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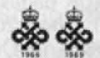

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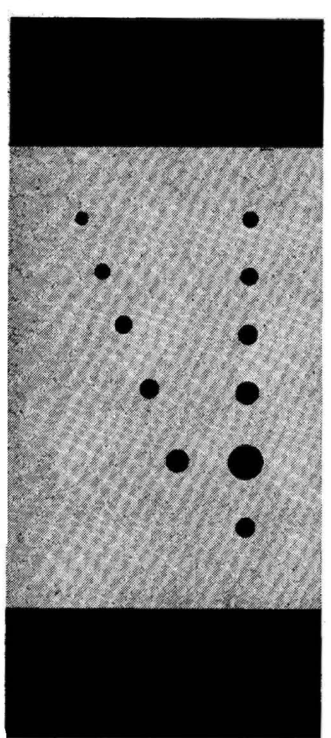

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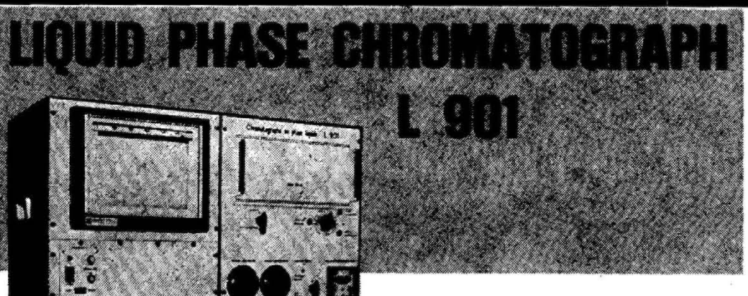

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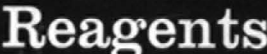

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ESTIMATION OF THE SURFACE AREA OF ADSORBENTS FROM THE THIRD GAS–SOLID VIRIAL COEFFICIENT

W. RUDZIŃSKI, A. WAKSMUNDZKI, Z. SUPRYNOWICZ AND J. RAYSS
Department of Physical Chemistry, Institute of Chemistry UMCS, Lublin, Nowotki 12 (Poland)

(First received February 18th, 1972; revised manuscript received May 23rd, 1972)

SUMMARY

A new method is proposed for the estimation of the surface area of adsorbents from the second and third gas–solid virial coefficients. The method is based on a three-dimensional model of physical adsorption, and is in addition an extremely simple one. The data needed can be easily obtained by gas chromatography.

INTRODUCTION

Virial expansion is at the present time the most promising and descriptive formula in the theory of physical adsorption of gases on solids. Virial expansion of the adsorption isotherm is the kind of expansion most often considered. This is due to the fact that the relationship obtained when the number of molecules adsorbed is treated as a function of the adsorbate density is the relationship most often investigated in experimental works concerning adsorption phenomena.

In the virial treatment of physical adsorption, the number of adsorbed molecules is expanded in a power series with respect to the density of the adsorbate phase, the coefficient at the $(n - 1)$th power of the density being called the nth gas–solid virial coefficient.

In the theoretical and experimental work done in this field so far, only the first two terms of this expansion are taken into account, these being the second and the third gas–solid virial coefficients, respectively.

The second gas–solid virial coefficient introduces the effect of the interaction between a single molecule and the adsorbent surface, while the third represents the effect of the interaction between two adsorbate molecules and the adsorbent surface. Since virial treatment is a most accurate expression, the properties of the adsorption systems determined on using this equation should also be accurate. In particular, one would expect that the use of the virial equation to estimate the surface area of an adsorbent will lead to good results.

The first theoretical papers along these lines were by BARKER AND EVERETT[1], followed by SAMS *et al.*[2]. They have shown how the second and third gas–solid virial coefficients may be used to determine the surface area of an adsorbent, and also to

obtain information about the forces between the adsorbed particles. They were, simultaneously, the first to investigate three-body effects, *i.e.* of two adsorbate molecules plus adsorbent, in the virial data, with respect to the problem of estimation of surface area.

The theoretical calculations of SINANOGLU AND PITZER[3], YARIS[4] and MCLACHLAN[5] have shown that three-body effects can contribute considerably to the total interactions between the adsorbed molecules.

All three derivations lead to the same conclusion, *viz.* that the third-order interaction can be taken into account by introducing an additional term proportional to r^{-3} in the pair interaction potential valid for the bulk phase. The fluctuation, or dispersion part, of this third-order interaction is shown by them to yield a repulsion between the two molecules in a monolayer that amounts to 20–40% of the gas phase LJ (12, 6) potential minimum. The same energy, yields an additional attraction of about 10–20% LJ (12, 6) potential minimum when the two molecules are on top of one another, as in multilayer formation.

KRIZAN AND CROWELL[6] were the first to investigate the contribution of the three-body effects to the thermodynamic properties of adsorption systems. They rather considered adsorption systems as two-dimensional ones, the deviations from planar configurations of molecules being treated as a small parameter only.

JOHNSON AND KLEIN[7] also have presented analyses of experimental data in terms of a two-dimensional model, using the LJ (12,6,3) potential derived by SINANOGLU AND PITZER. The difference between the two papers lies only in the mode of evaluation of the proportionality constant η in the SINANOGLU AND PITZER calculations. The calculations of η made by KRIZAN AND CROWELL are of a purely theoretical nature, whereas JOHNSON AND KLEIN found η by using a "best-fit" procedure with experimental data.

Comparison of the two lots of result shows that the values of η calculated by KRIZAN AND CROWELL are in general two to five times higher than the best-fit values obtained by JOHNSON AND KLEIN. Furthermore, the surface area calculated from these data seems to depend strongly on the factor η. Thus, there should be strong discrepancy between the surface areas obtained by means of the two procedures mentioned above.

The procedure of JOHNSON AND KLEIN has been next criticized by WOLFE AND SAMS[8] on the basis that the best-fit values of η are probably quite sensitive to one's choice of potential parameters for the bulk phase. Their object was to investigate the consistency between theoretical results obtained in three different ways, and the results obtained by using the best-fit procedure. The most interesting result obtained by them is that, although η is extremely sensitive to the choice of bulk potential parameters, any reasonable choice of the bulk parameters will lead to substantially the same value for the surface area.

Moreover, they are of the opinion that the three-body effects in physical adsorption may be smaller than was previously supposed.

The last conclusion is in strong contradiction to the earlier suggestions of KRIZAN AND CROWELL and JOHNSON AND KLEIN. Thus, it seems that the determination of the surface area from the third gas–solid virial coefficient still remains an unresolved problem because of the difficulties involved due to the three-body effects.

In the current paper we once more consider this problem, using a three-dimen-

sional model of physical adsorption. The existence of the third-order interaction permits some approximations which then lead to an exceptionally simple expression for the surface area. The experimental data needed can be easily obtained by gas chromatography.

THEORETICAL

It has been shown in our previous paper[9] that the second and third gas–solid virial coefficients B_{2s} and B_{3s} may be written in the following form:

$$B_{2s} = \int\limits_{V_s}\left\{\exp\left[\frac{W_1(\boldsymbol{r}_1)}{-kT}\right]\right\}\mathrm{d}\boldsymbol{r}_1 \tag{1}$$

$$B_{3s} = \iint\limits_{V_s}\left\{\exp\left[\frac{W_1(\boldsymbol{r}_1) + W_1(\boldsymbol{r}_2)}{-kT}\right]\right\} \times \left\{\exp\left[\frac{\omega_2(\boldsymbol{r}_1,\boldsymbol{r}_2)}{-kT}\right] - 1\right\}\mathrm{d}\boldsymbol{r}_1\mathrm{d}\boldsymbol{r}_2 \tag{2}$$

where

V_s = volume of surface phase

$W_1(\boldsymbol{r}_i)$ = potential energy of adsorption of a single molecule, whose centre is at point $\boldsymbol{r}_i$

$\omega_2(\boldsymbol{r}_1, \boldsymbol{r}_2)$ = interaction energy between two adsorbate molecules in the presence of solid

k = Boltzmann constant

T = absolute temperature.

Next, by using an algebraic method, the following expression has been obtained for the average density ϱ_1 in the adsorbed phase[10]:

$$\bar{\varrho}_1 = \frac{\bar{P}}{kT} + \frac{b_2}{b_1^2}\left(\frac{\bar{P}}{kT}\right)^2 + \ldots \tag{3}$$

where $\bar{P}$ is the average pressure in the adsorbed phase and b_1 and b_2 are cluster-type integrals, dependent on configuration integrals[11]. For example:

$$b_1 = \frac{1}{V_s} B_{2s} \tag{4}$$

$$b_2 = \frac{1}{2V_s} B_{3s} \tag{5}$$

The basic idea of our method is to consider the retention data from a region of as small as possible concentration of solute. We have suggested in our previous paper that for this region the simplest form of the retention equation of CONDER AND PURNELL[12] may be used. Thus:

$$jV_R = V_f + V_s \frac{\bar{\varrho}_1}{\varrho_0} \tag{6}$$

where

j = James–Martin compressibility factor

V_f = free gas phase volume

ϱ_0 = adsorbate density in free gas phase.

Using this expression, the following relationship between the adsorbate density ϱ_0 and the retention data has been developed, and is believed to be valid at very small concentrations of solute:

$$jV_R - V_f = B_{2s} + \varrho_0 B_{3s} \tag{7}$$

However, the above equation is in fact valid only for the case of infinite dilutions of solute. To establish the connection between the retention data and the gas–solid virial coefficients for the case of finite concentrations of solute, we consider the retention equation of CONDER AND PURNELL[12] in the form valid for all concentrations of solute:

$$V_R - V_f = F \cdot y \cdot V_s \frac{\partial \bar{\varrho}_1}{\partial \varrho_0} \tag{8}$$

where

F = compressibility factor depending upon column conditions

y = mole fraction of solute in free gas phase.

We then follow the procedure described in our previous paper. According to this procedure, we substitute the term $\bar{P}/kT$ in eqn. 3 with its value for the adsorbate phase considered to be that for an ideal gas, *viz.*

$$\frac{\bar{P}}{kT} = B_{2s} \frac{P_0}{kT} \tag{9}$$

where P_0 is the average pressure in the free gas phase. Next, we assume that at very small concentrations of solute, the free gas phase can still be considered as an ideal one. Inserting eqn. 9 into eqn. 3, we get a first correction for the non-ideality of the adsorbed phase. We are of the opinion that this correction is satisfactory for the region of very small concentrations considered here. Thus, we get:

$$\bar{\varrho}_1 = \frac{B_{2s}}{V_s} \varrho_0 + \frac{B_{3s}}{2V_s} \varrho_0^2 + \ldots \tag{10}$$

Inserting eqn. 10 into eqn. 8 we get:

$$V_R - V_f = F \cdot y \cdot (B_{2s} + \varrho_0 B_{3s}) \tag{11}$$

It follows from eqn. 11 that the plot $(V_R - V_f)$ *versus* ϱ_0 should yield a straight line, having a slope of FyB_{3s}, and intercept FyB_{2s}.

Now, we want to show how the surface area S can be calculated from the virial coefficients B_{2s} and B_{3s}. To do this, we assume the adsorption surface to be a plane (let it be an xy plane), and the adsorption potential $W_1(\boldsymbol{r}_i)$ to depend on the distance z from the surface. Next, we approximate the adsorption potential $W_1(\boldsymbol{r})$ by the following square-well function:

$$W_1(\boldsymbol{r}) = W_1(z) = \begin{cases} +\infty & \text{for } 0 \leqslant z < a \\ W_{\text{min.}} & \text{for } a \leqslant z < b \\ 0 & \text{for } b \leqslant z < +\infty \end{cases} \tag{12}$$

It is well-known that the above function is a good approximation for adsorption potentials when the parameters a, b, and $W_{\text{min.}}$ are suitably chosen. In addition, the above function allows for a very precise definition of the surface volume V_s, and

therefore for its accurate determination. This problem has been largely discussed in our previous paper.

Next, we shall consider the interaction energy to have the form:

$$\omega_2(\boldsymbol{r}_1,\boldsymbol{r}_2) = \begin{cases} +\infty & \text{for } O \leqslant |\boldsymbol{r}_1 - \boldsymbol{r}_2| < D \\ \omega_{\text{min.}} & \text{for } D \leqslant |\boldsymbol{r}_1 - \boldsymbol{r}_2| < RD \\ O & \text{for } RD \leqslant |\boldsymbol{r}_1 - \boldsymbol{r}_2| < +\infty \end{cases} \tag{13}$$

Let us now use functions 12 and 13 to evaluate coefficients B_{2s} and B_{3s}. We must first remark, however, that the result of the integration depends on the relations between the parameters $(b - a)$, D, and R. We shall later assume that $(b - a) \approx 0.1\, D$, and $R \approx 2$ (ref. 13). This is in accordance with the true physical situation in the majority of adsorption systems investigated in practice. Let us introduce the following notations:

$$\Omega_{1D} = 6D^2(b-a)^2 - (b-a)^4$$

$$\Omega_{1RD} = 6R^2D^2(b-a)^2 - (b-a)^4$$

$$\Omega_{2D} = 3D^3(b-a) - \frac{7}{2}D^2(b-a)^2 - \frac{5}{3}D(b-a)^3 - \frac{1}{4}(b-a)^4$$

$$\Omega_{2RD} = 3R^3D^3(b-a) - \frac{7}{2}R^2D^2(b-a)^2 - \frac{5}{3}RD(b-a)^3 - \frac{1}{4}(b-a)^4 \tag{14}$$

With the above notation, integration of eqns. 1 and 2 yields:

$$B_{2s} = S(b-a)\exp\left(\frac{W_{\text{min.}}}{-kT}\right) \tag{15}$$

$$B_{3s} = -\left\{\frac{\pi S}{6}\Omega_{1D}\exp\left[\frac{2W_{\text{min.}}}{-kT}\right] + \frac{\pi S}{3}\Omega_{2D}\exp\left[\frac{W_{\text{min.}}}{-kT}\right]\right\} + \left\{\frac{\pi S}{6}(\Omega_{1RD} - \Omega_{1D})\exp\left[\frac{2W_{\text{min.}}}{-kT}\right] + \frac{\pi S}{3}(\Omega_{2RD} - \Omega_{2D})\exp\left[\frac{W_{\text{min.}}}{-kT}\right]\right\}\left\{\exp\left[\frac{\omega_{\text{min.}}}{-kT}\right] - 1\right\} \tag{16}$$

Considering the second term in eqn. 16, let the first $W_{\text{min.}}$ be unperturbed, *i.e.* the same as in the bulk phase, then for the majority of the adsorption systems investigated in practice $\omega_{\text{min.}}/-k$ is from about 100° K, for simple gases, to about 400° K, for many organic compounds[14]. The adsorbates which are most often used in surface area measurements are simple gases, so that we shall later use $(\omega_{\text{min.}}/-k) = 100°$ K in our considerations. In addition, according to the theoretical results of SINANOGLU AND PITZER, the value $\omega_{\text{min.}}$ in the adsorbed phase should be about 50% smaller than that for the bulk phase, because of the third-order interactions. The last argument decreases our estimated value of $\omega_{\text{min.}}/-k$ to about 50° K.

The temperature regions which are most often investigated in practice are close to room temperature, *i.e.* they are about 300° K. Thus, to underestimate the most probable value of exp $[\omega_{\text{min.}}/-kT]$, we can reckon that $(\omega_{\text{min.}}/-kT) \approx 1/6$, so that $\exp[\omega_{\text{min.}}/-kT] \approx 1.10$, and the total factor $\{\exp[\omega_{\text{min.}}/-kT] - 1\}$ will be of the order 10^{-1}. The remaining factor in the second term in eqn. 16 is of the same

order as the first term in the equation. Therefore, we shall neglect the second term in comparison with the first.

Now consider once more the first term in eqn. 16. The value $W_{\text{min.}}/-k$ for the majority of simple gases on typical adsorbents is of the order of about 6000° K (ref. 13). Thus, at room temperature the total factor $\exp[W_{\text{min.}}/-kT]$ will be of the order 10^5. This enables us to neglect the second component in the first term of eqn. 16, by comparison with the first, containing the second power of $\exp[W_{\text{min.}}/-kT]$. With the above approximations, the expression for B_{3s} reduces to the following:

$$B_{3s} = -S\pi D^2(b-a)^2 \exp\left[\frac{2W_{\text{min.}}}{-kT}\right] \tag{17}$$

Dividing B_{3s} given as above by B_{2s}^2 given by eqn. 15, we get:

$$S = -\pi D^2 \frac{B_{2s}^2}{B_{3s}} \tag{18}$$

Thus, we have obtained an extremely simple formula for the surface area, and the presence of the third-order interaction is here rather a favourable circumstance.

The value D which is used in eqn. 18 remains to be discussed, since third-order interaction effects also change the slow-collision diameter, though only to a small extent according to SINANOGLU AND PITZER's theory. The above assumptions are additionally supported by the results obtained by WOLFE AND SAMS. Accordingly, the value for D for the bulk phase may be used in eqn. 18, which (apart from simplicity) is an additional advantage of this equation.

EXPERIMENTAL

To illustrate our method, we have measured the surface area of the Schuchardt silica gel, which is acceptable for cyclohexane and carbon tetrachloride. In these measurements we used a 2 m-long chromatographic column, containing 4 g of 80–120 mesh silica gel, produced by Schuchardt in Munich, G.F.R.

To plot $V_N = V_N(\varrho_0)$, we used the following sample sizes of solute: 0.1 μl, 0.2 μl, 0.3 μl, 0.5 μl, 0.8 μl, and 1.0 μl. It was found that the concentrations of solute in our chromatographic column, obtained with these sample sizes vary from about 10^{-5} to about 10^{-4} mole/l.

The measurements were performed with the Polish apparatus "Chromatoprep N-502", using a katharometer as detector. Hydrogen was used as carrier gas, with a flow-rate of about 50 ml/min.

RESULTS AND DISCUSSION

The results of our measurements and calculations are presented in Tables I and II.

First, we shall explain how the values B_{2s} and B_{3s} were obtained. The experimental function $V_N = V_N(\varrho_0)$ was interpolated by means of a polynome of the second order, *viz.* $V_N = B_{2s} + B_{3s}\varrho_0 + B_{4s}\varrho_0^2$. The calculations were performed numerically, and the procedure of the smallest areas was included in the program, to

TABLE I

RESULTS OF MEASUREMENTS AND CALCULATIONS FOR CYCLOHEXANE ADSORBED ON SCHUCHARDT'S SILICA GEL

Temperature (°K)	B_{2s} *(l/g)*	B_{3s} *(l²/mole·g)*	B_{2s}^2/B_{3s} *(moles/g)*	*Surface area from virial expansion (m²/g)*	*Surface area from BET method (m²/g)*
359.8	$2.73 \cdot 10^{-2}$	−10.59	$-7.03 \cdot 10^{-5}$	58.6	11.9
368.9	$2.19 \cdot 10^{-2}$	− 5.13	$-9.30 \cdot 10^{-5}$	77.5	16.1
374.5	$1.93 \cdot 10^{-2}$	− 4.33	$-8.58 \cdot 10^{-5}$	71.5	18.3
383.9	$1.54 \cdot 10^{-2}$	− 2.63	$-9.02 \cdot 10^{-5}$	75.2	15.6
410.5	$8.53 \cdot 10^{-3}$	− 0.94	$-7.77 \cdot 10^{-5}$	64.7	13.2
426.2	$6.49 \cdot 10^{-3}$	− 0.59	$-7.05 \cdot 10^{-5}$	58.7	12.4
Average values (over temperature)			$-8.12 \cdot 10^{-5}$	67.7	14.6

analyse experimental deviations. The function $V_N = V_N(\varrho_0)$ was measured chromatographically, using the method recommended by CONDER[15], *i.e.* by considering the positions of peak maxima.

Next the values B_{2s}^2/B_{3s} for different temperatures were considered. According to the results obtained in the theoretical section, they should be temperature-independent ones. It can be seen from Tables I and II that there is no regular relationship between these values and the absolute temperature. The differences between them are probably due to experimental uncertainties. Thus, even when temperature dependence exists, it is smaller than the changes due to experimental errors.

Finally, we have calculated the surface areas S, and compared them with the areas calculated by the BET method, being the one most widely known and most often used so far.

Before presenting the results of these calculations, we want to explain how the values of D for carbon tetrachoride and cyclohexane were found. The value of D used for carbon tetrachloride was $D = 6.1$ Å, found earlier by LAUGER[16] from the transport properties of gaseous carbon tetrachloride. The appropiate value for cyclohexane was calculated from liquid densities, by using the WASSENBERG–BALLANDIN formula[17], and has been found to be equal to 6.6 Å.

TABLE II

RESULTS OF MEASUREMENTS AND CALCULATIONS FOR CARBON TETRACHLORIDE ADSORBED ON SCHUCHARDT'S SILICA GEL

Temperature (°K)	B_{2s} *(l/g)*	B_{3s} *(l²/mole·g)*	B_{2s}^2/B_{3s} *(moles/g)*	*Surface area from virial expansion (m²/g)*	*Surface area from BET method (m²/g)*
359.8	$4.30 \cdot 10^{-2}$	−20.77	$-8.9 \cdot 10^{-5}$	62.6	13.1
374.5	$2.85 \cdot 10^{-2}$	− 8.87	$-9.1 \cdot 10^{-5}$	64.0	13.9
393.0	$2.25 \cdot 10^{-2}$	− 2.94	$-9.9 \cdot 10^{-5}$	69.6	14.3
410.5	$1.20 \cdot 10^{-2}$	− 2.09	$-7.0 \cdot 10^{-5}$	49.2	9.9
419.5	$9.85 \cdot 10^{-3}$	− 1.24	$-7.8 \cdot 10^{-5}$	54.7	12.0
426.2	$8.69 \cdot 10^{-3}$	− 0.79	$-9.5 \cdot 10^{-5}$	66.8	13.6
Average values (over temperature)			$-8.9 \cdot 10^{-5}$	62.7	12.8

To use the BET method, we first calculated the adsorption isotherms for each temperature by considering them to be equal to ($B_{2s}\varrho + B_{3s}\varrho^2 + B_{4s}\varrho^3$). In this way six adsorption isotherms were obtained both for cyclohexane and for carbon tetrachloride. The BET values of the areas, calculated from these isotherms, are presented in Tables I and II. It is seen that like the virial expansion values, they are not temperature-dependent, though they do differ a little because of experimental errors.

One would expect the virial expansion values for surface area to be greater than those calculated by the BET method. The explanation for this is very simple as in the BET theory the lateral interactions are completely neglected. These lateral interactions have an effect of decreasing the effective area of adsorbent, since the repulsive part of these interactions contributes predominantely to the total interaction effect. This effective area is identified with the real area in the BET theory.

The most interesting result obtained here is, in our opinion, the degree of influence of the lateral interactions on the calculated surface areas. It seems that this effect is much greater than previously supposed.

TABLE III

RESULTS OF MEASUREMENTS AND CALCULATIONS FOR THE DATA OF SAMS *et al.*[2] CONCERNING ADSORPTION OF ARGON ON GRAPHITIZED CARBON BLACK P33.

Temperature (°K)	B_{2s} *(cm³/g)*	B_{3s} *(cm⁶/mole·g)*	*Surface area from three-dimensional treatment according to eqn. 18 (m²/g)*	*Surface area from two-dimensional treatment of* SAMS *et al. (m²/g)*
240.019	0.0812	− 91.1	—	
220.393	0.1158	− 123.5	—	
207.773	0.1508	− 136.5	—	
175.082	0.3650	− 425.7	—	
166.135	0.4933	− 442.9	—	
158.077	0.6720	− 248.7	—	
150.140	0.9421	631.9	—	
145.114	1.1850	2052.0	—	
140.607	1.4802	5215.0	15.8	9.0

Our results are now compared with the results obtained by SAMS *et al.* for argon adsorbed on graphitized Carbon Black P33 (ref. 2). The calculations of SAMS *et al.* are based on a two-dimensional model where lateral interactions are taken into account. They are included in Table III. In addition, the ratio B_{2s}^2/B_{3s} has been plotted as a function of absolute temperature in Fig. 1. It is seen from this figure that one may observe a temperature dependence of B_{2s}^2/B_{3s} when investigating wider temperature regions, especially at low temperatures. This is due to the fact that at low temperatures the second term in eqn. 16, which includes the factor $\{\exp[(\omega_{\min.}/-kT)] - 1\}$ cannot be neglected. On the other hand, at higher temperatures, the value B_{2s}^2/B_{3s} in Fig. 1 depends only slightly on temperature, *i.e.* this is the temperature region for which our assumptions are valid. One may extract from this a simple test for choosing the temperature region which is appropriate for surface area measurements, when using eqn. 18. This is the region of the plateau in the plot B_{2s}^2/B_{3s} *versus* T. However, it is not recommended that regions of very high temperatures are

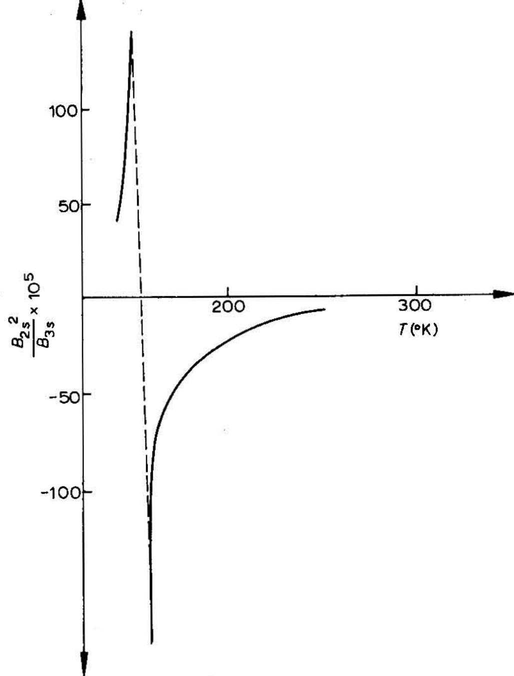

Fig. 1. Plot of B_{2s}^2/B_{3s} *versus* T for the data of SAMS *et al.* obtained for adsorption of argon on graphitized Carbon Black P33.

investigated, since the accuracy of the measurements decreases because of the small retention volumes.

If we now calculate the surface area of graphitized Carbon Black P33, using our method and the data of SAMS *et al.*, for the highest temperature under investigation, we get the value 15.8 m^2/g, whereas the value obtained from the Kirkwood–Miller formula (second-order interaction) gives a value of 8.64 m^2/g. It is of additional interest to compare our value with that obtained by SAMS *et al.* on the basis of the two-dimensional model, with lateral interactions taken into account. The value obtained by SAMS *et al.* is about 9 m^2/g. It follows that there is a considerable difference between our results, obtained by using the three-dimensional model of adsorption, and the results of SAMS *et al.* obtained using the two-dimensional model.

It is obvious that the finite thickness of the adsorbed layer considered by us introduces a much greater effect of repulsive interactions than is the case in a two-dimensional layer.

It now remains for us to decide which values for the surface areas are more suited to the real physical situation. In our opinion, in spite of a number of approximations made by us, our values should be closer to the true physical ones, since the three-dimensional model of adsorption is generally assumed to be the most realistic picture of physical adsorption.

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THE QUANTITATIVE ASSESSMENT OF FLUORESCENCE ON THIN MEDIA CHROMATOGRAMS

A THEORETICAL STUDY

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SUMMARY

Owing to inherent wavelength conversion in fluorescence analysis, the measuring signal is much less affected by optical background noise than is the case in absorptiometric measurements by both transmittance or reflectance procedures. This explains the higher sensitivity and accuracy obtained in fluorescence analysis. A further advantage of fluorimetry is the much wider range of concentrations over which the response is nearly linear. Measurements from either side of the medium are shown to be nearly equivalent in this respect. More light is, however, available if the measurements are carried out from the illuminated side.

INTRODUCTION

In several recent papers[1–3], we discussed the theory of the transmission and reflection of light in a dispersive medium based upon the simplified theory of KUBELKA AND MUNK[4]. The treatment was oriented towards the requirements of quantitative thin media chromatography (and electrophoresis) by photodensitometric methods. Fluorescence measurements, which in this field are equally important, were not covered. The present paper, therefore, corrects that omission and concentrates upon an application of the general theory to fluorescence methods.

In the case of fluorescence analysis, measurements are carried out at a wavelength of light different from the illuminating wavelength; this represents the fundamental difference between conventional transmittance or reflectance measurements and fluorescence procedures. In a sense, there is an analogy between fluorescence techniques and the use of frequency conversion in the common superheterodyne radio receiver. In this type of receiver, now used almost universally, the frequency of the

received signal is, at the input of the receiver, converted to a different wavelength; further processing of the signal is achieved in the new spectral position. The main advantages of this technique are better selectivity and higher sensitivity, the latter due to a reduction in noise.

