9	0.85	+ 2
I^-	0.80	+15	0.84	+11	0.82	+12
SCN^-	0.85	+15	0.87	+13	0.82	+ 2
ClO_3^-	1.0	58	1.0	60	1.0	71
BrO_3^-	0.91	37	1.0	40	1.0	53
IO_3^-	0.88	0	0.80	5	0.85	16t
ClO_4^-	0.88	61[a]	0.78	65[a]	0.86	71[a]
IO_4^-	—	—	—	—	—	—
ReO_4^-	0.76	37	0.73	42	0.68	49
$HCOO^-$	1.0	+2	1.0	+2	1.0	0
CH_3COO^-	1.0	+2	1.0	+2	1.0	0
Ox^{2-}	—	—	—	—	—	—
$Cr_2O_7^{2-}$	0.68	40t	0.70	46	0.65	49
CrO_4^{2-}	—	—	—	—	—	—
$S_2O_3^{2-}$	1.0	0	1.0	+4	0.90	0
$S_2O_8^{2-}$	1.0	58	1.0	64	1.0	68
SeO_3^{2-}	0.87	+6	0.87	+4	0.81	0
$Fe(CN)_6^{3-}$	—	—	—	—	—	—
SO_4^{2-}	1.0	14	1.0	19	1.0	0(?)

[a] Anion used as reference.
t = tailing.

TABLE V

R_F VALUES OF INORGANIC ANIONS ON STRONGLY BASIC ANION-EXCHANGE RESIN PAPER AMBERLITE SB-2 IN THE NITRATE FORM

The last column gives the R_F values in 1 *N* KNO_3 on cellulose Whatman No. 1 paper to show where adsorption on cellulose occurs. The values for KNO_3 are taken from ref. 5.

Anion	*Eluent*									
	KNO_3			$Mg(NO_3)_2$			$Al(NO_3)_3$			KNO_3
	0.1 N	*0.5 N*	*1 N*	*0.1 N*	*0.5 N*	*1 N*	*0.1 N*	*0.5 N*	*1 N*	*1 N*
Cl^-	0.22	0.51	0.66				0.30	0.60	0.72	1.0
Br^-	0.10	0.33	0.43	0.12	0.36	0.52	0.12	0.36	0.50	1.0
I^-	0.03	0.07	0.12	0.02	0.08	0.15	0.03	0.06	0.15	0.84
SCN^-	0.02	0.06	0.10	0.01	0.08	0.14	0.02	0.06	0.12	0.81
ClO_3^-	0.12	0.34	0.49	0.14	0.30	0.49	0.11	0.35	0.44	1.0
BrO_3^-	0.28	0.60	0.75	0.36	0.67	0.81	0.34	0.70	0.82	1.0
IO_3^-	0.60	0.87	0.88	0.65; 0.95	0.85; 1.0	0.93	0.64; 0.92	0.80; 0.92	1.0	1.0
IO_4^-	0	0.05	0.07	0.01	0.04	0.08	0.02	0.04	0.09	1.0
$Cr_2O_7^{2-}$	—	—	—	0	0.01	0.03	0.01	0.02	0.03	—
CrO_4^{2-}	0.03	0.06	0–0.17	0	0.01	0.03	0.01	0.02	0.03	1.0
$S_2O_3^{2-}$	0.08	0.43	0.83	0.16	0.17 (0.70)	0.95	0.17	0.73; 0.93	1.0	1.0
$Fe(CN)_6^{3-}$	0	0	0	0	0	0	0	0	0.01	1.0

to that of the halides. This was also noted in previous studies on the ion pairing of cobalt(III) complexes[1]. With cadmium nitrate as electrolyte which acts mainly as a complexant unlike the previous electrolytes which should essentially form outer-sphere complexes, we obtained again interesting separations (Table IV). Halides move cationically, presumably as CdX^+. There is an excellent separation of oxyhalides with a tendency of the heavier being being most retarded. Some divalent anions such as persulphate and dichromate are moving anionically while others, thiosulphate and selenite, are almost isoelectric.

These results clearly show that excellent separations can be obtained with complexing agents and that a study of other complexing cations may be rewarding.

Chromatography on ion-exchange resins

One of us (L.O.) had published the R_F values of a series of inorganic anions on Amberlite SB-2 paper developing with 0.1, 0.5 and 1 *N* KNO_3 (ref. 8). Table V

TABLE VI

R_F VALUES OF INORGANIC ANIONS ON MACHEREY, NAGEL & CO. STRONGLY BASIC QUARTERNARY AMMONIUM CELLULOSE PAPER IN THE NITRATE FORM

Eluents: ammonium, magnesium and aluminium nitrates.

Ion	*Eluent*								
	NH_4NO_3			$Mg(NO_3)_2$			$Al(NO_3)_3$		
	0.1N	*0.5N*	*1N*	*0.2N*	*1N*	*2N*	*0.15N*	*0.4N*	*0.9N*
F^-	0.83 (0.50)	0.90	0.88	—	—	—	—	—	—
Cl^-	0.43	0.74	0.80	0.56–0.84	0.86	0.85	0.59–0.74	0.87	0.87
Br^-	0.57	0.73	0.80	0.56	0.78	0.84	0.56	0.80	0.81
I^-	0.45	0.73	0.77	0.45	0.68	0.73	0.49	0.69	0.75
SCN^-	0.47	0.70	0.76	0.45	0.64	0.74	0.46	0.63	0.66
ClO_3^-	0.44	0.71	0.77	0.64	0.84	0.85	0.72	0.83	0.83
BrO_3^-	0.47	0.76	0.83	0.67	0.81	0.87	0.68	0.83	0.86
IO_3^-	0.40–0.54	0.78	0.82	0.86	0.81	0.90	0.90	0.87	0.91
ClO_4^-	0.27	0.56	0.62	0.47	0.73	0.73	0.47	0.65	0.67
IO_4^-	0.42	0.73	0.85	0.61	0.76	0.90	—	—	—
ReO_4^-	0.32	0.46	0.55	0.33	0.57	0.66	0.32	0.49	0.55
$HCOO^-$	0.75	0.80	0.84	0.90	0.90	0.91	—	—	—
CH_3COO^-	0.80	0.90	0.91	0.82–0.88	0.88–0.96	0.88–0.93	—	—	—
OK^{2-}	0.86	0.93	0.89	—	—	—	—	—	—
$Cr_2O_7^{2-}$	0.18	0.40	0.53	0.12	0.37	0.50	0.18	0.43	0.41
CrO_4^{2-}	0.15	0.37	0.52	0.12; 0.41	0.37; 0.82	0.44; 0.83	0.20	0.45	0.42
$S_2O_3^{2-}$	0.90	0.89	0.90	0.87	0.89	0.89	0.95	—	—
$S_2O_8^{2-}$	0.10	0.51	0.67	0.25	0.63	0.75	0.27	0.60	0.68
SeO_3^{2-}	0.80	0.84	0.86	0.72	0.88	0.91	0.76	0.82	0.82
$Fe(CN)_6^{3-}$	0.12	0.30	0.47	0.08	0.47	0.67	0.15	0.62	0.48
Pyrophosphate	0.96	1.0	1.0	—	—	—	—	—	—
PO_4^{3-}	0.85	0.95	1.0	—	—	—	—	—	—
$H_2PO_4^-$	0.82	0.88	0.88	—	—	—	—	—	—
$HAsO_4^{2-}$	0.85 (0.38)	0.85	0.88	—	—	—	—	—	—
TeO_4^{2-}	0.82	0.87	0.93	—	—	—	—	—	—

nitrate as developing solvents. There seems to be little difference between K^+, shows the R_F values on the same resin paper using magnesium nitrate and aluminium Mg^{2+} and Al^{3+} as eluent, the R_F values of the halides and oxyhalides being almost identical in all three.

Chromatography on anion-exchange cellulose paper

In this section we report for the first time data for a range of inorganic anions on an anion-exchange cellulose (Macherey, Nagel & Co. quarternary ammonium cellulose paper). From an analytical point of view the results are rather disappointing as most substances have rather high R_F values. Also there are no large differences between halides or between oxyhalides.

TABLE VII

R_F VALUES OF INORGANIC ANIONS ON MACHEREY, NAGEL & CO. STRONGLY BASIC QUARTERNARY AMMONIUM CELLULOSE PAPER IN THE NITRATE FORM

Eluent: cadmium nitrate.

Anion	*Normality*		
	0.1	*0.5*	*1*
F^-	0.85	0.88	0.88
Cl^-	0.70	0.74	0.75
Br^-	0.77	0.79	0.81
I^-	0.77	0.80	0.80
SCN^-	0.56	0.76	0.80
ClO_3^-	0.67	0.76	0.83
BrO_3^-	0.86	0.86	0.90
IO_3^-	0.88	0.92	0.90
ClO_4^-	0.45	0.64	0.70
ReO_4^-	0.31	0.48	0.53
$Cr_2O_7^{2-}$	0.10	0.36	0.42
$S_2O_3^{2-}$	0.72	0.87	0.89
$S_2O_8^{2-}$	0.18–0.35	0.60	0.67
SeO_3^{2-}	0.80	0.85	0.85

Table VI further shows that generally there are no marked differences between R_F values in ammonium, magnesium and aluminium nitrates. Chromatography with a complexing cation, *i.e.* with cadmium, does not change the picture very much either (see Table VII).

DISCUSSION

The work reported here shows that improvement of electrophoretic separations of anions may be obtained if electrolytes of suitable metal ions capable of ion pairing are employed. Highly hydrated monovalent anions do not separate better, but oxyhaides which are less hydrated are subject to ion pairing as are polyvalent anions.

A rather unexpected result of this work was that the cation of the developer had little effect in ion-exchange chromatography. This seems to be due to the fact

that in the law of mass action equation the activity of the polyvalent metal ion is diminished by ion pairing with the nitrate (used as eluent), and thus although there is also ion pairing with the anion under study the two effects cancel each other. What is surprising however is that there are not even small differences. From studies in solution it can also be assumed that the quarternary ammonium groups of the resin form ion pairs with anions. It is equally remarkable that there is no effect due to the differences in ion pairing ability of different anions with the groups of the resin and the metal ions in solution.

Complexing with cadmium produces rather spectacular results in paper electrophoresis with sequences that could have been predicted in most cases from the complex chemistry of cadmium. Ion-exchange chromatography on cellulose anion-exchange paper yields rather poor results as the complexed anions are all moving with high R_F values.

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CHROM. 4786

CRYSTALLINE INSOLUBLE ACID SALTS OF TETRAVALENT METALS

X. FIBROUS THORIUM PHOSPHATE, A NEW INORGANIC ION-EXCHANGE MATERIAL SUITABLE FOR MAKING (SUPPORT-FREE) INORGANIC SHEETS

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SUMMARY

Some investigations on the preparative procedure of fibrous thorium phosphate are reported. This new inorganic ion exchanger has been characterized on the basis of chemical analysis, thermal stability and ion-exchange properties with alkali metal ions. The ion-exchange capacity for Na^+ is 3.7 mequiv./g.

Fibrous thorium phosphate has been employed to prepare porous, support-free, inorganic ion-exchange sheets suitable for chromatographic experiments.

INTRODUCTION

In a previous paper[1] the preparation and some ion-exchange properties of a fibrous cerium(IV) phosphate were reported. This compound, besides possessing good ion-exchange capacity, has the property of giving flexible sheets similar to cellulose paper[2]. Since these inorganic ion-exchange sheets are very interesting both for theoretical studies and for their potential practical applications in chromatography[3], electrophoresis, ion-exchange membranes, etc., research was carried out to obtain other fibrous inorganic ion exchangers of the cerium(IV) phosphate type. In the present study the synthesis and some ion-exchange properties of a fibrous thorium phosphate are reported.

EXPERIMENTAL

All reagents were C. Erba R.P. products. The molar ratio of PO_4 to Th was determined as follows: 100–200 mg of sample were dissolved in 6 ml of hot concentrated HNO_3. The solution was then diluted to 100 ml with distilled water. Phosphates were determined colorimetrically in 2 ml of this solution, as previously described[1], while thorium was precipitated in the remaining solution as oxalate and determined as ThO_2. X-ray analysis, titration and weight-loss curves were obtained as described in previous papers of this series[1,4,5].

RESULTS AND DISCUSSION

It is known that when an acidic solution of a thorium salt is mixed (at room temperature) with a solution of phosphoric acid, amorphous thorium phosphate is obtained. This material shows a variable PO_4/Th ratio (depending on the preparative conditions) and a poor ion-exchange capacity (about 1 mequiv. of Na^+ per g) (ref. 6).

To obtain a fibrous material we have studied the effect of the concentration of the reagents, temperature and digestion time on the formation of the precipitate. It was found that the formation of fibrous thorium phosphate is favoured by a high PO_4/Th ratio, high temperature and a long digestion. Temperature and digestion time are interdependent; the lower the temperature the longer the digestion must be to obtain a good fibrous material for the preparation of inorganic sheets (Table I). A low yield or no precipitate is obtained (depending on the temperature) when the

TABLE I

PO_4/Th RATIO OF VARIOUS SAMPLES OF FIBROUS THORIUM PHOSPHATE PREPARED AT DIFFERENT TEMPERATURES, PO_4/Th RATIOS IN SOLUTION AND DIGESTION TIMES

$Th(NO_3)_4$ solution is 0.1 *M* in 1 *M* HNO_3.

H_3PO_4 (molarity)	*PO_4/Th (in solution)*	*Temperature (°C)*	*Digestion time (h)*	*PO_4/Th (in solid)*	*Remarks*
1	10	25		1.5[a]	No sheets obtained
1	10	60	3	1.6	Friable sheets obtained
1	10	60	20	1.8	Friable sheets obtained
1	10	90	3	—	Friable sheets obtained
1	10	90	10		Not very flexible sheets obtained
1	10	90	40	1.9[b]	Flexible sheets obtained
1	10	100	3		Friable sheets obtained
1	10	100	10		Not very flexible sheets obtained
1	10	100	20	1.9[b]	Flexible sheets obtained
2	20	90	10		Not very flexible sheets obtained
2	20	90	40	2.1[b]	Flexible sheets obtained
2	20	100	24		Flexible sheets obtained
2	20	100	40	2.0[b]	Flexible sheets obtained
3	30	100	—	—	No precipitation occurs

[a] Amorphous material.
[b] Fibrous material.

phosphoric acid concentration is higher than 2 *M* and the PO_4/Th ratio is higher than 30. This fact is most likely concerned with the well-known formation of soluble thorium complexes with phosphoric acid.

The fibrous phosphate employed in this work was prepared as follows. A volume of a 0.1 *M* thorium nitrate solution in 1 *M* HNO_3 was added dropwise (~1 ml/min) to a well-stirred volume of 2 *M* phosphoric acid. The temperature was maintained at about 100°. The precipitate so obtained was stirred for about 40 h at this temperature, then filtered, washed with distilled water until reaching pH 3–4 and dried in a desiccator over saturated sodium chloride. By filtering the precipitate under the same conditions described for cerium(IV) phosphate flexible inorganic

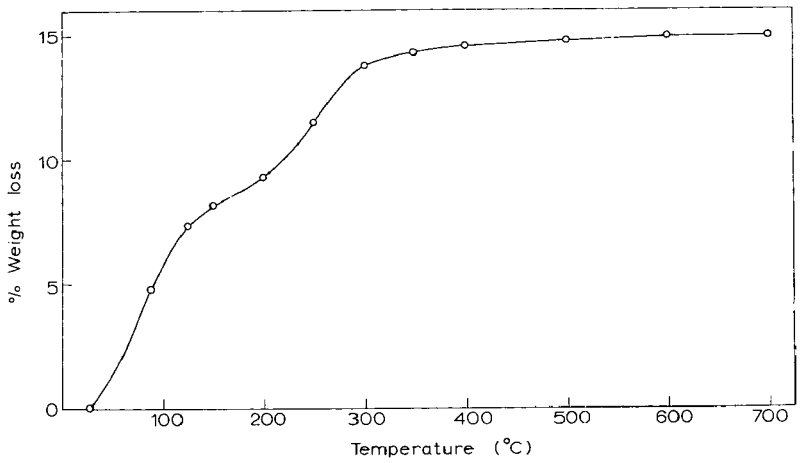


Fig. 1. Percentage weight loss of fibrous thorium phosphate, dried over a saturated NaCl solution at different temperatures.

sheets were obtained and it could be seen under the microscope that thorium phosphate is a fibrous material of cerium(IV) phosphate type[7].

As regards its chemical composition solid fibrous thorium phosphate has a PO_4/Th ratio of about 2, and when it is heated to 800° it loses 4 moles of water for each gramatom of thorium. Two moles of water are lost at 120° (or when dried over P_4O_{10} under vacuum); the third is lost between 120 and 240°, while the material is practically anhydrous at 500° (Fig. 1). Fibrous thorium phosphate can thus be represented as $ThO_2 \cdot P_2O_5 \cdot 4H_2O$. Its X-ray diffraction pattern is reported in Table II.

TABLE II

d VALUES FROM X-RAY POWDER PATTERN OF FIBROUS THORIUM PHOSPHATE IN H^+ FORM

d (Å)	*I*
11.47	S
5.38	W
4.95	VW
3.79	VW
3.54	VW
2.91	VW
2.80	VW
2.05	VW
1.56	W

We have investigated the ion-exchange properties of fibrous thorium phosphate with alkali metal ions. Fig. 2 shows the uptake curve of Na^+ and the phosphate loss curve at different pH values. From the sodium uptake and the mmoles of phosphate released to the solution at pH 11, one can derive an experimental ion-exchange capacity of $3.68 + 0.17 = 3.85$ mequiv./g; a value close to 4.18 mequiv./g is calculated assuming two exchangeable hydrogen ions per formula weight of exchanger.

In Table III the saturation capacity and the phosphate loss, obtained with

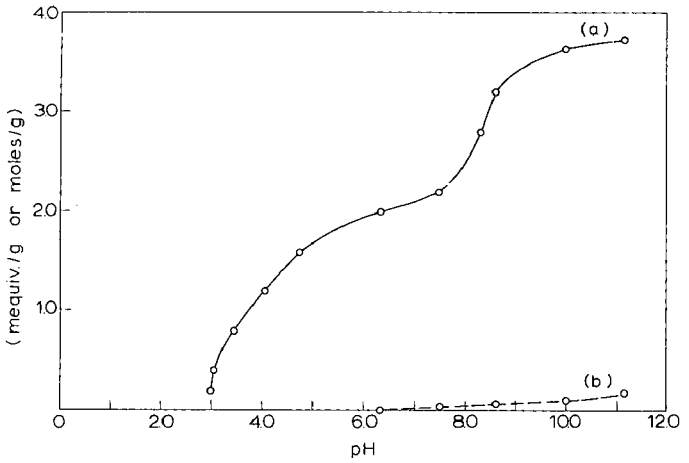


Fig. 2. Sodium uptake (a) and hydrolysis (b) curves of fibrous thorium phosphate titrated with 0.1 *M* (NaCl + NaOH).

batch experiments, are reported for the alkali metal ions. For Na^+, K^+ and Cs^+, the saturation capacity decreases with increasing crystalline ionic radius, while the reverse occurs for the phosphate loss. This fact can be accounted for by considering steric effects or electrostatic interactions in the exchanger. The relatively high phosphate loss in the Li^+–H^+ process can be explained by the macroscopic swelling observed in the material when converted into the Li^+ form.

As already mentioned, fibrous thorium phosphate can be employed to prepare inorganic ion-exchange sheets similar to those obtained with fibrous cerium(IV) phosphate. Because of their practical applications, it seems convenient to compare these inorganic ion-exchange sheets. Thorium phosphate sheets compare favorably with those of cerium(IV) phosphate as regards stability to strong reducing agents.

TABLE III

CAPACITY AND PHOSPHATE LOSS OF FIBROUS THORIUM PHOSPHATE (DRIED OVER SATURATED NaCl SOLUTION) FOR VARIOUS ALKALI METAL IONS

Ion	*Equilibrium pH*	*Capacity (mequiv./g)*	*Phosphate loss (mmoles/g)*
Li^+	10.7	3.2	0.7
Na^+	10.8	3.7	0.2
K^+	10.9	3.1	0.4
Cs^+	11.3	1.9	0.6

This property may be important in chromatography where reducing agents are often used as eluents or spot-test reagents. Furthermore, cerium(IV) phosphate decomposes at temperatures higher than 500° (for the Ce(IV)–Ce(III) reduction), while this does not occur for fibrous thorium phosphate. On the other hand, thorium phosphate sheets are radioactive and have a lower flexibility than cerium(IV)

phosphate sheets. For these reasons, work is being pursued in our laboratory to obtain non-radioactive but chemically stable new inorganic ion-exchange sheets.

ACKNOWLEDGEMENT

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EFFECT OF RESIN CROSS-LINKING ON THE ANION-EXCHANGE SEPARATION OF RARE EARTH–EDTA COMPLEXES

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SUMMARY

The anion-exchange separation of rare earth complexes with EDTA on Dowex 1 resins with 2, 4, 8, 10, and 16 nominal cross-linking, respectively, was investigated with loads at tracer levels. Selectivity coefficients, separation factors, plate heights, and resolutions for adjacent peaks were calculated from the elution curves. Although the separation factors increased rather uniformly with the increase in resin cross-linking, the best separations were obtained with Dowex 1 X4 due to the most promising value of the plate height. Plate height, which was the lowest for the X4 resin, was found to increase both with increase and decrease of resin cross-linking. A change from an X4 to an X16 resin resulted in a drastic increase of plate height of nearly two orders of magnitude. It was shown that the results obtained cannot be interpreted on the grounds of the classical GLUECKAUF equation. The introduction of a new term, accounting for longitudinal diffusion in the resin phase, into GLUECKAUF's equation was suggested and the qualitative agreement between theoretical prediction and experimental data was demonstrated.

The existence of a sieve effect in the case of the anion exchange of rare earth–EDTA complexes on highly cross-linked resins was also pointed out.

INTRODUCTION

The effect of resin cross-linking on ion-exchange separations has not been investigated in detail. It is generally assumed in the literature[1,2] that an increase in cross-linking results in a rise of resin selectivity (as expressed in terms of selectivity coefficient), especially at tracer level loadings. The quality of a separation of two elements under investigation depends, however, on the value of the separation factor (ratio of distribution coefficients) rather than on the absolute values of the selectivity coefficients. Although an increase in the separation factor with an increase of resin cross-linking was observed in many cases[3–9], a survey of other data[10–12] shows that this situation is by no means universal. Furthermore, the quality of separation depends on the dynamics of the column process, which should also change with the change in resin cross-linking. In an attempt to gather more experimental data in order to make some generalizations possible the effect of resin cross-

linking on the separation of several elements in various systems was investigated. In this paper, the first of a series, the results of the separation of rare earth complexes with EDTA on strongly basic anion-exchange resins are presented.

EXPERIMENTAL

Resins

Dowex 1 resins with quaternary ammonium functional groups were employed. Methods of grinding, fractionating and conditioning the resins, as well as microscopic determination of particle size, have been described earlier[13,14].

The exchange capacity of strongly basic groups (Z_s) was determined by converting a known amount of resin first to the [OH^-]-form with 1 *N* NaOH and then to the [Cl^-]-form with an excess of 1 *N* NaCl solution. After washing the column with deionized water until free from chlorides, Cl^- ions were eluted with 1 *N* NaOH solution and determined titrimetrically[15].

The total exchange capacity (Z_c) was determined in a similar manner except that the resin was converted to the [Cl^-]-form with 1 *N* HCl solution and the column was washed with absolute ethanol instead of water[16].

To determine the exchange capacity of the resins in ethylenediaminetetraacetate ([H_2Y^{2-}])-form, the resin ([Cl^-]-form) was converted into the [H_2Y^{2-}]-form with a large excess of 0.2 *M* Na_2H_2Y solution until no chlorides could be detected in the effluent. After washing with deionized water, H_2Y^{2-} ions were displaced from the resin with 1 *M* NaCl solution and determined by titration with a standard solution of magnesium chloride in the presence of Eriochrome Black T. All the values of exchange capacity were expressed in terms of a number of mequiv./1 g of dry (dried at 105° to a constant weight) resin in a specified ionic form.

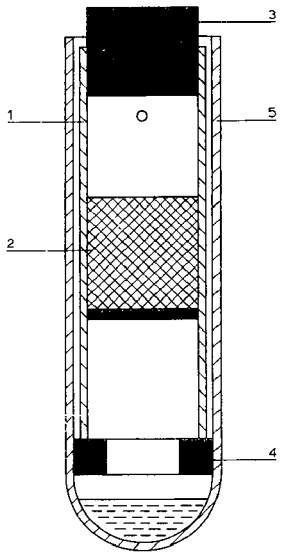


Fig. 1. A device for the determination of the water content of the resin by centrifugation. 1 = Glass tube with a sintered glass disc (G4) in the middle; 2 = ion-exchange resin bed; 3 = rubber bung; 4 = rubber collar; 5 = plastic centrifuge tube.

The water contents of swollen resins were determined by a modified method of PEPPER *et al.*[17]. Approximately 4–5 g of water swollen resin (0.10 mm $\leq \varnothing \leq$ 0.30 mm) was placed in a glass vessel with a fritted glass dicc (*cf.* Fig. 1) and centrifuged at 3000 r.p.m. (approx. 900 *g*) for 15 min. After centrifugation the bottom of the vessel touching the rubber ring was placed in contact with blotting paper to remove water droplets adhering to the glass. The vessel with the resin was then weighed, dried at 105° to constant weight, and weighed again. These data together with the weight of empty vessel enabled the calculation of the percentage of water in a swollen resin and its water content (g H_2O per g of the dry resin). The data were corrected for the residual liquid retained at the interface of the beads. This correction (0.034 g H_2O/ml of the bed) was determined by centrifuging glass beads (60 mesh) under the same conditions as stated above and was in a good agreement with the data published in the literature[18].

The density of the dry resin (d_s) was determined picnometrically using the resin dried at 105° and *n*-heptane as the picnometric fluid[19]. The density of the swollen resin (d_j) was determined in a similar manner using fully swollen resin (and water)[20], and introducing the correction for the water held at the resin bead interface.

Bed density (d_z) (g of dry resin per ml of the bed) was determined by introducing a known amount of the resin in deionized water into graduated cylinder and measuring the volume occupied by the resin bed after settling.

Radioactive tracers and reagents

The following radioactive tracers were used: $^{140}La(T_{\frac{1}{2}} = 40.2$ h); $^{142}Pr(T_{\frac{1}{2}} = 19.2$ h); $^{147}Pm(T_{\frac{1}{2}} = 2.6$ y); $^{153}Sm(T_{\frac{1}{2}} = 1.96$ d); $^{152,154}Eu(T_{\frac{1}{2}} = 13$–$16$ y); $^{160}Tb(T_{\frac{1}{2}} = 73$ d); $^{165}Dy(T_{\frac{1}{2}} = 2.3$ h); $^{166}Ho(T_{\frac{1}{2}} = 27.3$ h); $^{170}Tm(T_{\frac{1}{2}} = 127$ d); $^{177}Lu(T_{\frac{1}{2}} = 6.8$ d); $^{46}Sc(T_{\frac{1}{2}} = 85$ d); $^{90}Y(T_{\frac{1}{2}} = 65$ h); $^{134}Cs(T_{\frac{1}{2}} = 2.3$ y).

^{147}Pm and $^{152,154}Eu$ were supplied by The Centre for Production and Distribution of Isotopes, Świerk. The other rare earth tracers were prepared by neutron irradiation of the appropriate spectrally pure grade of oxides or nitrates in the Polish reactor EWA. The ^{134}Cs was prepared by irradiation of CsCl of spectral purity followed by purification on an ion-exchange column[21] to remove ^{32}P and ^{32}S formed as result of the reaction of ^{35}Cl with fast neutrons.

Disodium ethylenediaminetetraacetate was prepared by purification of commercial $Na_2H_2Y \cdot 2H_2O$ according to BLAEDEL AND KNIGHT[21a]. An 0.2 *M* stock solution of Na_2H_2Y was made and other concentrations were prepared from the stock solution by dilution. The exact concentration was checked in each case by titration with standard magnesium chloride solution in the presence of Eriochrome Black T.

All other reagents were of analytical grade.

Apparatus and procedure

Jacketed glass columns of approximately 2 mm internal diameter were employed. Constant temperature ($\pm$ 0.2°) was maintained by passing water from a Höppler ultrathermostat through the jacket of the column.

The solution of radioactive tracers of rare earths in HCl together with a small quantity of ^{134}Cs was evaporated to dryness in a glass crucible, then enough Na_2H_2Y solution to complex all the rare earth ions was added and the solution gently evapo-

TABLE I

FUNDAMENTAL PROPERTIES OF THE RESINS EMPLOYED

Resin	*Functional groups*	*Exchange capacity (mequiv./g dry resin [Cl⁻])*				*Density ([Cl⁻]-form)*			*Maximum water content*			*Normality of the resin phase (Z_s/W_{H_2O})*	*Particle size of the fraction used for column studies*
		Z_s	Z_e	Z_1[a]	Z_2[b]	d_s	d_j	d_z	% H_2O	W_{H_2O} (g H_2O/g dry resin [Cl^-])	W_{H_2O}/Z_s (g H_2O/mequiv.)		
Dowex 1 X2	$-N(CH_3)_3^+Cl^-$	4.08	4.12	4.66	4.71	1.09	1.04	0.166	72.53	2.641	0.645	1.55	6 $\mu \leq \varnothing \leq$ 38 μ
Dowex 1 X4	$-N(CH_3)_3^+Cl^-$	3.92	3.95	4.61	4.70	1.06	1.07	0.253	58.36	1.403	0.357	2.80	11 $\mu \leq \varnothing \leq$ 42 μ
Dowex 1 X8	$-N(CH_3)_3^+Cl^-$	3.24	3.24	4.50	4.68	1.07	1.11	0.413	36.12	0.566	0.175	5.72	17 $\mu \leq \varnothing \leq$ 44 μ
Dowex 1 X10	$-N(CH_3)_3^+Cl^-$	2.83	2.83	4.45	4.67	1.07	1.10	0.436	35.74	0.556	0.195	5.11	17 $\mu \leq \varnothing \leq$ 46 μ
Dowex 1 X16	$-N(CH_3)_3^+Cl^-$	1.96	1.97	4.30	4.63	1.04	1.11	0.448	19.03	0.240	0.122	8.17	20 $\mu \leq \varnothing \leq$ 43 μ
Amberlite IRA-400	$-N(CH_3)_3^+Cl^-$	3.70	3.76	—	—	1.02	1.09	0.340	44.30	0.795	0.220	4.55	

[a] Calculated by assuming that each aromatic ring originating from styrene carries one functional group.

[b] Calculated by assuming that each aromatic ring originating both from styrene and divinylbenzene, respectively, carries one functional group.

rated to dryness. The residue was dissolved in 75 μl of the eluant (Na_2H_2Y solution of the proper concentration) and 25 μl of this solution was pipetted onto the top of the Dowex 1 column ($[H_2Y^{2-}]$-form). The total rare earth content in the "load" did not exceed 0.3% of the exchange capacity of the resin in the column. The mixture was eluted at a constant flow rate, the effluent being collected in drops on a moving paper band. The drops were then automatically dried under infrared lamps, cut out of the band and counted with a G.M. or scintillation counter. The details of the apparatus and procedure have been described earlier[13,14].

RESULTS

Physico-chemical characterization of the resin

The fundamental properties of the resins used in this study are summarized in Table I. The data for Amberlite IRA-400 employed in earlier work[13,22] are also included.

As can be seen all the resins studied can be regarded as monofunctional. The exchange capacity was found to be considerably lower than that calculated, assuming that each aromatic ring carries one functional group. The differences between the theoretical and experimental values, which are especially notable for resins of higher cross-linking, were considerably greater than those observed for sulfonic acid cation-exchange resins[23].

This evidently reflects the much greater complexity of the production of anion-exchange resins (chloromethylation followed by amination) compared with the sulfonation reaction used to prepare cation exchangers from the same styrene-divinylbenzene copolymer. It is probable that side reactions accompanying the

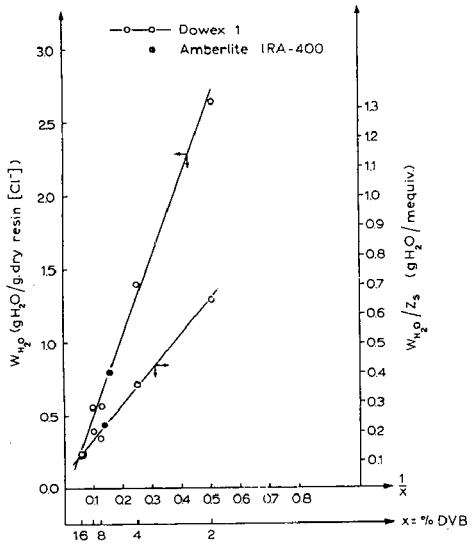


Fig. 2. Water content (W_{H_2O}) and specific water content (W_{H_2O}/Z_s) of Dowex 1 resins as a function of the reciprocal of the nominal divinylbenzene content.

chloromethylation and resulting in the formation of additional cross-linkings *via* methylene bridges are to some extent responsible for this effect[24].

When attempting to correlate the quality of separation with resin cross-linking it is desirable to express the latter not in terms of "nominal divinylbenzene content" (mole per cent of DVB in the polymerization mixture)[1], which does not necessarily reflect the actual cross-linking in the resin, but in terms of some readily measurable physical property of the exchanger. As can be inferred from Table I, the density of both the dry and swollen ion exchanger is characteristic for the kind of exchanger rather than its cross-linking. On the other hand such properties as bed density, maximum water content and normality of the resin phase change relatively regularly with the increase in cross-linking and can be used for its characterization, as has also been done by other workers[17,24–27].

In Fig. 2, the water content (W_{H_2O}) and the specific water content (W_{H_2O}/Z_s), respectively, are plotted *vs.* reciprocal of the nominal DVB content. As can be seen there is almost exactly linear dependence between W_{H_2O} or W_{H_2O}/Z_s and $1/X$ (X = % DVB). Slight deviations observed for X8–X10 resins might conceivably stem from some inhomogeneities of the resin phase in these exchangers.

Similar straight-line dependence was obtained on plotting ($V_j/V_s - 1$) (where V_j and V_s are the equivalent volumes of the swollen and dry resin, respectively) *vs.* $1/X$ according to the method of BOYD AND SOLDANO[28].

Basic relationships

Weight distribution coefficients of the rare earths (amount per g of dry resin/amount per ml of solution) were calculated from elution curves by means of the equation[22]

$$\lambda_{LnY^-} = \frac{U_{max.(LnY^-)} - (U_0 + V)}{m_j} = \frac{U_{max.(LnY^-)} - U_{max.(Cs^+)}}{m_j} \qquad (1)$$

where:

$U_{max.}$ = volume of the eluant at which the concentration of a given ion reached its maximum (ml)
U_0 = dead volume of the column
V = free volume of the resin bed
m_j = weight of the dry ion-exchange resin in the column (g)

From these data selectivity coefficients, $k^{LnY^-}_{H_2Y^{2-}}$, and "corrected" selectivity coefficients $k'^{LnY^-}_{H_2Y^{2-}}$, for the ion-exchange reaction:

$$1/2R_2H_2Y + LnY^- \rightleftharpoons RLnY + 1/2\ H_2Y^{2-} \qquad (2)$$

were calculated by the formula[22,29]

$$k^{LnY^-}_{H_2Y^{2-}} = \frac{\lambda_{LnY^-} \cdot m^{1/2}{}_{H_2Y^{2-}} \cdot d}{C_r} \qquad (3)$$

where

$m_{H_2Y^{2-}}$ = molality of eluant (Na_2H_2Y) solution

d = density of eluant solution

C_r = concentration of the resin phase (mmole per g of dry resin [H_2Y^{2-}])

and

$$k'^{LnY^-}_{H_2Y^{2-}} = k^{LnY^-}_{H_2Y^{2-}} \frac{\gamma^{3/2}_{Na_2H_2Y}}{\gamma^2_{NaLnY}} \quad (4)$$

where γ denotes the mean activity coefficients which can be derived from the tables published by LIETZKE[30]. Separation factors of adjacent elements are given by:

$$\alpha_1^2 = \frac{\lambda_{LnY^-\,(2)}}{\lambda_{LnY^-\,(1)}} \quad (5)$$

In addition plate heights[31] are given by:

$$H = \frac{L \cdot W^2}{8(U_{max.} - U_0)^2} = \frac{L \cdot \sigma^2}{(U_{max.} - U_0)^2} \quad (6)$$

where

L = length of the resin bed

W = width of the peak for the $M = \frac{M_{max.}}{e} = 0.368\, M_{max.}$ ordinate

$\sigma = \frac{W}{2\sqrt{2}}$ = standard deviation of chromatographic peak.

The resolution[31] was also calculated from:

$$R_3 = \frac{U_{max.(2)} - U_{max.(1)}}{3 \cdot (\sigma_1 + \sigma_2)} \quad (7)$$

Analysis of the separations

Typical elution curves of the rare earth ethylenediaminetetraacetates at 25° and 75° from the five resins employed are presented in Figs. 3–6. In each series the dimensions of the resin bed were nearly identical for all exchangers and the concentrations of the eluant solution were so chosen that the peak elution volumes of one of the middle elements were approximately the same (at 25°).

As the particle size of the resins and the flow rates were also approximately equal, these elution curves are directly comparable. Plate heights, separation factors, and resolutions for adjacent peaks were calculated with the aid of eqns. 6, 5 and 7 respectively and are also listed in the figures.

One can see at once from Figs. 3–6 that the separating ability of the resin depends very much on its cross-linking, the separation being quite unsatisfactory with the highest cross-linkings.

However, the separation factors of the rare earths do even increase (more or less regularly) with a decrease in specific water content, *i.e.* with an increase of resin cross-linking (see Fig. 7). The origin of the changes in the degree of separation must therefore lie elsewhere.

As was pointed out previously, resolution, which is a quantitative measure of separation efficacy, is a function of separation factor and of plate height[31], *viz.*

$$R_3 = \frac{(\alpha_1^2 - 1) \cdot \sqrt{L}}{3(\alpha_1^2 + 1) \cdot \sqrt{\bar{H}}} \quad (8)$$

where $\bar{H}$ is the mean plate height.

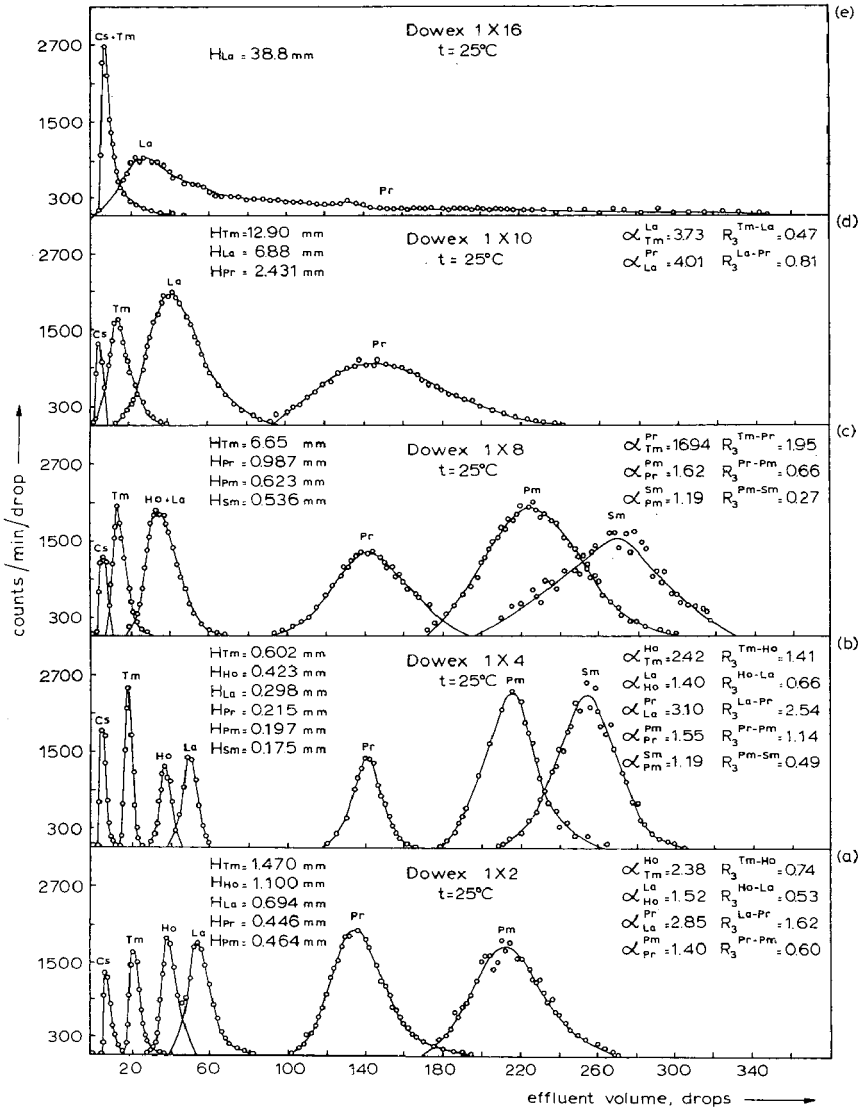


Fig. 3. Effect of resin cross-linking on the separation of Tm, Ho, La, Pr, Pm and Sm ethylenediaminetetraacetates at 25°. (a) Dowex 1 X2 [H_2Y^{2-}] (6 $\mu \leq \varnothing \leq$ 38 μ); column: 5.30 cm × 0.0310 cm²; eluant: 0.011 *M* Na_2H_2Y; flow rate: 0.91 cm/min. (b) Dowex 1 X4 [H_2Y^{2-}] (11 $\mu \leq \varnothing \leq$ 42 μ); column: 5.30 cm × 0.0310 cm²; eluant: 0.036 *M* Na_2H_2Y; flow rate 1.19 cm/min. (c) Dowex 1 X8 [H_2Y^{2-}] (17 $\mu \leq \varnothing \leq$ 44 μ); column: 5.30 cm × 0.0310 cm²; eluant: 0.078 *M* Na_2H_2Y; flow rate: 1.22 cm/min. (d) Dowex 1 X10 [H_2Y^{2-}] (17 $\mu \leq \varnothing \leq$ 46 μ); column: 5.30 cm × 0.0310 cm²; eluant: 0.096 *M* Na_2H_2Y; flow rate: 1.17 cm/min. (e) Dowex 1 X16 [H_2Y^{2-}] (20 $\mu \leq \varnothing \leq$ 46 μ); column: 5.30 cm × 0.0310 cm²; eluant: 0.029 *M* Na_2H_2Y; flow rate: 1.21 cm/min.