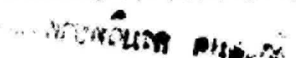
Similar causes are responsible for the advantages of fluorimetric methods compared with conventional photodensitometry. The sensitivity of all photometric methods is basically determined by the background noise[5-7]. In the common types of photometric instrumentation as used in chromatographic evaluation, this background noise consists of two components. One is electrical noise originating in the photo-detector element and in the input stages of the associated amplifier chain. The other is optical noise caused by random fluctuations of the optical transfer of the chromatographic medium. If sufficient light is available, the first component can be made negligibly small and so the sensitivity, accuracy and resolving power of the method are principally determined by the optical background noise.

The principal advantage of fluorescence procedures rests with the low level of optical background noise. Ideally, the medium itself should not fluoresce at all. As only the intensity of light at the wavelength of fluorescence is evaluated, the medium affects the received signal only in those areas where a fluorophore is present. Optical background noise is consequently generated only within the boundaries of the zone of separated fluorophore. Its intensity is, therefore, much smaller than in conventional photodensitometric evaluation because in this situation optical noise is generated all over the scanned area of the medium.

In practice, of course, this ideal situation is never achieved and some spurious fluorescence is encountered all over the medium, giving rise to an optical noise signal similar in character to that encountered in direct photodensitometry. Fortunately, however, the intensity of this signal is relatively weak in comparison with that encountered without wavelength conversion. It is this factor that makes fluorescence methods attractive as a simple and sensitive approach not requiring sophisticated instrumentation. This does not imply, of course, that the results obtained in fluorimetry could not be improved if more sophisticated instrumentation were to be used. It should be noted that the above argument applies only to direct fluorescence measurements and not to indirect methods such as quenching.

Recently, rather sophisticated and refined instruments for the photodensitometric evaluation of thin media chromatograms have been developed[8,9]. The basic principle in these cases has been to apply two light beams of different wavelengths, followed by a suitable combination of the two signals in such a way as to remove much of the background optical noise. Depending upon the amount of noise cancellation, these instruments produce results that are comparable with those obtained in simple fluorescence measurements.

Most of the versions of the double-beam principle as used in conventional photodensitometry are not very efficient when applied to fluorimetry because the reference beam tends to acquire noise information from parts of the medium outside the fluorescing zone. This information is not compensated for by a corresponding noise signal in the measuring beam and so, instead of an improvement in the signal to noise ratio, a deterioration often occurs. Moreover, noise due to spurious fluorescence outside the fluorescing zone is not affected at all. There are certain modifications of the double-beam principle, however, which will avoid the first of the above problems



while maintaining the principal advantages. Spurious fluorescence is, in this case, best reduced by computer processing based upon its statistical properties. On balance, therefore, an evaluation of the amount of a separated fluorophore in applicable cases, using proper instrumentation, still appears to offer the lowest obtainable sensitivity thresholds.

THE KUBELKA AND MUNK THEORY APPLIED TO FLUORIMETRY

When a beam of light impinges upon an optically turbid medium, part of it is absorbed and converted into heat, and part is scattered at the particle boundaries in the medium in all directions. To determine the optical transfer of such a medium is a complex and mathematically extremely difficult task. Fortunately, however, for many cases of technical importance a simplified theory developed by KUBELKA AND MUNK[4] gives adequate results. The basic assumption of this theory is that the light inside the medium propagates only in the forwards *and* in the backwards directions perpendicular to the boundary surfaces of the medium, which are assumed to be plane parallel.

When the conclusions obtained from the KUBELKA AND MUNK theory are verified experimentally by measuring the optical transfer of the medium, it has to be kept in mind that the above assumption applies only to points inside the medium. Surface points can, for most practical purposes, be considered as omnidirectional point sources of light. The KUBELKA AND MUNK theory gives with good approximation the total intensity of these fictitious light sources. The intensity of light radiated by them in a given direction is proportional to the cosine of the angle between that direction and the surface plane of the medium. The intensity decreases with the square of the distance from the radiating surface element. The measured optical transfer values, *e.g.*, optical density and coefficient of reflectance, are, therefore, strongly dependent upon the distance between medium and optical pick-up system and upon the angle of inclination of its optical axis against the surface plane of the medium (Fig. 1).

In the KUBELKA AND MUNK theory, two parameters are used to characterize the optical response of the medium. One is the coefficient of absorbance, K, and the

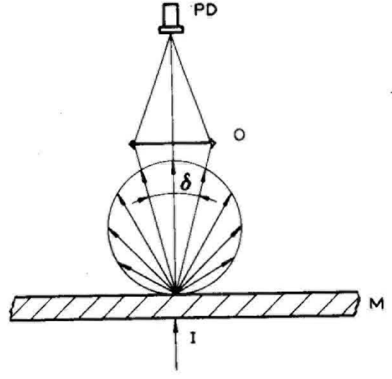


Fig. 1. Directional diagram of transmitted scattered radiation. M = medium; I = illuminating beam; O = optical pick-up system; PD = photo-detector.

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other the coefficient of scattering, S. If the theory is to be applied quantitatively, the numerical values of these parameters have to be known or they must be determined independently.

The theory further assumes that the medium can be considered as a sheet with plane parallel surfaces and that the direction of illumination is perpendicular to the surface. Between the boundary surfaces the medium is assumed to be homogeneous. This means that neither K nor S is a function of the distance x from the illuminated surface.

In chromatography, the concentration of a separated substance is very frequently a function of the distance from the surface. It is then mainly the coefficient of absorption, K, that changes. The resulting error is usually negligible if the concentration of the separated substance is small. The error encountered at higher concentrations depends upon the non-uniformity of the concentration profile, the values of the optical constants of the medium and the mode of photometric determination used.

Part of the light impinging upon the surface of the medium is immediately reflected from the surface without entering the medium to any appreciable depth. This "specular" reflection is not affected by the possible presence of the separated substance in the medium and, therefore, does not convey any information about its interior. From a chromatographic point of view, specularly reflected light is a loss and has to be discounted from the total intensity of illuminating light.

In fluorescence measurements, the wavelength of the primary illuminating beam is usually in the ultraviolet region and is, therefore, invisible to the naked eye. When fluorescence is encountered, part of the primary radiation is converted to a different wavelength, usually in the visible range. This energy is lost to the primary beam. The loss is irreversible very much like the loss by absorption. The proportion of primary energy converted at any given point inside the medium to a different wavelength can be designated the coefficient of fluorescence, F, which is, of course, proportional to the local concentration, C, of fluorescing substance:

$$F = \alpha \cdot C \tag{1}$$

The KUBELKA AND MUNK theory assumes that the medium is homogeneous; F is, therefore, considered to be independent of the distance from the surface. This assumption is, of course, a simplification, as the concentration profile of the separated substance will usually vary with depth.

The fundamental equations of the KUBELKA AND MUNK theory can be written in the following form (see ref. 1 for further details):

$$\begin{aligned} \frac{d^2 j(x)}{dx^2} &= \gamma^2 j(x) \\ \frac{d^2 r(x)}{dx^2} &= \gamma^2 r(x) \end{aligned} \tag{2}$$

Here $j(x)$ is the intensity of light travelling in the forwards direction and $r(x)$ that of the backwards travelling component, and x is the distance from the illuminated surface (see Fig. 2). For convenience, it is usual to assume that the thickness of the medium is equal to unity:

$$0 \leqslant x \leqslant 1 \tag{3}$$

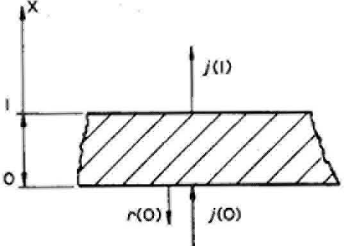

Fig. 2. Schematic representation of the KUBELKA AND MUNK hypothesis. $j(0)$ = incident light intensity (specular reflection discounted); $j(1)$ = transmitted light intensity leaving the medium with the angular distribution shown in Fig. 1; $r(0)$ = backscattered (reflected) light intensity.

The coefficient γ in eqn. 2 is very approximately equal to the optical density of the medium provided that the mentioned distance effects are discounted. It is, however, expressed in natural logarithmic units whereas conventional optical density is measured in logarithmic units to the base ten. In the solution of the above equations as derived in ref. 1, another coefficient, designated ϱ, appears. ϱ is the coefficient of re-reflexion for a medium with very large (theoretically infinite) optical density, and is measured on a linear, that is non-logarithmic, scale. It can be shown that

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

Both γ and ϱ can be determined from the optical constants, K and S, of the medium:

$$\gamma = \sqrt{K(2S + K)} \tag{5}$$

$$\varrho = \frac{K - \gamma}{K + \gamma} \tag{6}$$

From eqn. 6, it can be seen that ϱ does not depend upon the thickness of the medium.

The effect of fluorescence upon the propagation of the primary radiation is tantamount to an increase in absorbance, that is, an increase in energy loss of the exciting radiation:

$$\gamma_p = \sqrt{(K + F)\,(2S + F + K)} \tag{7}$$

$$\varrho_p = \frac{K + F - \gamma_p}{K + F + \gamma_p} \tag{8}$$

The total energy density of the exciting radiation at any point x in the interior of the medium is equal to the sum of the forwards and backwards travelling components:

$$v(x) = r_p(x) + j_p(x) \tag{9}$$

Addition of eqn. 2 gives:

$$\frac{\mathrm{d}^2 v(x)}{\mathrm{d}x^2} = \frac{\mathrm{d}^2}{\mathrm{d}x^2}[r_p(x) + j_p(x)] = \gamma_p{}^2[r_p(x) + j_p(x)] = \gamma_p{}^2 v(x) \tag{10}$$

Eqn. 10 is essentially identical to the well known equation describing an electrical transmission line with purely resistive parameters. A transmission line of

this type is easily implemented and can, therefore, serve as a convenient model to simulate the optical behaviour of turbid media[1]. The general solution to eqn. 10 is well known (see ref. 10 for details) and consequently it has not been listed here.

The intensity of fluorescence $f(x)$ excited at any particular point in the interior of the medium is proportional to the total energy there and, of course, to the coefficient of fluorescence F, which was assumed to be constant throughout the medium. This yields

$$f(x) = F\,v(x) \tag{11}$$

The propagation of the light created by fluorescence inside the medium can be described by a set of equations identical with those illustrated in eqns. 2. These two equations can again be combined into a single second-order differential equation of the same type as that listed in eqn. 10. The constants γ and ϱ appearing in the solutions are defined by eqns. 5 and 6, and they therefore do not contain the coefficient F. No index will be used to designate them.

It should be noted that the coefficients of absorbance and scattering are, in general, wavelength dependent. For chromatographic purposes, however, it is desirable to use a medium which is "gray" throughout the range of wavelengths employed, that is, a medium where the values of K and S change very little within this range. If F is then small compared with K, the coefficients γ and ϱ are essentially the same for both the primary and the excited radiations and the subscript p can, therefore, be deleted:

$$\begin{aligned} \gamma &\approx \gamma_p \\ \varrho &\approx \varrho_p \end{aligned} \tag{12}$$

The length of the optical path, which has to be traversed by the excited fluorescence before reaching the surface, is obviously dependent upon the coordinate x of the point where it was generated. There is no obvious reason why any particular direction of propagation should have preference over another. It can, therefore, be assumed that half of the generated intensity of fluorescent light travels in the forwards direction and the other half in the backwards direction. It should be noted that these are the only two directions permitted by the KUBELKA AND MUNK theory.

Let the transmittance of a sheet of medium thickness x now be $A_T(x)$. The fluorescent light generated at point x arrives, therefore, at the two surfaces with the intensities (see Fig. 3)

$$i(x)_{\text{far}} = \frac{F\,v(x)}{2} \cdot A_T(1 - x) \tag{13}$$

$$i(x)_{\text{near}} = \frac{F\,v(x)}{2} \cdot A_T(x) \tag{14}$$

The subscript "near" designates the illuminated surface and "far" the opposite one. The total intensity of fluorescence generated at point x is, of course, determined by eqn. 11.

Now, each point along the axis x contributes its share to the total intensity of fluorescence observed at the surface. This intensity is, therefore, the result of the

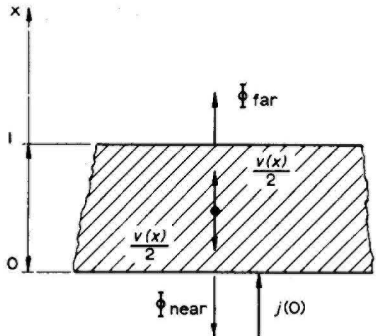

Fig. 3. The propagation of fluorescence radiation excited in the bulk of the medium. $v(x)$ = total energy density of primary radiation at x; Φ = total amount of fluorescence reaching the surface of the medium for $j(0) = 1$.

superposition of all elementary contributions for values $0 \leqslant x \leqslant 1$. Mathematically, this amounts to integration:

$$\Phi_{\text{far}} = \int_0^1 i(x)_{\text{far}} \mathrm{d}x = \int_0^1 \frac{F\, v(x)}{2} \cdot A_T(1 - x)\mathrm{d}x \tag{15}$$

$$\Phi_{\text{near}} = \int_0^1 i(x)_{\text{near}}\, \mathrm{d}x = \int_0^1 \frac{F\, v(x)}{2} \cdot A_T(x)dx \tag{16}$$

The mathematics for solving eqns. 15 and 16 will be published in a separate paper. The complete solutions are much too complex for interpretation in practical terms. However, for several cases of practical importance, considerable simplifications are possible. The results obtained for these specific cases will be discussed in the following sections.

THE CASE OF BEER'S LAW

The first case to be considered is that of a medium with very little scattering, where Beer's exponential law holds with reasonable accuracy. With such a medium, the back-scattered component of the incident light is too weak to permit useful measurements in the straight "reflectance" mode. Fluorescence measurements can, however, still be carried out from either side of the medium.

In an as yet unpublished theoretical study, the intensity of fluorescence measured at the illuminated (*i.e.*, "near") side of the medium is determined by the expression:

$$\Phi_{\text{near}} = \frac{F}{2K + F} \{1 - \mathrm{e}^{-(2K+F)}\} \tag{17}$$

At dilute concentrations of fluorophore it can usually be assumed that $F \ll K$, and eqn. 17 can then be simplified to:

$$\Phi_{\text{near}} \approx \frac{F}{2K} (1 - \mathrm{e}^{-2K}) \tag{17a}$$

If, in addition, the optical density of the medium is very low, a further simplification is possible:

$$1 - e^{-2K} \approx 2\,K; \qquad K \ll 1$$
$$\Phi_{near} \approx F \tag{17b}$$

In the opposite case, when the concentration of a strongly fluorescing substance is large, eqn. 17 becomes:

$$F \gg K$$
$$\Phi_{near} \approx [1 - e^{-F} \cdot e^{-2K}] \tag{17c}$$

For measurements from the far side of the medium, the expression is:

$$\Phi_{far} = e^{-K}(1 - e^{-F}) \tag{18}$$

At low concentrations of fluorophore, eqn. 18 reduces to:

$$F \ll 1$$
$$\Phi_{far} \approx F \cdot e^{-K} \tag{18a}$$

Comparison of eqns. 17 and 18 shows that both methods give, in most cases, comparable results. Measuring from the near side has the obvious advantage that the available intensity of light is larger. This factor only becomes important, however, in the case of media with very high optical density or when detecting equipment of low sensitivity is used.

TURBID MEDIUM WITH MEDIUM TO LARGE OPTICAL DENSITY

The next case to be considered is much more general and covers most media of practical importance. It is assumed that the blank medium has appreciable scattering and at the same time a transmittance lower than approximately 0.5 optical density units. Another simplifying assumption is that the optical parameters of the medium are very nearly the same for the exciting wavelength and the fluorescent response. In other words it is assumed that the medium is "gray" until far into the ultraviolet region and that the coefficient of fluorescence F is small compared with K (see eqn. 12).

The above assumptions are, of course, only approximately valid. They are, however, justified because of the considerable simplification to the resulting relations without producing excessive errors.

For the intensity measured at the near end of the medium the expression obtained (theoretical studies to be published) is:

$$\Phi_{near} \approx F \cdot \frac{1 - \varrho^2}{2\gamma} \tag{19}$$

Under the conditions assumed above, $e^{-\gamma}$ is very nearly equal to the transmittance of the medium for the incident radiation. $0.4\,\gamma$ is approximately equal to the optical density of the medium, if the distance effects mentioned earlier in this paper are discounted.

At the far side of the medium, the intensity of fluorescent light is:

$$\Phi_{far} \approx F \cdot \frac{e^{-\gamma}}{2\gamma} \cdot (1 - \varrho^2)\,(1 + \varrho) \tag{20}$$

In most practical cases, $\varrho^2 \ll 1$ so that the term $1 - \varrho^2$ in eqns. 19 and 20 can usually be neglected. It can also be shown (see eqn. 6) that the coefficient ϱ is independent of the thickness of the medium and, therefore, does not carry noise produced by random changes in the thickness of the medium.

Comparison of eqns. 19 and 20 shows that the intensity of fluorescent light is, in general, much higher at the near side than at the far side. The former is also less influenced by thickness variations of the medium and, therefore, less noisy.

A decided advantage of fluorescence measurements compared with absorptiometry is the extremely good linearity of response in terms of concentrations. Eqns. 15 and 20 show that the range of linearity for fluorescence measurements extends to much higher concentrations than is the case for conventional transmittance or reflectance measurements. This fact is, of course, empirically well known to chromatographic workers. Only at fairly high concentrations do deviations from linearity begin to appear. Fluorescence measurements can be made from either side of the medium without affecting the linearity.

Eqns. 19 and 20 also indicate why conventional double-beam systems are not very effective in further reducing the residual optical noise encountered when scanning a fluorescing zone. The expressions for conventional transmittance and reflectance measurements, as derived in ref. 1, differ considerably from the corresponding expressions developed in this paper for fluorescence intensity. A non-converted reference beam is, therefore, influenced by the optical background noise of the medium in a different way and noise cancellation by either difference or ratio forming of the two beam signals is possible only to a limited extent.

ACKNOWLEDGEMENTS

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CHROM. 6053

UNE MÉTHODE DE CHROMATOGRAPHIE GAZEUSE DE HAUT POUVOIR DE RÉSOLUTION, FACILE À AUTOMATISER

LA CHROMATOGRAPHIE À PHASE RÉTROGRADE À TROIS PRÉLÈVEMENTS

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SUMMARY

A gas chromatographic method with high resolving power, which can easily be automated: reversed-phase chromatography with three collectors

The fundamentals of a special method of gas chromatography are described. The solid phase, which is usually stationary, is made to retrograde and two differential detectors are connected at three points placed along the column. The device can be stabilised due to a simple control system and the percentage of a component present can be monitored continuously, or the full analysis of samples continuously taken may be obtained at very short intervals.

The resolving power of a column set up in this way is higher than that of the same column operated by the usual methods.

NOS BASES DE DÉPART

Depuis déjà plus de quinze ans FREUND *et al.*[1], BENEDEK ET SZEPESY[2,3], ou PICHLER ET SCHULZ[4] ont présenté une méthode de chromatographie gazeuse préparative où la phase adsorbante, au lieu d'être "stationnaire" comme dans les colonnes habituelles, est animée d'un mouvement en sens contraire à celui du gaz vecteur.

En reprenant les notations de LITTLEWOOD[10], considérons la masse de phase adsorbante par unité de longueur de la colonne. Cette masse adsorbante est ici animée d'une vitesse $\dot{x}$ par rapport à la paroi, en sens inverse du débit volumique $\dot{V}$ de gaz porteur entraînant un débit volumique $\dot{U}$ de l'échantillon, que nous supposerons un mélange des corps $a, b, \ldots. p$. Ainsi, pour les besoins de nos calculs ultérieurs nous définissons le gaz circulant dans la colonne comme un mélange de gaz porteur et des corps $a, b, \ldots. p$, à raison d'une masse $C_a, C_b, \ldots.. C_p$ par unité de volumes.

A l'équilibre la phase adsorbante capterait une masse q_n du corps n par unité de masse, et la loi de Henry s'écrit:

$$q_n = \beta_n C_n$$

Cette équation reste valable en régime dynamique si les vitesses d'adsorption sont grandes. Le débit global massique de la colonne en corps n vaut donc

$$\dot{w}_n = C_n \, (\dot{V} - \beta_n \, m \, \dot{x}) \tag{1}$$

Cette formule n'est toutefois valable que si elle conduit à une valeur positive de $\dot{w}_n$; si non

$$\dot{w}_n = 0 \text{ et } C_n = 0 \tag{2}$$

Le montage de FREUND *et al.*[1] et de BENEDEK ET SZEPESY[2,3] permet par conséquent de diviser un échantillon en deux de façon continue; l'une des fractions comporte tous les composants assez adsorbés par la phase solide traversant la colonne pour circuler dans le sens de cette dernière; l'autre fraction comporte tous les composants assez peu adsorbés pour sortir avec le gaz porteur.

L'expérience montre que cette séparation n'est pas très précise, parce que ces auteurs se contentent de laisser tomber en chute libre la phase adsorbante composée de granulés de charbon actif. Même en supposant que ce charbon était originellement d'une granulométrie rigoureusement régulière, par attrition certains grains s'allègent en fonctionnement plus que d'autres et leur vitesse limite de chute devient plus faible, tandis que le gaz porteur entraîne des très fines particules de charbon. Néanmoins ce montage peut rendre des services en préparative.

Pour aller plus loin, il faut adopter des colonnes-révolver comme celles décrites par BARKER ET HUNTINGTON[5] ou par TARAMASSO *et al.*[6,7]. Elles sont composées de petits segments de colonne disposés en série autour d'un support tournant et reliés par un distributeur à des arrivées de fluides alimentaires fixes.

Si les tronçons de colonne étaient très courts et très nombreux, tout se passerait dans les colonnes-révolver comme dans le montage décrit en premier lieu, mais avec une définition bien plus rigoureuse de la vitesse $\dot{x}$ de la phase adsorbante. Or on peut montrer qu'il n'est pas nécessaire en fait que le nombre des tronçons soit tellement élevé pour que ce résultat soit atteint avec une bonne approximation. Si on arrive à vaincre les difficultés matérielles nouvelles amenées par la présence d'un distributeur tournant, les montages de BARKER ET HUNTINGTON[5] ou de TARAMASSO *et al.*[6,7] présentent donc un avantage de précision sur celui de FREUND *et al.*[1] et BENEDEK ET SZEPESY[2,3], et ceci va nous permettre d'exploiter la colonne d'une façon assez particulière, par différence de composition.

PRINCIPE DE LA MÉTHODE

Sur une colonne à phase rétrograde, effectuons en un point donné B, un soutirage de débit $\dot{B}$. Le débit de gaz porteur va passer en ce point de $\dot{V}$ à $\dot{V} - \dot{B}$.

Si la formule 1 conduit pour un composant m de l'échantillon à une valeur positive de $\dot{w}$ en amont du soutirage, elle conduit en général aussi à une valeur de $\dot{w}$ positive en aval; la concentration C_n en corps n est alors la même dans le gaz situé dans la colonne en amont de la fuite, dans le gaz soutiré et dans le gaz situé dans la colonne

en aval de la fuite: la fuite n'a aucune action sur la composition du gaz circulant dans la colonne, même si la valeur de $\dot{w}$ n'est que faiblement positive.

Si la formule 1 conduit en aval de la fuite pour un composant n de l'échantillon à une valeur négative, de si peu que ce soit, la formule 2 doit être appliquée; la fuite produit donc une discontinuité de composition du gaz circulant dans la colonne, puisque la teneur C_n en composant n de l'échantillon, qui était restée constante depuis l'injection de l'échantillon jusqu'à ladite fuite, devient brusquement nulle en aval.

La méthode d'exploitation que nous proposons, pour des colonnes d'un type déjà connu, repose entièrement sur cette discontinuité de composition autour d'une fuite[8].

EXPLOITATION DE LA COLONNE

Il suffit alors de prélever un petit débit $\dot{A}$ en amont de B et un petit débit $\dot{C}$ en aval de B et d'installer un détecteur différentiel sur ces deux débits pour que l'indicateur y de ce capteur donne

$$y = \left[\sum_{n=a}^{p} C_n \right]_A - \left[\sum_{n=a}^{p} C_n \right]_C = C_m \tag{3}$$

On voit que si la vitesse $\dot{x}$ de rétrogradation de la colonne varie continûment de 0 à $\dot{X}$, le détecteur différentiel donne une série de pics correspondant à chacun des composants $a, b, \ldots. p$, la hauteur des pics (et non leur surface) étant proportionnelle à $C_a, C_b, \ldots. C_p$.

On peut automatiser l'appareil et supprimer l'effet d'une dérive progressive des qualités du garnissage de la colonne en plaçant un second détecteur différentiel sur les prélèvements $\dot{B}$ et $\dot{C}$ qui fournit une donnée supplémentaire z (voir Fig. 1).

Le rapport z/y des indications des deux indicateurs, soit

$$\frac{z}{y} = \frac{\left[\sum_{n=a}^{p} C_n \right]_B - \left[\sum_{n=a}^{p} C_n \right]_C}{\left[\sum_{n=a}^{p} C_n \right]_A - \left[\sum_{n=a}^{p} C_n \right]_C} \tag{4}$$

varie de 1, valeur qu'il prend pendant la période de croissance de y, à 0 pendant sa période de décroissance, si bien qu'en règlant la vitesse $\dot{x}$ de la colonne en fonction de z avec pour valeur de consigne $z = 1/2$, y se stabilise à sa valeur maximale correspondant au milieu de la vague. Entre deux pics le rapport z/y, en principe indéterminé, retourne en fait de 0 à 1.

Avec ce montage, l'appareil donne en permanence la teneur C_m de l'échantillon en corps m.

Un autre système d'exploitation automatique du montage (voir Fig. 2) consiste à envoyer en permanence y dans un enregistreur à bande déroulante, et à déclancher l'application de la plume sur le papier seulement quand $z = 1/2$ par valeur décroissante. La vitesse $\dot{x}$ de la colonne variant lentement de 0 à X à la commande d'une horloge, puis s'annulant brusquement et augmentant de nouveau jusqu'à X, etc. ..., l'enregistreur trace par points une courbe $C_m = f(t)$ pour chaque composant de l'échantillon; la seule difficulté est d'identifier ces courbes, et d'étalonner l'appareil pour chacun de ces corps, surtout s'il apparaît une courbe nouvelle lors de la sur-

veillance d'un échantillon prélevé en permanence sur une installation industrielle, par suite de l'apparition d'un composant non prévu à l'avance.

POUVOIR DE RÉSOLUTION

Pour calculer le pouvoir de résolution nous devons examiner ce qui se passe lorsque la vitesse de transfert de matière entre phase adsorbée et phase gazeuse n'est plus très grande. Appelons x l'abscisse curviligne d'un point courant de la colonne. Le bilan-matière pendant un temps dt dans un petit segment dx de colonne, pour le corps n s'écrit (en omettant l'indice n qui surchargerait l'écriture) pour le gaz

$$\dot{V}\, C\, \mathrm{d}t = V\left(C + \frac{\partial C}{\partial x}\, \mathrm{d}x\right) \mathrm{d}t - K\,(\beta C - q)\, m\, \mathrm{d}x\, \mathrm{d}t + \frac{\partial C}{\partial t}\, a\, \mathrm{d}x\, \mathrm{d}t \tag{5}$$

K étant la vitesse d'adsorbtion suivant LITTLEWOOD[10] et pour le solide

$$\dot{x}\, m\left(q + \frac{\partial q}{\partial x}\, \mathrm{d}x\right) \mathrm{d}t - K\,(\beta\, C - q)\, m\, \mathrm{d}x\, \mathrm{d}t = \dot{x}\, m\, q\, \mathrm{d}t + \frac{\partial q}{\partial t}\, m\, \mathrm{d}x\, \mathrm{d}t \tag{6}$$

d'où, en tirant q de la première équation et en portant sa valeur dérivée dans la seconde

$$(m\, \dot{x}\, \beta - V)\, \frac{\partial C}{\partial x} - \left(m\, \beta + \frac{a}{K}\right) \frac{\partial C}{\partial t} - \frac{1}{K}\,(a\, \dot{x} - V)\, \frac{\partial^2 C}{\partial x \partial t} - \frac{\dot{x}\, \dot{V}}{K}\, \frac{\partial^2 C}{\partial x^2} -$$

$$- \frac{a}{K}\, \frac{\partial^2 C}{\partial t^2} = 0 \tag{7}$$

Cette équation se simplifie en régime permanent

$$(m\, \dot{x}\, \beta - V)\, \frac{\partial C}{\partial x} - \frac{\dot{x}\, \dot{V}}{K}\, \frac{\partial^2 C}{\partial x^2} = 0 \tag{8}$$

avec

$$K\,(\beta\, c - q) - \frac{\dot{V}}{m}\, \frac{\partial C}{\partial x} = 0. \tag{9}$$

Sa solution avec P et Q comme constantes d'intégration est:

$$C = P\, \frac{\dot{x}\, \dot{V}}{K\,(m\, \dot{x} - \dot{V})}\, \mathrm{e}^{K \frac{m\, \beta\, \dot{x} - \dot{V}}{\dot{x}\, \dot{V}} x} + Q \tag{10}$$

$$q = \frac{P\, \dot{V}}{K\, m} \left(\frac{m\, \beta\, \dot{x}}{m\, \beta\, \dot{x} - \dot{V}} - 1\right) \mathrm{e}^{K \frac{m\, \beta\, \dot{x} - \dot{V}}{\dot{x}\, \dot{V}}} + Q \tag{10}$$

On voit que si $m\, \dot{x} - \dot{V}$ est négatif, c tend rapidement vers des valeurs très petites après la fuite, mais ne s'annule jamais complétement sur une longueur finie L (appelons η l'erreur relative correspondante):

$$L = \frac{\dot{x}\, \dot{V}}{K\,(m\, \beta\, \dot{x} - \dot{V})}\, \log \eta \tag{11}$$

En conséquence deux corps m et n ayant des coefficients de partage voisins ne sont séparés que si

$$\dot{V} > \frac{\beta_n}{\beta_m - \beta_n} (\dot{A} + \dot{B} + \dot{C}) \tag{12}$$

$\dot{A}$, $\dot{B}$ *et* $\dot{C}$ étant le débit des détecteurs A, B *et* C.