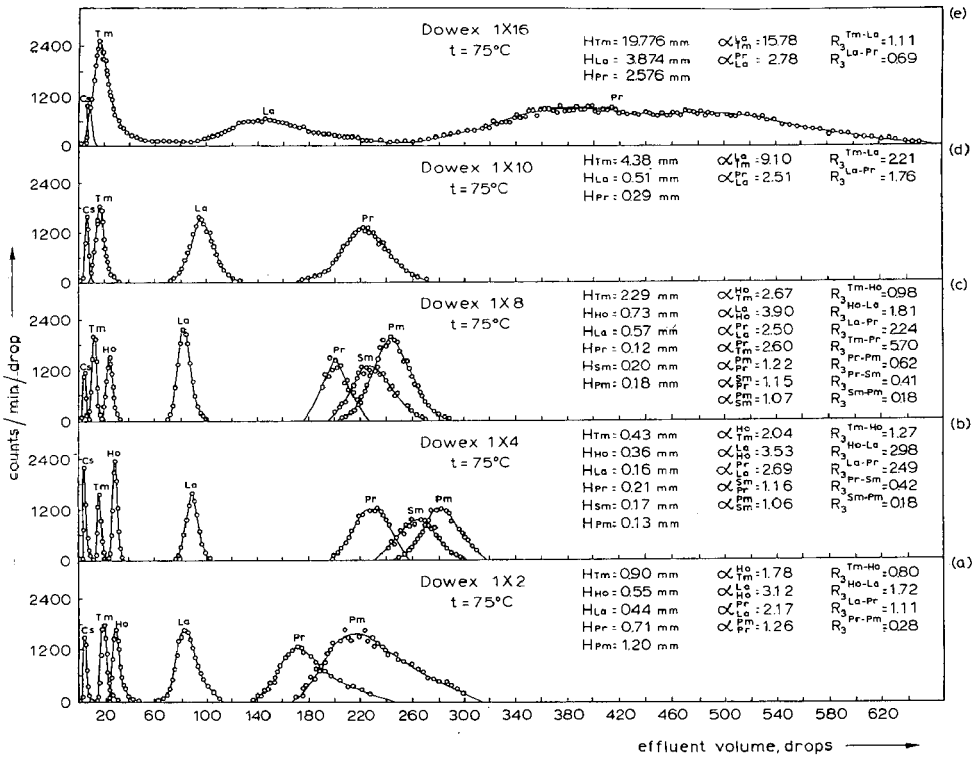


Fig. 4. Effect of resin cross-linking on the separation of Tm, Ho, La, Pr, Pm and Sm ethylenediaminetetraacetates at 75°. (a) Dowex 1 X2 [H_2Y^{2-}] (6 $\mu \leq \varnothing \leq 38\ \mu$); column: 5.30 cm × 0.0310 cm²; eluant: 0.011 *M* Na_2H_2Y; flow rate: 1.21 cm/min. (b) Dowex 1 X4 [H_2Y^{2-}] (11 $\mu \leq \varnothing \leq 42\ \mu$); column: 5.30 cm × 0.0310 cm²; eluant: 0.036 *M* Na_2H_2Y; flow rate: 1.12 cm/min. (c) Dowex 1 X8 [H_2Y^{2-}] (17 $\mu \leq \varnothing \leq 44\ \mu$); column: 5.30 cm × 0.0310 cm²; eluant: 0.078 *M* Na_2H_2Y; flow rate: 1.15 cm/min. (d) Dowex 1 X10 [H_2Y^{2-}] (17 $\mu \leq \varnothing \leq 46\ \mu$); column: 5.30 cm × 0.0310 cm²; eluant: 0.096 *M* Na_2H_2Y; flow rate: 1.15 cm/min. (e) Dowex 1 X16 [H_2Y^{2-}] (20 $\mu \leq \varnothing \leq 46\ \mu$); column: 5.30 cm × 0.0310 cm²; eluant: 0.029 *M* Na_2H_2Y; flow rate: 1.16 cm/min.

The theoretical expression for the plate height in ion-exchange chromatography as derived by GLUECKAUF[32] and with subsequent refinements[1,33] is given by:

$$H = 1.64 \cdot r_0 + \frac{\lambda' \cdot 0.142 \cdot r_0^2 \cdot u}{(\lambda' + i)^2 \cdot \bar{D}} + \frac{(\lambda')^2 \cdot 0.266 \cdot r_0 \cdot u}{(\lambda' + i)^2 (1 - i) \cdot D \cdot (1 + 70 r_0 u)} + \frac{D \cdot i \cdot \sqrt{2}}{u} \qquad (9)$$

where

r_0 = mean radius of resin particles

$\bar{D}$ and D = diffusion coefficients in the resin and in solution phases, respectively

λ' = volume distribution coefficient ($\lambda' = \lambda \cdot d_z$)

i = fractional free volume of the resin bed

u = linear flow rate of eluant solution

It follows from this equation that the plate height depends on the distribution coefficient of the eluted species if the particle diffusion mechanism is predominant or at least significant. The plate height should then increase with the increase of $1/(\lambda' + i) \cong 1/\lambda'$.

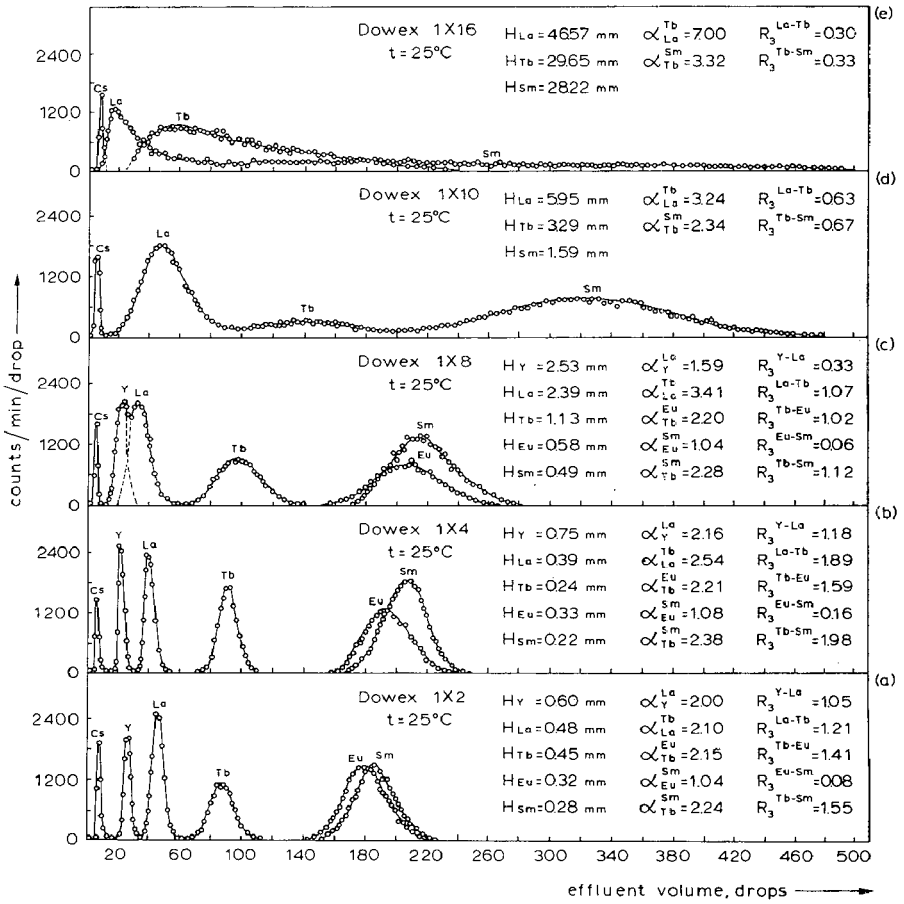


Fig. 5. Effect of resin cross-linking on the separation of Y, La, Tb, Eu and Sm ethylenediaminetetraacetates at 25°. (a) Dowex 1 X2 $[H_2Y^{2-}]$ ($6\ \mu \leq \varnothing \leq 38\ \mu$); column: 4.90 cm × 0.0305 cm²; eluant: 0.011 *M* Na_2H_2Y; flow rate: 0.95 cm/min. (b) Dowex 1 X4 $[H_2Y^{2-}]$ ($11\ \mu \leq \varnothing \leq 42\ \mu$); column: 4.90 cm × 0.0305 cm²; eluant: 0.036 *M* Na_2H_2Y; flow rate: 1.32 cm/min. (c) Dowex 1 X8 $[H_2Y^{2-}]$ ($17\ \mu \leq \varnothing \leq 44\ \mu$); column: 4.95 cm × 0.0305 cm²; eluant: 0.078 *M* Na_2H_2Y; flow rate: 1.33 cm/min. (d) Dowex 1 X10 $[H_2Y^{2-}]$ ($17\ \mu \leq \varnothing \leq 46\ \mu$); column: 5.10 cm × 0.0305 cm²; eluant 0.063 *M* Na_2H_2Y; flow rate: 1.18 cm/min. (e) Dowex 1 X16 $[H_2Y^{2-}]$ ($20\ \mu \leq \varnothing \leq 43\ \mu$); column: 4.90 cm × 0.0305 cm²; eluant: 0.048 *M* Na_2H_2Y; flow rate: 1.31 cm/min.

This is indeed the case when separating rare earth ethylenediaminetetraacetates (see Fig. 8). Therefore comparisons made in order to evaluate the effect of resin cross-linking on plate height should be made not only at constant r_0 and u but also at constant λ' (which can be read from such a plot as that presented in Fig. 8).

In Fig. 9 the plate height, normalized for the value of a volume distribution coefficient of $\lambda' = 20$, is plotted as a function of specific water content. As can be seen the dependence of H on the cross-linking is unexpectedly great.

The most favourable (the lowest) H value is achieved at relatively low cross-linking ($X = 3$–4). When changing to highly cross-linked resins ($X = 16$), the plate height is increased by more than two orders of magnitude (at 25°). The plate height

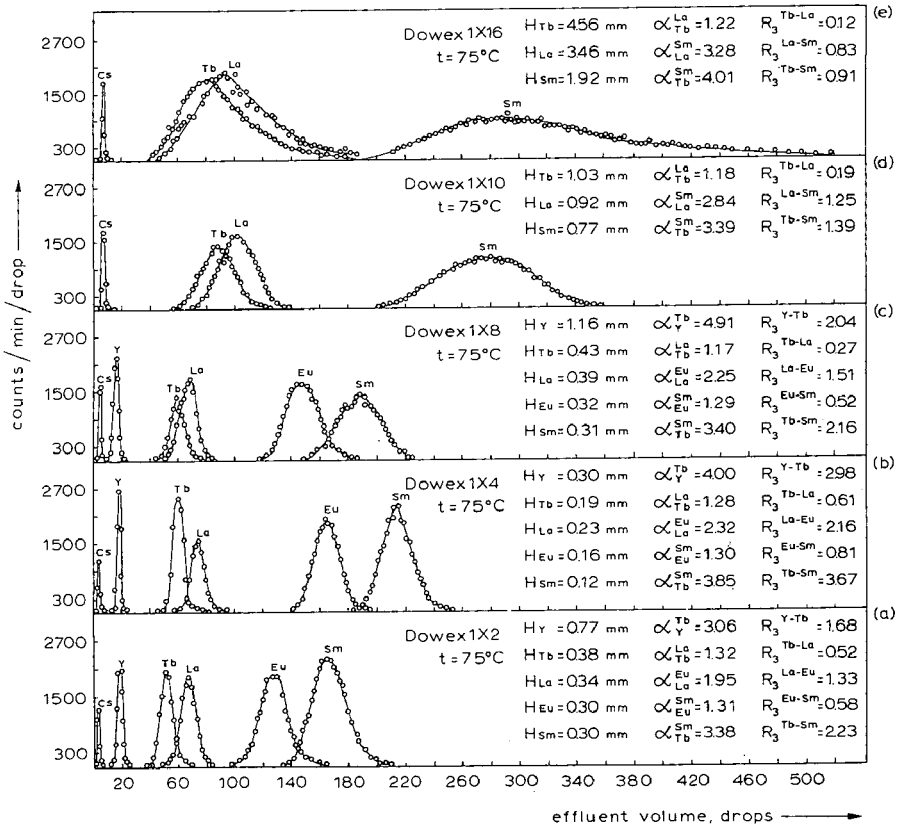


Fig. 6. Effect of resin cross-linking on the separation of Y, La, Tb, Eu and Sm ethylenediaminetetraacetates at 75°. (a) Dowex 1 X2 [H_2Y^{2-}] (6 $\mu \leq \varnothing \leq 38\ \mu$); column: 4.90 cm × 0.0305 cm²; eluant: 0.011 M Na_2H_2Y; flow rate: 1.18 cm/min. (b) Dowex 1 X4 [H_2Y^{2-}] (11 $\mu \leq \varnothing \leq 42\ \mu$); column: 4.85 cm × 0.0305 cm²; eluant: 0.036 M Na_2H_2Y; flow rate: 1.26 cm/min. (c) Dowex 1 X8 [H_2Y^{2-}] (17 $\mu \leq \varnothing \leq 44\ \mu$); column: 4.80 cm × 0.0305 cm²; eluant: 0.078 M Na_2H_2Y; flow rate: 1.29 cm/min. (d) Dowex 1 X10 [H_2Y^{2-}] (17 $\mu \leq \varnothing \leq 46\ \mu$); column: 4.95 cm × 0.0305 cm²; eluant: 0.063 M Na_2H_2Y; flow rate: 1.23 cm/min. (e) Dowex 1 X16 [H_2Y^{2-}] (20 $\mu \leq \varnothing \leq 43\ \mu$); column: 4.90 cm × 0.0305 cm²; eluant: 0.048 M Na_2H_2Y; flow rate: 1.33 cm/min.

also increases when going to lower ($X = 2$) cross linkings.

As a corollary, the resolution of rare earth pairs is best with Dowex 1 X4 and decreases with both an increase and decrease of cross-linking (see Fig. 10), despite the other trend in the separation factors (see Fig. 7).

DISCUSSION

As can be seen from Fig. 8 the plate height for Dowex 1 X16 is a linear function of $1/\lambda'$ which indicates that the particle diffusion mechanism (the second term in eqn. 9) is predominant. On going to lower cross-linkings some deviations from linearity of the H–$1/\lambda'$ plot appear and for Dowex 1 X2 the plot is distinctly curvilinear especially at low $1/\lambda'$ values. It is thus clear that the relative contributions to the

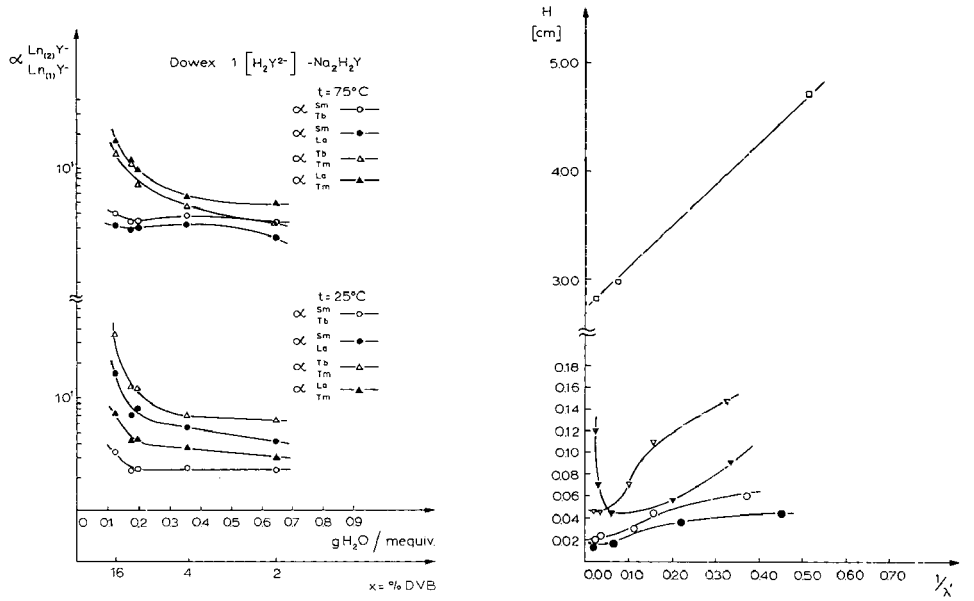


Fig. 7. Separation factors of some rare earths (present as LnY^- anions) on Dowex 1 as a function of the specific water content of the resin.

Fig. 8. Plate height as a function of the reciprocal of bed distribution coefficient for resins of various degrees of cross-linking. —▽—▽—, Dowex 1 X2 [H_2Y^{2-}], temp. 25°; —▼—▼—, Dowex 1 X2 [H_2Y^{2-}], temp. 75°; —○—○—, Dowex 1 X4 [H_2Y^{2-}], temp. 25°; —●—●—, Dowex 1 X4 [H_2Y^{2-}], temp. 75°; —□—□—, Dowex 1 X16 [H_2Y^{2-}], temp. 25°.

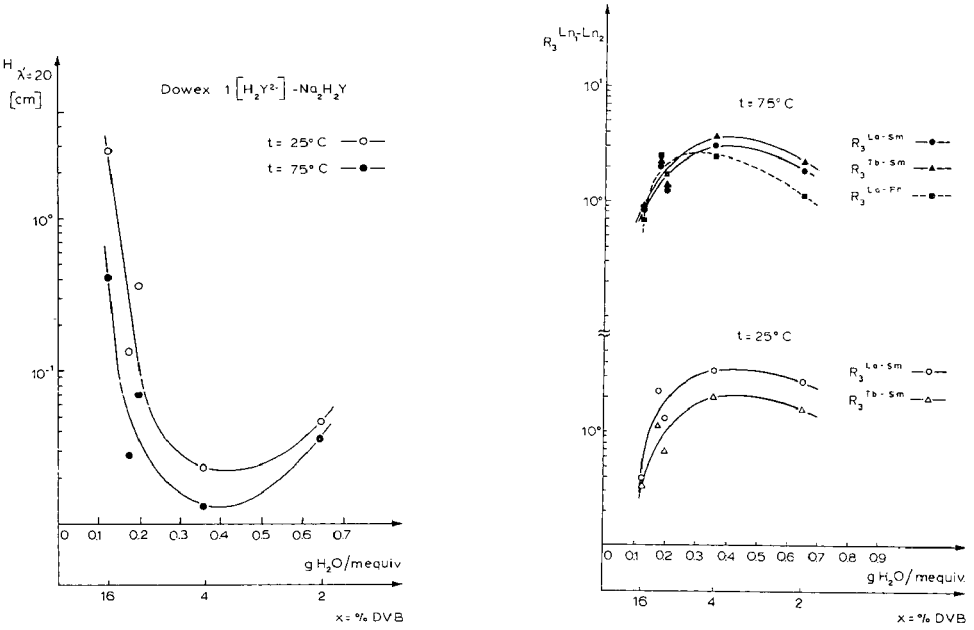


Fig. 9. Plate height, normalized for a bed distribution coefficient value of $\lambda' = 20$, as a function of the specific water content of the resin.

Fig. 10. Resolutions for some rare earth ethylenediaminetetraacetates in the system: Dowex 1 [H_2Y^{2-}]–Na_2H_2Y as a function of the specific water content of the resin.

total plate height of the four terms in eqn. 9, which describe four mechanisms of zone spreading, must change with the variation of cross-linking.

These four terms (from left to right) reflect eddy diffusion, diffusional mass transfer in the resin phase (or "particle diffusion"), diffusional mass transfer in the liquid film (or "film" diffusion), and longitudinal diffusion in the mobile phase, respectively.

Eqn. 9 alone, however, cannot altogether account for such a dependence of the plate height upon resin cross-linking, as was observed experimentally (see Fig. 9). One can easily see that when r_0, u, and λ' are constant, $\bar{D}$ is the only variable that influences the plate height when a given resin is substituted for another resin of different cross-linking. The variations of the diffusion coefficient in the liquid phase, D, due to the different concentrations of eluant solution employed are usually small, and in the first approximation can be neglected.

As is known from published data[28,34] diffusion coefficients in the resin phase as a rule decrease with increase in resin cross-linking. The magnitude of this decrease depends on the kind and ionic form of the resin, kind of counterion, its charge, temperature, etc.

At any rate, while one could expect a decrease of plate height with an increase of specific water content (decrease in resin cross-linking) (see Fig. 9), the occurrence of the minimum and the subsequent increase of H with further increase of water content in the resin cannot be explained on the grounds of eqn. 9.

It seems logical, then, to assume that not all the significant mechanisms of zone spreading have been taken into account.

GIDDINGS[35] pointed out that under some circumstances longitudinal diffusion in the stationary phase also contributes a significant plate height.

This contribution, denoted here as H_s, is given by the equation[35]:

$$H_s = \frac{2\gamma_s \cdot \bar{D} \cdot (1-R)}{u \cdot R} \tag{10}$$

where γ_s = obstruction factor which accounts for the fact that diffusion in the stationary phase cannot take place along a direct unobstructed route; and R = ratio of zone velocity to that of mobile phase (= equilibrium fraction of solute in the mobile phase).

Applying the classical MARTIN AND SYNGE equation[36] written in terms of the symbols used in ion-exchange chromatography:

$$R = \frac{V}{V + \lambda' \frac{m_j}{d_z}} \tag{11}$$

we obtain:

$$H_s = \frac{2\gamma_s \cdot \bar{D} \cdot \lambda' \cdot m_j}{u \cdot V \cdot d_z} = \frac{2\gamma_s \cdot \bar{D} \cdot \lambda'}{u \cdot i} \tag{12}$$

In this expression the plate height is directly proportional both to the diffusion coefficient in the resin (stationary) phase and to the distribution coefficient.

The separate contributions to zone spreading due to the various mechanisms are additive, so the equation for the plate height in ion-exchange chromatography

can be finally written as:

$$H = 1.64r_0 + \frac{\lambda' \cdot 0.142 \cdot r_0^2 \cdot u}{(\lambda'+i)^2 \cdot \bar{D}} + \frac{(\lambda')^2 \cdot 0.266 \cdot r_0 \cdot u}{(\lambda'+i)^2(1-i) \cdot D(1+70r_0 \cdot u)} + \frac{D \cdot i \cdot \sqrt{2}}{u} + \frac{2\gamma_s \cdot \bar{D} \cdot \lambda'}{u \cdot i} \quad (13)$$

Some important experimental facts observed in the present work can be explained on the basis of this equation.

(1) It would be expected that longitudinal diffusion in the resin phase would contribute more to the total plate height, the lower flow rate, the higher distribution coefficient of the eluted species and the lower resin cross-linking (high $\bar{D}$).

Also, an increase in temperature, which is known to increase the diffusion coefficients[28,31], should make the significance of this term more pronounced. If we look at Fig. 8 it is easy to see that these expectations seem to be fulfilled.

While there are no signs of the H_s contribution in the case of Dowex 1 X16, the curves for the lower cross-linked resins become distinctly flattened at low $1/\lambda'$ values, *i.e.* in the region where longitudinal diffusion in the stationary phase becomes most significant. The effect of temperature is particularly characteristic. At high $1/\lambda'$ values where the particle diffusion mechanism still predominates an increase in temperature from 25° to 75° results in a decrease of the plate height for the X2 and X4 resins. As the $1/\lambda'$ diminishes the curves for 25° and 75° come closer together and in the case of Dowex 1 X2 a sharp increase of H at very low $1/\lambda'$ values is even observed. This is a conclusive evidence that some mechanism of zone spreading that is directly proportional to distribution coefficient must be operative.

(2) To evaluate the effect of resin cross-linking on the zone spreading under the conditions when λ', r_0, u, i, D, and γ_s, are approximately constant, it is convenient to rewrite eqn. 13 in the form:

$$H = A + \frac{B}{\bar{D}} + C + E + F \cdot \bar{D} = (A + C + E) + \frac{B}{\bar{D}} + F \cdot \bar{D} \quad (14)$$

where A, B, C, E and F are constants under these circumstances.

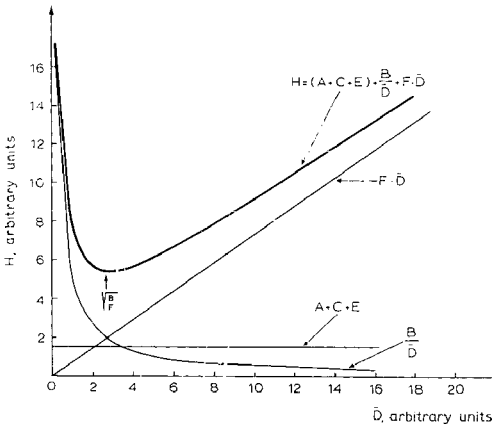


Fig. 11. Schematic representation of the dependence of plate height on the diffusion coefficient in the resin phase.

The schematic representation of the function described by eqn. 14 and its components is given in Fig. 11. As can be seen the curve with a minimum at $\bar{D} = \sqrt{B/F}$ is obtained. Theory thus predicts that with an increase of the diffusion coefficient in the resin phase the plate height at first decreases, passes through a minimum and then increases with the further increase of $\bar{D}$. To compare this theory with experimental data the functional dependence of the diffusion coefficient on the water content of the resin or some other property which can serve as a measure of cross-linking, would have been needed. Although this is not available it is reasonable to assume that diffusion coefficient increases approximately linearly with the specific water content. The striking similarity of the hypothetical curve on Fig. 11 with the plot of H *vs.* W_{H_2O}/Z_s (see Fig. 9) shows that this assumption is probably not far from being true. The shape of the curves shown in Fig. 9 confirms the validity of eqn. 13 and is conclusive evidence that the contribution of the longitudinal diffusion in the stationary phase may be significant in ion-exchange chromatography.

The origin of the great changes in plate height with resin cross-linking in the system examined is of interest. In Fig. 12 the corrected selectivity coefficients for the ion-exchange reaction described by eqn. 2 (where: Ln = Tm, La and Sm, respectively) are shown as a function of specific water content. $k'^{LnY^-}_{H_2Y^{2-}}$ at 25° at first regularly increases (Sm, La) or remains constant (Tm), but at higher cross-linkings a sudden drop in the selectivity coefficient values occurs.

This can be interpreted as a result of a sieve effect, *i.e.* partial exclusion of the ions when their dimensions begin to exceed the "pore" dimensions in the resin phase. It is easy to see that the percentage decrease of $k'^{LnY^-}_{H_2Y^{2-}}$ when changing from X8

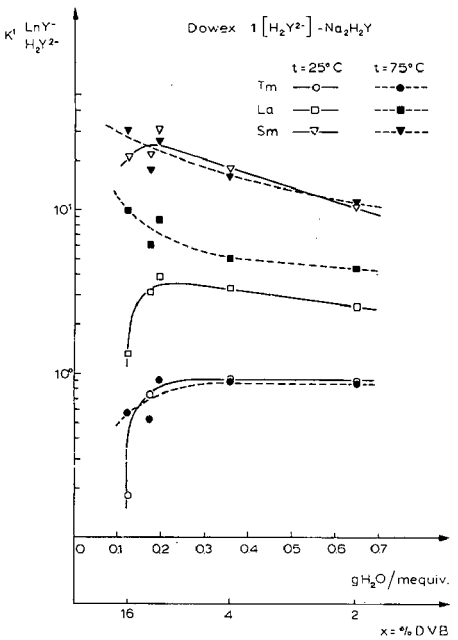


Fig. 12. Corrected selectivity coefficients for the exchange reaction: 1/2 $R_2H_2Y + LnY^- \rightleftharpoons RLnY +$ 1/2 H_2Y^{2-} as a function of specific water content of the resin.

to X16 resin increases in the series: Sm < La < Tm (see Fig. 12), *i.e.* in the order of increasing hydrated ionic radii[13,22,29].

At 75°, the selectivity coefficients for Dowex 1 X16 increase considerably (see Fig. 12). In the case of Tm, even at this temperature, $k'^{LnY^-}_{H_2Y^{2-}}$ for Dowex 1 X16 is still lower than for medium cross-linked resins, but for La and Sm $k'^{LnY^-}_{H_2Y^{2-}}$ increases over the whole range of cross-linkings (X2–X16) examined.

This is probably caused by the changes in the structure of complex ions with temperature[29] and their dehydration, as well as by an increase in elasticity of the polymeric network that increases effective pore dimensions at higher temperatures.

Additional data for the existence of a sieve effect can be derived from Table II. In this table values determined experimentally for exchange capacity in the $[H_2Y^{2-}]$-form are compared with those calculated on the basis of the Z_s values in chloride form (*cf.* Table I). For X2 and X4 resins the differences between the calculated and experimental values are practically within the limits of experimental error, which is of the order of ± 0.04 mequiv./g[15]. This difference, however, increases with the rise in resin cross-linking and for Dowex 1 X16 amounts to as much as 0.23 mequiv./g, *i.e.* 14% of the exchange capacity.

TABLE II

EXPERIMENTAL AND CALCULATED[a] ION-EXCHANGE CAPACITIES OF ANION EXCHANGERS IN THE ETHYLENEDIAMINETETRAACETATE FORM

Resin	Z_s *(mequiv./g dry resin* $[H_2Y^{2-}]$*)*	
	Calculated	*Determined experimentally*
Dowex 1 X2	2.82	2.76
Dowex 1 X4	2.74	2.68
Dowex 1 X8	2.40	2.28
Dowex 1 X10	2.16	2.06
Dowex 1 X16	1.61	1.38

[a] Calculated from Z_s values determined experimentally for the resins in question in $[Cl^-]$-form (*cf.* Table I).

That means that ~14% of the exchange sites in this resin are completely inaccessible for the H_2Y^{2-} anion. One can expect that LnY^- ions which should be at least as large as H_2Y^{2-} anions will be even more strongly excluded from the resin phase.

An even more pronounced sieve effect was observed in a similar system with rare earth 1,2-diaminocyclohexanetetraacetates[37].

The results of the present study clearly show the significance of the proper choice of resin cross-linking in order to realize practical separations.

In the same system a given type of ion-exchange resin may, depending on its cross-linking, yield either excellent separation or virtually no separation at all (*cf.* Figs. 3 and 5) although the trend in the values of separation factors is quite the opposite.

This indicates that the common laboratory practice of inferring the possibility

of a separation on the grounds of separation factor values alone, may lead to fallacious conclusions if the interrelation between resin cross-linking and dynamics of chromatographic process is not taken into account.

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Notes

CHROM. 4752

A rapid method of determining isothermal vapour–liquid equilibria

Several articles have appeared in the literature on the determination of vapour–liquid equilibria by gas chromatography[1–5]. WICHTERLE *et al.*[4,5] have designed an all-glass isothermal vapour–liquid equilibrium cell which is attached directly to the gas chromatograph. With the aid of this cell it is possible to determine these equilibria much more rapidly and with very much smaller samples than by the classical distillation methods. Partial pressures have also been determined by the same means[5].

We have found an even simpler method of determining isothermal vapour–liquid equilibria. The system involves the use of a lockable gas-tight syringe and an ampoule fitted with a silicone rubber septum. The results obtained are in good agreement with those obtained by WICHTERLE AND BOUBLIKOVA[5].

Apparatus

The equilibrium vessel used in this work was made by sealing off the bottom end of a Quickfit* ST 10/2T sleeve to give an ampoule having a screw thread at its upper end which can be sealed with a silicone rubber septum by means of the plastic screw cap supplied with the Quickfit sleeve. Such ampoules are also commercially available.

The sampling device is a 1 ml series A Pressure-Lok** gas syringe. This syringe is claimed to be pressure tight to 250 p.s.i. and can be sealed by twisting the needle. This permits a sample of vapour to be drawn into the syringe and locked in it prior to withdrawing the needle from the ampoule. However, as the syringe is at ambient temperature there is a tendency for part of the vapour to condense, so that it is not possible to inject the vapours directly into the gas chromatograph. To overcome this difficulty a small quantity of solvent was placed in the syringe prior to sampling. The vapours are dissolved in the solvent and the solution was then analysed.

Procedure

Approximately 1 ml of the mixture whose isothermal vapour–liquid equilibrium is to be determined is placed in the ampoule together with a small Teflon coated magnet. The ampoule is sealed with a silicone rubber septum by means of the plastic screw cap. As the latter has a relatively large orifice it is advisable to place a washer having a small central hole above the septum to keep it from being blown out in the event of pressure build-up. The sealed ampoule is placed vertically in a thermostatted bath so that it is almost totally immersed and the contents stirred magnetically.

A quantity of 0.1 ml of a solvent (preferably with a higher boiling point than the materials to be analysed) is placed in the 1 ml Pressure-Lok syringe, in the following manner:

* Quickfit and Quartz Ltd., Stone, Staffordshire, Great Britain.
** Catalog No. 010033, Precision Sampling Corp., Baton Rouge, La., U.S.A.

The needle of the Pressure-Lok syringe is removed by unscrewing the locking mechanism and withdrawing the needle from the Teflon plug at the bottom of the syringe. The plunger is set slightly above the 0.1 ml calibration mark, the syringe inverted and 0.1 ml of the solvent injected into the syringe through the hole in the Teflon plug with the aid of another syringe. The needle is now replaced with the syringe still inverted and the syringe locked. This method of loading the syringe prevents contamination of the needle with solvent and therefore avoids any possibility of contaminating the sample in the equilibrium cell.

When the ampoule and its contents have reached the desired temperature the needle of the syringe is forced through the septum into the vapour space of the test tube, the needle allowed to warm up and the plunger raised to the 0.8 ml graduation mark. This creates a vacuum in the syringe and on opening the locking device vapours are drawn into the syringe. The needle is left open for a few seconds, relocked and withdrawn. The syringe is shaken thoroughly so that the vapours dissolve in the solvent and the contents are then ejected into a small test tube and analysed in the usual manner by gas chromatography. The solvent peak is of course not involved in the calculations; it must, however, be well separated from the other peaks.

As initially there is a tendency for liquid to condense on the cold needle it is advisable to penetrate the septum at an angle so that if a drop of liquid forms at the tip of the needle it can be removed by touching the needle tip on the ampoule wall.

Discussion and results

As it is only necessary to determine the ratio of the components, the volumes of solvent and vapour taken are not critical. Nor is it necessary to remove air from the ampoule. This can, however, be done by freezing the ampoule contents and penetrating the septum with a needle attached to a vacuum line.

The results for *n*-hexane–toluene at 70° obtained by this method using *m*-xylene as solvent are given in Table I and plotted together with those obtained by WICHTERLE AND BOUBLIKOVA[5] in Fig. 1.

TABLE I

VAPOUR–LIQUID EQUILIBRIUM DATA OF THE *n*-HEXANE–TOLUENE SYSTEM AT 70°

x_1	y_1
0.000	0.000
0.083	0.292
0.169	0.533
0.258	0.558
0.352	0.677
0.449	0.736
0.548	0.799
0.654	0.837
0.764	0.906
1.000	1.000

An attempt to determine partial pressures by the use of a solvent containing an internal standard proved unsuccessful. Although the amount of solvent in the

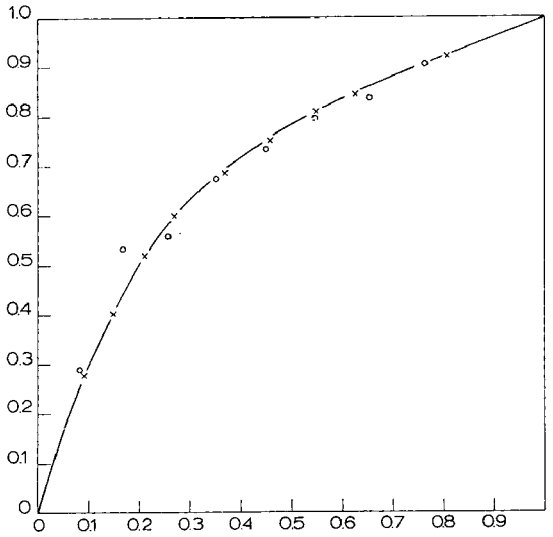


Fig. 1. x–y diagram of n-hexane–toluene system at 70°. ○, This work; ×, results obtained from ref. 5.

syringe can be measured accurately the volume of vapour sampled cannot be determined. The fact that the syringe is at ambient temperature and that it contains a solvent for the vapours causes vapour to condense in the syringe during the sampling. As a result the volume of vapour that enters the syringe is larger than the volume set by the piston of the syringe.

This system is therefore only suitable for the determination of the relative vapour composition but for this purpose it is much simpler than the equilibrium cell used by WICHTERLE AND BOUBLIKOVA[5].

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CHROM. 4826

N,O-Dipivalyl methyl esters of the thyroid hormones and related compounds: Synthesis, purification and gas chromatography

The classical methods for the detection and separation of non-radioactive iodoamino acids involve paper or thin-layer chromatography prior to their final quantitative determination by the ceric sulfate–arsenite color reaction. Only in recent years has gas chromatography been applied here. For this purpose heat-stable volatile derivatives would be needed. For instance, the N,O-bistrifluoroacetyl methyl esters derivatives were prepared[1], but no details on either yield or minimum detectable limit were given. With the availability of BSA, a powerful silylating agent, the TMS derivatives of the thyroid hormones and other iodoamino acids were made in macro quantities and separated by gas chromatography[2–4]. These derivatives can be easily prepared, but are not stable in air, and, therefore, would be difficult to use as precise standards. The N,O-dipivalyl methyl esters have recently been prepared in small quantities, tested by GC[5,6] and found to be particularly well suited for analyzing hormones in serum[7]. Pure derivatives in macro quantities were not isolated, however. These derivatives are more difficult to prepare than the TMS derivatives. On the other hand, however, they are quite stable under atmospheric conditions.

In this work we will describe the synthesis and purification of the N,O-dipivalyl methyl esters of MIT, DIT, T_2, T_3, T_4 and DBrT in weighable quantities.

Halogenated amino acids were esterified with methanolic hydrochloric acid in the usual way and dipivalyl derivatives were made from the crude methyl esters following essentially the published method[8]. Pure substances were isolated by adsorption chromatography on Florisil. The purity of each derivative was checked by melting point, elemental analysis, TLC and GC.

Experimental

Synthesis and purification. The halogenated amino acid (500 mg) was esterified by heating at 70° for 1 h with dry methanolic hydrochloric acid (25%, 20 ml) in a tightly closed screw cap vial. (All substances dissolved completely except T_4.) The crude methyl ester was checked by TLC (Silica Gel GF_{254}) using solvent system I, chloroform–methanol–formic acid (80 : 15 : 5)[5].

The dry crude ester was taken up in dry tetrahydrofuran (20 ml). Triethylamine (1 ml) and pivalic anhydride (1 ml) were added. The mixture was refluxed under nitrogen for 2 h. After cooling, the precipitated amine hydrochloride was removed by filtration and the filtrate evaporated to dryness under nitrogen, yielding a thick brown oil which did not solidify in the cold.

This oil was dissolved in a minimum quantity of benzene and chromatographed on a Florisil column, mesh 60–100, prepared in hexane. Elution was started with hexane–benzene (1 : 1) (100 ml), followed by benzene (100 ml) and 2% absolute ethanol in benzene (100 ml). 10 ml fractions were collected and tested on TLC (Silica Gel GF_{254} using solvent system II, isooctane–chloroform–formic acid (10:20:1)[5]). The fractions containing the largest amount of material as viewed under UV light on the TLC plates were further tested by GC to determine the degree of purity.

The derivatives were usually eluted with 2% ethanol in benzene. The relevant fractions were pooled and evaporated under a nitrogen stream. A light colored oil was obtained in all cases. This oil was triturated with light petroleum ether in the cold until it solidified. Recrystallization was performed by dissolving in dry benzene and adding light petroleum ether. In some cases charcoal was employed for decolorization. Preparative GC for purification was not successful.

Gas chromatography. F & M Model 609 with 400 oven and flame ionization detector was used.

Column: 6 ft. × 4 mm I.D. Pyrex column packed with 1% OV-1 coated on 80–100 mesh acid washed dimethylchlorosilane treated Chromosorb G. Column temperature: 200° for iodotyrosines, 250° for iodothyronines.

Helium was used as carrier gas with a flow rate of 80 ml/min. Gases used for the detector were: air and hydrogen at flow rates of 450 ml/min and 45 ml/min, respectively.

TABLE I

ELEMENTAL ANALYSIS OF N,O-DIPIVALYL METHYL ESTER OF HALOGENATED AMINO ACIDS

Amino acid	*Empirical formula*	*M.P.*	*Mol.wt.*		*Analysis (%)*		
					C	*H*	*Halogen*
MIT	$C_{20}H_{28}O_5NI$	80	489.33	Calc.	49.09	5.77	25.94
				Found	49.49	5.91	26.01
DIT	$C_{20}H_{27}O_5IN_2$	58–60	615.23	Calc.	39.04	4.42	41.25
				Found	38.88	4.47	41.22
T_2	$C_{26}H_{31}O_6NI_2$	93–95	707.32	Calc.	44.14	4.42	35.88
				Found	44.40	4.38	35.72
T_3	$C_{26}H_{30}O_6NI_3$	86–87	833.21	Calc.	37.48	3.63	45.69
				Found	37.83	3.75	45.66
T_4	$C_{26}H_{29}O_6NI_4$	103–106	959.10	Calc.	32.56	3.05	52.92
				Found	33.05	3.12	52.68
DBrT	$C_{20}H_{27}O_5NBr_2$	101–103	521.26	Calc.	46.08	5.22	30.66
				Found	46.36	5.50	30.59

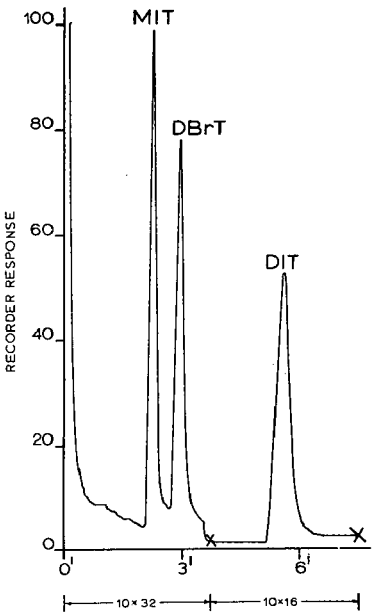
Results

Table I indicates melting points and elemental analysis of derivatives. Figs. 1 and 2 show the type of separation obtained by GC, isothermally at two different temperatures, of mixtures of the purified derivatives of the three halogenated tyrosines and of the three iodothyronines.

Table II summarizes GC data for all six compounds.

Discussion

A quantitative estimation of thyroid hormones and their precursors and metabolites from biological systems such as thyroid, and serum, has so far been difficult due to the similarity of many of their chromatographic properties as well as to their presence in extremely small quantities. In gas chromatography one of the difficulties has been lack of suitable, thermally stable, volatile derivatives in the pure state to be used directly as standards. The N,O-dipivalyl methyl ester derivatives have been employed for measuring thyroid hormones in serum by GC with the help



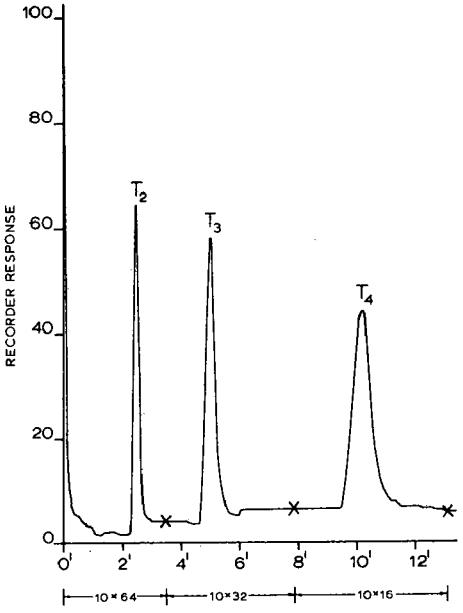


Fig. 1. Gas chromatogram of the N,O-dipivalyl methyl esters of MIT, DBrT and DIT, isothermal at 200°. About 10 μg of each compound injected.

Fig. 2. Gas chromatogram of the N,O-dipivalyl methyl esters of T_2, T_3, and T_4, isothermal at 250°. About 10 μg of each compound injected.

of the electron capture detector[7]. Very small amounts could be detected, but precise answers were difficult to obtain due to the non-availability of the pure derivatives. We have been successful now in isolating these derivatives in macro quantities and in pure form. These substances are solid at room temperature and can be weighed without special precautions. With these pure standards, the analysis of biological samples by GC and the ^{63}Ni-electron capture detector will be possible. The DBrT derivative, which would not be found in biological samples, has a retention time between that of MIT and DIT and would probably be a suitable internal standard for the estimation of these two iodoamino acids. The T_2-derivative, presumably also not found in biological samples, may serve to quantitate T_3 and T_4. Studies along those various lines are in progress.