Puisque l'asservissement stabilisant la colonne assure la relation

$$\beta\, m\, \dot{x} - \dot{V} = \dot{A} + 0.5\, \dot{B} \tag{13}$$

on voit que si $\dot{A} = \dot{B} = \dot{C}$, il faut que

$$L > 2\, \frac{\dot{x}}{K} \frac{\beta_n}{\beta_m - \beta_n} \log \eta \tag{14}$$

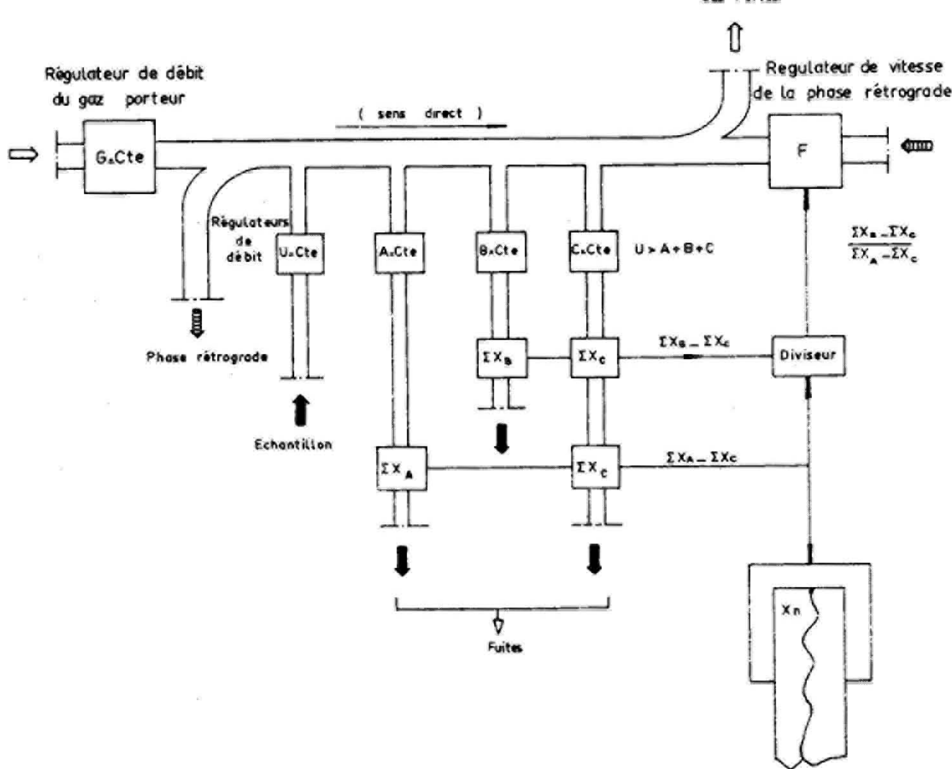

Fig. 1. Dosage continu d'un composant pas différence. La colonne de chromatographie est schématisée par le conduit horizontal, portant à gauche le régulateur de débit de gaz porteur, et à droite le régulateur de vitesse de la phase rétrograde.

Entre la sortie de phase rétrograde, à gauche, et la sortie de gaz porteur, à droite, on voit, régulièrement espacés, l'entrée continue de l'échantillon, puis les trois prélèvements.

Un détecteur différentiel est placé sur les prélèvements B et C et donne directement la variation de la propriété mesurée par le détecteur entre B et C, $\Sigma X_B - \Sigma X_C$; un second détecteur différentiel basé sur la mesure de la même propriété, placé sur les prélèvements A et C, donne $\Sigma X_A - \Sigma X_C$. Cette dernière grandeur est d'une part inscrite en bas et à droite, sur un enrégistreur à bande déroulante, d'autre part comparée à $2(\Sigma X_B - \Sigma X_C)$ par le "Diviseur": si elle est plus grande, le diviseur donne l'ordre au régulateur F de diminuer la vitesse de la phase rétrograde; si elle est plus petite, l'effet du régulateur s'inverse.

Dans ces conditions l'enrégistreur inscrit en permanence la valeur de X_n pour un même composant de l'échantillon prélevé de façon continue, même si les propriétés du garnissage dérivent dans le temps.

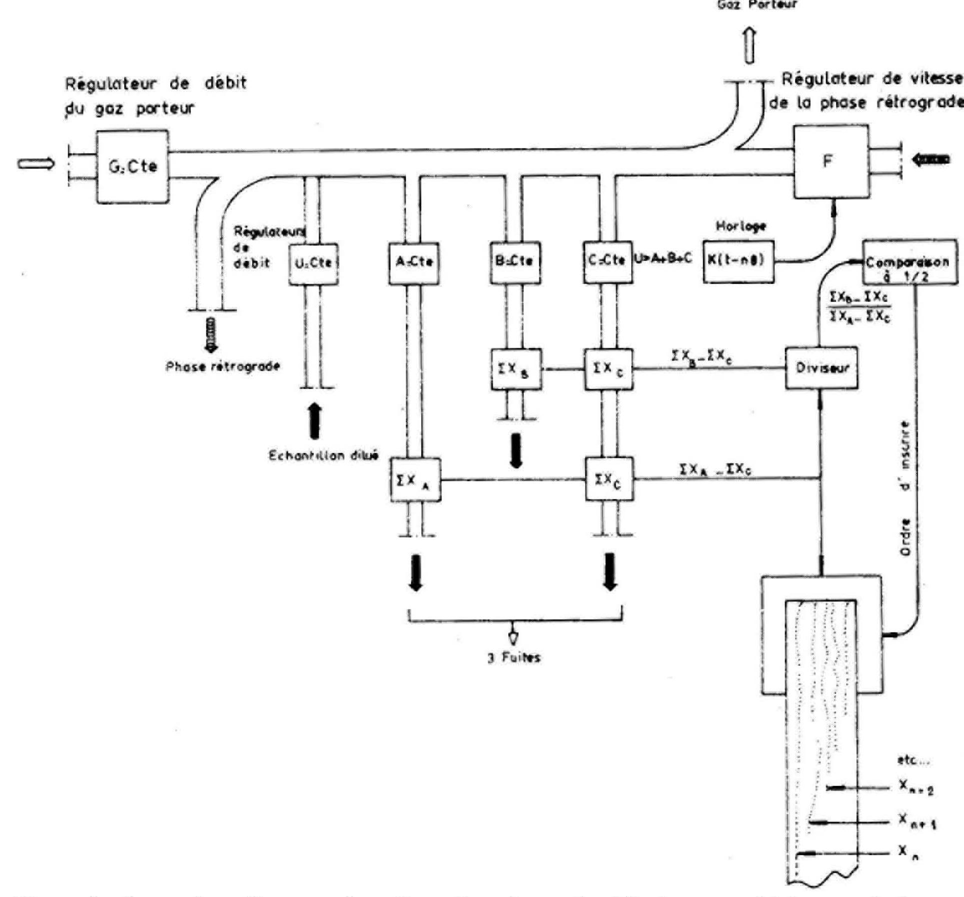

Fig. 2. Analyse automatique quasi-continue. La colonne, les détecteurs, enrégistreurs, régulateurs, sont disposés de la même façon que sur la Fig. 1, mais exploités autrement. De plus une horloge est ajoutée au montage. Elle donne au régulateur de vitesse de la phase rétrograde une valeur de consigne augmentant linéairement avec le temps; puis retombant brusquement à zéro, puis augmentant de nouveau, etc., en dents de scie.

Au lieu d'appuyer en permanence sur le papier, la plume de l'enrégistreur ne le fait que sur ordre donné par le diviseur lorsque

$\Sigma X_A - \Sigma X_C$ est égal à $2[\Sigma X_B - \Sigma X_C]$

Dans ces conditions l'enrégistreur trace autant de courbes pointillées que de composant séparable de l'échantillon, chacune indiquant la teneur d'un composant; le numéro de chaque composant peut être repéré en augmentant la vitesse de déroulement du papier, pour rendre perceptible l'ordre dans lequel les courbes sont tracées; en général on n'effectuera cette opération que s'il apparaît une courbe nouvelle, correspondant à un composant dont la teneur était jusqu'alors trop faible pour qu'il soit séparé. Le montage permet donc de surveiller une manipulation de chimie de longue durée, sans laisser échapper des composants imprévus ou de présence fugitive.

ce qui permet d'évaluer la taille à donner à la colonne pour obtenir une résolution donnée.

Pour fixer les idées on peut calculer que pour séparer la 2,5-xylidine de la 2,6-xylidine sur squalane correspondant à

$$\frac{\beta_n}{\beta_m - \beta_n} = 450 \qquad (15)$$

avec des détecteurs demandant un débit $\dot{A} = 1$ cm³/min avec un facteur de précision de 10^{-4}, il faut un débit de gaz porteur $\dot{V} = 900$ cm³/min et une vitesse de rétrogradation du garnissage adsorbant $\dot{x} = 110$ cm³/min; la section de la colonne doit atteindre environ 0.4 cm². La longueur entre le point d'injection du porteur et la première fuite A, ou entre A et B, ou entre B et C, ou entre C et la sortie serait de 1 m, et la longueur totale de la colonne, rinçage compris, de 6 m, donc relativement faible pour un tel pouvoir de résolution.

Nous ne nous étendrons pas sur les autres causes d'erreur. En effet la principale est le nombre limite de segments élémentaires entre deux trous du distributeur relié à la colonne; les calculs d'erreur sont trop longs pour être exposés ici, mais montrent qu'il est inutile de prévoir dans l'exemple précédent des tronçons élémentaires d'une longueur inférieure à 10 cm.

Signalons seulement que la diffusion longitudinale de l'échantillon dans le gaz porteur n'a pratiquement aucune influence. La forme des pics n'ayant aucun rapport avec le régime (continu) d'injection de l'échantillon, une des causes importante[9] d'erreur sur les colonnes exploitées de façon habituelle se trouve *ipso-facto* éliminée.

RÉSUMÉ

On décrit le principe d'une méthode particulière de chromatographie gazeuse, consistant à faire rétrograder la phase solide habituellement stationnaire, et à placer deux détecteurs différentiels branchés en trois points échelonnés sur la colonne. L'appareil obtenu peut être stabilisé grâce à un asservissement simple et donne soit en continu la teneur en un composant, soit à intervalles rapprochés répétitifs l'analyse compléte d'un èchantillon prélevé de façon continue.

On montre que le pouvoir de résolution d'une colonne ainsi équipée est supérieur à celui que donnerait la même colonne exploitée suivant les méthodes habituelles.

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DIE GENAUIGKEIT DER GASCHROMATOGRAPHISCHEN ANALYSE VON FETTSÄUREN

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SUMMARY

Accuracy in gas chromatographic analysis of fatty acids

The results of a collaborative study made by ten Dutch laboratories showed that in gas chromatographic analyses of fatty acids in three test samples systematic errors were made. The data obtained with the third sample investigated were, in general, less dispersed than those obtained with the first. Yet the systematic errors were not eliminated and deviations of 10% and more still occurred. Comparison with an American collaborative study showed no difference in the accuracy of the results. From the results it is possible to estimate the reliability of the analysis of a specific component at a certain concentration level.

EINLEITUNG

Viele Laboratorien in der Lack- und anverwandten Industrie führen regelmässig gaschromatographische (GC) Analysen von Fettsäuren durch, die als solche oder in der Form von Ölen verwendet werden. Vor einigen Jahren wurde von Seiten der niederländischen Industrie die Frage nach der Zuverlässigkeit der in den Laboratorien durchgeführten Bestimmung gestellt.

Die Faktoren welche die quantitative GC Bestimmung der Fettsäuren beeinflussen wurden schon von verschiedenen Forschern nachgegangen[1-3]. Es gibt jedoch noch verhältnismässig wenig Veröffentlichungen über Ringversuche. Erst neuerdings wurden von HERB UND MARTIN[4] die Resultate eines Ringversuches von 30–40 amerikanischen Laboratorien bekannt gegeben. Deshalb wurde damals von zehn niederländischen Laboratorien beschlossen einige Testgemische gemeinschaftlich zu analysieren.

Eine erste Voraussetzung zur Erzielung von statistisch wertvollen Resultaten ist eine genügend genormte Untersuchungsmethode. Dies war leider nicht möglich, da jedes Laboratorium seinen eigene Typ Gaschromatograph, ausgestattet mit Flammionisation- oder Katharometerdetektion, hatte. Ausserdem verwenden die Laboratorien verschiedene Trennsäule und hat jedes seine eigene Analysenmethode. Eine zusammenfassende Übersicht wird in Tabelle I gegeben. Aus diesen Gründen wurde

TABELLE I

ARBEITSWEISE BEI DER GASCHROMATOGRAPHISCHEN ANALYSE VON FETTSÄUREMETHYLESTER

Lab.	*Detektor*	*Trennsäule*					*Trennbedingungen*				
		Träger-material	*Trenn-füllung*	*Fülling/Träger (%)*	*Länge (cm)*	*Durch-messer (cm)*	*T Proben-eingang (°C)*	*T Säule (°C)*	*Träger-gas*	*Gasdurch-fluss (ml/min)*	*P (kg/cm²)*
4	FID	Diat. S 80–100	PEGA	5	120	0.4	215	180	N_2	60	1.1
1	FID	Diat. S 60–80	DEGS	10	180	0.46	290	175	He	40	—
3	FID	Embacel 60–80	DEGS	15	300	0.6	300	200	N_2	65	—
5	Wärmeleitf.	Embacel	DEGS	30	225	0.6	200	180	H_2	83	1.05
6	Wärmeleitf.	Diat. W	DEGS	25	350	0.6	260	200	H_2	40	—
2	FID	Chrom. W 60–80	BDS	15	200	0.5	260	200	N_2	30	0.75
7	FID	Gesil. Supercel 22	EGS	15	170	0.4	225	195	He	30	—
9	FID	Chrom. W 60–80	DEGS	20	200	0.6	270	15–225	He	75	—
8	FID	Chrom. W 60–80	DEGS	10	250	0.6	290	200	N_2	25	—
10	Wärmeleitf.	Chrom. W 60–80	DEGS	20	250	0.6	300	150–220	H_2	80	—

von einer Normung abgesehen und wurden die Laboratorien gebeten die Analysen auf gewohnte Weise durchzuführen.

Es wurden von uns drei Testgemische zur Analyse angeboten und zwar (in chronologische Folge): (i) Ein Gemisch von Methylestern von Leinölfettsäuren, von uns aus Leinöl mit Hilfe von Natriummethanolat in Methanol hergestellt (Testgemisch 1). (ii) Ein Gemisch von Methylestern von Fettsäuren, von uns durch Einwage der GC reinen Komponenten hergestellt (Testgemisch 2). (iii) Ein Öl, von uns an die zehn Laboratorien geschickt und von diesen in eigener Weise umgesetzt in das Gemisch von Fettsäuremethylester und analysiert (Testgemisch 3).

Da aus dem Gemisch der eingewogenen Methylester die meisten Schlüsse über die Genauigkeit der Analyse gezogen werden können, werden die Resultate dieser Untersuchung zuerst besprochen.

Auf die, bei der Bearbeitung der Resultate gebrauchten statistischen Methoden (u.a. Varianten analyse und Teste nach STUDENT UND SCHEFFÉ), wird nicht eingegangen. Nur sollen die Resultate dieser Bearbeitung besprochen werden.

ANALYSERESULTATE UND DISKUSSION

Analyse vom Gemisch Fettsäuremethylester mit bekannter Zusammensetzung (Testgemisch 2)

Die Ergebnisse der GC Analyse vom Testgemisch 2 sind in Tabelle II gegeben. Aus der statistischen Bearbeitung dieser Resultate ergibt sich folgendes:

(1) Die Unterschiede zwischen den Mittelwerten der Laboratorien sind reell und nicht zufällig.

(2) Der Gesamtmittelwert von zehn Laboratorien pro Komponente unterscheidet sich wesentlich von der eingewogenen Menge.

TABELLE II

ERGEBNISSE DER ANALYSE EINES GEMISCHES EINGEWOGENER FETTSÄUREMETHYLESTER (TESTGEMISCH 2)[a]

Komponente[b]	*Einwaage*	*Labor*										*Gesamt-mittel-wert*
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	
14:0	8.61	8.1	7.2	8.41	9.3	7.7	6.9	9.3	10.0	9.7	9.5	
		9.0	7.3	8.65	9.0	7.6	6.9	9.6	9.8	9.6	9.6	8.7
		9.4	7.4	8.54	8.7	7.7	6.9	9.0	10.3	9.6	9.6	
16:0	17.40	16.9	16.5	17.76	18.1	17.7	15.6	17.7	19.4	18.7	18.9	
		17.5	16.6	17.96	18.1	16.8	16.5	17.5	19.5	18.8	19.3	17.9
		17.7	16.3	17.82	17.9	17.3	16.0	17.7	20.0	18.9	19.3	
18:0	27.13	27.2	28.5	27.95	26.9	28.4	27.6	28.4	28.8	28.0	27.5	
		25.6	28.1	28.04	27.0	27.7	27.2	28.6	28.5	28.0	28.1	27.8
		25.6	28.5	28.25	27.0	28.2	26.9	28.7	28.1	28.0	27.5	
18:2	28.58	29.0	29.6	28.50	28.1	28.9	28.5	28.2	27.5	27.4	27.5	
		28.7	29.6	28.42	28.2	30.0	28.6	28.4	27.8	27.5	27.1	28.4
		28.8	29.5	28.38	28.8	29.3	28.4	28.5	27.5	27.4	27.5	
18:3	18.28	18.8	18.2	17.38	17.6	17.3	18.7	16.3	14.3	16.2	16.5	
		19.2	18.4	16.94	17.7	17.9	19.2	16.0	14.4	16.0	15.9	17.1
		18.5	18.3	17.02	17.6	17.5	18.2	16.1	14.1	16.1	16.1	

[a] Gew. %.

[b] 14:0 = Myristinsäure; 16:0 = Palmitinsäure; 18:0 = Stearinsäure; 18:1 = Ölsäure; 18:2 = Linolsäure; 18:3 = Linolensäure.

(3) Es ist nicht möglich, eines oder mehr Laboratorien anzuweisen, deren Ergebnisse extrem abweichen, wodurch die Folgerungen (1) und (2) erklärt werden können. Es kann deshalb auch keine kleinere Gruppe von Laboratorien gefunden werden deren Ergebnisse unbedingt übereinstimmen.

(4) Keines der Laboratorien führt die Analyse für alle Komponenten fehlerfrei aus, d.h. dass die Differenz zwischen der eingewogenen und der gefundenen Menge nicht immer aus der Streuung der Resultate innerhalb eines Laboratoriums erklärt werden kann. Sogar das "beste" Laboratorium findet für zwei Komponenten noch ein wesentlich andere Quantität als eingewogen wurde. Das "schlechteste" Laboratorium hat keine einzige Menge genau gefunden.

In analytischer Hinsicht ist dieses Resultat enttäuschend, da die Streuung der Ergebnisse offenbar keine zufälligen Ursachen hat, sondern systematisch ist. Wie aus Tabelle III hervorgeht haben die Laboratorien 1 und 4 die besten Erfolge erzielt. Weiterhin zeigt die Tabelle, dass Abweichungen von mehr als 10% keine Ausnahme sind. Man könnte denken dass die Laboratorien 1 und 4 "gute" Erfolge durch ihre Arbeitsweise erzielt haben. Aus Tabelle I kann jedoch keine Beziehung zwischen dem Ergebnis und der Arbeitsweise gefunden werden.

TABELLE III

DIE ZUVERLÄSSIGKEIT DER LABORE

Labor	*Anzahl mittlerer Werte*[a] *innerhalb der angegebenen Grenze*[b]		
	±2½%	*±5%*	*±10%*
1	4	5	5
2	1	4	4
3	2	4	5
4	3	5	5
5	2	4	5
6	3	3	4
7	2	2	3
8	0	2	2
9	0	2	4
10	1	1	2

[a] Die Anzahl mittleren Werte (aus drei Analysen) ist über allen Komponenten summiert.
[b] Relative Abweichung der eingewogenen Menge.

Die geringe Übereinstimmung zwischen den Laboratorien könnte vielleicht u.a. verursacht sein durch die Art der untersuchten Verbindungen. Ob eine Verbindung bei der Analyse im Vergleich mit anderen Verbindungen Schwierigkeiten verursachen kann, zeigt vielleicht der Variationskoeffizient.

$$\text{Variationskoeffizient} = \frac{\text{Standardabweichung}}{\text{Menge}} \times 100$$

Bei vielen quantitativen chemischen Bestimmungen zeigt sich, dass die Streuung der Ergebnisse zunimmt, wenn die Menge des zu bestimmenden Stoffes grösser wird. Der Variationskoeffizient wird dabei kleiner oder bleibt mindestens gleich. Wenn nun für die verschiedenen Komponenten die Variationskoeffizienten berechnet werden und danach den zugehörenden Konzentrationen zugeordnet werden, so ergibt sich, dass

für Linolensäure (18:3) der Variationskoeffizient höher ist als auf Grund der Konzentration erwartet werden muss. Man kann also annehmen, dass bei dieser Komponente ein extra systematischer Fehler zwischen den Laboratorien auftritt, was bei den anderen Komponenten nicht der Fall ist.

Die Analyse von Gemischen von Fettsäuremethylestern bereitet aus Ölen (Testgemische 1 und 3)

Die Resultate der Analysen der Testgemische 1 und 3 sind in den Tabellen IV und V angeführt. Aus beiden Analysen können dieselben Schlussfolgerungen gezogen werden als bei der oben beschriebenen Untersuchung vom Testgemisch 2, und zwar:

(i) Es gibt wesentliche Unterschiede zwischen den Mittelwerten der zehn Laboratorien.

(ii) Es ist unmöglich durch ausser Betracht lassen der extremen Resultate, eine kleinere Gruppe zu finden derer Mittelwerte als gleich zu betrachten sind.

Ebenso wie bei der vorigen Untersuchung kann aus dem Unterschied in der Ausführungsweise keine Anweisung für dieses schlechte Resultat gefunden werden. Auch hier wurden für beide Analysen die Variationskoeffizienten berechnet. Auch hier ergab sich dass bei der Untersuchung des Testgemisches 1, sowie des Testgemisches 2, in der Bestimmung von Linolensäure (18:3) ein extra systematischer Fehler zwischen den Laboratorien gemacht sein muss. Beim Testgemisch 3 war dies nicht der Fall. Das bessere Ergebnis könnte darauf weisen dass die Bestimmung der Linolensäure genauer durchgeführt wurde.

Eine wichtige Anweisung für die mögliche Ursache der Streuung folgt aus einer Vergleichung von Gewichtsprozentwerten und Peakfläche-Prozentwerten, wie sie am Testgemisch 1 gefunden wurden. Wenn die Laboratorien mit den richtigen Korrekturfaktoren arbeiten, dürfte man erwarten dass die Streuung zwischen den Flächen-

TABELLE IV

ERGEBNISSE DER GASCHROMATOGRAPHISCHEN ANALYSE VON LEINÖLFETTSÄUREMETHYLESTER (TESTGEMISCH 1)[a]

Komponente[b]	*Labor*										*Gesamt-mittelwert*
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	
16:0	6.3	5.75	6.4	6.5	5.6	5.2	6.8	6.5	6.6	7.2	
	6.4	5.94	6.2	6.1	6.0	5.3	6.9		6.7	7.9	6.3
	6.9	5.98	6.6	6.1	5.5	5.7	6.5		6.8	7.0	
18:0	4.5	4.60	5.1	4.4	4.0	4.0	4.7	4.2	4.4	3.4	
	4.3	4.63	4.6	4.5	3.7	4.0	5.1		4.1	2.5	4.2
	4.3	4.62	5.2	4.3	3.4	4.2	4.5		4.5	2.5	
18:1	19.2	18.67	19.1	19.2	18.1	16.8	20.2	18.5	19.3	22.8	
	19.0	18.43	19.5	18.9	18.0	17.1	20.4		19.5	23.4	19.2
	19.2	18.46	19.4	18.8	18.1	17.2	19.9		19.1	23.1	
18:2	15.1	15.85	16.1	15.3	14.8	14.2	16.0	14.9	15.1	15.1	
	15.0	15.79	16.2	16.4	15.0	14.6	16.4		15.2	16.1	15.4
	15.4	15.79	15.9	15.9	14.7	14.2	16.0		15.2	15.8	
18:3	54.8	55.13	53.2	54.7	57.5	58.2	52.4	53.6	54.6	51.5	
	55.3	55.21	53.5	54.0	47.2	57.8	51.3		54.5	50.1	54.4
	54.2	55.15	53.0	54.8	58.3	56.2	53.0		54.4	51.5	

[a] Gew. %.
[b] Für eine Zeichenerklärung siehe Fussnote zu Tabelle II.

TABELLE V

ERGEBNISSE DER ANALYSE VON EINEM ÖL (TESTGEMISCH 3)

Komponente[b]	*Labor*					
	1	*2*	*3*	*4*	*5*	*6*[a]
	$NaOCH_3$ in Methanol	*Schwefelsäure in Benzol–Methanol (1:3)*	*Methanolyse*	*Dimethoxypropan/Methanol*	*Verest. mit Methanol/p-toluensulfonsäure*	*Verest. mit Methanol + 2% Schwefelsäure*
16:0	9.0	8.8	8.8	9.3	8.5	8.9
	8.8	8.3	8.9	9.5	8.4	7.8
	7.7	8.4	8.7	9.4	8.5	8.9
18:0	4.2	7.1	3.8	4.8	4.5	4.7
	4.1	7.2	3.7	4.7	4.5	4.5
	4.2	7.1	3.4	5.0	4.4	4.6
18:1	20.8	22.5	19.0	21.1	20.9	21.4
	20.6	22.5	19.1	21.1	21.3	21.5
	21.1	22.6	18.9	21.1	21.0	21.6
18:2	33.3	30.5	33.5	32.4	33.5	32.0
	33.3	30.5	33.4	32.1	33.8	33.3
	33.9	30.3	34.1	32.0	33.4	32.7
18:3	32.7	31.2	33.5	32.3	32.6	33.0
	33.2	31.6	33.6	32.6	32.0	32.9
	33.1	31.6	34.2	32.5	32.7	32.2

[a] Dieses Labor hat zwei Veresterungsmethoden verwendet.
[b] Für eine Zeichenerklärung siehe Fussnote zu Tabelle II.

Prozentwerten grösser ist als zwischen den Gewichtsprozentwerten. Tabelle VI zeigt dass dies nicht der Fall ist. Das bedeutet, dass für die Umrechung die Korrekturfaktoren gegen alle Erwartungen nicht richtig sind. Die Streuung innerhalb der einzelnen Laboratorien ist für Flächen- und Gewichtsmengen dieselbe; dies ist deutlich, weil jedes Laboratorium seine eigenen Korrekturfaktoren gebraucht.

Eine andere Quelle von systematischen Fehlern bei der Berechnung der Zusammensetzung aus dem Chromatogramm könnte die Messung der Peakfläche sein. Diese kann auf verschiedenen Weisen durchgeführt werden. Daher wurden die Peakflächen der Chromatogramme von Testgemisch 1 von fünf Personen aufs neue gemessen.