TABLE II

GAS CHROMATOGRAPHIC DATA FOR HALOGENATED AMINO ACIDS

N,O-Dipivalyl methyl ester	*Column temperature (°C)*	*Retention time*
MIT	200	2.3
DBrT	200	3.0
DIT	200	5.8
T_2	250	2.4
T_3	250	5.0
T_4	250	10.2

Abbreviations

BSA	N,O-bis(trimethylsilyl) acetamide
TMS	Trimethylsilyl
MIT	L-Monoiodotyrosine
DIT	L-3,5-Diiodotyrosine
T_2	L-3,5-Diiodothyronine
T_3	L-3,5,3'-Triiodothyronine
T_4	L-Thyroxine
DBrT	L-3,5-Dibromotyrosine
TLC	Thin-layer chromatography
GC	Gas chromatography

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CHROM. 4833

Rapid gas chromatographic separation of amino acid enantiomers using N-perfluoroacyl esters

Amino acid enantiomers can be resolved by gas chromatography using optically active stationary phases[1–5]. Stationary phases most commonly used are N-trifluoroacetyl (TFA)-L-valyl-L-valine cyclohexyl ester[3,5] and N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester[4]. N-TFA amino acid isopropyl esters have been utilized thus far as the most notable derivatives. However, the retention times were long, resulting in extended gas chromatographic runs. It was of interest to investigate the influence of perfluoroacyl derivatives of amino acid esters other than the trifluoroacetyl group with respect to retention times and resolution factors.

We wish to report the preparation of different N-pentafluoropropionyl (PFP), N-heptafluorobutyryl (HFB), and N-pentadecafluorooctanoyl (PDFO)-D,L-leucine

esters and their gas chromatographic behavior when used on the optically active stationary phase N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester.

Identical methods for the preparation of N-TFA and N-PFP leucine esters were used[4,6]. N-HFP and N-PDFO leucine esters were prepared by reaction of heptafluorobutyric or pentadecafluorooctanoic anhydride respectively in chloroform (ratios, 1 : 4) with the corresponding leucine ester in a sealed tube. Reaction time was 5 min at 100°. The gas chromatographic experiments were carried out with a Varian Aerograph 1200-1 instrument with attachments for capillary column and flame ionization detector. A 400 ft. × 0.02 in. I.D. stainless-steel column coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester was used.

Table I reveals that N-PFP and N-HFB leucine esters are in general more volatile than the corresponding N-TFA derivatives. They require about 20%–27%

TABLE I

COMPARISON OF GAS CHROMATOGRAPHIC DATA OF DIFFERENT N-PERFLUOROACYL LEUCINE ESTERS
Column: 400 ft. × 0.02 in. I.D. stainless-steel capillary coated with N.TFA-L-phenylalanyl-L-leucine cyclohexyl ester, 110° isothermal, carrier gas He, pressure 10 p.s.i.

Leucine ester	*TFA*[a]	$r_{L/D}$	*PFP*[a]	$r_{L/D}$	*HFB*[a]	$r_{L/D}$	*PDFO*[a]	$r_{L/D}$	*Time reduction*[b] *(%)*	
									N-PFP	*N-HFB*
Methyl	D 6.10	1.082	4.45	1.090	4.90	1.091	12.40	1.100	26.9	19.3
	L 6.60		4.85		5.35		13.65			
Isopropyl	D 7.85	1.127	5.70	1.123	6.20	1.129	15.30	1.137	27.6	21.0
	L 8.85		6.40		7.00		17.40			
tert.-Butyl	D 7.40	1.176	5.40	1.167	6.00	1.142	15.35	1.117	27.1	19.5
	L 8.70		6.30		6.85		17.15			

[a] Corrected retention volumes.
[b] Reference compound is always the corresponding N-TFA-leucine ester, arbitrarily set at 100%.

less time, depending on the ester used. N-PFP-leucine isopropyl ester shows the highest reduction in retention time, 21.6%. N-HFB leucine esters are less volatile than N-PFP derivatives, but still more volatile than N-TFA derivatives. On the other hand, N-PDFO derivatives require twice as much time as the corresponding N-TFA derivatives. Apparently, the highest volatility is reached with the pentafluoroacyl derivatives.

However, more significant is the result that the reduction in retention time does not effect the resolution factors of the L- and D-enantiomers $r_{L/D}$. On the contrary, N-PFP-leucine isopropyl esters show the same or a slight increase in the resolution factor. Fig. 1 shows the separation of N-TFA, N-PFP, N-HFB, and N-PDFO-D,L-leucine isopropyl esters injected together on a 400 ft. column coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester. All isomers are very well resolved with high efficiency in a short time.

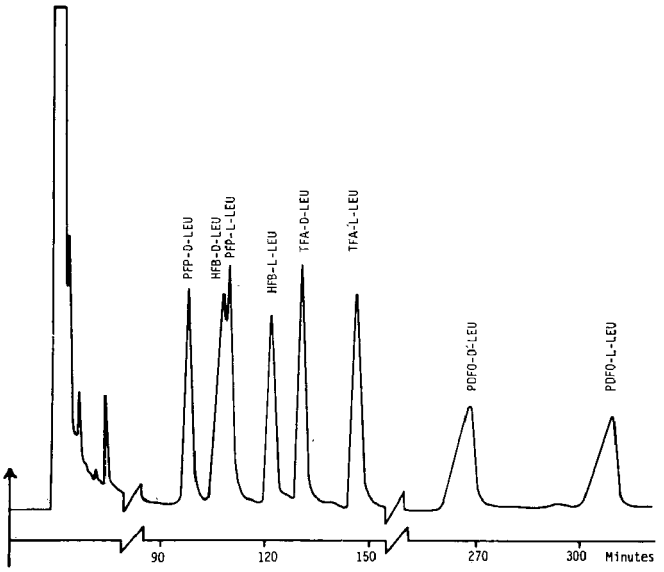


Fig. 1. Gas chromatogram of N-perfluoroacyl leucine isopropyl esters. Instrument, Varian-Aerograph 1200-1. Column, 400 ft. × 0.02 in. I.D., stainless-steel capillary, coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester. Temperature 110° isothermal, injector 190°, detector FID 280°. Carrier gas He, pressure 10 p.s.i.

The new derivatives, and especially PFP amino acid isopropyl esters, have two advantages. Due to their higher volatility, the PFP derivatives would allow the resolution of lower volatile amino acid enantiomers. On the other hand, a reduction in column temperature can be achieved in order to give better resolution of enantiomers. Further work on this subject is under investigation.

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CHROM. 4791

Preparative gas chromatography as a clean-up procedure in toxicology*

One basic problem inherent to toxicology is the difficulties encountered in extracting the toxic agent from tissue in amounts large enough and sufficiently pure for identification.

Several methods of purification of tissue extracts are available to the toxicologist. In general, however, those which give pure substances also give very low yields. Preparative gas chromatography (GC) has been examined as a technique for the purification of extracts and this communication relates some of its potential in toxicology.

Experimental

TABLE I

TECHNICAL DATA

Instrument:	Varian Aerograph Autoprep Model 705
Recorder:	Honeywell, 1.0 mV
Column:	3 m × 7 mm, aluminium
Support:	Chromosorb A, 45/50 mesh
Coating:	OV-1, 10% OV-17, 10%
Carrier gas:	Nitrogen; flow rate 140 ml/min, inlet pressure 60 lb.
Detector:	Flame ionisation
Split ratio:	1 : 4.3
Temperatures:	Inlet, 280° Column, varying Collector, 250°

The solution to be chromatographed was made up by dissolving the extract in ten parts of a suitable solvent, such as alcohol, chloroform or hexane. It was either injected in the conventional manner with a syringe, or where several injections were necessary, by means of the automatic injector. The latter allowed for the injection of up to 1.5 ml at one time and could be set to inject automatically at pre-determined intervals, overnight if necessary.

In most instances, the instrument was operated isothermally except where two compounds with great differences in molecular weight were run simultaneously, *e.g.* morphine and quinine or amitriptyline and perphenazine. In these cases the temperature was increased during the run so that the higher molecular weight compound was recovered in a reasonable time.

Results and discussion

Ordinarily, long columns (7–10 m) with high loading of coating material (20–

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30%) are used in preparative scale GC. In this study it was found, however, that good separations could be obtained with shorter columns (3 m) and lower loading (5–10%) in much shorter time. This also allowed the application of this method of purification to certain high molecular weight drugs (*e.g.* some of the phenothiazines), which would not elute from the high load columns at reasonable temperatures or at acceptable retention times. Another difficulty encountered with the high load columns was the contamination of the column with the extraneous material in the extract. In some cases the instrument had to run at high temperatures for 6–8 h before the column was ready for another injection. This did not occur with the shorter, lower load columns. Furthermore, contamination of the collected materials with silicone grease occurred less often with the low load columns.

Columns of a wider diameter than 7 mm were tried but since these had to be constructed so that both ends were reduced to 7 mm in order to fit the instrument, a "dead volume" was created at both these ends. This not only caused broadening of the peaks but also, in several instances, the appearance of substances injected days before as "ghost" peaks in the middle of another run. Needless to say, the recoveries in these cases were lower than usual.

Although a small part of the effluent is directed into the flame ionisation detector, the main portion is led out of the instrument and the desired material collected in specially designed bottles[1]. Most of the neutral and acidic drugs could be collected with fairly good yield without cooling of the effluent. However, nearly all of the basic drugs required cooling of the collection bottles in order to trap them in good yields. This was especially evident with the phenothiazine derivatives. This group of drugs and some others tend to form a very fine aerosol or smoke which escapes easily through the glass wool traps making them difficult to collect. In general, therefore, it is recommended that the collection bottles be cooled in order to achieve maximum recovery. An ice–water mixture will do in many cases but a dry ice–acetone mixture is more efficient and gave better recoveries.

A preparative scale chromatogram is frequently of a very poor quality. The peaks are often broad and asymmetrical and show all the characteristics of an overloaded column. This is of little importance since the tracing is needed only to show where to start and discontinue collecting; the main objective is to obtain a pure

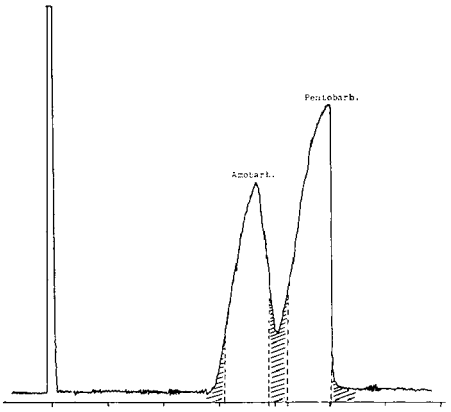


Fig. 1. Preparative GC separation of amobarbital and pentobarbital.

product. An example of this is shown in Fig. 1 for a mixture of amobarbital and pentobarbital. The collection of amobarbital was started approximately one-quarter up the first peak and stopped again before any contamination by pentobarbital could take place. The collection of the latter compound was started a short distance up the upstroke of the peak and discontinued just before the base line. The identification of the two barbiturates was accomplished by means of crystal test, analytical GC and IR spectrophotometry. Both analytical GC and TLC showed that separation of the drugs was complete.

Fig. 2 shows the UV spectra obtained before and after a preparative GC clean-up

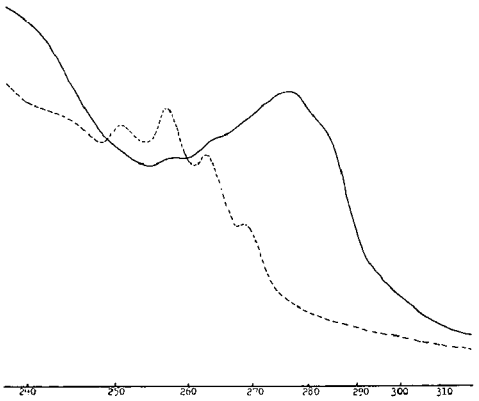


Fig. 2. UV spectrum of glutethimide. Case No. 884-67 LC liver. Solvent: ethanol. ———, Neutrals from 5-way split; – – – –, neutrals after preparative GC clean-up.

of a neutral fraction from a routine five-way extraction of a liver. The history of the case indicated suicide by ingestion of glutethimide (Doriden). Due to partial decomposition of the organ the resulting extract was very impure and no satisfactory thin-layer or gas chromatogram could be obtained. Only the UV spectrum gave a slight indication of the presence of the drug in the extract. The clean-up resulted in a crystal-

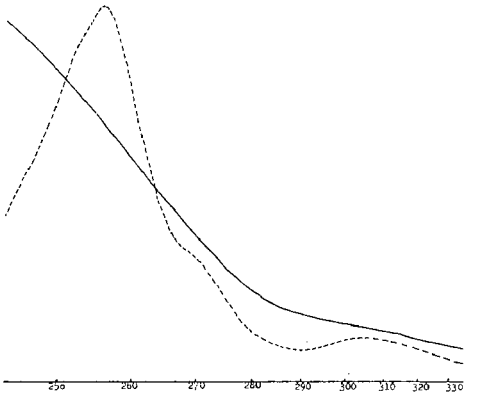


Fig. 3. UV spectrum of perphenazine. Case No. 1546-69 JK liver. Solvent: ethanol. ———, Phenothiazine extraction; – – – –, after preparative GC clean-up.

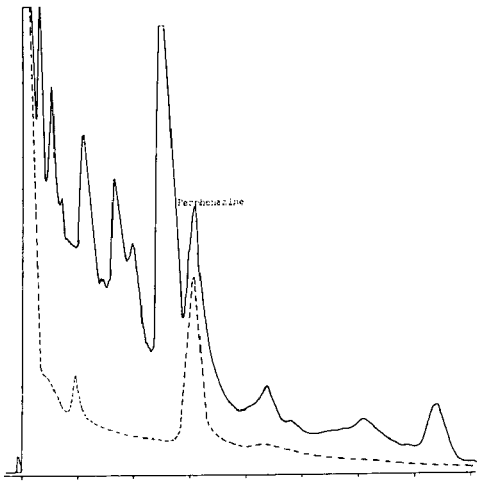


Fig. 4. Gas chromatogram of perphenazine. Case No. 1546-69 JK liver. OV-1, 250°, FID. ———, Phenothiazine extraction; – – – –, after preparative GC clean-up.

line colourless material, which could easily be identified by conventional, analytical methods.

Figs. 3, 4 and 5 show the results of chromatographing basic fractions from a putrefied fatty liver. Since suicide by means of Etrafon D (a mixture of perphenazine and amitriptyline) was indicated, the isolation of perphenazine according to the method of CURRY[2] was attempted. This produced a very impure extract as indicated by the UV spectrum and gas chromatogram in Figs. 3 and 4. During this run it was essential to cool the collection flask in a dry ice–acetone bath to avoid perphenazine escaping as a fine aerosol. Pure amitriptyline was isolated from the basic fraction of the regular five-way extraction (Fig. 5).

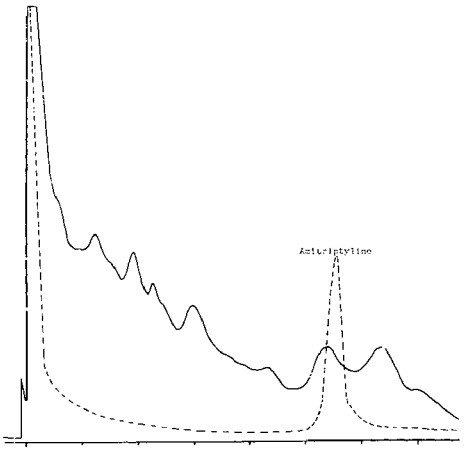


Fig. 5. Gas chromatogram of amitriptyline. Case No. 1546-69 JK liver bases. OV-17, 220°, FID. ———, Bases from 5-way split; – – – –, bases after preparative GC clean-up.

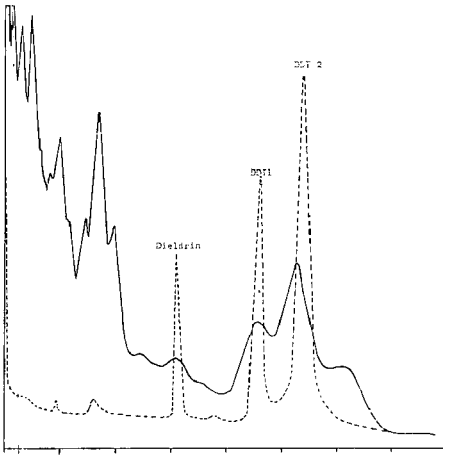


Fig. 6. Gas chromatogram of Dieldrin and DDT. Case No. 1144-67 RS liver neutrals. SE-30, 190°, EC. ———, Conventional clean-up; – – – –, preparative GC clean-up.

Fig. 6 shows the results of a clean-up of another neutral fraction of a putrefied fatty liver. Conventional purification procedures with an activated alumina column and dimethylformamide partition[3] gave only a slight indication of the pesticides suspected.

In none of the cited cases was any quantitative estimation of the poison encountered attempted. Work along these lines is in progress. Preliminary recovery studies from the injection of 1 mg quantities are given in Table II.

TABLE II

RECOVERY DATA

Drug	% *Recovery*
Barbiturates	95–98
Doriden	90–95
Meprobamate	90–95
Librium	82–85
Phenothiazine	70–75

The losses of material are mainly due to the column and the collection technique. The data do not represent recovery from tissue, but give only the recovery when a known amount of the substance is injected and collected.

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GLC diastereomerer Benzaldehydcyanhydringlycoside

Bisher sind wenig Angaben über die GC cyanogener Glycoside gemacht worden[1–3]. Bis auf eine Arbeit[3] ist hierbei der Nachweis und die Abtrennung diastereomerer Formen unterblieben. Prunasin (D-Mandelsäurenitril-β-D-glucosid) und sein Diastereomeres (L-Mandelsäurenitril-β-D-glucosid) kommen beide in Pflanzen vor[4]. Das Gemisch beider Formen, Prulaurasin, wird als Isolierungsartefact betrachtet[5]. Amygdalin (D-Mandelsäurenitril-β-D-gentiobiosid) wurde ebenfalls häufig aus Pflanzen isoliert. L-Mandelsäurenitril-β-D-gentiobiosid (Neoamygdalin) und das Gemisch beider Formen (Isoamygdalin) wurden bisher in der Natur nicht gefunden[4,6].

Bei Einwirkung von Alkali, z.B. 0.005 *N* Ammoniak, isomerisieren die reinen D- und L-Formen leicht zum Gemisch, das je nach Dauer der Einwirkung verschiedene Zusammensetzung zeigt und sich in *ca.* 12 h auf ein Gleichgewicht einstellt. Bei unseren Arbeiten werden Amygdalin und Prunasin nach Silylierung gaschromatographisch bestimmt. Diastereomere Formen als Isolierungsartefacte oder natürlich vorkommende Verbindungen sollten dabei erkannt werden.

Experimentelles

Herstellung der Glycoside. Ausgegangen wurde von Amygdalin (Handelspräparat der Fa. Roth, Karlsruhe), das mehrfach aus Äthanol umkristallisiert wurde. Fp. 213–215°. Prunasin wurde aus Amygdalin nach FISCHER[7] hergestellt. Fp. 148°. Isoamygdalin wurde aus 4 mg Amygdalin durch Behandeln mit 5 ml 0.005 *N* Ammoniak über Nacht hergestellt. Der getrocknete Ansatz wurde direkt silyliert[8]. Prulaurasin wurde aus Prunasin ebenso wie Isoamygdalin dargestellt[9].

Silylierung. 1–2 mg Glycosid, bzw. der Isomerisierungsansatz wurden in 0.7 ml wasserfreiem Pyridin gelöst, mit 0.2 ml Hexamethyldisilazan und 0.1 ml Trimethylchlorsilan versetzt und geschüttelt. Hinzugefügt wurden etwa 1 mg Triphenylbenzol (TPB) als innerer Standard. Die Ansätze blieben 10 min stehen. 1–2 μl wurden injiziert.

Gaschromatographie. Die gaschromatographischen Bedingungen waren wie folgt:

Gerät:	Varian Aerograph, Modell 1200		
Säulen:	SE-30, 3%	OV-1, 3%	OV-17, 3%
	Stahl, 2.50 m	Stahl, 3.00 m	Stahl, 3.00 m
	1/8 in.	1/8 in.	1/8 in.
	jeweils auf Chromosorb AW-DMCS 80–100 mesh		
Trägergas:	Stickstoff,		
	25 ml/min	30 ml/min	30 ml/min
Detektor:	FID, jeweils 320°		
Injektor:	jeweils 240°		
Temperatur:	190–270°, 2°/min	190–280°, 1°/min	195–255°, 1°/min
Papiervorschub:	20 in./h	10 in./h	10 in./h

Resultate und Diskussion

Zur Analytik schwer flüchtiger Stoffe wie TMS-Amygdalin zusammen mit TMS-Prunasin waren temperaturstabile Trennphasen notwendig. An SE-30 wurde

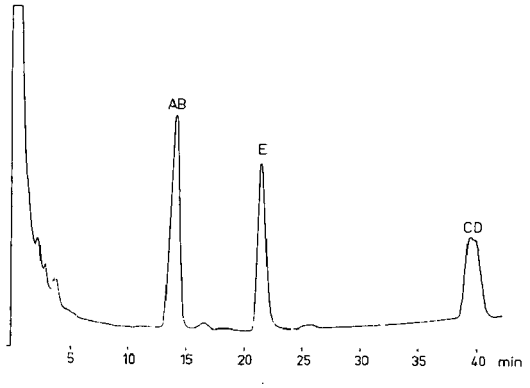


Fig. 1. Gaschromatogramm von TMS-Prulaurasin (AB) und TMS-Isoamygdalin (CD) an SE-30. Innerer Standard TPB (E).