TABELLE VI

VARIANZE DER ERGEBNISSE VOR UND NACH KORREKTION (TESTGEMISCH 1)

Komponente	*Varianz des Peakflächeprozentsatzes*		*Varianz des Gewichtsprozentsatzes*	
	Innerhalb Lab.	*Zwischen Lab.*	*Innerhalb Lab.*	*Zwischen Lab.*
16:0	0.05	0.02	0.05	0.03
18:0	0.05	0.07	0.05	0.07
18:2	0.09	0.16	0.09	0.17
18:3	0.59	10.4	0.59	10.6

	7	8[a]		9	10	*Gesamtmittelwert*
Verest. mit Dimethoxypropan	*Verest. mit $NaOCH_3$*	*Verest. mit Dimethoxypropan*	*Verest. mit $NaOCH_3$*	*Verest. mit BF_3/Methanol*	*Verest. mit Methanol + 2% Schwefelsäure*	
8.7	8.10	9.05	8.98	9.3	9.1	
9.0	8.08	9.09	8.79	9.3	9.1	8.8
	8.56	8.73	8.44	9.3	9.2	
4.9	4.22	4.34	4.72	3.9	5.3	
4.5	4.51	4.57	4.36	4.1	4.7	4.6
	4.48	4.41	4.33	4.2	4.1	
19.6	21.19	20.92	21.67	20.9	21.9	
19.8	21.10	21.11	21.46	21.4	21.4	21.0
	20.73	21.37	21.61	20.7	20.9	
30.6	33.27	33.78	33.44	34.6	31.1	
31.7	34.07	33.28	33.21	33.7	32.4	32.9
	33.37	34.15	33.72	33.6	33.5	
36.2	33.23	31.90	31.18	31.3	32.6	
34.9	32.24	31.94	32.17	31.5	32.4	32.6
	32.86	31.34	31.90	32.2	32.3	

Berechnet wurde das Produkt von Höhe und Breite des Peaks in halber Höhe. Die Mittelwerte der fünf Messungen (eine Messung je Person) wurden berechnet. Diese Mittelwerte wurden als die Oberflächen des betreffenden Peaks betrachtet.

Aus der Vergleichung der Streuung der hieraus berechneten Flächenprozentwerte mit der Streuung in den Flächenprozentwerten der Laboratorien selbst kam hervor dass diese von gleicher Grössenordnung sind und dass daher die Art der Messung der Oberflächen nicht die Ursache der schlechten Übereinstimmung zwischen den Laboratorien sein kann.

Wie gross ist die Genauigkeit der erhaltenen Resultaten?

Aus dem Vorhergehenden geht hervor, dass die festgestellten systematischen Fehler für die Beantwortung dieser Frage einen ernsthaften Handikap bedeuten. Eine verantworte statistische Behandlung bringt uns deshalb nicht viel weiter.

Ein anderes Problem ist, dass bei der ersten und letzten Untersuchung die wirklichen Mengen nicht genau bekannt sind. Einen Anhaltspunkt gibt Tabelle VII, in der für das dritte Testgemisch angegeben ist welcher Prozentsatz der Messungen pro Komponente innerhalb bestimmten Grenzen vom Gesamtmittelwert der Laboratorien liegt. Wie aus Tabelle VII ersichtlich weichen mehrere Laboratorien für bestimmte Komponenten mehr als 10% vom Mittelwert ab. Im günstigsten Fall liegt 75% der Resultate innerhalb 2½% des Mittelwertes, was bedeutet dass auch in diesem

TABELLE VII

DIE ZUVERLÄSSIGKEIT DER ANALYSE PRO KOMPONENT (TESTGEMISCH 3)

Komponente	*Gesamtmittelwert (Gew. %)*	*Prozentsatz der mittleren Ergebnisse*[a]*, innerhalb die Angegebene Grenze*[b]		
		$\pm 2\frac{1}{2}\%$	$\pm 5\%$	$\pm 10\%$
16:0	8.8	33	83	100
18:0	4.6	33	41	75
18:1	21.0	75	75	92
18:2	32.9	75	83	100
18:3	32.6	75	92	100

[a] Summiert über alle Labore.
[b] Relatieve Abweichung vom Gesamtmittelwert.

Fall zwei Ergebnisse noch 5% von einander abweichen können. Dazu ist ausserdem zu beachten, dass nicht sicher ist, ob die gefundenen Gesamtmittelwerte mit den wirklichen Mengen übereinstimmen. Über diese Frage können vielleicht die Ergebnisse der Laboratorien 1 und 4, die besten bei der Untersuchung der eingewogenen Methylester, Auskünfte geben. Es zeigt sich, dass der Mittelwert der zehn Laboratorien beim dritten Testgemisch sehr nahebei dem der Laboratorien 1 und 4 liegt, so dass nahezu sicher ist dass die Streuung um den Gesamtmittelwert derselben Grösse ist wie die um den wirklichen Gehalt. Es ist zu bemerken dass die Streuungen beim ersten und zweiten Testgemisch viel grösser waren, obwohl beim Dritten auch noch eine Umesterung durchzuführen war.

Die Ergebnisse von Tabelle VII wurden verglichen mit denen von HERB UND MARTIN, die ebenso in einem Ringversuch eine grosse Anzahl Öle und einige Methylestergemische bekannter Zusammensetzung analysiert haben[4]. Bei diesen Untersuchungen wurden bei den Ergebnissen annähernd gleiche Streuungen gefunden. Auch ergab sich der Eindruck, dass der Mittelwert der Laboratorien dicht beim wirklichen Gehalt liegt.

Die Frage bleibt noch wie gross die Genauigkeit einer individuellen Bestimmung ist (wie weit liegt der gefundene Wert vom wirklichen?). Dies lässt sich von der gefundenen Standardabweichung (SA) der Gesamtmittelwerte ableiten. Diese Standardabweichung hängt jedoch von der Konzentration ab. Darum wurde für jede Komponente bei den drei gemeinschaftlichen Analysen die Variationskoeffizient berechnet. Die gefundenen Werte sind in Fig. 1 gegen die Menge ausgesetzt, ungeachtet der Art der Komponente. Bei der Berechnung der Variationskoeffizienten sind statistisch abweichende Werte vernachlässigt. Die gezogene Linie zeigt den Zusammenhang zwischen Variationskoeffizient und Konzentration gefunden von HERB UND MARTIN. Die Übereinstimmung ist ausgezeichnet. Die Ergebnisse in der amerikanischen Untersuchung streuen in demselben Mass um der Linie als in unserem Fall. Die genauigkeit der Variationskoeffizient ist also nicht sehr gross insbesondere nicht bei kleinen Mengen. Dennoch gibt die Kurve einen Eindruck über die Genauigkeit der Bestimmung. Mit dem gefundenen Gehalt kann man aus der Figur die Variationskoeffizient ablesen und hieraus lässt sich die zugehörende Standardabweichung berechnen.

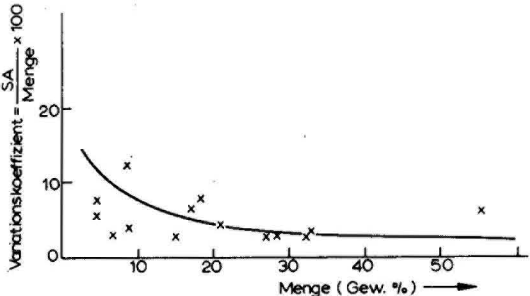

Fig. 1. Zusammenhang zwischen Variationskoeffizient und Menge.

SCHLUSSFOLGERUNGEN

Das Ergebnis der quantitativen GC Analyse von Fettsäuremethylestern bleibt auch nach dem dritten Ringversuch noch unbefriedigend und zwar deshalb, weil die Streuungen zwischen den Laboratorien sich nicht erklären lassen aus der Streuung innerhalb der Laboratorien. Ursache können nur systematische Fehler in der Bestimmung sein.

Keine Anweisung wurde gefunden, dass in der Durchführung der Analyse Fehler gemacht werden. Ebenso wenig sind sie zurückzuführen auf die Messung der Peakflächen. Es bleibt nur die Möglichkeit dass Fehler in den Korrekturfaktoren gemacht werden. Für eine Verbesserung der Ergebnisse ist es unseres Erachtens notwendig dass grösste Sorgfalt auf die Bestimmung dieser Faktoren gelegt wird.

ZUSAMMENFASSUNG

Aus den Ergebnissen eines Ringversuches, an dem zehn Laboratorien beteiligt waren, ergab sich, dass bei der gaschromatographischen Analyse von Fettsäuren in drei Testgemischen systematische Fehler gemacht wurden. Obwohl im Vergleich zum zuerst analysierten Testgemisch die Ergebnisse der Laboratoria untereinander beim dritten Testgemisch im algemeinen besser übereinstimmend homogener geworden waren, verschwanden die systematischen Fehler nicht, und kamen Abweichungen von mehr als zehn Prozent vom Gesamt-Mittelwert noch vor. Aus einer Vergleichung mit einer ähnlichen amerikanischen Untersuchung stellte sich heraus, dass die Genauigkeit der Bestimmung in beiden Fällen dieselbe war. Es wird eine Möglichkeit gegeben die Genauigkeit der Analyse einer bestimmten Komponente bei einer bestimmten Konzentration zu schätzen.

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THE DETERMINATION OF PART-PER-BILLION LEVELS OF CITRIC AND NITRILOTRIACETIC ACIDS IN TAP WATER AND SEWAGE EFFLUENTS*

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SUMMARY

Citric and nitrilotriacetic acids can be determined at the 1–10,000 p.p.b.** levels in aqueous systems ranging from tap water to sewage effluents by use of anion-exchange clean-up, derivatization with butanol–HCl and gas chromatography. A variety of metals present at legal tolerance limits do not interfere. The two esterified acids separate well on a special gas chromatographic phase; however, citric acid can also be separated from nitrilotriacetic acid by ion exchange prior to derivatization, if so desired.

INTRODUCTION

Both the "natural" citric and the man-made nitrilotriacetic acid (NTA) are efficacious complexing agents that can be used as "builders", *i.e.*, detergent additives that soften the water. The fate and effects of NTA have been the subject of much controversy, focusing on its biodegradability, its toxicological effects, and its role in the transport of heavy metals in natural bodies of water (see, for instance, refs. 1–13). The use of NTA in detergents is currently permitted in Canada and prohibited in the United States; however, recent U.S. Government decisions make its re-introduction under restricted conditions almost certain[14].

Analytical methods for NTA and citric acid abound[1,2,15–55]. The zinc–Zincon[2], polarographic[21–29] and gas chromatographic (GC) techniques[30–33] are most often used for NTA, while citric acid is usually determined by either liquid chromatography[36–44] or GC[45–55].

The reliable determination of complexing agents present at very low levels in motley waters involves the elimination of inorganic interferences as the main problem. Soft waters are fairly easy to handle but waters with a high degree of hardness or significant levels of heavy metal ions are difficult to analyze by all the methods listed.

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** Throughout this article the American (10^9) billion is meant.

That metal ions may interfere with analytical methods based on chelation equilibria is fairly obvious. However, gas chromatographic methods suffer no less. The hardness of Columbia water, for instance, effectively prevents the esterification of NTA even at the relatively high 1 p.p.m. level, and ion-exchange clean-up consequently becomes necessary. Other chelating agents, such as naturally occurring carboxylic, hydroxy-carboxylic or amino acids, as well as NTA metabolites, can also interfere with both types of methods. Furthermore, acidification of turbid or contaminated water samples is often necessary to release the full amount of chelating agents for analysis.

Under these circumstances, the concomitant use of two disparate techniques, *e.g.*, polarography and GC, undoubtedly improves analytical reliability. This study is concerned with the GC method for NTA and citric acid, the improvement of its accuracy and sensitivity down to the lower p.p.b. ranges, using aqueous systems with high levels of inorganic and organic contaminants. The need for these improvements arose during an analytical project involving 300 samples of tap water, river water and sewage treatment plant influents and effluents.

For this project in particular, and for similar problems in general, the limits of detection were in need of improvement. Minute traces of NTA can occur in drinking water in regions where it is used as a detergent additive. Citric acid is a common metabolite whose presence in water is interesting with regard to biological activity. Both compounds, of course, strongly influence metal equilibria in aqueous systems. Consequently, precise concentration data are necessary to estimate the relative amounts of metals that are chelated, non-chelated, or, for that matter, may be solubilized from sediments under equilibrium conditions.

NTA is frequently monitored in the input and output of sewage treatment plants. Interest in these data is based on the bacterial degradation of NTA in the activated sludge and the role of residual NTA in the transport of heavy metals through the treatment into the plant effluent. Citric acid can be present in these effluents at fairly high levels and, unfortunately, its ester derivatives interfere with those of NTA on most GC phases.

Consequently, one of the first problems approached in this study was the separation of the NTA and citric acid esters by gas–liquid chromatography (GLC). A second problem involved the removal of materials that interfered with the derivatization. Initial experiments in our laboratory had shown that not all methods described in the literature gave satisfactory results with Columbia water, which is hardly surprising in view of its hardness (Table I); however, we felt that this very fact gave us a chance to study a particularly difficult matrix. In a third set of experiments, several metals with high chelate formation constants (Table II) were added to Columbia water and the analytical technique adjusted accordingly in order to exclude their interference. Finally, we searched for particular conditions under which most of the citric acid could be separated from NTA by ion exchange prior to GC. Although our GC separation of the two derivatives was adequate at comparable concentrations, a great excess of the earlier-eluting citric acid would have interfered with the NTA determination.

Much of the analytical technique used in this study is based on the work by CHAU AND FOX[32] and WARREN AND MALEC[33]. Quite helpful was the methodologically related knowledge on the determination of amino acids, directly available to us from GEHRKE's group (*e.g.*, ref. 56). The packing material used for GC originated from one

TABLE I

COLUMBIA TAP WATER ANALYSIS

Component	Amount (p.p.m.)	Component	Amount (p.p.m.)
Bicarbonates	357	Calcium	59
Carbonates	0	Magnesium	27
Silica	8	Nitrate	0.3
Iron	0.02	Sulfate	16
Aluminum	0.1	Chloride	33
Manganese	0	Fluoride	1.2
Sodium	46		
Potassium	5.6		
	Total residue	424	

TABLE II

METAL IONS ADDED TO COLUMBIA TAP WATER

Metal	Added as	Metal concentration (p.p.m.)
Lead	$Pb(NO_3)_2$	0.10[a]
Copper	$Cu(NO_3)_2$	0.02[a]
Nickel	$Ni(NO_3)_2$	0.80[a]
Zinc	$ZnCl_2$	0.10[a]
Chromium	$CrCl_3$	1.00[a]
Cadmium	$CdCl_2$	0.01[a]
Mercury	$Hg(NO_3)_2$	0.005[b]
Iron	$Fe(NO_3)_3$	1.00[c]

[a] Legal limit in Missouri water, adopted by the State Pollution Control Board, 1971.
[b] Tentative standard, U.S. Department of Health, Education and Welfare.
[c] Arbitrary value, no legal limit set.

of our own studies of non-extractable polymers on diatomaceous supports[57].

EXPERIMENTAL

General procedure

The water samples can be stabilized with 1% formaldehyde if they are not processed immediately. The analysis starts with the addition of 2 ml of 4 *M* formic acid to 50 ml of sample. The pH is checked and lowered to 2.5 by the addition of more formic acid if necessary. The 125 ml erlenmeyer flask containing the sample is then put into a water-bath at 60° and purified nitrogen is bubbled through the solution from a PTFE capillary for 30 min. After coming to room temperature, the sample can be transferred to the reservoir of the ion-exchange column.

Bio-Rad AG1-X2, 50–100 mesh (Bio-Rad Laboratories, Richmond, Calif.) is used to pack a 2-in. resin bed into a 150 mm × 5 mm I.D. column (Fischer and Porter Co., Warminster, Pa.). (Solvent reservoirs were blown from some of these columns to speed up the handling of multiple samples.) Before runs, 10 ml of 16 *M* formic acid are passed through the resin followed by de-ionized water up to a pH of 5–6.

The sample is poured into the reservoir and allowed to flow through the resin at full speed (*ca.* 3 ml/min for tap water). It is followed by two 5 ml portions of 0.1 *M*

formic acid, and then the NTA and citric acid are eluted from the column with 16 M formic acid, of which 8 ml are collected in a 16 × 75 mm culture tube with a PTFE-lined screwcap. (These culture tubes are boiled for 2 h in concentrated HCl and rinsed with de-ionized water before re-use.)

The formic acid is evaporated from the culture tube in a tube-heating block under a stream of nitrogen. The block maintains the tubes at 85° for 2 h and is then allowed to cool while the evaporation continues overnight.

A 2 ml volume of dry 3 N HCl–butanol[56] is added to the dried samples and the culture tubes are capped tightly and placed for 25 min in an ultrasonic water-bath at 75°. The ultrasonic stirring is used only for the first 5 min of reaction time. After esterification, the tubes are cooled, opened and the excess of reagent removed by a slow stream of nitrogen in the tube-heating block. The evaporation starts at 85°, but the heat is turned off after a few minutes and the tubes are removed immediately when they appear to be dry.

Just prior to GC analysis, 100 μl of dry acetone are added to the esterified acids for a 2 μl injection into the gas chromatograph. The 5 ft. × 4 mm I.D. glass column contains a non-extractable packing derived from Carbowax 20M on Celite 545, 100–120 mesh (details of the preparation of this particular column will be published separately[57]. Its performance is roughly comparable to that of a well coated, well conditioned 0.3% Carbowax 20M on well acid-washed Chromosorb W). The Microtek 220 injection port is maintained at 230° and on-column injection is used for isothermal chromatography at 183°. Calculations are based on peak heights.

Tap water calibration curve

Citric acid and nitrilotriacetic acid trisodium salt were added to Columbia tap water in amounts corresponding to 1, 3, 10, 30, 100, 300, 1000, 3000 and 10,000 p.p.b. each of the free acids and the samples were analyzed as described above.

Metal ions in water

A number of metals likely to interfere with the analytical procedure were added to tap water (Columbia municipal water supply) at levels corresponding to legal limits, as shown in Table II. There is no legal limit for iron in Missouri and its concentration was arbitrarily chosen. The resulting water was spiked with NTA and citric acid at the 1, 3, 10, 30 and 100 p.p.b. levels and analyzed as described under *General procedure*.

Sewage samples

Effluent from the Columbia sewage treatment plant was spiked with NTA and citric acid at the 1, 3, 10, 30 and 100 p.p.b. levels, and analyzed as described under *General procedure*.

Removal of citric acid by ion exchange

Columbia tap water was spiked with 1–100 p.p.b. of NTA, accompanied by a 100-fold excess of citric acid thus ranging from 100 to 10,000 p.p.b. The general procedure was followed; however, the resin was washed with two 5 ml portions of 2 M formic acid after the sample had passed through. This fraction (which contains up to 99% of the citric acid) can be discarded. NTA was then eluted with 16 M formic acid and derivatized.

RESULTS AND DISCUSSION

The general procedure described above has been developed in response to analytical problems that arose during a sampling project. The additional experiments on citric acid and metal interference were conducted to ensure a broader applicability of the method.

The ion-exchange and derivatization steps are modifications of the procedures described by CHAU AND FOX[32], WARREN AND MALEC[33] and GEHRKE and coworkers (*e.g.*, ref. 56). The purpose of most of the procedural details is self-evident, but two points may warrant additional discussion.

The initial sample heating at 60° for 30 min in formic acid of pH 2.5 under nitrogen serves several purposes. First, it avoids the formation of bubbles in the later ion-exchange chromatography during elution with 16 *N* formic acid, thus circumventing an upset of the resin bed. Second, a certain acid concentration is necessary to prevent heavy metals from interfering with the analytical method. This was established during initial studies of metal interferences, which involved the application of a neutral sample to the ion-exchange column. In this event, almost no acid derivatives were found by GC analysis. Third, the repeated analyses of sewage samples from different locations with varying acid concentrations in the initial step showed that often, but not always, the highest amount of NTA was released by high acid concentrations. It has not been established whether this effect arose from conjugate hydrolysis, desorption from particulates, dissolution of suspended materials, and/or release of chelated metals. It should be stressed at this point that we do not filter turbid samples as is common practice in NTA analysis. Our own choice of acid concentration clearly represents a compromise engendered by the disparity of samples. It should be optimized whenever NTA or citric acid levels are studied in one particular type of water.

The second point of interest concerns the choice of a final solvent for injection into the gas chromatograph. Initially, methylene chloride was used in analogy to amino acid analysis. However, our flame ionization detectors slowly became noisy and lost sensitivity, presumably from carbon deposits. Changing to Freon 113[33] avoided the deposits, but the NTA derivative was increasingly decomposed in the gas chromatograph, despite the on-glass column injection, when greater numbers of samples were processed in succession. Therefore we made the somewhat unlikely choice of acetone. For some time, *n*-octacosane was used as internal standard in a manner similar to literature methods, but was abandoned as its use did not result in improved precision.

Fig. 1 shows the separation of NTA and citric acid butyl ester derivatives from Columbia tap water spiked with 100 p.p.b. each of the free acids. Fig. 2 is a calibration curve of the two acids in Columbia tap water. The lower part of this calibration curve is enlarged in Fig. 3, and the results of experiments 3, 4 and 5 are superimposed. Columbia sewage effluents contained a peak corresponding to 10 p.p.b. of NTA derivative and the respective results therefore trail off at this level. All other results fall reasonably close to the two lines.

The results indicate that the method works well with Columbia tap and sewage effluent water and could be expected to do likewise with a variety of other waters. (Sewage influent, however, still presents problems in the lower concentration ranges.)

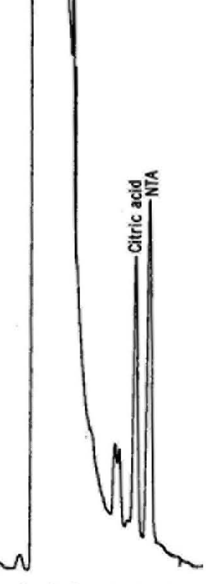

Fig. 1. Typical chromatogram from water spiked with 100 p.p.b. each of NTA and citric acid.

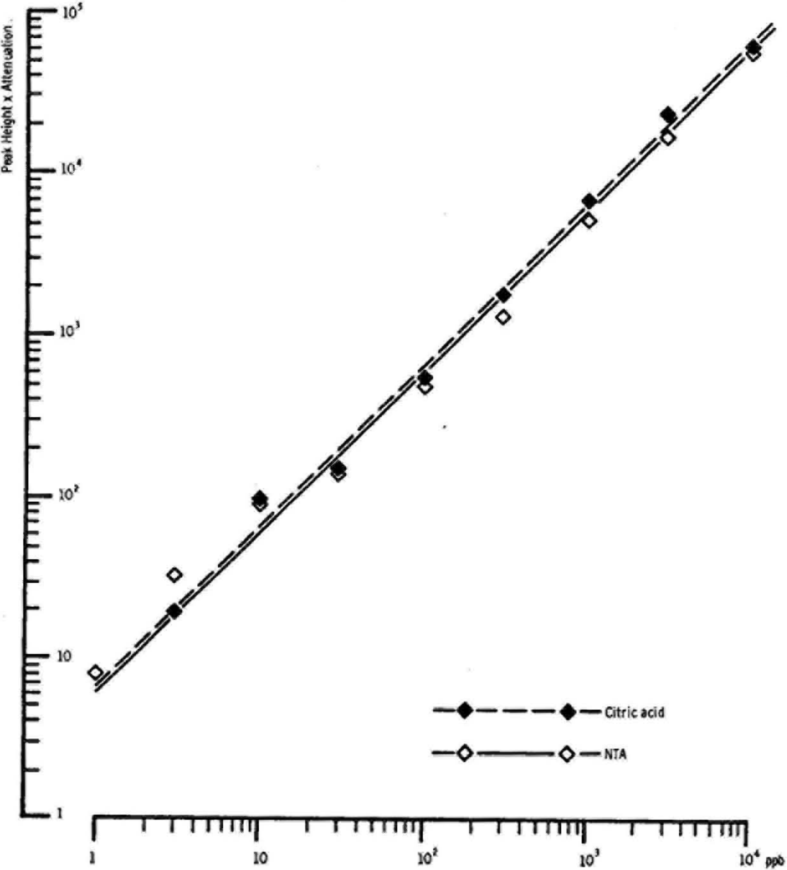

Fig. 2. Columbia tap water calibration curve.

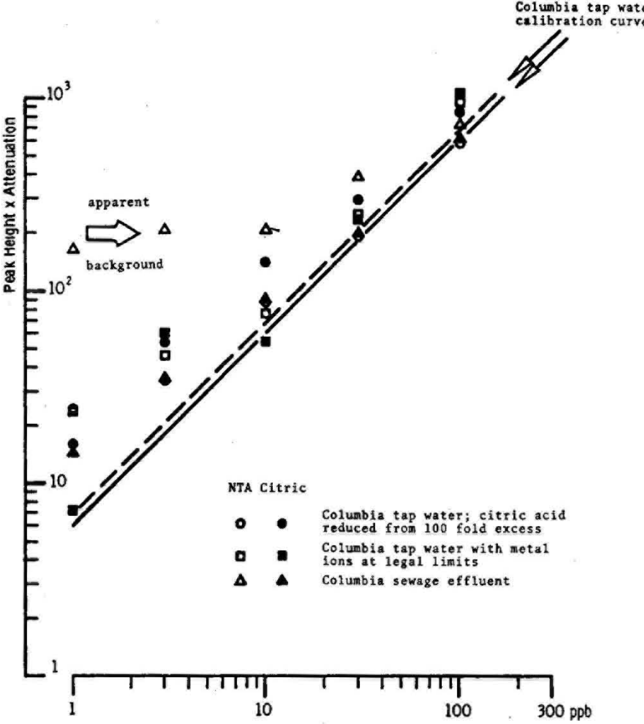

Fig. 3. Determination of NTA and citric acid under various conditions in the lower p.p.b. range.

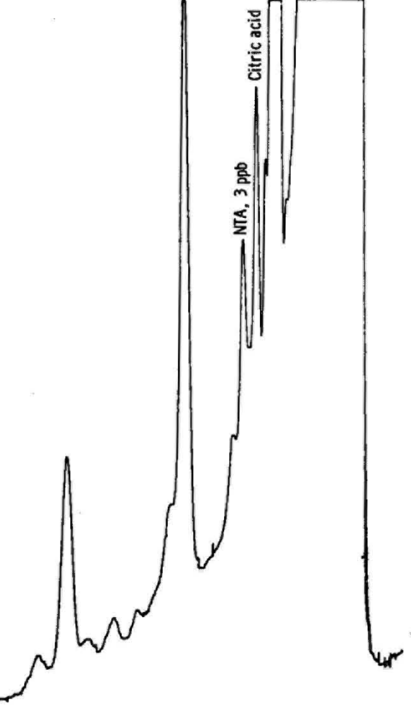

Fig. 4. Analysis of Columbia tap water containing 3 p.p.b. of NTA and 300 p.p.b. of citric acid, the latter reduced by ion exchange.

The metals selected did not interfere at the chosen conditions. The modified ion-exchange procedure worked well, reducing citric acid to approximately 1–2% of its original value. Fig. 4 shows a chromatogram of 3 p.p.b. of NTA determined in the presence of (originally) 300 p.p.b. of citric acid.

The method, as described, lowers the former limits of detection considerably, to about 1 p.p.b. of either acid. There is little interference from heavy metals and, generally, few extraneous peaks show up in the gas chromatogram. It should be noted, however, that the accuracy of the results, as is characteristic of most types of trace analyses in the p.p.b. range, could vary widely with waters of greatly different compositions. The analytical results obtained from such systems should therefore be interpreted with caution.

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SEPARATION AND IDENTIFICATION OF HALOCYCLOALKANES BY GAS CHROMATOGRAPHY. PART II

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SUMMARY

Mixtures of isomeric dihalo- and halomethylcyclopentanes and of dihalo-,halomethyl- and halocyanocyclobutanes have been separated by gas chromatography on a tritolylphosphate column. The order of elution is the same for each set of isomeric disubstituted cycloalkanes. The relative retention volumes can be used to determine rapidly the structure of a given isomer and distinguish it from other stereoisomers. An attempt is made to rationalise the retention volume data in terms of the structural properties of the cycloalkanes.

INTRODUCTION

Chlorination or bromination of a substituted cyclopentane or cyclobutane produces a set of five isomeric disubstituted cycloalkanes. These isomers must be separated and identified in order to obtain a fuller understanding of the halogenation process[1]. The isomer configurations can be established by the usual physico-chemical methods, once pure samples can be obtained.

In this paper we report a method of separation which is appropriate for a wide variety of disubstituted cycloalkanes, and show that an isomer's configuration can be established from its relative retention volume.

EXPERIMENTAL

Materials

Methylchlorocyclobutane isomers were prepared by gas-phase chlorination of methylcyclobutane at 80°, using ten parts methylcyclobutane to one part chlorine. The products were distilled through Carbosorb to remove HCl and unreacted chlorine, giving a mixture of the five methylchlorocyclobutanes, with the chloromethyl isomer, in a solution of unreacted methylcyclobutane[2]. Chlorocyanocyclobutane isomers were prepared by gas-phase chlorination of cyanocyclobutane[2]. Bromochloro- and dibromocyclobutane isomers were prepared by gas-phase bromination of chlorocyclobutane and bromocyclobutane, respectively.

Methylchloro- and chlorofluorocyclopentane isomers were prepared by gas-phase chlorination of methylcyclopentane and fluorocyclopentane, respectively. Methylbromo-, bromofluoro-, bromochloro-, and dibromocyclopentane isomers were prepared by gas-phase bromination of methylcyclopentane, fluorocyclopentane, chlorocyclopentane, and bromocyclopentane, respectively.

Product isomers were identified by injecting a sample of each mixture into a 15% w/w tritolylphosphate (TTP) column, housed in a Perkin-Elmer F11 gas chromatograph, maintained at a suitable temperature. The effluent from the column was led via a single stage Bieman separator into the source of an A.E.I. MS12 mass spectrometer. Mass spectra were thus obtained for the separated isomers, which could then be identified.

Method

The analytical data were obtained by a method similar to that described previ ously[3]. A 15% w/w tritolylphosphate column, having a resolution of about 1100 theoretical plates, was employed. The retention volumes were measured relative to that of *trans*-1,2-dichlorocyclohexane, whose suitability as a standard has already been established for a variety of chromatographic conditions.

The isomers were separated using one of the following sets of conditions. The particular conditions for a given set of isomers are indicated in the appropriate table in the RESULTS section. (a) Column temperature, 70°; nitrogen flow rate, 60 ml/min; inlet pressure, 8.5 lbs./in.^{2}; (b) column temperature, 75°; nitrogen flow rate, 55 ml/min, inlet pressure, 7.9 lbs./in.^{2}; (c) column temperature, 115°; nitrogen flow rate, 75 ml/min; inlet pressure, 12 lbs./in.^{2}; (d) column temperature, 115°; nitrogen flow rate, 71 ml/min; inlet pressure, 10.2 lbs./in.^{2}; (e) column temperature, 100°; nitrogen flow rate, 50 ml/min; inlet pressure, 7.2 lbs./in.^{2}; (f) column temperature, 100°; nitrogen flow rate, 71 ml/min; inlet pressure, 10.2 lbs./in.^{2}.

RESULTS AND DISCUSSION

The retention volumes of the isomers of methylchloro-, chlorocyano-, bromochloro-, and dibromocyclobutane, relative to *trans*-1,2-dichlorocyclohexane, are given in Table I. The relative retention volume data for methylchoro-, chlorofluoro-,

TABLE I

RELATIVE RETENTION VOLUMES (V'_R) OF DISUBSTITUTED CYCLOBUTANES ON 15% TTP

trans-1,2-Dichlorocyclohexane was used as internal standard. Conditions are described in text under *Method*.

Compound	*Isomer*					*Conditions*
	1,1	*trans-1,2*	*trans-1,3*	*cis-1,3*	*cis-1,2*	
C_4H_6MeCl[a]	0.025	0.030	0.033	0.033	0.039	b
C_4H_6CNCl	0.377	1.43	1.56	2.80	4.94	e
C_4H_6ClBr	0.184	0.355	0.395	0.555	0.974	c
$C_4H_6Br_2$	0.395	0.751	1.12	2.60	—	c

[a] $C_4H_7CH_2Cl$, V'_R = 0.051.

TABLE II

RELATIVE RETENTION VOLUMES (V'_R) OF DISUBSTITUTED CYCLOPENTANES ON 15% TTP

trans-1,2-Dichlorocyclohexane was used as internal standard. Conditions are described in text under *Method*.

Compound	*Isomer*					*Conditions*
	1,1	*trans-1,2*	*trans-1,3*	*cis-1,3*	*cis-1,2*	
C_5H_8MeCl[a]	0.059	0.072	0.077	0.077	0.089	b
C_5H_8ClF	0.035	0.039	0.039	0.068	0.174	a
C_5H_8MeBr	0.263	0.304	0.333	0.333	0.377	f
C_5H_8BrF	0.112	0.183	0.183	0.395	—	f
C_5H_8ClBr	0.368	0.694	1.91	2.20	—	c
$C_5H_8Br_2$	0.681	0.874	1.39	2.61	—	d
$C_5H_8Cl_2$[b]	0.187	0.214	0.284	0.478	0.946	c
$C_7H_{12}Cl_2$[b]	1.16	2.05	3.18	3.94	6.13	c

[a] $C_5H_9CH_2Cl$, $V'_R = 0.124$.
[b] Corrected values for dichlorocyclopentane and dichlorocycloheptane isomers.

methylbromo-, bromofluoro-, bromochloro-, and dibromocyclopentane isomers are given in Table II.

The order of elution is the same for each set of isomers, *i.e.* 1,1; *trans*-1,2; *trans*-1,3; *cis*-1,3; *cis*-1,2. With several of the series the *trans*-1,3- and *cis*-1,3-isomers were eluted as a single peak. This order of elution is identical with that found previously for dihalocyclohexane and dihalocycloheptane isomers[3].

Two major trends in the results may be noted at once. The spread in the relative retention volumes for a set of chloro- or bromomethyl isomers is much less than the spread for a set of dihaloisomers. For instance in Table I, V_R' changes from 0.025 for 1-methyl-1-chlorocyclobutane to 0.039 for *cis*-1-methyl-2-chlorocyclobutane, whereas a much bigger change from 0.184 to 0.974 is observed for the corresponding bromochloroisomers. Secondly, the long relative retention times of the chlorocyanocyclobutanes, in relation to their molecular weight, are also of interest.

We previously proposed that the retention volume data could be rationalised in terms of the availability of the ring substituents to interact with the tritolylphosphate stationary phase[3]. With the *cis*-isomers both substituents were available on the same side of the molecule so that interaction was strong. For the *trans*-isomers the availability of both substituents becomes less, until for the *trans*-1,2- or 1,1-isomers only one substituent could interact with the stationary phase. The data reported here can also be interpreted in terms of the same proposal. For the methylhalo isomers only one halogen substituent is available for interaction with the stationary phase irrespective of the isomer configuration. The methyl substituent being less polar will interact only weakly. The small spread of values of the methylhalocycloalkane relative retention times is thus readily understood. The cyano substituent being even more polar than halogen, interacts more strongly so that the relative retention volumes of the halocyanocyclobutanes are very long. On going from the 1,1- to the *cis*-1,2-chlorocyanocyclobutane a chlorine substituent also becomes available to interact with the stationary phase. Hence the spread of relative retention volume values from the 1,1-

to the *cis*-1,2-chlorocyano isomers would be expected to be about the same as for a set of dihaloisomers; exactly as is observed.

A plot of log V_R' against boiling point is shown in Fig. 1 for all the isomers having known boiling points (refs. 4–6; other boiling points were determined in our laboratories), including those studied previously such as the dichlorocyclobutanes whose boiling points we have recently obtained. A general linear tendency can be observed, but the graph shows two kinds of structures as illustrated in Figs. 1a and 1b, respectively. The graph can be resolved into three straight lines: one line passing close to all the disubstituted cyclobutanes, one line representing the disubstituted cyclopentanes, and a third line correlating the cyclohexane isomers. The graph may also be resolved into a series of curves, each curve representing one set of isomers. The curvature is most marked for the *cis*-isomers, particularly the *cis*-1,2-isomers, indicating the increased interaction with the stationary phase shown by these isomers having two substituents available on the same side of the molecule.

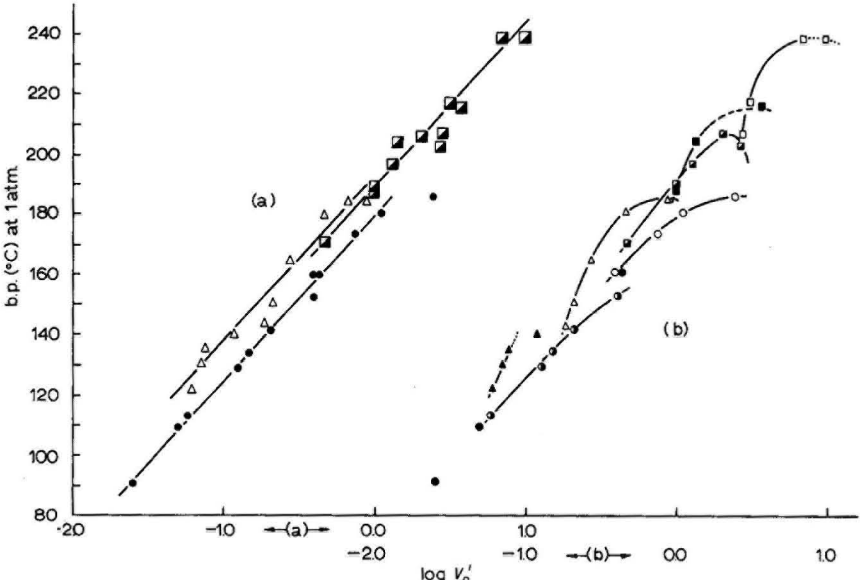

Fig. 1. Correlation of boiling point with log V'_R. (a) ●, Disubstituted cyclobutanes; △, disubstituted cyclopentanes; ◪, disubstituted cyclohexanes. (b) ●, Methylchlorocyclobutane isomers and *trans*-1-bromo-3-chlorocyclobutane; ◑, dichlorocyclobutane isomers; ○, dibromocyclobutane isomers; △, methylchlorocyclopentane isomers and $C_5H_9CH_2Cl$; ▲, dichlorocyclopentane isomers; ◪, dichlorocyclohexane isomers; ■, chlorobromocyclohexane isomers; □, dibromocyclohexane isomers.

A particularly interesting case is that of the methylchlorocyclopentanes. Since only one halogen substituent is available from all the isomers, little or no curvature would be expected. The 1,1- and *trans*-isomers clearly lie on a straight line, but unfortunately it was not possible to measure the boiling point of the *cis*-1-methyl-2-chlorocyclopentane, nor is a value for the pure material given in the literature.

In Fig. 2a a plot of log V_R' against molecular weight is shown for all the dihalocyclobutane and dihalocyclopentane isomers, excluding the fluorine containing com-

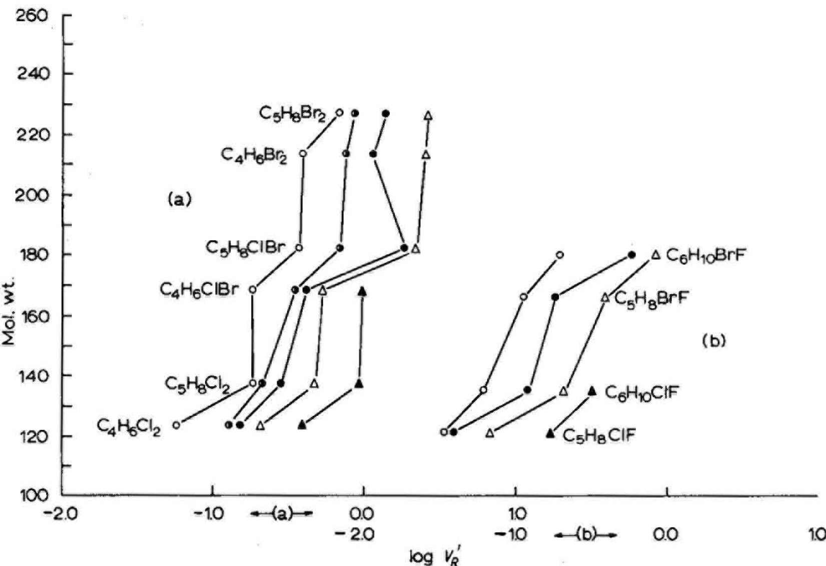

Fig. 2. Plot of log V'_R against molecular weight. (a) ○, 1,1-isomers; ◑, *trans*-1,2-isomers; ●, *trans*-1,3-isomers; △, *cis*-1,3-isomers; ▲, *cis*-1,2-isomers. (b) ○, 1,1-isomers; ●, *trans*-1,2- and *trans*-1,3-isomers; △, *cis*-1,3-isomers; ▲, *cis*-1,2-isomers.

pounds. A fairly regular progression from one set of isomers to another is generated, similar to that obtained previously with disubstituted cyclohexane isomers[3]. The fluorine containing isomers also form a regular progression within themselves (see Fig. 2b) but do not fit in well with the other series. The order of elution of these isomers is the same as for other dihalocycloalkanes, but the magnitude of their relative retention volumes is much less.

Irregularities in the correlation of log V_R' with molecular weight can probably be attributed to changes in the inter-halogen distance which occur for a given isomer structure. For instance, the inter-halogen distance in the *cis*-1,3-isomers may vary, not only with the type of halogen present, but also because of changes in ring size, and ring conformation. The number of stable isomer conformations depends on ring size and on the type of substituent present[7]. For the 1,1-isomers the cycloalkane ring might simply be regarded as a hydrocarbon "tail" attached to one active site capable of interaction with the stationary phase. Conformational and other changes in the ring as the substituents are varied, should have a relatively minor effect. A much more regular relationship of log V_R' with molecular weight would then be expected for the 1,1-isomers only.

Fig. 3 shows the plot obtained for all the 1,1-isomers containing chlorine and/or bromine. A very regular array is formed, the only serious deviation being that of 1,1-dibromocyclopentane. The relative retention volumes of this limited range of compounds are a simple function of the numbers of carbon, chlorine and/or bromine atoms present. This function obviously has a very limited range of application. The fluorine containing 1,1-compounds do not fit into the same sequences, although they probably form a similar array amongst themselves. The rudiments of this array can be observed in Fig. 2b for the 1,1-chlorofluoro- and bromofluorocyclopentane and cyclohexane

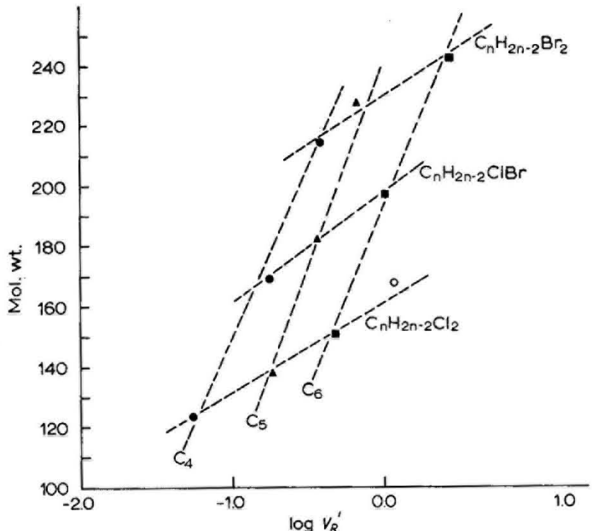

Fig. 3. 1,1-Dihalo isomers: plot of log V'_R against molecular weight. ●, Dihalocyclobutanes; ▲, dihalocyclopentanes; ■, dihalocyclohexanes; ○, 1,1-dichlorocycloheptane.

isomers. However, the data are not extensive enough to check this point. Attempts to prepare chlorofluoro- and bromofluorocyclobutane isomers have so far failed. The regular function may, however, be applicable to the cycloheptane series of chloro/bromo-1,1-isomers. Thus, the predicted relative retention volume for 1,1-dichlorocycloheptane is 1.5, which compares reasonably with the observed value of 1.2 (see Fig. 3).

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GAS CHROMATOGRAPHIC DATA FOR POLYCHLORINATED BIPHENYL COMPONENTS IN SIX AROCLORS®

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SUMMARY

Illustrations of gas chromatographic curves, relative retention times, and response data from 10% DC-200 and 1:1 15% QF-1/10% DC-200 on 80–100 mesh Chromosorb W HP columns with electron capture detection are compiled for six Aroclors®. Aroclors® are commercial mixtures of polychlorinated biphenyls commonly used as analytical references for polychlorinated biphenyl residue determination.

INTRODUCTION

The industrial chemicals known as polychlorinated biphenyls (PCB), which have become widespread environmental contaminants[1–3], are generally determined by essentially the same techniques used for organochlorine pesticides[4] and may act as interferences in the gas–liquid chromatographic (GLC) determination of pesticide residues. Because there is no standard PCB and individual chlorinated biphenyl compounds are not readily available, it is necessary to rely on Aroclors® as analytical references for PCB residue determinations.

Aroclor® is the general tradename for commercial mixtures of PCB manufactured in the United States by Monsanto Company. Each Aroclor is a mixture of chlorinated biphenyls (1200 series), chlorinated terphenyls (5400 series) or a combination of chlorinated biphenyls and terphenyls (4400 series). The last two digits of the identifying number indicate the percentage weight of chlorine, *e.g.*, Aroclors 1254 and 1260 are biphenyls containing 54 and 60% chlorine, respectively[5]. GLC patterns of PCB residues in environmental samples have generally resembled Aroclors 1254 and 1260, although the possibility exists that residues may derive from any of the Aroclors.

The GLC retention times, relative peak sizes, peak shapes, and overall peak pattern of PCB residues must be carefully compared to the corresponding data for the Aroclors in order to determine the Aroclor(s) that most closely resemble the residue. Because the magnitude of total area varies significantly for the same weight of different Aroclors, the quantitative value determined for the PCB residue depends

upon which Aroclor(s) is chosen for quantitation reference. The interpretation and evaluation of GLC chromatograms in PCB determinations is augmented by ready access to relative retention times, response data, and reference chromatograms of the various Aroclors.

Data for these GLC characteristics of six Aroclors, obtained by using two GLC columns regularly employed in our laboratories[6], are presented here. Data are not presented for Aroclors 1232, 1268, 5442, 5460, and 4465. Aroclor 1232 has been commercially prepared[7] by blending appropriate quantities of Aroclors 1221 and 1242 to obtain 32% total chlorine; chromatograms of material obtained from different lots revealed substantial differences between some lots. Aroclor 1268 was not available at the time of this work. GLC at the described conditions of Aroclors 5442 and 5460 (chlorinated terphenyls) and 4465 (mixture of PCB and chlorinated terphenyls) resulted in multicomponent chromatograms with peaks emerging at retention times extending to several hours; several hundred nanograms were required for detection of late-eluting constituents. These GLC parameters are considered unsuitable for detection and measurement of the polychlorinated terphenyls. A system utilizing parallel columns with liquid phases of 1% OV-101 and 3% Dexsil 300 operated at 240° has been devised for GLC of both the polychlorinated terphenyls and PCB[8].

EXPERIMENTAL

GLC data were obtained with a gas chromatograph equipped with an electron capture detector and 4-mm × 6-ft. glass columns, packed both with 10% DC-200 on 80–100 mesh Chromosorb W HP and a 1:1 mixture of 15% QF-1 plus 10% DC-200 on 80–100 mesh Chromosorb W HP[9]. Operating conditions: nitrogen, 120 ml/min; column and detector temperature, 200°; injection temperature, 225°. The concentric design electron capture detector was operated at a d.c. voltage to produce half full scale recorder deflection for 1 ng heptachlor epoxide when full scale deflection is 1×10^{-9} A. Recorder speed was ½ in./min.

Different concentrations of Aroclors were injected at the conditions given to determine the quantity necessary for approximately half full scale recorder response or enough response to illustrate all the characteristic constituents of a particular Aroclor. Retention times were measured in millimeters from the leading edge of the response to the solvent and reported relative to the retention time of the pesticide aldrin. These conditions and manner of reporting GLC data for Aroclors were chosen to conform to GLC data compiled in the Food and Drug Administration *Pesticide Analytical Manual*[6] for a large number of pesticides.

RESULTS

The following information has been compiled in the tables and figures to be a source of reference and comparison for the GLC behavior of PCB: retention times, response data and illustrations of chromatograms for six Aroclors from two GLC columns. Table I lists the quantities necessary for approximately half full scale

TABLE I

QUANTITIES OF AROCLORS NECESSARY FOR APPROXIMATELY HALF FULL SCALE RECORDER RESPONSE[a]

Aroclor	*Nanograms for 1/2 f.s.r.*	
	DC-200[b]	*QF-1/DC-200*[b]
1221	80	60
1242	40	40
1248	40	30
1254	30	30
1260	20	20
1262	20	20

[a] Quantities of Aroclor mixtures to produce approximately half full scale recorder response (1/2 f.s.r.) for major Aroclor components or sufficient response to detect all the characteristic Aroclor constituents.

[b] GLC column and detector conditions are given under EXPERIMENTAL.

TABLE II

GLC RETENTION TIME DATA FOR SIX AROCLORS

Support material: Chromosorb W HP, 80–100 mesh; stationary phase: 10% DC-200 (6 ft. × 4 mm I.D. column); carrier gas: nitrogen, 120 ml/min; temperature: 200°; detector: electron capture (tritium); sample size: quantities necessary for approximately half full scale response (see Table I); data given in: retention sequence relative to aldrin from solvent peak.

Aroclor 1221	*Aroclor 1242*	*Aroclor 1248*	*Aroclor 1254*	*Aroclor 1260*	*Aroclor 1262*
0.21					
0.26					
0.31					
0.36					
0.39	0.39				
0.52	0.51	0.51			
0.59	0.57	0.57			
0.64					
0.69	0.67	0.67			
0.75	0.72				
		0.80			
0.88	0.86	0.85	0.87		
0.99	0.96	0.96	0.98		
	1.03	1.03	1.05		
1.27	1.22	1.23	1.27	1.28	1.26
1.42	1.39	1.39			
1.52	1.49	1.49	1.52	1.50	1.50
1.76	1.74	1.74	1.78		
1.86	1.83	1.84	1.88	1.86	1.85
2.08				2.07	2.07
2.22	2.20	2.20	2.20	2.21	2.22
	2.56	2.54			
2.65			2.63	2.63	2.61
				2.84	2.82
3.10		3.04	3.08	3.08	3.06
			3.60	3.50	3.50
			4.10	4.10	4.10
			4.30		
			4.90	4.90	4.90
			5.80	5.80	5.80
					6.40
				6.50	6.60
				7.80	7.80
				9.10	9.10

recorder response or for enough response to illustrate all the characteristic constituents of a particular Aroclor. Tables II and III give the retention times relative to aldrin for all the peaks present in six different Aroclors. These tables are arranged to readily show similarity and differences in the GLC elution pattern of the various Aroclors. Peaks with similar relative retention times in the different Aroclors are not necessarily responses to the same compound. Figs. 1–3 are gas chromatograms of six Aroclors from the 10% DC-200 column; Figs. 4–6 are gas chromatograms of six Aroclors from the 1:1 15% QF-1/10% DC-200 column at the specified conditions.

The GLC data for PCB presented here should be a useful reference for evaluation and interpretation of GLC chromatograms for PCB residue determinations.

TABLE III

GLC RETENTION TIME DATA FOR SIX AROCLORS

Support material: Chromosorb W HP, 80–100 mesh; stationary phase: 1:1 15% QF-1/10% DC-200 (6 ft. × 4 mm I.D. column); carrier gas: nitrogen, 120 ml/min; temperature: 200°; detector: electron capture (tritium); sample size: quantities necessary for approximately half full scale recorder response (see Table I); data given in: retention sequence relative to aldrin from solvent peak.

Aroclor 1221	*Aroclor 1242*	*Aroclor 1248*	*Aroclor 1254*	*Aroclor 1260*	*Aroclor 1262*
0.20					
0.29					
0.36					
0.39	0.41				
0.53	0.53	0.53			
0.57	0.61	0.61			
0.71	0.72	0.72			
0.78					
	0.81	0.81			
0.88	0.90	0.90	0.87		
1.02	1.02	1.02	1.02		
1.10	1.09	1.09			
1.31	1.34	1.34	1.32	1.31	1.31
1.53	1.52	1.52	1.52	1.53	1.53
1.82	1.82	1.82	1.82		1.84
				1.86	
1.96	1.97	1.97	1.96		
2.08					2.08
				2.14	
2.26				2.26	2.24
2.34	2.36	2.36	2.34		
2.68			2.68	2.66	2.66
2.84	2.80	2.80	2.80	2.88	2.88
3.22	3.24	3.24	3.20	3.22	3.22
			3.50	3.50	3.50
			3.90	3.90	3.90
			4.20	4.20	4.20
			5.00	5.00	5.00
			6.10	6.10	6.10
				6.50	6.50
					7.40
				8.00	
				9.50	9.30
					11.9

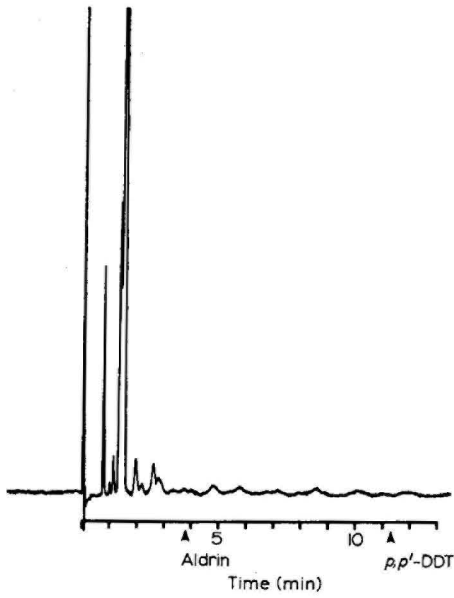

Fig. 1. GLC separation on 10% DC-200 column of 120 ng Aroclor 1221. GLC conditions are given under EXPERIMENTAL.

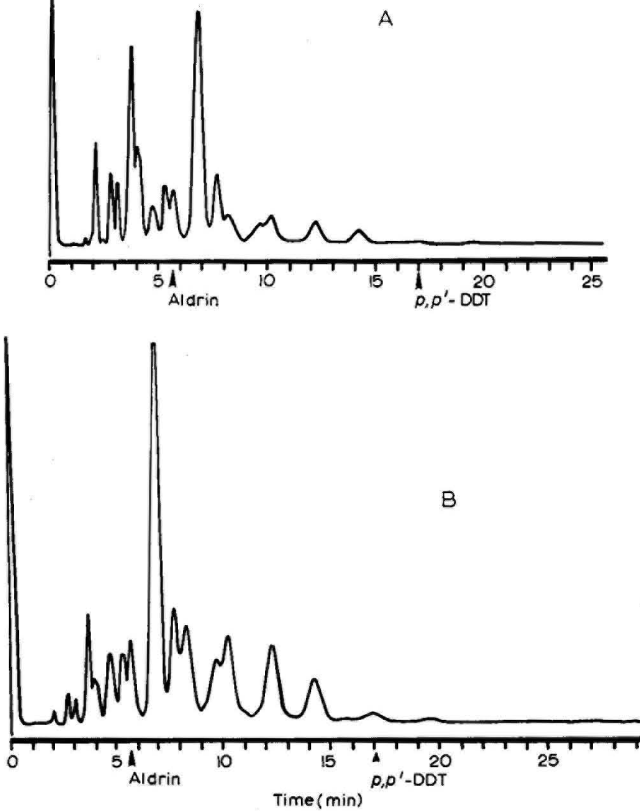

Fig. 2. GLC separation on 10% DC-200 column of (A) 50 ng Aroclor 1242 and (B) 50 ng Aroclor 1248. GLC conditions are given under EXPERIMENTAL.

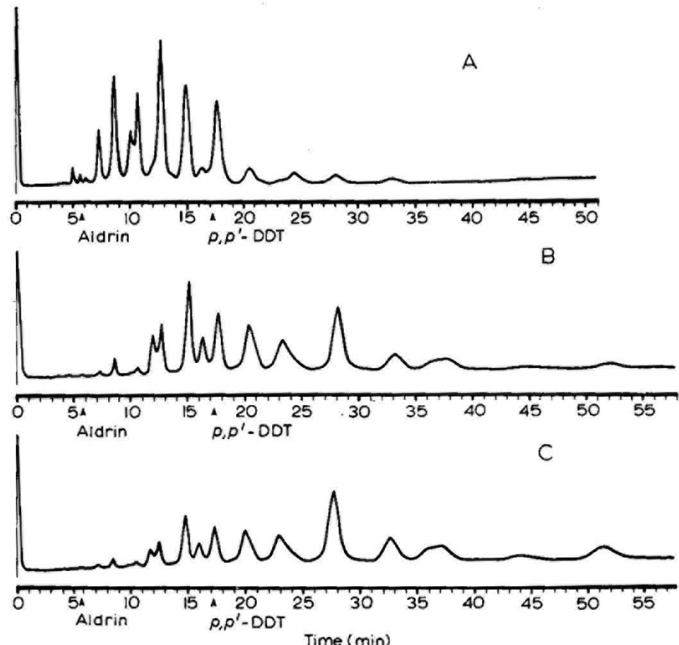

Fig. 3. GLC separation on 10% DC-200 column of (A) 32 ng Aroclor 1254, (B) 20 ng Aroclor 1260 and (C) 20 ng Aroclor 1262. GLC conditions are given under EXPERIMENTAL.