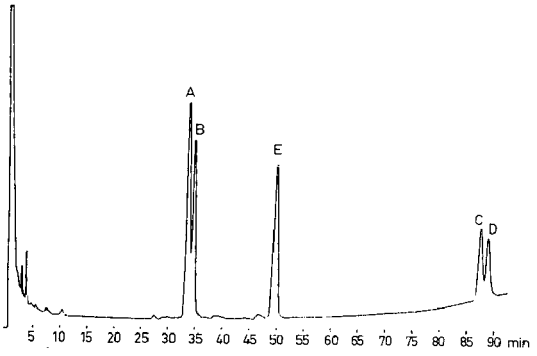


Fig. 2. Gaschromatogramm von TMS-Prulaurasin (Sambunigrin = A, Prunasin = B) und TMS-Isoamygdalin (Neoamygdalin = C, Amygdalin = D) an OV-1. Innerer Standard TPB (E).

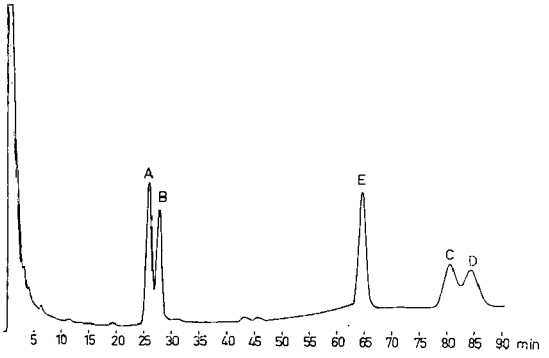


Fig. 3. Gaschromatogramm von TMS-Prulaurasin (Sambunigrin = A, Prunasin = B) und TMS-Isoamygdalin (Neoamygdalin = C, Amygdalin = D) and OV-17. Innerer Standard TPB (E).

Prulaurasin (D- und L-Mandelsäurenitril-β-D-glucosid) gar nicht, Isoamygdalin (D- und L-Mandelsäurenitril-β-D-gentiobiosid) nur andeutungsweise getrennt (Fig. 1).

An OV-1 konnten beide Diastereomeren-Gemische deutlich getrennt werden (Fig. 2). Gegenüber OV-1 zeigt OV-17 ab 250° eine deutlich höhere Flüchtigkeit (Fig. 3). Die Trennung der Amygdalin-Isomeren ist an OV-17 schlechter als an OV-1.

Additionsanalyse ergab, dass die Peaks mit kürzeren Retentionszeiten der L-, diejenigen mit längeren R_t-Werten der D-Form zuzuordnen sind. Die Einstellung eines Gleichgewichts bei der Isomerisierung verläuft bei beiden Glycosidgemischen zugunsten der L-Form. Dies wird für Amygdalin in der Literatur angegeben[8]. Amygdalin isomerisiert bereits in wässrigem Pyridin[8]. Eine ähnliche Beobachtung wurde von uns für Prunasin gemacht. Dies ist bei der praeparativen Papier- und Dünnschichtchromatographie mit dem häufig verwendeten Fliessmittel Butanol–Pyridin–Wasser (6 : 4 : 3) zu beachten.

Eine Kontrolle der Einheitlichkeit der Glycoside ist mit der angegebenen Methode leicht möglich.

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Effect of liquid phase loading and temperature on the relative retentions in the gas chromatography of methyl parathion and parathion

Differences in equilibrium constants of various solutes on a particular liquid phase are directly related to the separation achieved. The determination of equilibrium constants has limited value in the practical utilization of gas chromatography in comparison to theoretical studies, because their calculation requires precise knowledge of values such as amount of liquid phase, its density at operating temperatures, and its surface area, all of which are usually unknown and cumbersome to determine. A practical, simple operational substitute is the determination of relative retention time or volume in which retention of one solute is measured relative to that of a

reference solute on the same column and under the same operating conditions.

However, in the GLC of methyl parathion and metabolites relative retentions of P = O analogs were observed to be higher on low-loaded columns than on high-loaded columns[1]. KEULEMANS[2] explained that adsorption by the support of tube wall affects retention values particularly if polar solutes are analyzed on a column with a non-polar stationary phase, and such effects may be observed with other phases as well, and that absence of these effects can be verified when retention values are identical on two or more columns containing increasing amounts of stationary phases[3].

GIUFFRIDA *et al.*[4] evaluated the effect of liquid phase loading (20, 10, and 5% DC-200) on the relative retentions of various pesticides, including DDT, which is nonpolar, and observed a most significant effect due to loading. They evaluated these effects at different temperatures, but when they transposed these data they rightly concluded that liquid phase loading has no effect and the most significant differences are due to temperature, a fact well in agreement with theory[5].

In this communication are examined the effects of liquid phase loading and temperature on the relative retentions of methyl parathion and parathion, both nonpolar solutes, on Apiezon L liquid phase, which is classified as a nonpolar phase[6], in order to substantiate whether this general theory as developed for saturated hydrocarbons applies to these and related compounds. Although these compounds are termed nonpolar, they also have oxygen functions which may interact with the support or with the wall of the column.

Experimental

The GLC analyses were carried out with a Hewlett-Packard, Model 402, high-efficiency gas chromatograph equipped with two 2 ft. × 4 mm I.D. U-shaped glass columns with liquid phase loading of 3% and 12% Apiezon L on Gas-Chrom Q 80/100. The columns were conditioned at 240° for one week. Nitrogen, hydrogen, and air flow were identical and precisely controlled through flow controllers (Brooks Instrument Corp.). The hydrogen flame detectors were modified for the thermionic detection of phosphorus through the mounting of KCl pellets on the burner jets.

Standard solutions of purified methyl parathion and parathion (American Cyanamid Co.) in acetone were prepared. Two or three μl of 5 ng/μl of each were injected separately as well as in mixture. A given temperature was equilibrated for at least 1 h.

Results and discussion

Limited changes in retention time normally occur for various reasons[2]. In addition to adsorption by solid support and tube wall, variation can result from over-loading of the column due to large sample size, from changes in the chemical composition of the liquid phase, or some unresolved impurity in the solute peak.

Another factor can be an on-column reaction. The eluted peak of the product formed in such a reaction is 6–10 sec delayed in comparison to that when the product itself is injected. This phenomenon is discussed in detail by JAGLAN *et al.*[7].

The relative retentions of methyl parathion and of parathion are given in Table I. At a given temperature, the differences in the relative retention are small in comparison to the differences between temperatures. According to theory[5] the

TABLE I

COMPARISON OF RETENTION TIMES OF METHYL PARATHION AND PARATHION WITH DIFFERENT LIQUID PHASE LOADINGS AND TEMPERATURES

Column, Apiezon L on Gas-Chrom Q 80/100, 2 ft. × 4 mm I.D.; temperature of flash heater and detector, 210°; flow rates of nitrogen, hydrogen, and air, 40, 21, and 300 ml/min, respectively.

Temperature (°C)	*3% Apiezon L*			*12% Apiezon L*		
	Methyl parathion	*Parathion*	*Ratio of relative retentions*	*Methyl parathion*	*Parathion*	*Ratio of relative retentions*
185	1.45	2.20	1.517	7.0	10.50	1.500
195	1.05	1.55	1.476	5.1	7.5	1.471
205	0.8	1.15	1.437	3.7	5.25	1.419

liquid phase loading has no effect on relative retention when nonpolar solutes are analyzed on nonpolar phases. This is true for highly nonpolar compounds alone. Because the relative retention is higher on low liquid phase loading, probably adsorption with support does take place with these compounds. Therefore, it is concluded that it is fallacious to assume that these compounds are nonpolar in the classical sense, since these compounds are hundreds of times more soluble in this liquid phase than are the saturated hydrocarbons.

The data also verify that relative retention increases with decreasing temperature. The temperature dependence of the relative retention has been expressed as $\log \alpha = (a/T) + b$, where α is the relative retention, T is the absolute temperature, and a and b are constants[8]. Very often $a > 0$ and the relative retention increases with decreasing temperature. When a is approximately equal to zero the relative retention remains practically the same when the temperature is changed, and this is noticed with a number of compounds[9]. Infrequently a/T becomes smaller than b when the temperature is changed; thus the order of peaks can be reversed.

The variation of relative retention of the solutes with temperature is similar to changes in their equilibrium constants or their retention volumes. Therefore, linear plots of log retention time *versus* reciprocal absolute temperature can be obtained as reported previously[10].

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Chromatographic behavior of silicic acid on Sephadex columns

Gel chromatography has developed into a useful tool for the separation of organic macromolecules and for the estimation of their molecular weight. In recent years gel chromatography has been used for the investigation of inorganic polymers such as polyphosphates[1–4], polymeric ferric hydroxide[5,6] and polymeric molybdates[7].

When a sodium silicate solution, which is highly alkaline, is neutralized or acidified, monosilicic acid* polymerizes with time, but it is not clear whether polysilicic acid of low molecular weight can exist in a stable state. The present paper describes the application of gel chromatography to the study of polymerization of silicic acid.

Experimental

Sample solutions and reagents. All reagents used were reagent grade. A silicic acid solution was made by fusing 0.500 g of anhydrous silica with 4 g of sodium carbonate. The melt was dissolved in distilled water and diluted to 1000 ml. Blue Dextran 2000 solution was 0.2%. 0.1 *M* sodium chloride solution (pH 2) was adjusted with hydrochloric acid to make the eluent.

Sephadex column. Sephadex G-25 (50–150 μ) and G-100 (40–120 μ) were used after swelling for at least 48 h in distilled water. The column was a 1.5 × 60 cm glass tube with a porous polystyrene disc at one end. The column was wet-packed in the usual manner, using just enough gel to bring the packing to constant volume marked on the column at 57 cm. In addition, a small disc of filter paper served to reduce disturbance at the top of the bed.

Procedure. Experiments were carried out as follows. The eluent was passed through the column. An aliquot of alkaline silicic acid solution was neutralized by adding hydrochloric acid. After standing for a definite period, the pH of the sample solution was adjusted to 2 with hydrochloric acid. One ml of the sample solution made as described above was placed on the column bed just as the last layer of eluent soaked into the bed. Then the eluent was applied when the last portion of the sample solution soaked into the bed. Using a fraction collector, the effluent was collected in fractions of 1 ml, with flow rates of 20–26 ml/h (Sephadex G-25) and 5–6 ml/h (Sephadex G-100). The effluent fraction was transferred into a platinum crucible, and 0.2 ml of a 0.1 *M* sodium carbonate solution was added. The solution in the crucible was heated nearly to dryness on a sand bath. Polysilicic acid was thus converted to monosilicic acid. Distilled water was added to the crucible, and monosilicic acid in this solution was measured by colorimetry.

Results and discussion

Effect of pH on the polymerization of silicic acid. The rate of polymerization of

* In this paper, silicic acid which reacts with molybdate reagent in acid solution and gives a yellow color is defined as monosilicic acid for convenience. The reaction of silicic acid with molybdate reagent is used for a colorimetric determination of monosilicic acid. Silicic acid which does not react with molybdate reagent is defined as polysilicic acid.

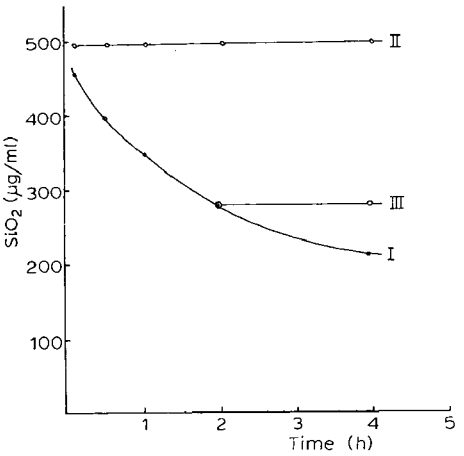


Fig. 1. Effect of pH on the polymerization of silicic acid. Sample I: neutral solution. Sample II: pH 2 solution. Sample III: pH 2 solution adjusted 2 h after neutralization.

silicic acid differs with the pH of solution. In a strongly alkaline solution, monosilicic acid does not polymerize, but polysilicic acid rapidly forms in neutral solution. The polymerization of silicic acid is least rapid in an aqueous solution at pH 2. Below about pH 2, the rate of polymerization increases with greater acidity[8,9]. An example is shown in Fig. 1. The ordinate is the concentration of monosilicic acid, determined by a colorimetric method. The abscissa is the time after the pH is adjusted. Monosilicic acid has decreased rapidly in the pH 7 solution (Sample I). In the pH 2 solution, monosilicic acid has not decreased at least within 10 h (Sample II), but polysilicic acid has formed very gradually over a long period. In the case of Sample III, 2 h after neutralization the pH of the sample solution was adjusted to 2. Polysilicic acid rapidly formed until 2 h after neutralization, but monosilicic acid did not decrease within a few hours after adjustment to pH 2. From the results mentioned above, it can be assumed that the state of silicic acid in the solution is just "fixed" when the pH of solution is adjusted to 2.

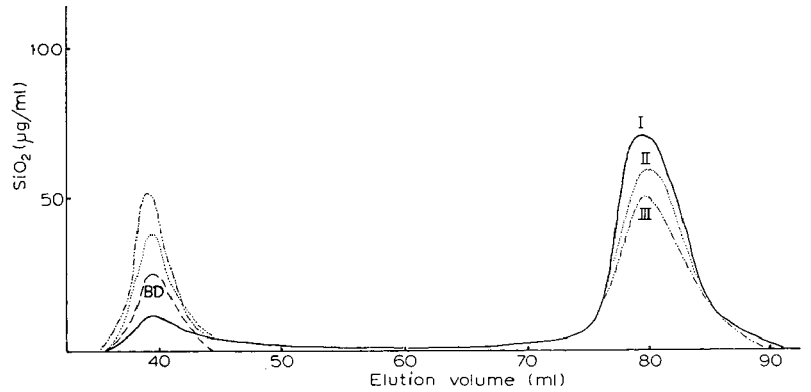


Fig. 2. Elution curves of silicic acid chromatographed on a column of Sephadex G-25. Sample I: 1 h after neutralization. Sample II: 2 h after neutralization. Sample III: 4 h after neutralization. BD: Blue Dextran.

Sephadex G-25. Fig. 2 shows typical chromatograms for silicic acid samples obtained at different periods after neutralization on Sephadex G-25. Two peaks were obtained. Peaks on the right in Fig. 2 were due to monosilicic acid, and peaks on the left were due to polysilicic acid. The volume for polysilicic acid appeared to be identical to that for Blue Dextran. It can be assumed that the elution volume of Blue Dextran is equal to the void volume of the bed.

An approximate separation range (shown by molecular weight) for Sephadex G-25 measured by peptides and globular proteins is given as 1000–5000 (ref. 10). If it can be assumed that polysilicic acid has spherical particles and that an approximate separation range for silicic acid is the same as that for globular proteins, the molecular weight of the peak given by polysilicic acid is over 5000. An absence of any peak between two peaks shows that the particle size of polysilicic acid grew rapidly and that polysilicic acid of a low molecular weight (under 5000) could not exist in a stable state. The results described above suggest that monosilicic acid can be separated from polysilicic acid using a Sephadex column.

Sephadex G-100. Fig. 3 shows the elution profiles of silicic acid chromatographed

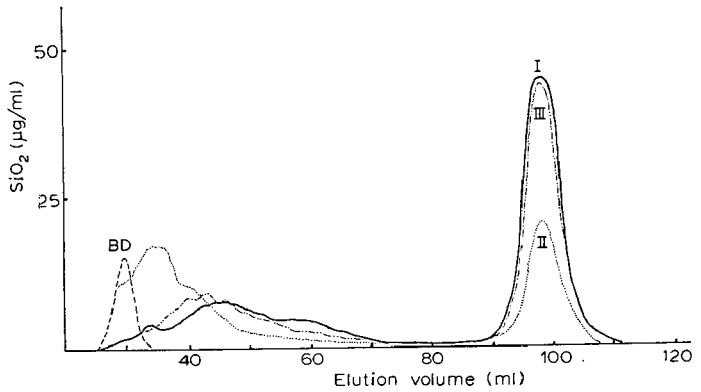


Fig. 3. Elution curves of silicic acid chromatographed on a column of Sephadex G-100. Sample I: 2 h after neutralization. Sample II: 48 h after neutralization. Sample III: adjusted to pH 2 2 h after neutralization and allowed to stand 12 days. BD: Blue Dextran.

on Sephadex G-100. Peaks on the right in Fig. 3 were due to monosilicic acid, and broad peaks on the left were due to polysilicic acid. An approximate separation range for Sephadex G-100 is given as 4000–150 000 (ref. 10). Peaks due to polysilicic acid indicate a broad distribution of molecular weight (size). It is seen that the molecular weight of polysilicic acid in Sample I ranges from 5000 to 150 000 because the lower limit for the molecular weight of polysilicic acid was given by the results obtained for Sephadex G-25.

The molecular weight distribution of polysilicic acid in Sample II shifted to 150 000 or larger compared with that of Sample I. It seems reasonable to assume that the broad distribution of molecular size, indicated by peaks due to polysilicic acid, arises from a random condensation of all the polymeric species.

The shape of the curve for Sample III is similar to that for Sample I. This

means that aggregation of polysilicic acid as well as polymerization of monosilicic acid in the pH 2 solution is strongly retarded.

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Resolution of some closely related prednisolone derivatives by thin-layer chromatography

One of the most important tests of steroidal drugs is the related foreign steroids test[1–3]. In this test use is made of thin-layer chromatography (TLC) to detect the presence of structurally related compounds that may exist in the therapeutically active steroid. As a result of the interest in this laboratory in the determination of steroid purity and absence of closely related compounds by TLC, solvent systems were reported previously for the separation of some estrogens[4] and some closely related hydroxycorticosteroids[5]. The semiquantitation of the closely related steroids as well as the estimation of flurandrenolone acetonide purity by TLC, were also achieved[6]. Several TLC systems for cortical steroids have been reported[7–16]. None of these were found to be completely adequate for the complete resolution of a mixture of closely related prednisolone derivatives in which we were interested. This paper describes a new developing system—and its application—for the complete resolution of this particular mixture by TLC on silica gel.

Materials

Reagents. All solvents and chemicals were reagent grade.

Developing system. Ethyl propionate.

Spray reagent. Methanolic sulfuric acid, prepared as mentioned previously[4].

Equipment. Pre-coated 250 μ thin-layer plates (Silica Gel F_{254}) supplied by Brinkmann Instruments Inc. Micro-pipets, Microcaps (Drummond Scientific Compa-

ny). Chromato-vue equipped with short and long wavelength UV lamp (about 254 and 366 mμ, respectively) by Ultra-Violet Products Inc.

Procedure

The TLC chamber was lined with filter paper and allowed to equilibrate with the developing solvent for 30 min to achieve complete saturation. The solutions of the prednisolone derivatives (Table I) made up (1 mg/ml) in a mixture of chloroform-methanol (1:1) were spotted on the plate using a micro-pipet. Spots of 5 μg steroid were used. The plate was developed in the chamber allowing the solvent front to travel 15 cm after passing through the point of application. It was then removed from the chamber and the solvent allowed to evaporate at room temperature (25°) for 5 min. A second development of the plate in the same developing solvent was necessary to obtain adequate resolution of the zones. The plate was allowed to dry again at room temperature and the spots were detected using a short wavelength UV light and marked.

Results and discussion

Each of the prednisolone derivatives listed in Table I was run singly and in combination with other compounds listed. Measurements were made from the point of application to the center of the spot and the R_F value of each steroid was calculated as the observed movement of the spot after the second run divided by the distance of a single development (15 cm) (ref. 18). The average value for five determinations of the R_F (× 100) for each of these steroids is given in Table I.

TABLE I

R_F (× 100) VALUES FOR THE PREDNISOLONE DERIVATIVES DEVELOPED TWICE BY ETHYL PROPIONATE
Each figure represents the average of five independent determinations.

No.	*Steroid*[a]	R_F (× 100)
1	6α-Fluoro-16α-methyl prednisolone (paramethasone)[17]	21
2	6α-Fluoro-16α-methyl prednisolone-21-acetate (paramethasone acetate)[17]	51
3	6α-Fluoro-16α-methyl prednisolone-11,21-diacetate	57
4	6α-Fluoro-16α-methyl prednisone-21-acetate	63

[a] All steroids in this work were obtained from Syntex Corporation, Palo Alto, Calif., U.S.A.

The solvent system reported in this paper has been used satisfactorily in this laboratory for the semiquantitative determination of the closely related foreign steroids (Table I, Nos. 1, 3 and 4) that may be present in the raw material of paramethasone acetate. In this case the TLC plate was divided into four equal sections. One and 3 μg of each of the related foreign steroids were applied on sections 1 and 3, respectively. On section 2, 100 μg of paramethasone acetate sample was applied. Section 4 was considered as the plate blank. The plate was developed and visualized as mentioned before. For the evaluation of the plate in order to determine the percentage of related foreign steroids, each extra spot—in section 2—other than the main compound was compared with the spot having the same R_F value (mobility) in

sections 1 and 3 containing, respectively, 1 and 3% level of related foreign steroids. The visual semiquantitation under short wavelength UV light is facilitated by having the sample between two levels of related foreign steroids, namely 1 and 3%. It was found that better detection of the 1 μg spot was achieved by applying the spray reagent then by heating at 105° for 5 min and observing the plate under long wavelength UV light.

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Beziehungen zwischen physikalisch-chemischen Eigenschaften und Wirksamkeit bei Steroiden

V. Papierchromatographisches Verhalten und Aktivität anabol wirksamer Steroide*

Das papierchromatographische Verhalten einer Verbindung wird bekanntlich durch ihren Verteilungskoeffizienten (α) in dem angewandten Laufmittelsystem bestimmt (Verteilungschromatographie)[2]. Daher kann umgekehrt aus der Laufstrecke die Verteilung zwischen mobiler und stationärer Phase abgeleitet werden.