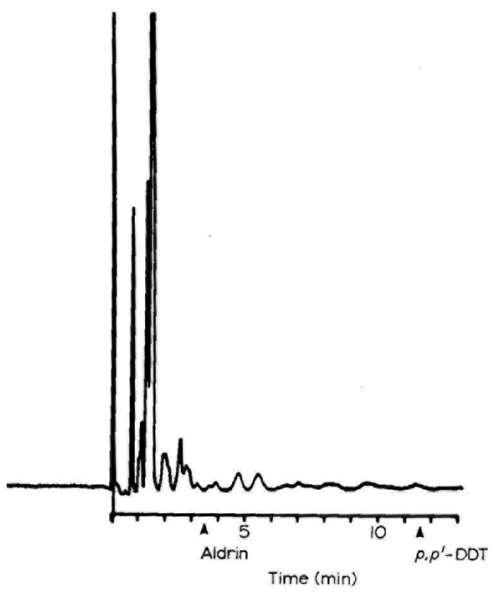

Fig. 4. Gas chromatographic separation on 1:1 15% QF-1/10% DC-200 of 120 ng Aroclor 1221. GLC conditions are given under EXPERIMENTAL.

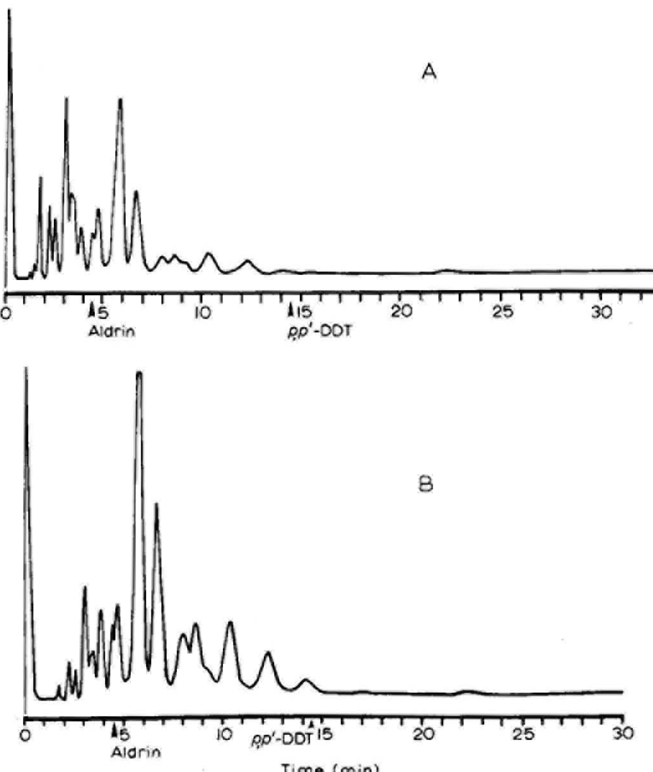

Fig. 5. Gas chromatographic separation on 1:1 15% QF-1/10% DC-200 of (A) 50 ng Aroclor 1242 and (B) 50 ng Aroclor 1248. GLC conditions are given under EXPERIMENTAL.

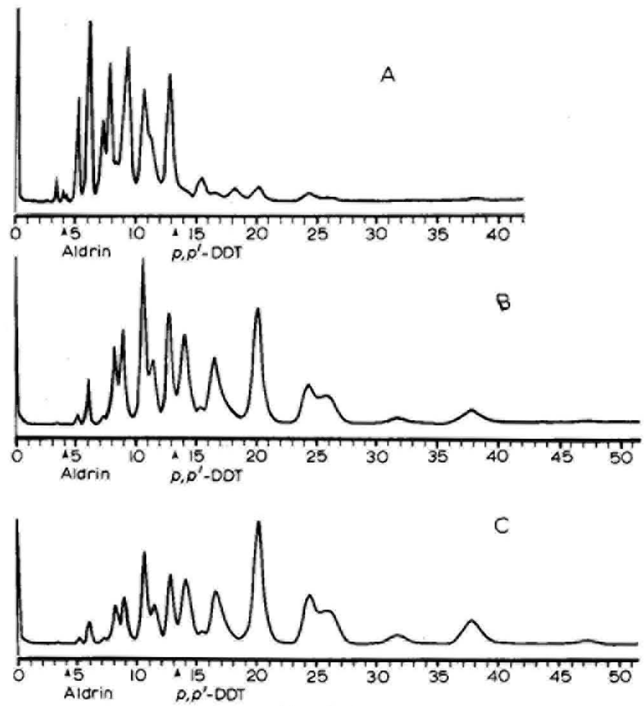

Fig. 6. Gas chromatographic separation on 1:1 15% QF-1/10% DC-200 of (A) 32 ng Aroclor 1254, (B) 20 ng Aroclor 1260, and (C) 20 ng Aroclor 1262. GLC conditions are given under EXPERIMENTAL.

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CHROM. 6203

PROBLEMS OF BACKGROUND CONTAMINATION IN THE ANALYSIS OF OPEN OCEAN BIOTA FOR CHLORINATED HYDROCARBONS

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SUMMARY

Contamination from chemicals, materials and equipment used in the analysis of open ocean samples for chlorinated hydrocarbons in the parts per billion range is a serious problem. Where applicable, heating the materials, equipment and chemicals at 300–350° overnight was effective in removing the contaminants responding to the electron capture detector. With this and other precautions, background contamination can be reduced to an extremely low level and this permits more accurate determination of the chlorinated hydrocarbons in open ocean samples.

INTRODUCTION

With the introduction of tritium and then ^{63}Ni electron capture detectors in gas chromatographs, residue analysts have been pushing the limits of detectability from parts per million (p.p.m.) to parts per billion*** (p.p.b.) level. In the past few years, persistent chlorinated hydrocarbons, mainly DDT, its metabolites and polychlorinated biphenyls (PCBs) have been detected in fish and wild life[1–5]. The distribution of these compounds is world-wide. Thus, the monitoring of these contaminants in open ocean biota is of great importance. From the data gathered, the distribution pattern, transport mechanism and food chain accumulation of these chlorinated hydrocarbons can be formulated.

The residue levels of these persistent chlorinated hydrocarbons in open ocean biota, especially those occupying lower positions in the food chain, are expected to be low, generally in the p.p.b. range. Due to the limitations of sample availability and handling problems, composite samples for analysis are usually in the range of 25–100 g. Thus, sample extracts have to be concentrated to a small volume (often from about 500 ml to less than 5 ml); also the gas chromatograph has to be operated at very high sensitivity in order to detect such low levels of chlorinated hydrocarbons. At these levels, the background contamination from chemicals, materials and equipment used becomes a major problem, especially for the identification and quantification of PCBs,

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*** Throughout this article the American (10^{-9}) p.p.b. is used.

because any extraneous peaks introduced into the chromatogram during the manipulation will interfere with the identification of PCBs, or even give false identification.

This paper describes the solution to some of the problems of background contamination which arise in the analysis of open ocean biota samples. Some of these background contamination problems have been reported in the literature[6,7]; however, to our knowledge, detailed discussions of the problems of low level background contamination, as applied to the analysis of open ocean biota, are scarce.

EXPERIMENTAL

A Tracor gas chromatograph (Model MT 220) equipped with electron capture (^{63}Ni) detector and U-shaped glass column (6 ft. × 1/4 in. O.D.) packed with 5% DC-200 on HP Chromosorb W (80–100 mesh) was used. Nitrogen was used as the carrier gas, at a flow-rate of 60 ml/min. The injector, oven and detector temperatures were 225°, 200°, and 275°, respectively. The operating sensitivity was about 0.05 ng heptachlor epoxide giving 50% full scale deflection (f.s.d.).

In all the following experiments, except where otherwise indicated, duplicate runs were performed. Prior to use, all glassware and other equipment were thoroughly rinsed with nanograde solvents (acetone and petroleum ether, b.p. 30–60°) and the final rinse concentrated to 2–3 ml, of which 10 μl was injected into gas–liquid chromatographic (GLC) column. If any peak greater than 2.5% f.s.d. occurred, the whole rinsing procedure was repeated until the final rinsing concentrate showed no interfering peak.

Solvents

Commercial nanograde solvents for pesticide analysis are generally of sufficient purity for routine pesticide analysis without further purification. However, trace amounts of impurities are still present in these solvents, *e.g.* Mallinckrodt Chemical Company's nanograde solvents were specified at maximum GLC interferences of 10 ng heptachlor epoxide per liter of solvent. A simple redistillation through a fractionating column in an all glass system will not normally remove all the remaining contaminants, for some of the organic contaminants will codistil over with the solvent.

Fig. 1 shows the chromatograms of nanograde petroleum ether which had been doubly-distilled through a 30-cm fractionating column in an all glass system. The solvent thus obtained was of very high purity, and a 200-fold (100 ml to 0.5 ml) concentration did not give any interfering peak at the operating instrumental sensitivity of 0.05 ng heptachlor epoxide for 50% f.s.d. (Fig. 1a). However, very minute traces of interfering organic contaminants were still present. This was evidenced by Fig. 1b, when a 1000-fold (100 ml to 0.1 ml) concentration was reached. The level of contaminants was estimated at 1–2 ng of heptachlor epoxide per liter of solvent. It is obvious therefore that more elaborate solvent purification methods have to be used if a concentration factor greater than 500-fold is required.

Distilled water

In the method of PORTER *et al.*[8] for the analysis of fish tissue for chlorinated pesticide residues, about 900 ml of distilled water is used in the extraction of a sample. The final sample extract is generally concentrated to about 5 ml, giving a concentra-

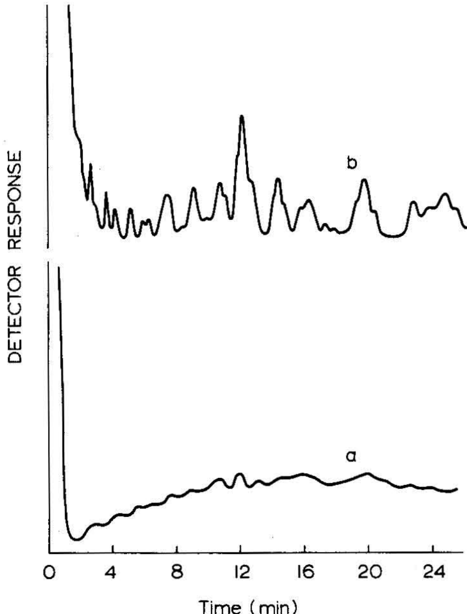

Fig. 1. Gas chromatograms of doubly-distilled petroleum ether. (a) 100 ml concentrated to 0.5 ml, 10 μl injected; (b) 100 ml concentrated to 0.1 ml, 10 μl injected.

tion factor of 180, and the purity of the distilled water used becomes a factor of prime importance. BEVENUE *et al.*[6] have discussed the contamination problems associated with the use of distilled water. We found that multiple distillations (up to four times, with the first 200 ml being discarded each time) through a 30-cm fractionating column in an all glass system did not remove all the contaminants. In order to remove the minute traces of organic contaminants, the water has to be triply-distilled in the presence of potassium permanganate (about 0.1–0.2 g of potassium permanganate for every 3 l of distilled water).

Glassware and Teflon equipment

The contamination problems associated with glassware and Teflon equipment have been discussed in the literature[7]. We found that we could remove most of the contaminants from glassware by washing it thoroughly with detergent and tap water, then rinsing with distilled water and acetone, and finally heating at 200° (method 3 in ref. 7) or 300° (we preferred 300°) overnight. Then, prior to use, we rinsed the glassware two or three times, first with acetone and then with the extraction solvent (petroleum ether or hexane) to remove the last traces of contaminants. Periodic washing of the polytetrafluoroethylene (PTFE) stoppers and stopcocks with chromic acid ensures the removal of organic contaminants from the PTFE surfaces. We also recommend soaking the PTFE material in acetone and then in petroleum ether or hexane for 10 min just prior to use.

Blender

The decontamination of the Waring stainless-steel high-speed blender posed

some problems. Due to the delicate fluorosilicone or PTFE gaskets the blender could not be heat-treated. Repeated washings with detergent and water could not generally remove the last traces of contaminants. However, we found that treatment of the blender with 100-ml portions of solvents (first acetone, then extraction solvent) at high speed several times (for about 1–2 min each time) effectively removed the last traces of contaminants.

Florisil

Florisil gave several early emerging peaks in the chromatogram that would interfere with the identification of PCBs, especially PCBs with low chlorine content, *e.g.* Aroclor 1242 and 1248. The contaminants could be removed by heating the Florisil at 300–350° for several hours or by washing the Florisil with 150 ml of petroleum ether prior to use. Fig. 2 shows the Florisil contaminants present in the 150-ml petroleum ether wash of 30 g of Florisil. No extraneous peak was observed after the 150-ml petroleum ether elution.

Sodium sulfate and sodium chloride

Reagent grade sodium sulfate and sodium chloride contain some volatile electron capturing contaminants. 30 g of reagent grade sodium sulfate (or sodium chloride) was extracted with 100 ml of petroleum ether in a 250-ml mixing cylinder

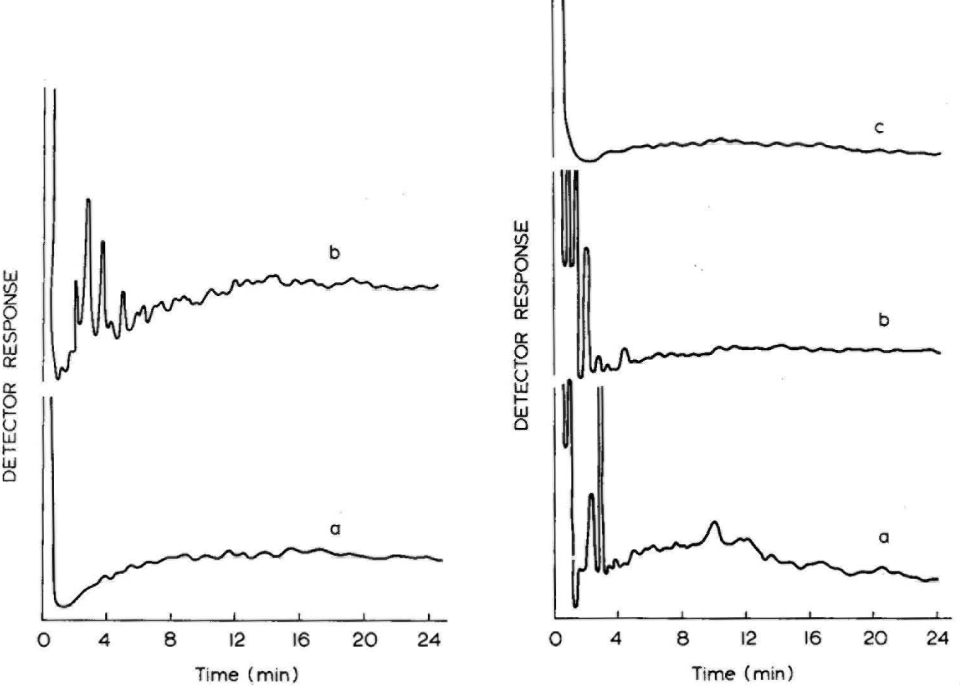

Fig. 2. (a) Gas chromatogram of petroleum ether extract of heat-treated Florisil; (b) gas chromatogram of impurities in Florisil.

Fig. 3. Gas chromatograms of petroleum ether extracts of sodium sulfate and sodium chloride. (a) Original sodium sulfate; (b) original sodium chloride; (c) heat-treated sodium sulfate.

for 1 h (with intermittent shaking). The petroleum ether extract was then concentrated to 4 ml, of which 10 μl was injected into the column. The resulting chromatogram showed several interfering peaks, as shown in Fig. 3a (or 3b). All of the contaminants in the petroleum ether extract could be removed by elution through a Florisil column (gas chromatogram not shown, but similar to Fig. 3c). Another procedure used to remove contaminants involved the heating of sodium sulfate (or sodium chloride) in a 300–350° oven overnight, prior to use. Fig. 3c shows the petroleum ether extract of 30 g of heat-treated sodium sulfate. No interfering peak was observed.

Sharkskin filter paper

Three different boxes of sharkskin papers were examined. Four pieces of filter paper were randomly selected from each box, and separately extracted with 100 ml of petroleum ether in 250-ml mixing cylinders with intermittent shaking for 1 h. Each extract was then concentrated to 4 ml, of which 10 μl was injected into the GLC column. All filter papers from the three boxes examined contained electron capturing contaminants. However, the composition and the level of contaminants seemed to vary from box to box. Thus the filter papers from one box gave only four minor interfering peaks, while filter papers from another box had a complex array of contaminants, as shown in Fig. 4a. Unlike sodium sulfate and sodium chloride, some of the contaminants in filter paper could not be removed by a Florisil column. Fig. 4b shows the chromatogram of a 6% diethyl ether in petroleum ether eluate from the Florisil column of the filter paper extract of Fig. 4a.

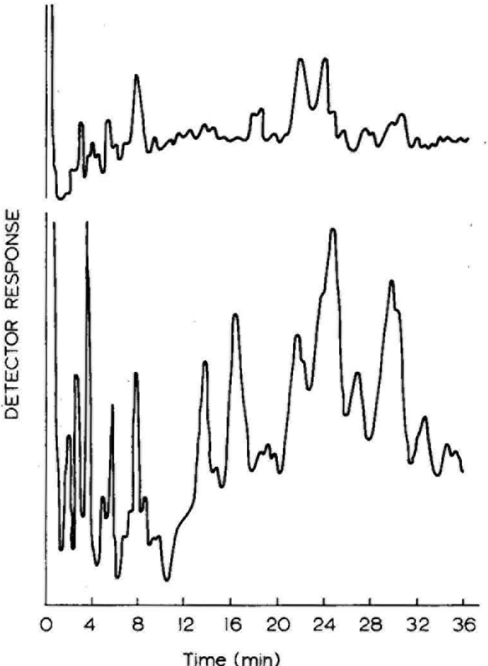

Fig. 4. Gas chromatograms of petroleum ether extracts of sharkskin papers. (a) Original petroleum ether extract concentrate; (b) 6% diethyl ether in petroleum ether eluate from Florisil column of extract concentrate from (a).

Most of the contaminants in the filter paper could be eliminated by extraction with petroleum ether or hexane. This could be done with a Soxhlet extractor or by soaking the filter papers in the solvent in a beaker for 3–4 h, and changing the solvent every hour.

Glasswool

Glasswool is used to plug the end of the Florisil and anhydrous sodium sulfate columns[8]. On some occasions it is substituted for sharkskin paper in the filtration process. An amount of glasswool equivalent to that normally used in analyzing a sample was extracted with 100 ml of petroleum ether in a 250-ml mixing cylinder and then concentrated to 4 ml, of which 10 μl was injected into the GLC column. A huge peak and several smaller peaks were observed in the resulting chromatogram (Fig. 5).

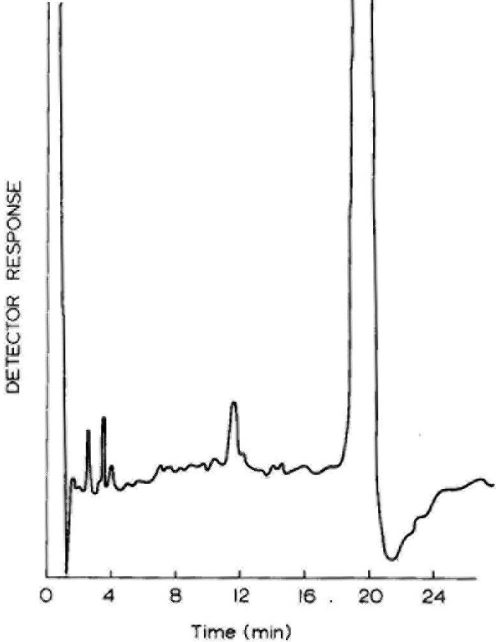

Fig. 5. Gas chromatogram of petroleum ether extract of untreated glasswool.

Heating the glasswool at 300–350° overnight was effective in removing all the electron capturing contaminants. The contaminants were also removed by passing the petroleum ether extract of the glasswool through a Florisil column.

Aluminum foil

Aluminum foil is used to line the caps of glass jars for storing samples. If a rubber stopper is used for the buchner funnel in the filtration process, it is also advisable to wrap the rubber stopper in aluminum foil, so as not to let the rubber stopper come in contact with the neck of the filtration flask or the organic solvent.

Three different boxes of aluminum foil were examined for electron capturing

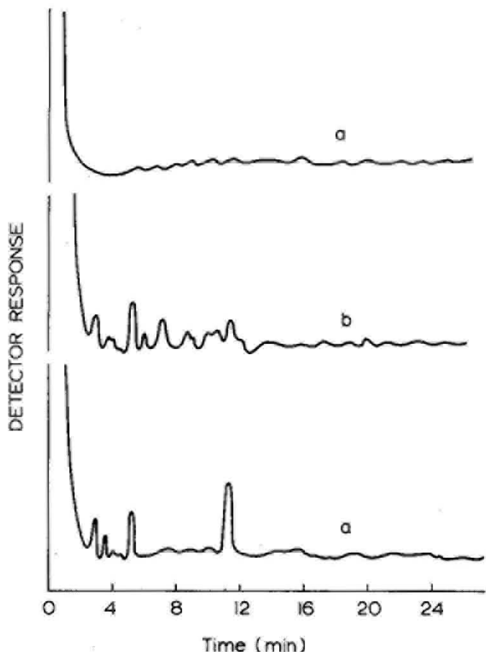

Fig. 6. Gas chromatograms of petroleum ether extracts of aluminum foil. (a) Original aluminum foil from box A; (b) original aluminum foil from box B; (c) heat treated aluminum foil, boxes A and B combined.

contaminants. A rectangular piece of approximately 5 in. × 7 in. was taken from each box and separately extracted with 100 ml of petroleum ether in a 250-ml mixing cylinder for about 1 h. Each of the extracts was then concentrated to 4 ml and 10 μl of this concentrate was injected into the column. As in the case of the filter papers, the composition and level of contaminants of the aluminum foil from each of the three boxes were different. However, only minor peaks were observed in all three cases. Two of the chromatograms are shown in Fig. 6 (a and b). Heating the aluminum foil in a 300–350° oven overnight effectively removed all the contaminants. Fig. 6c shows the petroleum ether extract of heat-treated aluminum foil.

PTFE–rubber laminated discs

The concentrated extract of a sample is usually stored in a 8–10 ml vial with the screw cap lined with a PTFE–rubber laminated disc (PTFE side facing the solution). The disc minimizes evaporation loss and thus the sample extract can be kept for some time without appreciable change in concentration. The PTFE surface is generally regarded as inert, and does not have any contaminant. However, caution is also needed in using the PTFE–rubber laminated discs.

We tested thirty of the PTFE–rubber laminated discs. The glass vials were first thoroughly washed with detergent, then chromic acid, tap water, and then rinsed with distilled water and heated overnight at 200°. The vials were then equipped with screw caps lined with PTFE–rubber laminated discs, and rinsed first with acetone and then twice with petroleum ether (by shaking about 4 ml of solvent in the vial for 1 min).

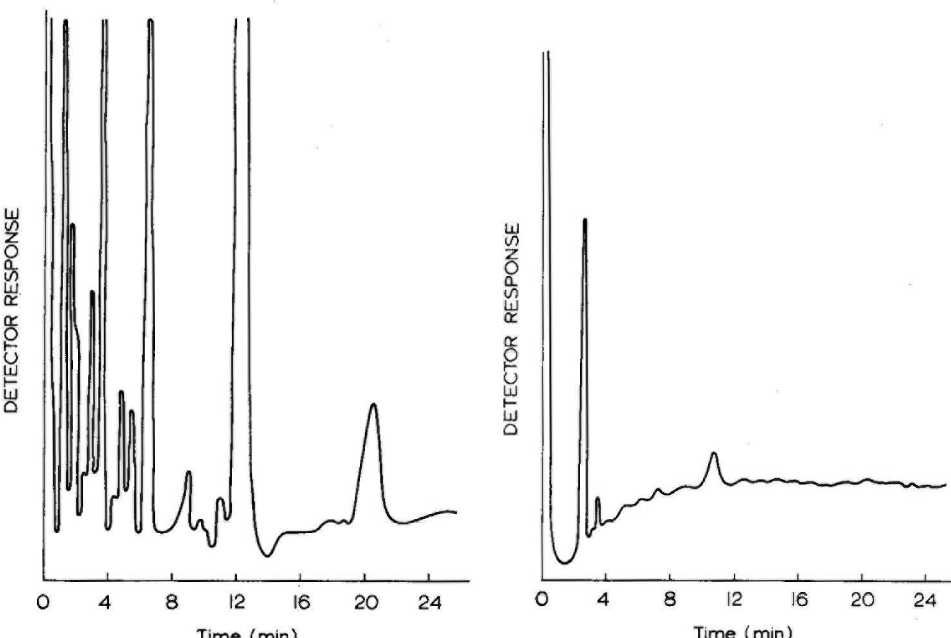

Fig. 7. Gas chromatogram of electron capturing contaminants from PTFE–rubber laminated disc.

Fig. 8. Gas chromatogram of electron capturing contaminants from screw cap with paper liner.

Then the vials were filled 3/4 full (about 6 ml) with petroleum ether. After two days 10 μl of the solvent in each vial were separately injected into the GLC column. No peak was detected in the solvent from any of the vials. The injections were repeated after six weeks. No peak was detected in the solvent from most of the vials. However, the solvent from two vials exhibited sizable amounts of contaminants, one was especially high, as shown in Fig. 7. The contaminants were not from the screw caps, as evidenced by observations from two control vials. In the two control vials, the original screw caps with paper liner were used. After six weeks, identical chromatograms were obtained from the solvents in the two vials. Only three peaks were observed (Fig. 8), indicating that the peaks originated from the cap.

The two PTFE–rubber laminated discs that gave off the electron capturing substances were observed to have some pinkish colorations on the PTFE sides. The contaminants may be from the PTFE, or it may be that there are some cracks in the PTFE layer, and that solvent vapor was able to penetrate through the PTFE and extract the electron capturing substances from the rubber. Although the majority of the PTFE–rubber laminated discs were good, the possible presence of a few defective discs would warrant some caution in their use. The problem could be minimized by running GLC measurements a few days after extraction of the sample.

RESULTS AND DISCUSSION

In the analysis of persistent chlorinated hydrocarbons (DDT, its metabolites

and PCBs) in open ocean biota, all the equipment and chemicals used are potential sources of contamination. If not properly eliminated or minimized, contaminants from these sources can contribute to false identification of certain chlorinated hydrocarbons, or the values obtained are higher than the actual residue levels. Such errors could lead to false alarms or conclusions. On the other hand, the low level of chlorinated hydrocarbons in open ocean biota should be accurately determined so that meaningful environmental control or improvement measures can be formulated. To this end, the analyst must be aware of the many problems associated with the analysis, the limitations imposed by the analytical method, and the meaningful detectability limits for the samples.

It is expected that the type and level of electron capturing contaminants in a given chemical (or material) will be different from batch to batch or from different

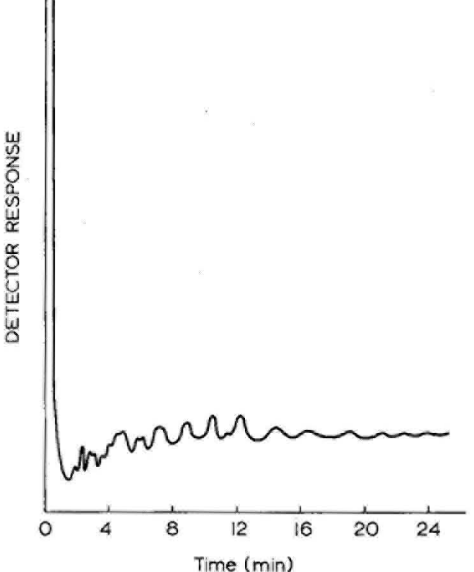

Fig. 9. Gas chromatogram of a blank run extract. 6% diethyl ether in petroleum ether eluate, concentrated to 5 ml, 10 μl injected.

sources. Although practically all the contaminants in the chemicals or materials can be removed by solvent extraction, we found that if the substance could be heated, heating in a 300–350° oven overnight was the most economical and convenient method.

Applying the precaution and the clean-up methods outlined in the EXPERIMENTAL section, background contamination can be reduced to very low levels. Fig. 9 shows the chromatogram of a typical blank run using the method of PORTER *et al.*[8] for the analysis of fish tissue. The background level of DDTs and PCBs for this particular blank run, based on a 50-g sample weight, is estimated at less than 0.05 p.p.b. for *p,p'*-DDE, less than 0.1 p.p.b. for *p,p'*-DDD and *p,p'*-DDT, and less than 1 p.p.b. for PCBs.