Der Verteilungskoeffizient besitzt andererseits auch für die Pharmakokinetik Bedeutung. Die Verteilung kommt für die Beeinflussung verschiedener Wirkungsfaktoren in Betracht:

(1) Resorption[3], Transport[4,5] und Ausscheidung[6],
(2) Anreicherung in verschiedenen Verteilungsräumen des Organismus[7],
(3) hydrophobe Bindung an Albumin und Ribonucleinsäure[8–11],
(4) freie Reaktionsenergie[12–14],
(5) Elektronen-Acceptor/Donator-Kapazität[15] und Bildung von Elektronenadditionsverbindungen[16],
(6) Metabolisierung[17], mikrosomale Oxydation[18] und biologische Halbwertszeit[19].

Zwischen α und papierchromatographischem (PC) R_F-Wert besteht die Beziehung[20,21]:

$$\alpha = \frac{A_M}{A_S}\left(\frac{1}{R_F} - 1\right).$$

Für den dekadischen Logarithmus des in Klammern stehenden Ausdrucks wurde der Begriff "R_M-Wert" eingeführt[22]:

$$R_M = \lg\left(\frac{1}{R_F} - 1\right).$$

Daraus folgt für den Zusammenhang mit α:

$$\alpha = \frac{A_M}{A_S} \cdot 10^{R_M}.$$

$$\lg \alpha = \lg A_M - \lg A_S + R_M.$$

Betrachtet man das Verhalten verschiedener Substanzen bei gleichem Phasenverhältnis, $A_M/A_S = \text{const.} = K$, so ergibt sich:

$$R_M = \lg \alpha + K'$$

Somit sind R_M und α nach einer einfachen logarithmischen Funktion voneinander abhängig. Da sich der R_M-Wert bei Einführung oder Eliminierung einer

* IV. Mitt., siehe Lit. 1.

TABELLE I

FLÜSSMITTELSYSTEME

System	*Stationäre Phase*	*Mobile Phase*	*Mischungsverhältnis*
A	Methanol–Wasser	Benzol	1 : 1 : 2
B	Methanol–Wasser	Toluol–Benzin	85 : 15 : 33 : 67
C	Methanol–Wasser	Benzin	17 : 3 : 20
D	Essigsäure (80%)	Benzol	Imprägnierung
E	Essigsäure (80%)	Benzin	Imprägnierung
F	1,2-Propylenglykol	Benzol	Imprägnierung
G	1,2-Propylenglykol	Toluol	Imprägnierung
H	Methanol–Wasser (chloroformgesättigt)	Methanol–Wasser (chloroformgesättigt)	Sättigung Chloroform–Methanol–Wasser (2 : 1 : 1)

Funktion in spezifischer Weise und nahezu unabhängig von der Grundstruktur ändert, also eine additive Grösse darstellt, kann das PC Verhalten eines Derivats ausserdem durch das Gruppeninkrement des R_M-Werts (ΔR_M) charakterisiert werden. Die Beziehung des ΔR_M zum α und zur Struktur wurde mehrfach überprüft[23].

Untersuchungen an Steroiden ergaben[24], dass zwischen der Wirkstoffverteilung in bestimmten Lösungsmittelsystemen und der biologischen Wirksamkeit ein Zusammenhang besteht. Wir erwarteten daher, auf Grund der genannten Relation zwischen α und R_F-Wert auch ein PC System zu finden, das physiologischen Bedingungen entspricht. Uns interessierte, inwieweit eine Beziehung des R_F- bzw. R_M-Werts zur Aktivität (anabol) wirksamer Steroide vorhanden ist.

Versuche und Ergebnisse

Wir benutzten wässrige Systeme vom Bush-Typ (A bis E), wasserfreie Systeme vom Zaffaroni-Typ (F und G)[25] und eine eigene Anordnung mit umgekehrtem Phasenverhältnis (H), in dem die schwach polare Phase zur Kammersättigung (mit Papier)

TABELLE II

UNTERSUCHTE VERBINDUNGEN UND IHRE hR_F-BZW. R_M-WERTE

No.	*Verbindung*	*Fliessmittelsystem*								
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	
I	17α-Methyl-testosteron (M)	89	45	22	86	61	69	74	44	hR_F
		−0.89	+0.09	+0.54	−0.79	−0.19	−0.35	−0.45	+0.10	R_M[a]
II	1(2)-Dehydro-M	87	29	08	85	62	61	65	53	hR_F
		−0.80	+0.38	+1.06	−0.77	−0.21	−0.19	−0.26	−0.05	R_M[a]
III	4-Chlor-M	90	59	33	89	60	77	84	27	hR_F
		−0.96	−0.15	+0.30	−0.91	−0.17	−0.52	−0.71	+0.42	R_M[a]
IV	11β-Hydroxy-M	61	08	01	30	15	21	21	86	hR_F
		−0.19	+1.04	+2.00	+0.37	+0.77	+0.56	+0.59	−0.87	R_M[a]
V	4-Chlor-11β-hydroxy-M	77	13	02	49	09	36	38	78	hR_F
		−0.47	+0.83	+1.72	+0.03	+1.01	+0.21	+0.22	−0.53	R_M[a]
VI	4-Chlor-1-dehydro-M	87	43	15	89	46	73	78	47	hR_F
		−0.82	+0.12	+0.78	−0.91	+0.08	−0.42	−0.55	+0.06	R_M[a]
VII	4-Chlor-11β-hydroxy-1-dehydro-M	73	14	02	40	04	36	36	85	hR_F
		−0.42	+0.79	+1.72	+0.18	+1.33	+0.24	+0.24	−0.75	R_M[a]

[a] Diese Werte stellen mittlere Grössen aus den einzelnen R_M-Werten dar und wurden nicht aus den mittleren hR_F-Werten berechnet.

TABELLE III

ΔR_M-WERTE ZUR GRUNDSTRUKTUR 17α-METHYLTESTOSTERON

Funktion	*Derivat*	*Fliessmittelsystem*							
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
1(2)-Dehydro	II	+0.09	+0.29	+0.52	+0.02	−0.02	+0.16	+0.19	−0.15
4-Chlor	III	−0.07	−0.24	−0.24	−0.12	−0.02	−0.17	−0.26	+0.32
11β-Hydroxy	IV	+0.70	+0.95	+1.46	+1.16	+0.96	+0.91	+1.04	−0.97
4-Chlor-11β-hydroxy	V	+0.42	+0.74	+1.18	+0.82	+1.20	+0.56	+0.67	−0.63
4-Chlor-1-dehydro	VI	+0.07	+0.03	+0.24	−0.12	+0.27	−0.07	−0.10	−0.04
4-Chlor-11β-hydroxy-1-dehydro	VII	+0.47	+0.70	+1.18	+0.97	+1.52	+0.59	+0.69	−0.85

und die polare Phase als Fliessmittel eingesetzt wurde. Die Systeme sind in Tabelle I beschrieben.

Alle Bestimmungen wurden mehrfach ausgeführt. Die mittleren hR_F-Werte in den Systemen A bis H sind in Tabelle II zusammengefasst. Aus den einzelnen hR_F-Werten folgen die darunter angegebenen R_M-Werte. Die ΔR_M-Werte werden in Tabelle III mitgeteilt.

TABELLE IV

VERGLEICH DER AKTIVITÄT MIT hR_F-bzw. R_M-WERTEN

Verbindung	*Relative anabole Aktivität (Methyltestosteron = 100)*	*Fliessmittel G*		*Fliessmittel H*	
		hR_F	R_M	hR_F	R_M
4-Chlor-M	50	84	−0.71	27	+0.42
4-Chlor-1-dehydro-M	65	78	−0.55	47	+0.06
Methyltestosteron (M)	100	74	−0.45	44	+0.10
1(2)-Dehydro-M	125	65	−0.26	53	−0.05
11β-Hydroxy-M	290	(21)	(+0.59)	(86)	(−0.87)
4-Chlor-11β-hydroxy-M	406	38	+0.22	78	−0.53
4-Chlor-11β-hydroxy-1-dehydro-M	1085	36	+0.24	85	−0.75

Vergleicht man das PC Verhalten mit den biologischen Aktivitäten der Verbindungen[26], so erhält man folgende Ergebnisse: Bei Anwendung der Fliessmittel A bis E treten zwischen beiden Parametern keine erkennbaren Zusammenhänge auf. Dagegen bestehen bei der Entwicklung der Chromatogramme in den Systemen F, G oder H zwischen den chromatographischen Kennzahlen und der anabolen Wirksamkeit gute Übereinstimmungen (Tabelle IV).

Da sich die Systeme F bzw. G und H in der Polarität der mobilen Phase grundsätzlich unterscheiden, zeigt die Abhängigkeit der beiden verglichenen Grössen erwartungsgemäss einen entgegengesetzten Verlauf. In einem System mit lipophiler mobiler Phase (F bzw. G) nehmen die hR_F-Werte beim Anstieg der anabolen Aktivität ab. Wird die Chromatographie mit einer hydrophilen mobilen Phase (H) durchgeführt, so erhält man eine Verteilung, bei der hohe hR_F-Werte einer hohen Wirksamkeit entsprechen. Die beachtlich gute Übereinstimmung zwischen den Ergebnissen in

TABELLE V

VERGLEICH DER AKTIVITÄTSANDERUNGEN MIT ΔR_M-WERTEN

Funktion	*ΔAnabole Aktivität, Rel.-% (Mittelwerte)*	*ΔR_M- und mittlere ΔR_M-Werte*	
		Fliessmittel G	*Fliessmittel H*
4-Chlor	− 19	−0.26; −0.29; −0.37 −0.31	+0.32; +0.11; +0.34 +0.26
1(2)-Dehydro	+ 74	+0.19; +0.02; +0.16 +0.12	−0.15; −0.22; −0.36 −0.24
11β-Hydroxy	+823	+1.04; +0.79; +0.93 +0.95	−0.97; −0.81; −0.95 −0.91

beiden (zueinander inversen) Systemen wird durch den Kurvenverlauf in Fig. 1 veranschaulicht.

Eine Ausnahme in der Reihenfolge ist unter den sieben verglichenen Verbindungen nur bei 11β-Hydroxy-methyltestosteron zu beobachten. Somit können innerhalb dieser oder ähnlicher Verbindungsreihen aus dem Verhalten in bestimmten PC Systemen relativ wahrscheinliche Aussagen über die pharmakologischen Eigenschaften neuer Wirkstoffe abgeleitet werden.

Eine ähnliche Beziehung ist zwischen der Änderung des R_M-Wertes (der Gruppenkonstante) und der prozentualen Änderung der Aktivität[26] zu erkennen. Beide Grössen ändern sich bei Einführung einer Funktion gegenüber verschiedenen Vergleichs- bzw. Ausgangsverbindungen in regelmässiger Weise. Besitzt die mobile Phase lipophilen Charakter (System F oder G), so nimmt der ΔR_M-Wert im gleichen Sinne wie die Aktivität zu. Benutzt man dagegen eine mobile Phase mit hydrophilen Eigenschaften (System H), so entspricht einer Erhöhung der anabolen Aktivität eine Herabsetzung des ΔR_M-Wertes (Tabelle V).

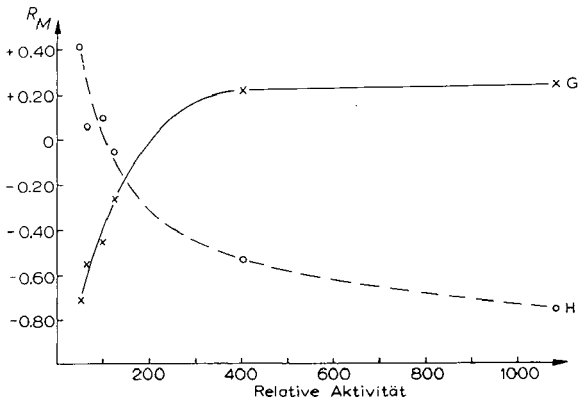


Fig. 1. Beziehung zwischen R_M-Wert und Aktivität.

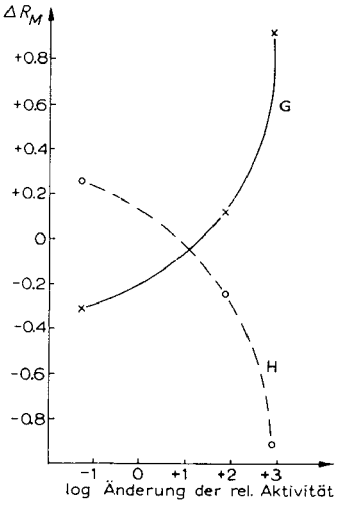


Fig. 2. Beziehung zwischen ΔR_M-Wert und Aktivitätsänderung.

Im vorliegenden Fall unterscheiden sich die mittleren ΔR_M-Werte der beiden Systeme G und H innerhalb der gegebenen Fehlergrenze nur durch das Vorzeichen. Die semilogarithmische Darstellung des Zusammenhangs veranschaulicht Fig. 2.

Aus den Ergebnissen folgt, dass bei einer Derivatbildung das PC Verhalten (unter bestimmten Entwicklungsbedingungen) und die biologische Aktivität gegenüber der Ausgangsverbindung so verändert werden, dass zwischen den Änderungen der beiden Eigenschaften ein Zusammenhang besteht. Diese Beziehung zwischen den Einflüssen einer Funktion auf R_F-Wert und Wirkung bildet die Grundlage für die Korrelation der Grössen im Gesamtmolekül.

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CHROM. 4794

Chromatographic isolation of 2-acetamido-1-(β'-L-aspartamido)-1,2-dideoxy-β-D-glucose from urine

Since the original report[1] on the occurrence of 2-acetamido-1-(β'-L-aspartamido)-1,2-dideoxy-β-D-glucose (AADG) in the urine of two mentally retarded siblings, further cases have been diagnosed[2]. For the correct identification of this relatively rare disease, isolation of AADG from the urine had to be established. A detailed description of the isolation and identification procedure is presented in this paper.

Experimental

Large volumes of untreated or concentrated urine were pipetted onto Whatman No. 3 MM chromatography paper sheets. Descending development of the sheets in *n*-butanol–water–acetic acid (60:25:15) lasted at least 48 h before they were dried. Narrow strips were cut from the sheets and stained with SCRIVER's[3] solution*. The strong brown colour indicated the location of AADG, and corresponding areas were cut from the remaining unstained paper. These strips were eluted with water, and the eluates were evaporated to dryness. After adding 1 ml of water and two drops of acetic acid to the dry material, the mixture was brought onto the top of a column packed with Dowex 50 W resin. Before that, the resin had been treated with 2 *M* acetic acid–pyridine buffer (pH 5.8) and equilibrated with water. The column was then eluted with 0.1 *M* acetic acid–pyridine buffer (pH 3.8), and fractions each 0.5 to 1 ml were collected[4].

A small sample from each fraction was pipetted onto a Whatman No. 1 chromatography paper sheet, and the sheet was developed as above. The pure AADG appeared as single brown spots, thus proving that no other ninhydrin-positive compounds were present. These pure fractions were pooled, evaporated to dryness, and dissolved in 1 ml of 1 *N* or 6 *N* hydrochloric acid. The solution was transferred into a hydrolysis tube, flushed with nitrogen, and sealed under vacuum. The tube was placed in an oven with a temperature of 100° for 1–16 h. It was then opened, the hydrochloric acid was evaporated, and the remaining acid was removed by washing three times with water. The dry residue constituted the hydrolysate of the purified AADG.

Column chromatography was performed using the Unichrom (Beckman) automated amino acid analyser. Analysis of the unhydrolysed material confirmed the presence of only one peak, close to the taurine peak, in the area of acidic amino acids. Column chromatography of the hydrolysed material showed two peaks, one in the area of acidic amino acids and the other in the area of more basic amino acids. The first peak coincided with that of aspartic acid and the second with that of glucosamine. A positive Morgan–Elson reaction confirmed the presence of glucosamine in the isolated material.

Discussion

Urine contains a number of peptides, *e.g.* 17 different peptides were discovered in 83 l of normal urine[5]. Antibiotics are a well-known source of extra spots on amino

* SCRIVER's solution: ninhydrin 1.25 g, isatin 0.05 g, lutidine 5 ml *ad* 500 ml of acetone.

acid chromatograms. It is therefore necessary to isolate a compound suspected to be an abnormal urine constituent caused by an inborn error of metabolism.

Since the development of the present isolation procedure for AADG, another method involving a combination of exclusion chromatography on Sephadex G-15 and ion-exchange chromatography on Zeo-Karb 225 (sulphonic acid type) ion-exchange resin followed by recrystallisation was published[6]. Although no comparison between that method and the present system was performed, both procedures obviously yield equally well-purified material and can be utilised if chromatographically pure AADG is needed for biochemical studies. Synthesis of AADG[7] is, of course, also possible but may be beyond the capacity of most clinical research laboratories.

The only hitherto known condition in man in which AADG is excreted in large quantities (up to 20 mg/100 ml) in the urine is aspartylglucosaminuria (AGU). Altogether 17 patients have been recorded[1,2]. However, the disease may appear to be more common if more attention is paid to the unknown "peptide" spots occasionally detected on amino acid chromatograms. The isolation method described here will serve as tool for the correct diagnosis.

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CHROM. 4788

Membrane chromatography of different molecular weight polyethylene glycols on polyvinylchloride ultrafilters

In a previous communication[1] we described the group separation of high- and low-molecular-weight polyethylene glycols (PEG) by membrane chromatography on cellulose nitrate. The behaviour of the PEG was generally similar to that of proteins and amino acids on the same carriers[2]. Quite recently, we have reported[3,4] that polyvinylchloride (PVC) membrane filters were suitable for use as chromatographic carriers and that they adsorbed proteins more firmly and over a much broader range of pH and molecular weights than nitrocellulose[5]. Furthermore, we noted that PEG 20 000 was also firmly adsorbed to PVC in a similar way.

In the present paper, we have investigated whether a quantitative microestimation of PEG could be achieved by membrane chromatography on PVC filters in a similar manner to that observed with proteins[3,4], and if so, whether there is any noticeable relationship between the molecular weights of different PEG fractions and the areas of the corresponding PEG layers. The last question was intended as a simple model for analoguous experiments with proteins.

Materials and methods

0.25% aqueous solutions of polyethylene glycols of average molecular weights 400, 600, 1500, 4000, 6000 and 20 000 (Hüls, Marl, G.F.R.) were used and applied to the starting edge of the chromatographic strips in volumes of 0.25–2 μl as described earlier[3,4]. Strips (3–4 mm × 10–20 mm) of PVC Sartorius membrane (Göttingen, G.F.R) SM 12801 were soaked with 40% ethanol, washed with distilled water, and were then used for one-dimensional ascending chromatography[3,4] with water as the developing agent. Chromatography lasted 1–3 min. The PEG was detected with Dragendorff reagent (see ref. 6). The stained areas were measured with a transparent millimeter scale and plotted against μg of PEG applied.

Results and discussion

Polyethylene glycols of molecular weight 1500, 4000, 6000 and 20 000 were adsorbed and spread as homogeneous and sharply outlined spots; however, the developed layer of PEG 1500 was markedly less stained with Dragendorff reagent. Samples of PEG 400 and 600 were not adsorbed and migrated freely with the front in one round, well defined spot in the same way as on nitrocellulose[1]. The calibration curves (Fig. 1) were linear within a 3% deviation about the mean but different for various PEG samples of different molecular weights; as was observed similarly with proteins on PVC membranes[3,4]. These results have shown that a simple and rapid microestimation of given fractions of PEG with molecular weights above 1500 can be achieved by membrane chromatography on PVC filters, and that a simple group separation of high- and low-molecular PEG fractions is possible on PVC as well as on nitrocellulose.

However, it was most interesting to note that the logarithms of the areas formed by equal quantities (in μg) of different PEG samples when plotted against

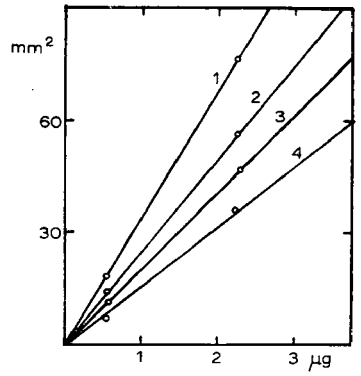


Fig. 1. Dependence of area on the amounts of different PEG samples. 1 = PEG 1500; 2 = PEG 4000; 3 = PEG 6000; 4 = PEG 20 000. Development in water on PVC membranes (Sartorius SM 12801) for 3 min.

the logarithms of the corresponding molecular weights, showed a nearly linear relationship (Fig. 2). This curve which obviously expresses an exponential function of a general formula $A = k/M^n$ can be used as a calibration curve for the estimation of the average molecular weights of PEG fractions in a given region. This fact can be interpreted by the formation of a very regular immobilised film of defined thickness for each PEG fraction on the surface of the microporous network of the particular PVC membrane filter. We have assumed that, in general, similar forces and mechanisms and principles of binding are probably involved here as was proposed for nitrocellulose membranes[6]. There also seems to be a certain distant analogy to the study of monomolecular films of proteins spread on the surfaces of liquid substrates[7].

We also attempted to construct an "ideal" calibration curve based on the following hypothetical and very simplified assumption: We considered that four groups of particles of a standard material having a standard density, are present as cubic particles of a relative volume (or molecular weight) ratio 20 000 n: 6000 n: 4000 n: 1500 n (*i.e.*, similar to the ratios of the PEG fractions tested) and are spread

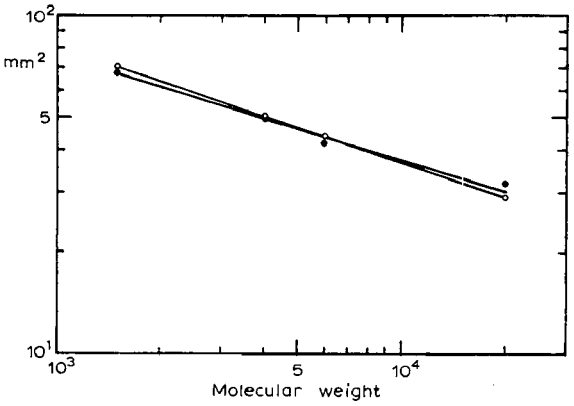


Fig. 2. Dependence of area on the molecular weight of the PEG. ● The calibration curve is valid for amounts of 2.0 µg PEG as derived from Fig. 1. ○ The hypothetical calibration curve (see text).

separately on a surface in monolayers with particles packed tightly side by side. The base areas x^2n of the cubes corresponding to the individual volumes x^3n were then calculated and related to one standard total volume, *e.g.* 20 000 n, in all cases. The ratio of the total areas covered by the hypothetical monolayers of an equal weight of standard particles differing only in their molecular size was 737 n: 1100 n: 1260 n: 1746 n, respectively. When n was taken as equal to 0.04 and the logarithms of the total areas were plotted *versus* the logarithms of the individual volumes a straight line was obtained (Fig. 2) which was very similar to that constructed from experimental data. This good analogy between hypothesis and experiment seems to verify the previous assumption and throws more light on the behaviour of the PEG fractions, and perhaps also of other substances, during membrane chromatography on PVC filters.

Further investigation dealing with a more detailed interpretation of our findings is in progress now. From preliminary results[8], it can be expected that the simple chromatographic principle used here for the experiments with PEG could also be applied to the determination of the molecular weights of proteins. Among the various techniques used for the estimation of the molecular weight of high-molecular substances, the proposed microchromatography on PVC membranes seems to be one of the most rapid, inexpensive and easy to perform.

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CHROM. 4834

Starch–iodide as a stain for peroxidases

The methods used for locating peroxidase bands after zone electrophoresis involve the peroxidation of chromogens such as benzidine and *o*-toluidine[1,2]. Many of these substances constitute health hazards because of their carcinogenic nature[3,4].

The oxidation of iodide to iodine is known to be accelerated by peroxidases[5]. In the presence of starch, iodine immediately forms an intense blue-black complex. This report describes how these reactions are adaptable to the detection of peroxidase isoenzymes after gel electrophoresis. Bands are sharper than those obtained with conventional chromogens in many cases and, of course, the reagents are completely innocuous.

For the study of this stain, zone electrophoresis of peroxidases from various sources was performed on horizontal starch gels (14%). Tris–citrate buffer (pH 7.0; $I = 0.018$ in gel and 0.27 in electrode buffer) was used, and a 150 V potential difference was applied for 18 h. After electrophoresis the gels were sliced horizontally; one section was stained by steeping in a solution of 0.02 *M* potassium iodide in 0.1 *M* phosphate citrate buffer (pH 5.4) containing 5×10^{-4} *M* H_2O_2, the other with one of the conventional peroxidase chromogens.

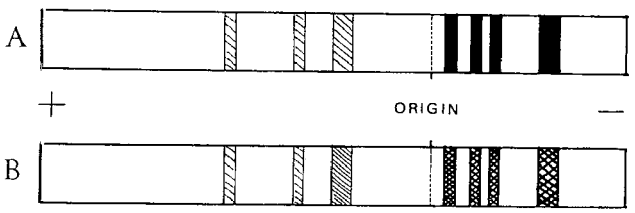


Fig. 1. Zymograms of horseradish peroxidase. (A) stained with starch–iodide; (B) stained with 3-amino-9-ethyl carbazole.

A typical result obtained with a commercial horseradish peroxidase preparation (Sigma Chemical Co., St. Louis, U.S.A.) is illustrated in Fig. 1. The four cathodic components were shown up very clearly by the starch–iodide stain which gave sharper bands than did other chromogens tested. The lack of detail from the anodic section of the gel is to be expected from a commercial preparation of horseradish peroxidase and has been reported previously[6].

Peroxidase preparations examined sometimes contained isoenzymes which were slower to react with iodide than with other chromogens, presumably a consequence of their having a relatively lower reactivity towards iodide. The starch–iodide stain proved particularly useful in studies on peroxidases from a number of algae. Some of these peroxidases (*e.g.* those from *Laminaria digitata* and *Enteromorpha linza*), which may play a role in biological iodination, displayed a much greater reactivity towards iodide than towards organic chromogens.

The iodide staining technique was also used to detect non-peroxidatic enzymes, the products of which may be coupled to a peroxidase reaction. For example, dipeptidases separated by gel electrophoresis were incubated with dipeptides and

L-amino acid oxidase, and the resultant hydrogen peroxide was coupled to horseradish peroxidase. The reactive zone was then very effectively located by the starch–iodide reaction. The technique may also prove applicable in polyacrylamide gel electrophoresis if a small percentage of soluble starch is incorporated in the polyacrylamide gel during its preparation.

I wish to thank Professor C. O'HEOCHA and Dr. P. O'CARRA for their helpful interest, and the Department of Education, Dublin, for a research maintenance grant.

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Densitometrische Bestimmung von Fe(II)–Fe(III)-Gemischen nach elektrophoretischer Trennung mittels N-(2-Hydroxyäthyl)iminodiessigsäure

Die in der Praxis bedeutsame Trennung von Eisen(II)- und Eisen(III)-ionen lässt sich u.a. auch mittels der Migrationsmethoden erreichen. Die Voraussetzungen für eine erfolgreiche Trennung sind vor allem durch die unterschiedliche Ladung sowie Tendenz zur Komplexbildung beider Valenzformen der Eisenionen gegeben. Man benutzte zu diesem Zweck Papierchromatographie[1], Ionenaustauschchromatographie[2] und in letzter Zeit auch Dünnschichtchromatographie[3]. Durch gleichzeitige Papierelektrophorese und Papierchromatographie erreichten LINGREN *et al.*[4] eine Fe(II)–Fe(III)-Trennung in verdünnter Schwefelsäure.

Mit der Einführung der N-(2-Hydroxyäthyl)iminodiessigsäure (HIDA) als einen breit anwendbaren komplexbildenden Elektrolyten für Trennung von anorganischen Ionen[5] wurde auch die Möglichkeit einer rein elektrophoretischen Trennung der Eisenionen gegeben. Tatsächlich erzielte man eine gute qualitative Separation eines äquimolaren Gemisches beider Valenzformen auf diesem Wege[6]. Weitere Untersuchungen der Möglichkeiten elektrophoretischer Ionentrennung in der Lösung von HIDA erwiesen[7], dass diese ohne jede Vortrennung bis zu einem Grenzverhältnis von etwa 1:500 durchaus erfolgreich verlaufen; die quantitative Bestimmung der ge-

trennten Ionen erfolgte dabei mittels einer modifizierten Methode der indirekten Densitometrie nach KEIL[8]. In der vorliegenden Mitteilung wird über die Trennung und Bestimmung annähernd äquimolarer Fe(II)–Fe(III)-Gemische sowie über die Bestimmung kleiner Mengen von Eisen(III)-ionen in Lösungen von Eisen(II)-salzen berichtet.

Methodik

Als Trägerelektrolyt diente eine 0.05 *M* Lösung des Mononatrium-hydroxyäthyliminodiacetats, deren pH-Wert durch Zusatz von Salpetersäure auf 1.6 eingestellt wurde; Ionenstärke der Lösung wurde mit Kaliumnitrat bei einem Wert $\mu = 0.1$ gehalten.

Die Standardlösungen für die Herstellung der Modellgemische erhielt man aus $Fe(NO_3)_3 \cdot 9H_2O$ und $FeSO_4 \cdot 7H_2O$. Die schwach angesäuerte Lösung von Eisen(II)-salz wurde in Gegenwart von Platinschwarz mit Wasserstoff reduziert bis zu einer negativen Reaktion auf Eisen(III) und nachher mit Stickstoff gesättigt. Der Eisengehalt beider Lösungen wurde komplexometrisch bestimmt.

Aus den Standardlösungen wurden zwei Reihen von Modellgemischen bereitet. Die eine enthielt Fe(II) und Fe(III) in einem Molarverhältnis ungefähr 1:2, 1:1 und 2:1. Die Konzentrationen wurden so gewählt, dass im aufzutragenden Volumen von 10 μl je 6–12 μg beider Formen vorhanden waren. Die andere Reihe von Proben enthielt 2–4 μg Fe(III) neben 200 μg Fe(II) in der Einzelauftragung, was einem Prozentgehalt von 1–2% Fe(III) in Fe(II) entspricht. Zu allen Proben wurde die Lösung des Na-HIDA in solcher Menge zugefügt, dass das Molarverhältnis Fe:HIDA gleich 1 war. Um die Oxidationswirkung des Luftsauerstoffs zu beschränken, wurden die Lösungen mit Stickstoff gesättigt und in gut verschlossenen Kölbchen im Kühlschrank aufbewahrt. Der Eisen(III)-gehalt der Gemische wurde in einstündigen Zeitabschnitten jodometrisch kontrolliert; es war jedoch binnen 5 Std. keine Erhöhung zu vermerken.

Die allgemeinen Arbeitsbedingungen für die Durchführung der Elektrophorese sind einer vorhergehenden Mitteilung[9] zu entnehmen. Alle Versuche haben wir mit dem Chromatographiepapier Whatman No. 2, bei einer Temperatur 15° und einem Spannungsgefälle 15 V/cm unternommen. Die Trenndauer in der ersten Reihe der Proben betrug 30 Min., in der zweiten 1 Std. Eine Erhöhung der Temperatur verursacht unerwünschte Diffusion der Zonen und verschlechtert die Trennwirkung. Auch eine Verlängerung der Versuchsdauer bei unverändertem Spannungsgefälle ist aus demselben Grunde nicht zweckmässig.

Auswertung

Bei jeder einzelnen Elektrophorese wurden zugleich mit den Proben zwei Standardlösungen mit bekanntem Eisengehalt aufgetragen. Die getrockneten Elektropherogramme wurden in eine 1%-ige Lösung des Diäthyldithiocarbaminsauren Natriums eingetaucht. Nach dem Austrocknen wurden die Elektropherogramme photographiert. Die Negative wurden densitometrisch ausgewertet mittels eines adaptierten und mit Linienschreiber gekoppelten Mikrophotometers (Schnellphotometer Zeiss, Jena), wie es ausführlicher in der Literatur[7] beschrieben wird. Die planimetrisch ermittelten Flächen der Maxima der densitometrischen Kurven wurden dann zur Konstruktion von Kalibrationskurven sowie zur Gehaltsbestimmung des zu bestimmenden Ions benutzt.

Ergebnisse und Diskussion

HIDA übt keine stabilisierende Wirkung auf die einzelnen Valenzformen des Systems Fe(III)–Fe(II) aus. Das geht unter anderem aus der Ermittlung der Redoxpotentiale hervor; diese werden durch Komplexbildung bei denselben Bedingungen, welche bei der Trennung auftreten, nicht beeinflusst. Trotzdem erscheint HIDA für die elektrophoretische Trennung ausserordentlich geeignet: das Reagens selbst sowie seine Metallchelate sind in wässrigen Lösungen gut löslich und die Gleichgewichte der Komplexbildung stellen sich rasch ein. Demzufolge bilden sich bei der Elektromigration im allgemeinen gut abgegrenzte und meist symmetrische Zonen.

Die Vorversuche ergaben, dass die besten Trennungen eines Fe(II)–Fe(III)-Gemisches bei einem pH-Wert des Trägerelektrolyten von 1.6 erreichbar sind. Wird bei einem höheren pH-Wert gearbeitet, so dehnt sich die Zone der Eisen(II)-ionen aus und mit deren steigender Konzentration wird dann der Trenneffekt beträchtlich herabgesetzt.

Ferner wurde das Grenzverhältnis der Konzentrationen untersucht, bei welchem noch kleine Mengen des Fe(III) von einem grossen Überschuss an Fe(II) einwandfrei abtrennbar sind. Unter unseren Versuchsbedingungen wurde als solches ein Verhältnis 1:100 gefunden. Was die absoluten Gewichtsmengen betrifft, erwies sich das Auftragen von ungefähr 2 μg Fe(III) mit 200 μg Fe(II) in einem Volumen von 20 μl als geeignet. Wurden dieselben Mengen in einem kleineren Volumen aufgetragen, so hatte dies ungünstige Auswirkungen auf die Qualität der Trennung.

Schliesslich wurde festgestellt, dass die Fläche der Maxima von densitometrischen Kurven eine lineare Funktion der Gewichtsmenge von Eisenionen in einem Bereich von 1–40 μg darstellt.

TABELLE I

BESTIMMUNG ANNÄHERND ÄQUIMOLARER Fe(III)–Fe(II)-GEMISCHE

Fe^{3+} : Fe^{2+}	*Fe(III)*				*Fe(II)*			
	Ber. (μg)	*Gef.* (μg)	*S* (μg)	*V* (%)	*Ber.* (μg)	*Gef.* (μg)	*S* (μg)	*V* (%)
1 : 2	6.08	6.55	0.31	4.73	12.60	12.82	0.40	3.10
1 : 1	9.12	9.63	0.27	2.80	9.40	9.72	0.37	3.80
2 : 1	12.17	12.32	0.35	2.84	6.30	6.75	0.36	5.30

Aufgrund dieser Erfahrungen wurden zuerst Analysen der Modellgemische der zu trennenden Ionen mit dem Molverhältnis ungefähr 1:2, 1:1 und 2:1 durchgeführt. Die in der Tabelle I angeführten Ergebnisse stellen die Mittelwerte von zehn parallelen Einzelbestimmungen dar; die Streuung der Methode wird zugleich mittels der Standardabweichungen *S* und Variationskoeffizienten *V* charakterisiert.

Die Reproduzierbarkeit der Methode (etwa 2–5%) ist gegenüber den Resultaten von POLLARD *et al.*[1,2] bedeutend niedriger. Die zitierten Autoren benutzten allerdings ein Elutionsverfahren, wobei die aufgetragenen Mengen von Eisenionen zwischen 25–2500 μg lagen. Die Trenndauer bei der Papierchromatographie nahm (samt Kammersättigung) ungefähr 3.5 Std. in Anspruch. Die elektrophoretische Trennung

TABELLE II

BESTIMMUNG DES Fe(III)-GEHALTES IN Fe(II)-SALZLÖSUNG

Fe(III)		*S*	*V*
Gegeben (%)	*Gefunden (%)*	*(%)*	*(%)*
1.01	0.98	0.11	11.2
1.51	1.55	0.03	1.9
2.02	1.99	0.09	4.5

dauert dagegen unter unseren Bedingungen nur etwa 30 Min. und diese Zeit könnte bei erhöhtem Spannungsgefälle ohne Schwierigkeiten noch herabgesetzt werden. Die Resultate der dünnschichtchromatographischen Trennung[3] sind mit unseren vergleichbar, wobei POKORNY für die quantitative Bestimmung ebenfalls Elutionsverfahren benutzt. Die von LINGREN *et al.*[4] erwähnte etwa 10%-ige Verschiebung des Konzentrationsverhältnisses zugunsten der Eisen(II)-ionen haben wir nicht beobachtet.

Die Ergebnisse der Analysen von Eisen(II)-lösungen mit Beimischung von etwa 1–2% Fe(III)-ionen sind aus der Tabelle II ersichtlich. Es handelt sich diesmal um Mittelwerte von je sechs parallelen Bestimmungen.

Die Genauigkeit ist in diesen Fällen begreiflicherweise niedriger, was auf das ungünstige Konzentrationsverhältnis zurückzuführen ist.

Bisher wurden höchstens Gemische 1:40 durch Elektrophorese qualitativ getrennt[4]. Eine quantitative papierchromatographische Trennung wurde im Verhältnis 1:25 durchgeführt[1]. Somit erscheint auch in diesem Falle die papierelektrophoretische Trennung der Gemische Fe(II)–Fe(III) unter Benutzung von HIDA als vorteilhaft.

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Report

CHROM. 4867

Gas Chromatography Discussion Group

The twelfth Annual General Meeting and Spring Informal Symposium was held on Friday, 10th April 1970 in the Assembly Hall of the International Students House, Great Portland Street, London. Nearly 200 members attended the symposium which was devoted largely to aspects of pollution, although several other interesting papers were also presented.

Mr. B. D. CADDOCK of the Shell Research Centre described a useful method for the characterisation of gasoline volatility based on current simulated distillation procedures. Instead of converting the chromatogram into an integral output as in simulated distillation, however, the separate peaks are integrated by dropping perpendiculars to the baseline at each trough and a boiling point is allocated to each peak from a calibration curve. The automation of the system by means of a digital voltometer provided with punched tape output, followed by computer interpretation of the data, was described.

Dr. R. KAISER of BASF, G.F.R. then discussed "Limitations of a Small Dedicated Satellite Computer for Quantitative Analytical and Process Gas Chromatography". The problems involved in the use of such systems were described and the advantages of several commercial units were compared.

Dr. M. B. EVANS of Hatfield Polytechnic provided some cause for consternation in his paper "Chemical Reactions on Gas Chromatography Columns" when he described some of the possible effects between common gas chromatographic supports and certain types of compound. The reactions he described all involved unsaturated hydrocarbons and appeared to take place via a protonation stage, initiated by Si–OH groups at the support surface. These could of course be removed or their effect reduced by various methods, including the use of polar liquid phases, and coating of the support surface with an inert film.

Three papers were given on different aspects of pollution. The first, entitled "Natural and Adventitious Flavour Contamination of Meat" by Dr. R. L. S. PATTERSON of the Meat Research Centre, described how a combination of gas chromatography and mass spectrometry had helped to identify several materials causing unpleasant odour or flavour in meat. The androgen steroid, 5α-androst-16-en-3-one, for example had been found to be responsible for a particularly offensive odour in the fat of the male pig, although only present to the extent of 1–2 p.p.m. This had so far prevented the raising of boars for fresh meat.

The second paper on pollution was given by Dr. D. GALL of the Warren Spring Laboratory. This was entitled "The Application of Gas–Liquid Chromatography to the Analysis of Air Pollutants" and consisted of a general description of the problems of air pollution, the types of pollutants, the methods of sampling and the gas chromatographic techniques used. This paper was well received in view of its topicality and general interest, but a suggestion by the author that gas chromatographers should provide inexpensive standardised equipment and simple techniques for the non-specialist user, met with the expected reaction.

The final paper "The Application of GLC to the Determination of Pollutants in Rivers" was given by Mr. A. W. DAVIES of the Lothians River Purification Board. This again was of general interest in view of the current problems of insecticide and oil pollution. The author described how GLC was employed as a purely fingerprint technique and a number of cases were cited where this had provided sufficient evidence to trace the source of the pollution.

The Annual General Meeting was held during an interval in the symposium. A notable feature of this meeting was the decision to form a new Sub-Group whose interests would be related to the use of computers in gas chromatographic analysis. Dr. R. KAISER was elected Chairman of this new group and Dr. D. R. DEANS, the Honorary Secretary.

The arrangements for the forthcoming General Symposium to be held in Dublin 28th September–2nd October, 1970 are going well. An interesting technical programme has been arranged as well as an enjoyable social programme, and a full attendance is anticipated.

Liquid Chromatography Sub-Group

An informal symposium of the Liquid Chromatography Sub-Group was held on May 6th, 1970 at the Royal Society of Tropical Medicine and Hygiene in London. The meeting was attended by about 85 delegates and four papers were presented.

The first paper was given by Dr. J. H. KNOX of the University of Edinburgh and was titled "Introduction to the theory of liquid chromatography". This paper outlined the basic theory of the chromatographic process, emphasising the important aspects in the case of liquid chromatography in columns. Dr. KNOX explained the advantages of the reduced plate height and velocity functions to enable a direct comparison to be made between columns, not only in liquid chromatography but also between columns in gas and liquid chromatography. Future trends of liquid chromatography in columns were also discussed.

In the second paper, Mr. G. LAWRENCE described a new technique for carrying out gradient elution. The technique involved the use of temperature programming to produce a polar gradient in the mobile phase (with respect to time) which is in axial equilibrium along the column, at all times throughout the analysis. This technique can be contrasted with conventional gradient elution methods where a polar gradient exists in the column during the elution process. Examples were shown of its application to several mixtures.

Mr. C. F. SIMPSON discussed "Chemically bonded Stationary Phases for Liquid Chromatography". The concept of these stationary phases was discussed in some detail. The support material used was silica gel onto which could be chemically bonded any organic molecule with a polar group which would chemically react with the hydroxyl group on the silica gel surface. These chemically bonded stationary phases behaved as a liquid–liquid partition system and this was illustrated by some experiments carried out by Mr. SIMPSON. These stationary phases were referred to as estersils and both gas and liquid chromatographic applications were discussed.

The final lecture by Dr. J. F. K. HUBER was concerned with the application of high-speed, high-efficiency liquid chromatography and a wide range of applications were illustrated and described from both the published literature and the authors own work. The chromatograms shown were of the analysis of multi-component (5–9

peaks) mixtures including some synthetic samples. These analyses were carried out in 15 min or less.

The meeting was concluded with an account of the latest available commercial equipment for liquid chromatography. Complete chromatographs as well as individual components were described with prices and distributors given, where the data was available.

Coal Tar Research Association,
Gomersal, Checkheaton, Yorkshire
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D. W. GRANT

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News

Apparatus

Disc Instruments, Inc., Santa Ana, Calif., U.S.A., has recently announced a newly designed *Integrator* for use with the Metrohm Labograph E478 Recorder. Designated the Model 267 Disc® Integrator, the new Integrator will supply peak area under each recorded peak by trace, with accuracies up to $\pm$ 0.1% full scale. By direct connection of the Disc Model 610 Automatic Printer, automatic digital printout of peak areas is available.

For further information apply to the publisher under reference No. Chrom. N-281.

Fractovap Model 1400 is a new gas chromatograph particularly suited for educational purposes. It is a simple rugged extremely low cost instrument still having full features of a dependable good gas chromatograph. Together with the instruction manual, Carlo Erba Technical Service provides a booklet of experimental exercises in order to make the teaching programme as fruitful as possible. A trouble shooting guide is also included allowing the student himself to repair the instrument in case of breakdown.

However, Fractovap Model 1400 can be used also in industrial laboratories for routine analyses of gaseous mixtures or low and medium boiling point liquids–switching column devices and full automatic sampling system are available.

For further information apply to the publisher under reference No. Chrom. N-280.

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