ACKNOWLEDGEMENT

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CHROM. 6179

A FLUORESCENCE DETECTOR FOR HIGH-SPEED LIQUID CHROMATOGRAPHY*

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SUMMARY

A simple, inexpensive and efficient detector for monitoring high-speed liquid chromatographic columns has been developed. The detector consists of a commercially available fluorimeter equipped with a small volume flow-through cell. The short-term noise obtained with this cell corresponds to 2 p.p.b. of quinine sulfate or $1.5 \cdot 10^{-11}$ g for a 7.5 μl cell volume. Variations in solvent velocity do not affect the baseline. The detector response is linear over a wide range for both peak height and peak area. Injections of 3 ng amounts of a fluorescent compound have been detected consistently.

INTRODUCTION

During the last 3–4 years, a large proportion of the studies concerned with high-speed liquid chromatography (HSLC) has been directed toward the development of new detection systems. Excellent discussions of various commercial detectors have appeared elsewhere[1–4]. Fluorescence detectors have been available commercially for some time[3], but there has been a definite lag in their application to the monitoring of HSLC columns. Fluorescence detectors are not as universal as other HSLC-detectors such as refractive index and UV detectors. This, coupled with the cost of such detectors, could be responsible for this lack of published data. However, a detector that shows some degree of selectivity can be useful for certain analytical problems. This work describes the conversion of a Turner fluorimeter into an HSLC detector and outlines some of the advantages associated with fluorescence detection. A flow-through cell is available for the Turner fluorimeter but is unsuitable for HSLC[5].

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* Presented at the 55th Canadian Chemical Conference, Quebec, Canada, 1972.

EXPERIMENTAL

Fluorescence detector

The detector used in this work consisted of a Turner 111 fluorimeter* equipped with a thin-layer chromatogram scanning door (Model 110-700). Fig. 1 shows a schematic diagram of the optics of the Turner fluorimeter. Radiation from the light source passes through the primary filter and onto the flow-through cell via the right-hand side of the hole in the door of the fluorimeter. Fig. 2 shows the position of the flow-through cell. The fluoresence produced in the cell is collected at the left-hand side of the hole by a leucite light-pipe, which directs it to a secondary filter placed in front of the phototube.

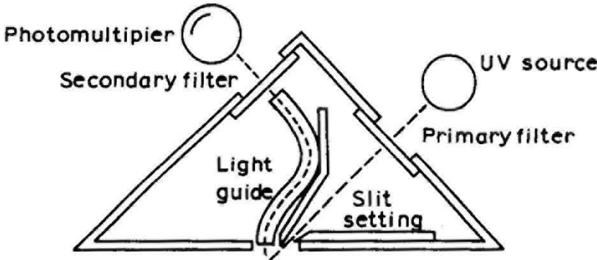

Fig. 1. Optical arrangement of detector.

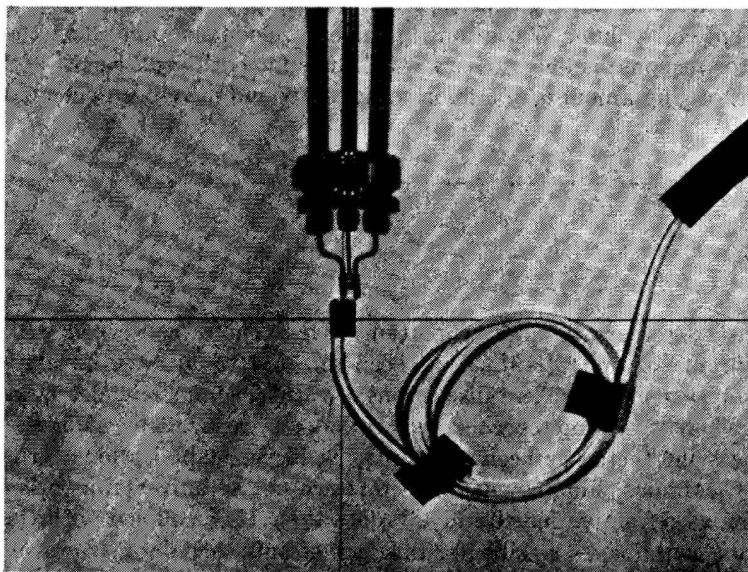

Fig. 2. Flow-through cell.

The connecting tubing was covered with several layers of black cloth to eliminate stray radiation. Fig. 3 shows the location of the detector in the chromatographic system.

* G. K. Turner, Palo Alto, Calif. 94303.

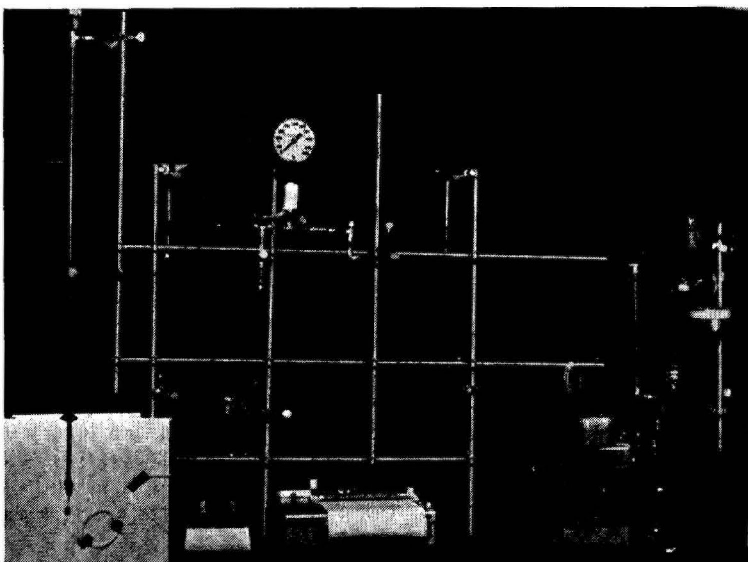

Fig. 3. High-speed liquid chromatograph.

Detector cell

The flow-through cell was made by drawing out soft glass tubing so that the final diameter was about 1/16 in. O.D. This tubing was bent into the desired shape and then connected to the 1/16 in. O.D. steel capillary tubing, extending from the chromatographic column, by means of a short piece of 1/16 in. I.D. PTFE tubing. Care was taken to ensure that the ends of the steel and glass tubing were in close contact. The cell exit was fastened to 1/16 in. I.D. PTFE tubing that directed the eluent into a flow-measuring device. As it was found that the PTFE tubing acted as a light-pipe and produced high and irregular backgrounds, about 2 ft. of this tubing was fastened to the detector in the form of a coil (Fig. 2) and covered with black cloth to avoid this effect.

Chromatographic system

The apparatus which was constructed for this work is shown in Fig. 3. The design of this equipment is based on that described elsewhere[6]. The injection port and safety relief valve have been described elsewhere[7]. Samples were injected directly on top of the column with a long-needle (10 cm) 10 μl Unimetric syringe*. The pump was a three-head diaphram pump (Type S4, Orlita K. G., Giessen, G.F.R.), which is capable of operating at pressures up to 350 atm. The detector output was connected to a Autolab 6300 digital integrator**. The signal from the integrator was amplified with a Beckman 73490 scale expander*** and then displayed on a Servogor strip-chart recorder§.

* 1010-T, Unimetrics Universal Corp., Anaheim, Calif. 92801.
** Vidar Autolab, Mountain View, Calif. 94040.
*** Beckman Instruments, Inc., Fullerton, Calif. 92634.
§ Carl Zeiss, Oberkochen, G.F.R.

The columns were made from 2.4 mm I.D. seamless stainless-steel* and were packed with uncoated Zipax®**. Two column lengths, 100 cm and 2.5 cm, were used. The short column served to study the band broadening caused by sample injection, cell flow patterns and the response time of the detector.

A UV detector*** was used for comparison purposes. The solvents were either spectranalyzed grade or re-distilled reagent grade.

RESULTS AND DISCUSSION

Detector characteristics

After an initial warm-up period of *ca.* 30 min, the detector exhibited a very stable baseline, as shown in Fig. 4. A similar baseline was obtained over much longer periods. The average peak to peak noise corresponds to 2 p.p.b. (parts per 10^9) of quinine sulfate in a 7.5 μl cell. This sensitivity compares favorably with that reported for a commercial fluorescence detector§, which has a noise level equivalent to 1 p.p.b. of quinine sulfate for a 10 μl cell.

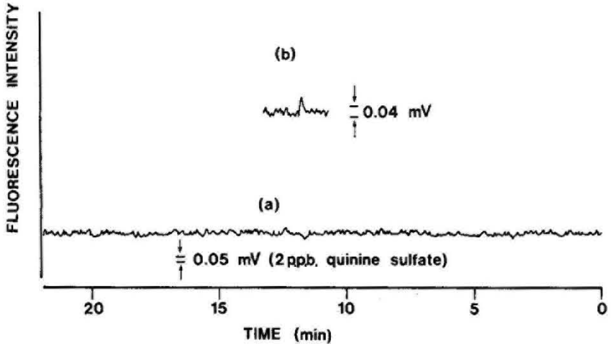

Fig. 4. (a) Background noise. Solvent, *n*-hexane. (b) Chromatographic peak corresponding to 3 ng of NBD ($k' = 0.18$). Solvent, *n*-hexane–acetone (8:2); flow-rate, 1.1 ml min^{-1}; 100 cm column.

The baseline shows no dependence on the flow-rate. The use of only one or two heads of the pump or a complete stop of flow did not have any visible effect on the baseline. This is an obvious advantage over refractive index and UV detectors, as simple and inexpensive pumps can be used without sophisticated damping devices. The response of the detector is also not affected by changes in solvent composition and consequently baseline stability will not be a problem for gradient elution techniques.

Samples containing 3 ng of 4-methylamino-7-nitrobenzo-2,1,3-oxadiazole (NBD) could be detected consistently.

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* American Instrument Co., Inc., Silver Spring, Md.
** E. I. du Pont de Nemours, Wilmington, Del.
*** Pharmacia Fine Chemicals Inc., Piscataway, N.J. 08854.
§ Laboratory Data Control, Inc., Riviera Beach, Fla. 33404.

NBD

A typical peak obtained for this sample is shown in Fig. 4. NBD is used for the fluorigenic labelling of pesticides[8]. The fluorescence intensity of NBD is nine times higher than that for quinine sulfate, as measured with an Aminco-Bowman spectrophotofluorimeter*. Consequently, the peak shown in Fig. 4 corresponds to the injection of 27 ng of quinine sulfate. However, it is assumed that the detection limit for NBD could be further reduced by using an excitation source that will produce more intense emission at the excitation wavelength for NBD (465 nm for 20% acetone in *n*-hexane). The light source used in this work was a general-purpose mercury lamp, which has a major emission at 360 nm as well as longer wavelength lines at 405, 436 and 546 nm. Such a lamp is ideally suited for quinine sulfate ($\lambda_{excitation} = 352$ nm). As the dilution factor for a sample passing through an HSLC column is of the order of 5–100 (ref. 9), the detection limit (twice the noise level) for the injection of quinine sulfate should be 0.2–4.0 ng.

In Fig. 5 it is shown that the chromatographic peaks produced by this detector are symetrical and reproducible. The response of the detector has a range of linearity that is greater than a factor of 340 for both peak area and height measurements (Fig. 6). This range can probably be extended to much higher concentrations, as the fluorimeter was set to maximum sensitivity in this work. The average relative devi-

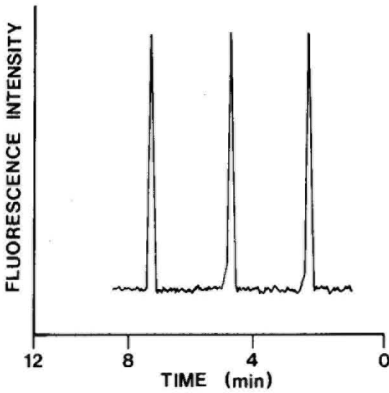

Fig. 5. Reproducibility of chromatographic peaks. NBD, 64 ng; flow-rate; 0.5 ml min^{-1}; 100 cm column.

* American Instrument Co., Inc., Silver Spring, Md.

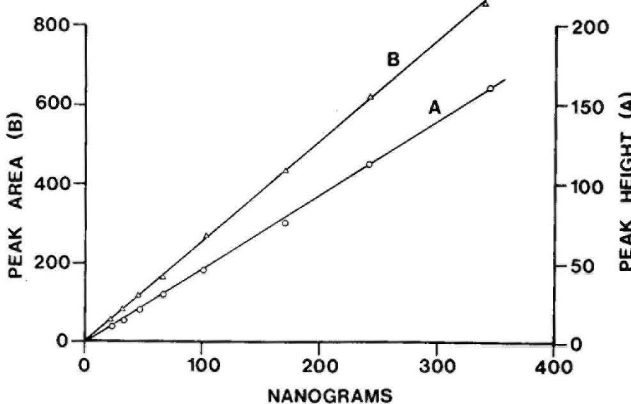

Fig. 6. Calibration curves. Sample, NBD; solvent, *n*-hexane–acetone (8:2); flow-rate, 0.5 ml min^{-1}; 100 cm column.

ations from the mean for the points shown in Fig. 5 (each point corresponds to the average of at least three samples) were 1.8% and 1.5% for peak area and peak height measurements, respectively.

Cell characteristics

The detector cells are made by drawing out glass tubing, and consequently very thin cell walls are obtained. This is an advantage with respect to light transmission and the production of different cell shapes. The physical stability of the cell, which is also important, seems to be entirely satisfactory. As both ends of the cell are fastened to PTFE tubing, there is some freedom of movement, which facilitates installation and removal and prevents breakage of the cell. At excitation wavelengths below 350 nm the cell should be constructed of quartz.

Because of this procedure used for making the cells, duplication of cell volumes is difficult, but this is not critical provided that the same cell is used for a particular set of experiments. As these cells are inexpensive and simple to make, it is simple to increase the cell volume for an enhancement sensitivity (larger cross-section) provided that the loss of resolution is not serious. Some studies have shown that an appreciable increase in cell volume can be tolerated with surprisingly little loss in resolution[9,10]. In the present work, it was found that an increase in the cell volume from 7.5 to 20 μl produced no noticable increase in band width for a 100 cm column.

The shape of the cell was found to be an important factor with respect to maximum efficiency. Fig. 7 shows the shapes of two cells that were studied. A number of other shapes were investigated but they gave no significant advantage. It was found that the use of the straight cell resulted in a 12% increase in band width compared with the bent tube (Fig. 7). This increase can be attributed to poor mixing in the straight cell. The small effluent stream from the steel capillary tube has a much faster velocity than the liquid in the larger glass cell and a considerable distance is required for complete mixing. The processes occurring in the two different cells are shown schematically in Fig. 5. A single bend is apparently sufficient to improve the radial mass transfer.

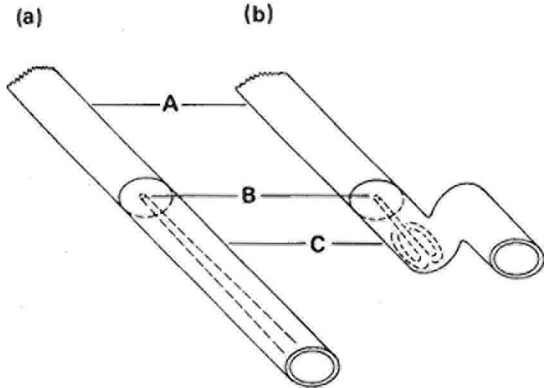

Fig. 7. Cell flow patterns. A, steel capillary tubing; B, exit from A; C, glass cell. Flow patterns are approximated by dashed lines.

The time variance of a chromatographic peak, σ^2_t, is given by the following equation:

$$\sigma_t^2 = (\sigma_c)^2_t + (\sigma_i)^2_t + (\sigma_d)^2_t \tag{1}$$

where $(\sigma_c)^2_t$ is the time variance due to processes occurring in the column, $(\sigma_i)^2_t$ is the time variance caused by sample injection and $(\sigma_d)^2_t$ is the time variance from processes occurring in the cell of the detector.

It is important that the contribution to band broadening by $(\sigma_{it})^2_t$ and $(\sigma_d)^2_t$ should be small compared with $(\sigma_c)^2_t$. An estimation of $(\sigma_d)^2_t$ was obtained by a procedure similar to that used by KIRKLAND[10]. By studying band broadening for a 100 cm and a 2.5 cm column and assuming that there is a linear relationship between $[(\sigma_c)^2_t + (\sigma_d)^2_t]$ and column length, it was found that $(\sigma_d)^2_t$ was 0.5 sec^2 for a flow-rate of 0.6 cm sec^{-1}. This value was corrected for $(\sigma_i)^2_t$. If a 5% increase in band width due to the detector is acceptable, then the minimum band width can be found as follows. The band width, W_t, caused by processes in the column is

$$W_t = 4(\sigma_c)_t \tag{2}$$

For a 5% increase in W_t due to $(\sigma_d)_t$

$$(W_t + 0.05\, W_t)^2 = 16(\sigma_c)^2_t + 16(\sigma_d)^2_t \tag{3}$$

Substitution of the experimentally determined value of $(\sigma_d)^2_t$ and eqn. 2 into eqn. 3 gives

$$17.6\,(\sigma_c)^2_t = 16\,(\sigma_c)^2_t + 8$$

$$(\sigma_c)_t = 5 \text{ sec} \tag{4}$$

$$W_t = 9 \text{ sec}$$

This means that the contribution to W_t by $(\sigma_d)_t$ for any peak with W_t smaller than 9 sec at a flow-rate of 0.6 cm sec^{-1} will be greater than 5% of W_t. However, the minimum W_t at this flow-rate (*i.e.*, $K' = 0$) is 26 sec. Consequently, the increase in W_t is negligible.

The value obtained for $(\sigma_d)^2{}_t$ corresponds to 0.2 cm^2 for $(\sigma_d)^2$. This agrees with previous results obtained under similar experimental conditions with a UV detector[10].

As a final check of the importance of $(\sigma_d)^2{}_t$, the cell was placed between the outlet of a 2.5 cm column and the inlet of a commercial UV detector. No increase in band broadening compared with that for the UV detector alone could be observed.

The above results suggest that moderate increases in cell volume, which will result in increased sensitivity, can be tolerated without serious loss in resolution.

Detector response

The disadvantage of this detector is the slow response time. On sudden application of a light signal, the detector required 5.5 sec to reach a constant maximum value. The time required for the response to decrease to the baseline value was 7.5 sec.

The effect of this slow response was noticed as an unusually rapid loss of efficiency or resolution when the solvent velocity, μ, was increased. The HETP *versus* μ curve (Fig. 8) has a sudden upward trend above 1.7 cm sec^{-1}. Such behavior is unusual and can be attributed only to the sluggish response of the detector. As W_t at this point is 10 sec, this is the minimum allowable peak width for maximum resolution at any velocity. A comparison of peak areas for the same sample at different velocities revealed that, after correcting for different residence times in the cell, the areas were identical until the peak width fell below 10 sec.

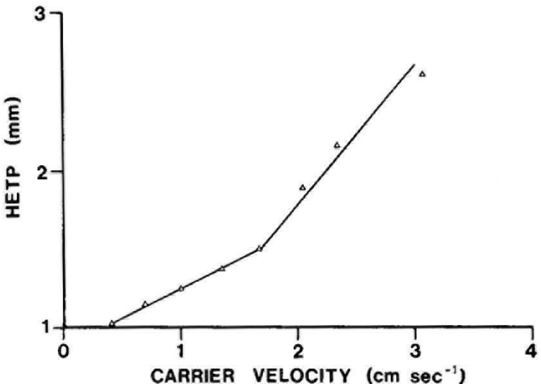

Fig. 8. Relationship between HETP and carrier velocity.

In the above investigation of band broadening processes, it was necessary to determine $(\sigma_i)^2{}_t$. A linear relationship was found between $(\sigma_i)^2{}_t$ and injection volume (1–10 μl) for the 2.5 cm column. The value of $(\sigma_i)^2{}_t$ for a solvent velocity of 0.6 cm sec^{-1} was 0.5 sec^2 per microliter injected. This means that the extra column variance $(\sigma_i)^2{}_t$ due to the injection process is as important (if not more so) than that due to the detector, $(\sigma_d)^2{}_t$. This agrees with results found elsewhere[10].

CONCLUSIONS

The detector described in this paper offers an inexpensive and selective means for monitoring high-performance liquid chromatographic columns. The Turner fluori-

meter equipped with the chromatogram scanning door is common to many laboratories and conversion to HSLC can be accomplished quickly and simply. The degree of selectivity and sensitivity can be modified by the appropriate choice of primary and secondary filters and light source. For certain analytical problems the enhanced selectivity of this detector, compared with UV and refractive index detectors, could be advantageous, especially when used in conjunction with one of these detectors. A particularly promising area of application is HSLC in conjunction with fluorigenic labelling procedures[11]. Such techniques would permit the expansion of the range of applicability of fluorescence detectors without serious losses in selectivity. The complete insensitivity of fluorescence detectors to flow patterns could permit the construction of low-cost HSLC instruments with simple pumping systems.

The response time of the detector is a disadvantage for faster solvent velocities. This is not a serious drawback, however, as velocities up to 2 cm·sec^{-1} can be used. The possibility of decreasing the response time is currently under investigation.

ACKNOWLEDGEMENTS

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CONTINUOUS DETECTION OF RADIOACTIVE EFFLUENTS IN LIQUID CHROMATOGRAPHY BY HETEROGENEOUS OR HOMOGENEOUS SCINTILLATION COUNTING

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SUMMARY

Beta radioactivity in liquid chromatography effluents can be monitored by either heterogeneous or homogeneous scintillation counting. The two systems have been compared and appropriate flow cells have been developed.

With heterogeneous counting, the solution is led through a U-shaped flow cell filled with glass scintillator beads. This system is best for preparative chromatography when activities are high and losses are unacceptable. The activity per peak should exceed 10 nCi for ^{14}C and 1 μCi for ^{3}H.

With homogeneous counting, part of the effluent is mixed with a scintillator solution and passed through an empty helical flow cell. This system is best for detection of ^{3}H, low activities, and in dual-label experiments, or when complete recovery is not vital. The activity per peak should exceed 2 nCi for ^{14}C and 5 nCi for ^{3}H.

INTRODUCTION

Recent advances in liquid chromatography include the development of various sensitive effluent detectors[1]. The continuous monitoring of ^{14}C- and ^{3}H-labelled compounds has, however, not yet reached the same degree of sophistication. We therefore investigated the methods reported in the literature and tried to optimize them and to overcome their particular disadvantages. We assumed that the best method of measuring radioactivity in solution is liquid scintillation counting.

Among the first to deal with the continuous detection of tritium and radiocarbon in solution were SCHRAM AND LOMBAERT[2]. Their cell consisted of polyethylene tubing filled with anthracene and bent into a spiral shape. The counting efficiencies for tritium and carbon-14 were 2% and 44%, respectively. Because anthracene and polyethylene were used, radioactivity could be measured in dilute aqueous solutions only. The applicability becomes more general when beads of lithium–cerium glass are used as the solid scintillator[3]. This material dissolves only in hydrofluoric acid. The counting efficiency, however, is low: 20% for ^{14}C and 0.3% for ^{3}H. A scintillator recommended for organic solvents is europium-activated calcium

fluoride in crystalline form[3,4]. The efficiencies cited are reasonable: 17–50% for ^{14}C and 1.6% for ^{3}H.

In the method of HUNT[5], the column effluent is mixed with a suitable liquid scintillator counting solution until a homogeneous mixture is obtained, and this mixture is passed through an empty cell. A disadvantage of this method is that the effluent is lost for further use. If, however, the eluted compounds must be recovered, a stream splitter can be used with a splitting ratio of, *e.g.*, 4:1. The mixing chamber used by HUNT[5] is rather large (about 1 ml), which may cause loss of resolution.

The only commercially available scintillation flow cells are of the anthracene-filled type with volumes of 1–4 ml (ref. 6). As these cells did not meet our requirements, we decided to develop our own flow cells. In doing so, we investigated the heterogeneous counting system, with various solid scintillators, and the homogeneous counting system, for both ^{14}C and ^{3}H activity. Important considerations are the size and the shape of the cell; the larger its effective volume, the higher is the sensitivity but the lower is the resolution. The optimum size will depend on the flow-rate and the proximity of the peaks. A practical minimum for the residence time in the cell is usually about half a minute.

METHODS

The counter was a Tracerlab Coruflow, Model SCE-542, with twin spectrometers and lin/log rate meters. It consists of a counting chamber with two low-noise, high-gain photomultiplier tubes in a lead shield. The photomultipliers are connected in a coincidence circuit. The instrument was used at room temperature. The dual spectrometer/rate meter unit has two independent channels, which have positions for display of linear, logarithmic or integrated counting rates. A two-pen recorder (Servogor 2, RE 520) was connected to the spectrometer and, if desired, to another flow detector, such as an ultraviolet monitor.

The counting efficiency (E) was calculated for each of the flow cells investigated with the aid of eqn. 1:

$$E\ (\%) = \frac{c}{d'} \cdot \frac{F}{V} \cdot 100 \qquad (1)$$

where c represents the integrated counts of a peak, d' the absolute activity (disintegrations/min), F the flow-rate (ml/min) through the cell and V the effective volume (ml) of the cell. If a solution of known activity per millilitre (D') is circulated through the cell, eqn. 1 simplifies to:

$$E\ (\%) = \frac{c'}{D'V} \cdot 100 \qquad (2)$$

where c' represents counts/min. The effective volume of a cell is determined from the weight of water required to fill it, or by the time of migration of the front of a coloured solution pumped through it at a known rate.

HETEROGENEOUS COUNTING SYSTEM

The heterogeneous counting system is shown schematically in Fig. 1. An ultra-

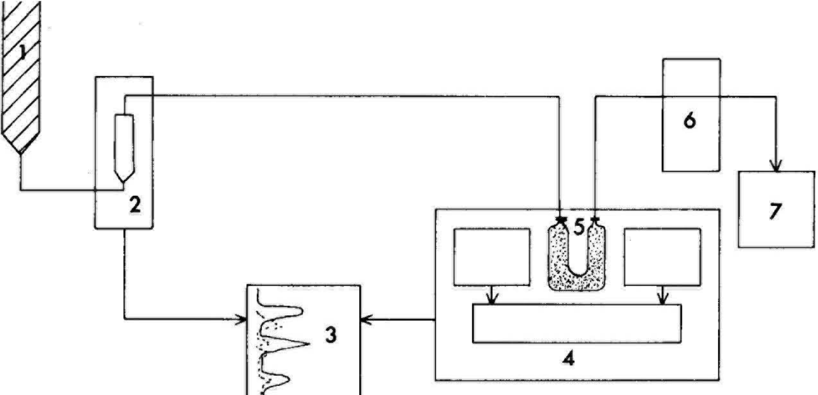

Fig. 1. Schematic representation of the heterogeneous counting system. 1 = Column; 2 = ultraviolet detector, 254 nm; 3 = two-pen recorder; 4 = liquid scintillation spectrometer; 5 = U-shaped flow cell filled with glass scintillator beads; 6 = pump (optional), 1.5 ml/min; 7 = fraction collector.

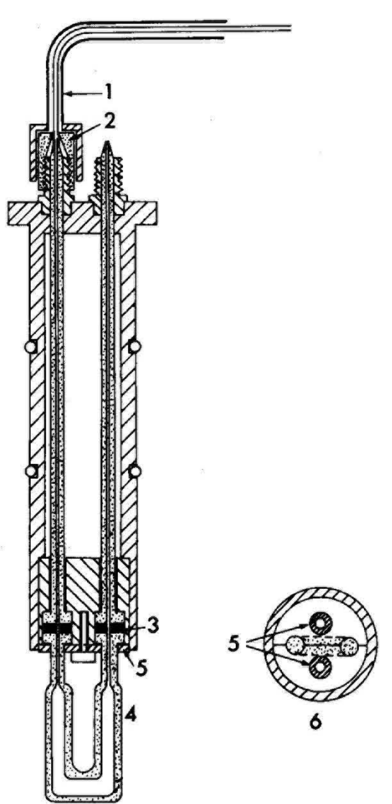

Fig. 2. Probe with scintillator flow cell for the heterogeneous counting system. 1 = Metal cap and bent sleeve for light-proofing; 2 = swage lock; 3 = Viton O-rings; 4 = interchangeable borosilicate glass cell; 5 = semi-circular collar disks; 6 = bottom view of cell and probe.

violet spectrometer (LKB Uvicord 4701 A) with a 0.1-ml cell is included as an independent flow monitor. The pump (LKB 12000) is optional, and may be included to ensure a constant flow-rate. All the flexible tubing, except that in the pump, is made of PTFE and all rigid tubing is made of glass. The probe with its dismountable flow cell is shown in Fig. 2. The cell can be detached by releasing the screws in the two semi-circular collar disks, which press the cell on to two Viton rubber O-rings. PTFE tubes are connected to the glass inlet and outlet tubes by swage locks. Close-fitting metal caps with bent sleeves preclude the entrance of light through the transparent tubes.

Various shapes were tried for the borosilicate glass cell. Coiled or helical cells were difficult to fill and, once filled, tended to become clogged. Flat, U-shaped cells were easy to handle and, moreover, gave the highest counting efficiencies without loss of resolution. The efficiencies of different cells filled with various solid scintillators are summarized in Table I.

TABLE I

COUNTING EFFICIENCIES FOR VARIOUS FLOW CELLS

Shape of cell	*Effective volume (µl)*	*Scintillator*	*Counting efficiency (%)*	
			^{14}C	^{3}H
U	350	Anthracene	37	1.0
U	160	Anthracene	31	1.0
Coil	500	Anthracene	20	0.6
U	350	PPO[a]	43	1.8
U	500	Butyl PBD[b]	40	1.7
U	480	Glass[c]	17	0.2
Coil	430	Glass[c]	5	<0.1
U	190	CaF_2 (Eu-activated)	38	0.5

[a] 2,5-Diphenyloxazole.
[b] 2-(4'-*tert*.-Butylphenyl)-5-(4''-biphenylyl)-1,3,4-oxadiazole.
[c] Cerium-activated lithium glass NE 901 (250–1000 µm), from Nuclear Enterprises Ltd., Edinburgh, Great Britain.

PPO and butyl PBD were dissolved in toluene. After evaporation of the solvent, the resulting dry cake was broken and sieved, and the 700–1000 µm particle fraction was funnelled into the cells. A small plug of quartz-wool was used to keep the scintillator in position.

Anthracene, PPO and butyl PBD are known to dissolve in organic solvents. Anthracene dissolves appreciably in 50% ethanol, while particles of PPO and butyl PBD are broken down by this solvent and clog the cell. PPO dissolved to some extent in 0.05 *N* HCl, as was evident from the ultraviolet spectrum of solvent that had passed the cell. Moreover, PPO adsorbed radioactive materials such as nucleotides, which resulted in tailing peaks and an increase in the background. Butyl PBD and anthracene dissolved slightly in 2 *N* HCl. Europium-activated calcium fluoride is known to dissolve in solutions of ammonium salts. Nucleotides were highly adsorbed by calcium fluoride; this is not unexpected, in view of the low solubility of calcium salts of nucleotides. The only universally applicable solid scintillator proved to be cerium-

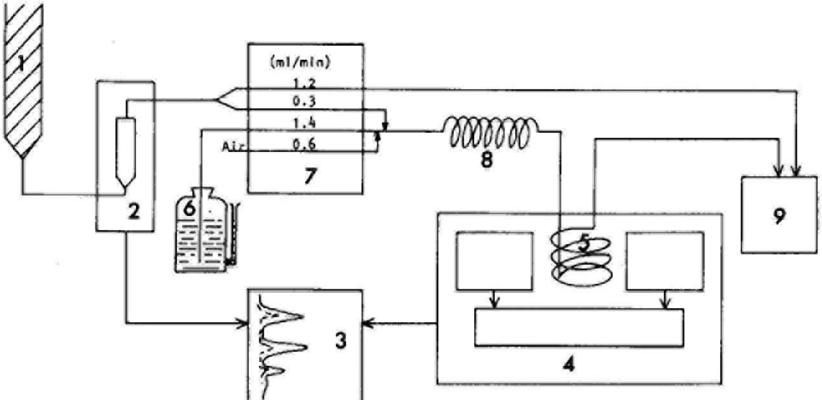

Fig. 3. Schematic representation of the homogeneous counting system. 1 = Column; 2 = ultraviolet detector, 254 nm; 3 = two-pen recorder; 4 = liquid scintillation spectrometer; 5 = helical flow cell; 6 = scintillator solution reservoir; 7 = proportioning pump; 8 = mixing spiral; 9 = fraction collector.

activated lithium glass beads, in spite of the relatively poor counting efficiency. No tailing of peaks or increase in background were observed. A minor inconvenience is the strong phosphorescence of the glass, which makes it necessary to store the cell in the dark for some days before use. Provided that excessive light is avoided during installation of the cell, adaptation is rapid. The resulting background is about 140 counts/min in the ^{14}C channel.

HOMOGENEOUS COUNTING SYSTEM

The system developed for homogeneous counting is shown in Fig. 3. Part of the column effluent is dissolved in a scintillator solution, which is subsequently passed through an empty flow cell. Emulsions of the type obtained in toluene–Triton X-100 mixtures are also regarded as being homogeneous in this context. A Technicon Auto-Analyzer proportioning pump, placed after the ultraviolet monitor, regulates the flow-rates. The total flow-rate of the effluent is 1.5 ml/min, of which 1.2 ml/min (80%) is usually collected unchanged. The remainder is added to a stream of scintillator solution (1:1 v/v toluene–Triton X-100 containing 8 g/l of PPO and 0.2 g/l of POPOP for aqueous effluents), which is interspaced with air bubbles to prevent tailing due to the difference in laminar flow-rates during transport. The scintillator solution and air were proportioned at flow-rates of 1.4 and 0.6 ml/min, respectively. The scintillator solution is transported through toluene-resistent Acidflex tubing (Technicon, Rotterdam). The mixture is homogenized in a mixing spiral, 70 cm long and of I.D. 2 mm. As mixing takes place between two successive air bubbles, *i.e.*, in about 30 μl of solution, resolution is not affected. The solution is then passed through a helical flow cell of I.D. 2 mm and volume 1.4 ml. The net flow-rate through the cell is 2.3 ml/min. The solution emerging from the flow cell can be collected if a more accurate determination of the radioactivity of the peak is required. One can, of course, use a different splitting ratio or even no splitting at all. For higher flow-rates, a larger cell should be used.

The counting efficiency with the toluene–Triton scintillator solution was about

80% for ^{14}C and 30% for ^{3}H. The background was about 65 counts/min. The system has also been used in combination with an AutoAnalyzer, in which event the counting mixture was passed through a 2.8-ml double-helical flow cell at a rate of 4.6 ml/min. A similar flow system has also been applied for the continuous detection of radioactive effluents from a gas chromatograph[7].

COMPARISON BETWEEN THE TWO COUNTING SYSTEMS

Liquid chromatography of an aqueous solution of [^{14}C]oxalic acid, [8-^{14}C]inosine 5'-monophosphate and [U-^{14}C]-guanosine 5'-monophosphate (each 80 nCi) over a strongly acidic cation-exchange resin (Dowex 50 X4 in the H^+ form, 450 mesh) was

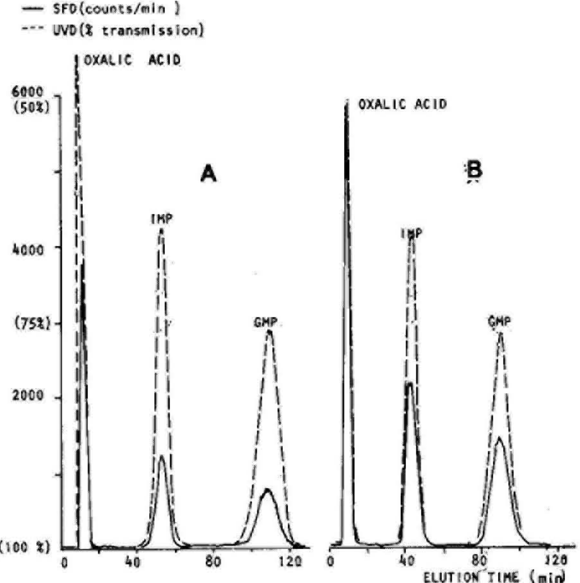

Fig. 4. Response to effluent of scintillation flow detector (SFD) and ultraviolet detector (UVD) after ion-exchange chromatography of ^{14}C-labelled oxalic acid, inosine 5'-monophosphate (IMP) and guanosine 5'-monophosphate (GMP). (A) Heterogeneous counting: 0.4-ml cell filled with glass scintillator beads, flow-rate 1.5 ml/min. (B) Homogeneous counting: 20% of effluent added to toluene–Triton scintillator solution; 1.4-ml cell, flow-rate 2.3 ml/min.

performed in duplicate and monitored with both the heterogeneous and homogeneous counting systems. The column was eluted with 0.05 *N* HCl. For heterogeneous counting (Fig. 1), the U-shaped flow cell was filled with cerium-activated lithium glass beads. The responses of the ultraviolet detector (UVD) and the scintillation flow detector (SFD) are plotted against elution time in Fig. 4A. For the homogeneous counting (Fig. 3) 20% of the column effluent was split off and added to a toluene–Triton X-100 scintillator solution. The plots of UVD and SFD response against elution time are shown in Fig. 4B.

For both homogeneous and heterogeneous counting, the correlation between the SFD and UVD responses was good. The SFD responses were of similar size for

the two systems. The lowest detectable ^{14}C-activity per peak was estimated at 10 nCi for both the heterogeneous system and the homogeneous system with an effluent splitting ratio of 4:1. In neither system was there any tailing or loss of resolution.

CONCLUSIONS

The main advantage of the heterogeneous counting system is that the effluent is fully recovered. Care should be taken in the choice of the solid scintillator; the only scintillator that does not dissolve in any common solvent and on which no materials are adsorbed is cerium-activated lithium glass. In view of the low counting efficiency of this scintillator, the system is most suitable for monitoring the separation of compounds of high activity, *e.g.*, in the purification of synthesized or commercial radioactive materials. The activity per peak should exceed 10 nCi for ^{14}C and 1 μCi for ^{3}H. In many isotope dilution analyses with ^{14}C-labelled compounds, the radioactivity will be high enough for this system.

In the homogeneous counting system, part of the effluent is "lost" when it is dissolved in the counting solution. The counting efficiency, however, is better than in the heterogeneous counting system, especially for ^{3}H. When no splitter is used, the activity per peak may be as low as 2 nCi for ^{14}C and 5 nCi for ^{3}H. This system can therefore be used with success in dual-label experiments, subject to the appropriate choice of spectrometer channels. Aqueous effluents can be dissolved in toluene–Triton X-100 and non-aqueous effluents in toluene scintillator solutions. When these solutions, interspaced with air bubbles, are pumped through toluene-resistent Acidflex tubing, no loss in resolution or tailing occurs. The homogeneous counting system is therefore most suitable for detection of ^{3}H, low activities, and for dual-label experiments.

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THE SEPARATION AND QUANTITATION OF CARBOHYDRATES ON CATION-EXCHANGE RESIN COLUMNS HAVING ORGANIC COUNTERIONS

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SUMMARY

The influence of organic base counterions and their degree of substitution on the separation of carbohydrates on strongly acidic cation-exchange resins with ethanol–water as mobile phase is described. Contrary to previous findings with inorganic counterions, increasing counterion size reduced the capacity ratios for the carbohydrates. The trimethylammonium form of the resin was found to give the best separations and the optimization of these separations with respect to mobile phase composition and column temperature is described. The common naturally occurring mono- and disaccharides (rhamnose, xylose, arabinose, mannose, glucose, galactose, maltose and lactose) can be rapidly separated and a quantitative method for their determination based on the modified moving wire/flame ionization detector is discussed.

INTRODUCTION

As the technological significance of the polysaccharide and individual carbohydrate composition of food products has become more apparent, the requirement within our laboratory for rapid qualitative and quantitative methods for the characterization of the carbohydrate components of food has increased. The upsurge of interest in liquid chromatographic techniques, with its attendant developments in pump, column packing, and detector technology, has led to many advances in the separation of low-molecular-weight carbohydrates. SAMUELSON and coworkers[1–3] have described separation methods based on both cation- and anion-exchange resins with ethanol–water as mobile phase, while other workers[4,5] have described the separation of borate complexes of simple carbohydrates on anion-exchange resins. Initial experiments in our laboratory with anion- (using both ethanol–water and borate techniques) and cation-exchange resins[6] suggested better and more rapid separations with the cation-exchange resins. The effect of counterion size on the separations indicated that organic bases might have useful properties as counterions. The investigation of the effects of organic bases as counterions with cation-exchange resins for the separation of carbohydrates is described.

A number of methods of detecting carbohydrates in a column effluent have been

TABLE I

EFFECT OF ORGANIC BASES ON THE CAPACITY RATIOS FOR CARBOHYDRATES

Aminex A-6 strongly acidic cation-exchange resin, 50 × 0.4 cm I.D. column; column temperature, 65°; mobile phase composition, 85% (w/w) ethanol in water.

Counterion	*Rhamnose*	*Ribose*	*Xylose*	*Arabinose*	*Fructose*	*Mannose*	*Glucose*	*Galactose*	*Sucrose*	*Maltose*	*Lactose*
Ammonium	4.19	6.49	7.04	9.47	10.73	11.50	11.43	14.10	[a]	21.08	37.62
Methyl ammonium	3.86	5.82	6.59	9.18	9.38	10.70	11.05	13.39	10.61	15.97	25.86
Dimethyl ammonium	2.77	4.16	4.91	6.06	6.06	6.96	7.41	8.62	6.36	9.27	14.11
Trimethyl ammonium	2.44	3.60	4.59	5.14	4.95	5.76	6.53	7.15	6.01	7.34	10.72
Tetramethyl ammonium	1.90	2.90	4.31	4.62	3.97	4.96	6.12	6.30	dec.[b]	7.24	10.46
Tetraethyl ammonium	1.92	2.86	3.68	3.68	3.46	4.24	4.56	4.92	4.59	5.01	6.17
Piperidinium	1.83	2.59	2.95	3.36	3.27	3.84	3.89	4.39	3.02	3.77	5.21
Hydroxy ammonium	3.27	4.13	4.63	5.00	7.48	7.17	8.93	9.43	[a]	16.30	26.03

[a] Not run.
[b] dec. = decomposition.

described. The most common, most complicated and probably most sensitive technique is based on a reaction with anthrone, orcinol, or cysteine, the carbohydrate derivative being monitored colorimetrically[4,5]. Other methods include direct weighing of the eluted carbohydrate[7], refractometric monitoring[8], direct combustion in a flame ionization detector[9], and discontinuous monitoring of carbon content[10]. HOBBS AND LAWRENCE[6] have described a quantitative method for the determination of lactose in milk. This method, based on the modified moving wire/flame ionization detection system[11], was used in the work described below.

EXPERIMENTAL

Apparatus

A standard liquid chromatographic system consisting of a solvent reservoir, pump, thermostatted column and continuous detector was used.

The pump, which was developed in our laboratory, was continuous, pulse-free, and capable of operating at up to 68 atm. The column flow-rate was temperature and composition dependent but was, typically, for a 100-cm column, 0.45 ml min^{-1} at a column temperature of 75° and inlet pressure of 30 atm with 85% ethanol in water as mobile phase.

The column (50 or 100 × 0.4 cm I.D.) was glass, fitted with a Pye GLC injection head (Pye Unicam, Cambridge). The column inlet was a 6.35-mm Kovar-to-glass seal, the injection head being slightly modified to take a compression fitting to the Kovar. The column outlet was a standard Pye GLC fitting connected directly to the detector. A water jacket, maintained at a constant temperature in the range 65 to 85° by circulation from a constant temperature water-bath, enclosed the column. A small cooling coil was wound round the column outlet connection to reduce solvent evaporation at the detector inlet.

A modified Pye moving wire detector[11] provided a direct trace of the column eluent composition on a potentiometric recorder, there being no necessity for coloured derivative formation as in methods previously described[2,4].

Materials

The carbohydrates used as standards were AnalaR grade (Hopkin and Williams, Chadwell Heath, Essex). The organic bases were obtained from B.D.H. (Poole, Dorset).

The resin (Aminex A-6, 17.5 μm particle size; Bio-Rad Laboratories, St. Albans, Herts.) was converted to the forms investigated (Table I) by repeated washings with 2 *N* solutions of the appropriate organic base. The packing procedure was to allow a slurry of the resin in the developing solvent to settle under gravity, aliquots of the slurry being added as necessary to fill the column.

Operating conditions

85% (w/w) ethanol in water was used as mobile phase to compare the effects of the organic base counterions. This composition was suggested by previous work (ref. 1, and Fig. 1 of ref. 6). A column temperature of 65° was chosen to compromise between carbohydrate solubility and column operating pressure. The shorter 50-cm column was used in each case.

For the fuller investigation of the trimethylammonium form of the resin, the column temperature was varied from 65 to 85° at mobile phase compositions from 80 to 87.5% (w/w) ethanol in water at both high and low flow-rates. The lower temperature limit was set by the mobile phase viscosity, the column inlet pressure to achieve adequate flow-rates at lower temperatures tending towards the safe limit for the column. Injection was from a 10-μl syringe, with the column flow stopped, into a 4-cm layer of fine glass beads above the column packing to give an even application to the resin.

RESULTS

Table I illustrates the effect of the resin form on the capacity ratios for the individual mono- and disaccharides. The capacity ratio (k') is related to the distribution volume (D_V, see ref. 1) and is given by:

$$k' = (V_R - V_0)/V_0$$

where V_R is the peak retention volume and V_0 is the column dead volume, which was the elution volume of the non-retained compound methyl palmitate.

The relative retentions of the individual carbohydrates on each resin form, the observed separations achieved, the total analysis times, and the effects of resin form on band broadening in the column showed that the best separation of mono- and disaccharides was achieved on the trimethylammonium form of the resin (Fig. 1).

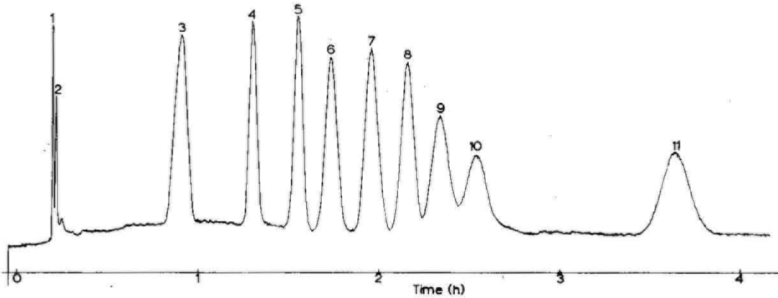

Fig. 1. Separation of mono- and disaccharides. Column, 100 × 0.4 cm I.D. Aminex A-6, trimethylammonium form; eluent, 85% (w/w) ethanol in water; temperature, 65°; flow-rate, 0.266 ml min^{-1}. Each peak corresponds to approximately 150 μg carbohydrate. 1 = Methyl palmitate; 2 = tetramethylglucose; 3 = rhamnose; 4 = ribose; 5 = xylose; 6 = arabinose; 7 = mannose; 8 = glucose; 9 = galactose; 10 = maltose; 11 = lactose.

The effects of column temperature, mobile phase composition, and flow-rate on the separation of carbohydrates on a 100-cm column of the trimethylammonium form of the strongly acidic cation-exchange resin were therefore investigated in order to optimize the separations obtained. The results obtained are summarized in Table II.

The effect of flow-rate was also considered. It was found that, within experimental error in the range 0.15 to 0.45 ml min^{-1}, the capacity ratio for each carbohy-

TABLE II

EFFECT OF SOLVENT COMPOSITION AND TEMPERATURE ON THE CAPACITY RATIOS FOR CARBOHYDRATES

oo × 0.4 cm I.D. column of trimethylammonium form of strongly acidic cation-exchange resin.

Carbohydrate	*Solvent composition % ethanol (w/w)* [a]											
	80.0			*82.5*			*85.0*			*87.5*		
	65°	*75°*	*85°*	*65°*	*75°*	*85°*	*65°*	*75°*	*85°*	*65°*	*75°*	*85°*
Rhamnose	2.06	1.93	1.86	2.20	2.17	2.08	2.44	2.30	2.23	3.30	3.17	3.04
Fucose	2.66	2.39	2.25	2.87	2.64	2.42	3.28	2.95	2.69	4.32	3.86	3.65
Ribose	3.02	2.83	2.66	3.22	3.14	3.01	3.60	3.33	3.20	4.91	4.62	4.33
Xylose	3.73	3.48	3.29	4.01	3.94	3.77	4.59	4.25	4.08	6.31	5.96	5.59
Arabinose	4.18	3.84	3.54	4.49	4.32	4.06	5.14	4.66	4.38	7.07	6.52	6.01
Mannose	4.40	4.17	3.94	4.89	4.83	4.64	5.76	5.36	5.15	8.30	7.91	7.42
Glucose	4.90	4.61	4.33	5.48	5.37	5.12	6.53	6.01	5.75	9.35	8.84	8.18
Galactose	5.33	4.94	4.55	5.94	5.70	5.34	7.15	6.33	5.99	10.08	9.36	8.67
Fructose	3.82	3.49	3.29	4.26	3.96	3.62d	4.95	4.51	4.09d	6.84	6.14d	dec.
Sucrose	3.80d	dec.	dec.	4.55d	4.44d	dec.	6.01	5.88d	dec.	9.26	8.38	8.04
Maltose	4.73	4.39	4.14	5.68	5.42	5.06	7.34	6.84	6.44	11.92	10.72	10.24
Lactose	6.71	6.01	5.56	8.23	7.56	6.86	10.72	9.55	8.80	17.84	15.36	14.02

[a] dec. or d. = total or partial decomposition.

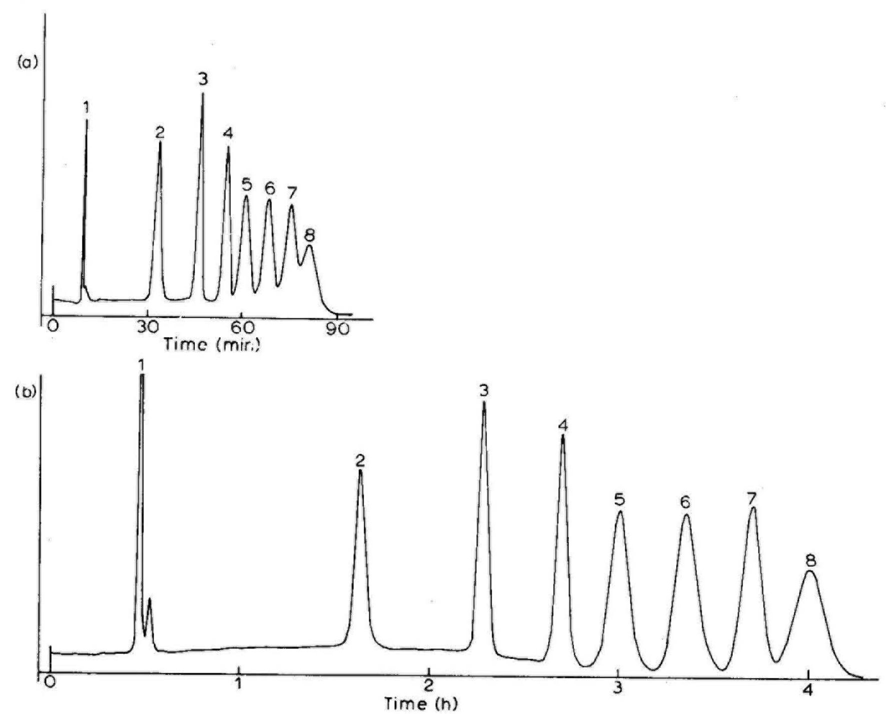

Fig. 2. Effect of flow-rate on the separation of monosaccharides. Flow-rate, (a) 0.49 ml min^{-1}; (b) 0.16 ml min^{-1}. For further conditions, see the legend to Fig. 1. 1 = Tetramethylglucose; 2 = rhamnose; 3 = ribose; 4 = xylose; 5 = arabinose; 6 = mannose; 7 = glucose; 8 = galactose.

drate was independent of flow-rate. However, it is apparent from Fig. 2 that flow-rate has a marked effect on resolution. This is particularly noticeable for glucose and galactose, it being possible to achieve their base-line separation if the flow-rate is low enough. The effect of temperature and mobile phase composition at constant flow-rate on the resolution of glucose and galactose is shown in Table III.

TABLE III

EFFECT OF SOLVENT COMPOSITION AND TEMPERATURE ON RESOLUTION OF GLUCOSE AND GALACTOSE

100 × 0.4 cm I.D. column of trimethylammonium form of strongly acidic cation-exchange resin.

Temperature (°C)	*Solvent composition % ethanol (w/w)*			
	80.0	*82.5*	*85.0*	*87.5*
65	1.238	1.183	1.187	1.143
75	1.238	1.112	1.113	1.037
85	1.058	1.007	0.965	0.854

Here, resolution $= (V_R^2 - V_R^1)/(P_W^2 + P_W^1)$, where the superscripts refer to individual peaks, V_R is defined above, and P_W is the peak width at 0.6065 of the peak height.

The mechanism of the separation system leads to chromatographically efficient columns, the theoretical plate height of the majority of carbohydrate peaks lying between 0.1 and 0.5 mm. Generally, sharper peaks are obtained at higher temperatures and higher water contents in the mobile phase.

DISCUSSION

Previous work[1,2] has shown that increasing the size of inorganic counterions used with strongly acidic cation-exchange resins increases the capacity ratios for individual mono- and disaccharides separated using them. However, the opposite trend is observed for alkyl-substituted ammonium ions as their size increases from ammonium, through the methylammonium forms to tetraethylammonium (Table I). The tetraethylammonium form gives a smaller difference from the tetramethylammonium form than might be expected from the change in ionic size. Another noticeable effect is that the hydroxyammonium form gives a good group separation of the pentoses, hexoses and disaccharides. As opposed to the inorganic counterions (including ammonium), the alkyl-substituted counterions separate mannose and glucose.

A number of effects may contribute to the separation of carbohydrates on ion-exchange resin columns. The most important are interaction with the counterion and partition between the water-rich stationary phase within the resin matrix and the ethanol-rich mobile phase[1,12]. Dispersion, electrostatic, and hydrogen bonding interactions with the resin matrix, the counterion, and the stationary phase may also influence the separation. The increasing hydrophobic character of the organic counterions with successive substitution of methyl or ethyl groups for the hydrogen of the ammonium ion will tend to reduce interaction with the hydroxyls of the carbohydrates and thus reduce capacity ratios. Similarly, the increasing hydrophobic character and decreasing charge density of the counterions will alter the relative amounts of ethanol

and water taken up by the resin, reducing water relative to ethanol and therefore decreasing the difference between the compositions of the mobile and stationary phases. The balance of the effects influencing the retention of the carbohydrates on the column will govern the separations obtained.

The effects of increasing water content of the mobile phase and increasing column temperature are to reduce the capacity ratios for the individual carbohydrates (Table II, trimethylammonium form). The effect of mobile phase composition changes is especially marked for disaccharides, which are generally less soluble in solutions of high alcohol content. The data in Table II may be used to predict the optimum conditions for the separation of most carbohydrate mixtures, base-line separation being achievable when the ratio of capacity ratios of any carbohydrate pair is greater than 1.08.

The implication of these temperature and mobile phase composition influenced variations in capacity ratios is that care must be taken in the initial selection of analysis conditions. If the carbohydrate mixture under analysis contains only monosaccharides, the mobile phase can have a much higher level of water, reducing the capacity ratios and giving a more rapid separation. However, if the disaccharides maltose and lactose are present, ' wer water contents are required to permit their elution after the monosaccharides (Table II). The advantages of higher water contents are evident from Table III, which illustrates that the resolution of the monosaccharide pair glucose and galactose increases with the water content of the mobile phase. In this case, the peaks become narrower more rapidly than their maxima approach and thus an enhancement in resolution is obtained.

It is evident from Fig. 2 that flow-rate influences peak resolution. However, in order to maximize the number of analyses, it is necessary to work at the highest possible flow-rate. This will be influenced by the resolution required between the carbohydrates present. For qualitative work, optimum resolution is not always necessary but for quantitative work it is advantageous to obtain complete resolution. The use of the modified moving wire detector, which permits the continuous monitoring of the column outflow and gives a reproducible, predictable response to carbohydrates, allows the carbohydrates present in a mixture to be readily quantified. The method of quantitation recommended is an extension of that described by HOBBS AND LAWRENCE[6], in which a carbohydrate not present in the mixture is added as an internal standard. In the majority of cases, ribose can conveniently be used. The use of this method with the moving wire detector gives an absolute measurement. The use of continuous refractive index monitoring for carbohydrate detection was studied but found to be less sensitive than the method described.

One minor disadvantage of the method described has been found to be ketose decomposition. This is particularly true for fructose at higher temperatures (>75°) and sucrose which hydrolysed at below 82.5% ethanol at all temperatures investigated. There are, however, a number of advantages in the method. The column itself, the trimethylammonium form of a strongly acidic cation-exchange resin, is the first cation-exchange resin column on which the separation of mannose and glucose has been reported, although it should be noted that similar separations were obtained with the other organic bases. The column system is flexible and gives rapid separations (*cf.* refs. 1, 4, and 5). Cation-exchange resins also have the advantage over anion-exchange resins that they are readily commercially available as smaller, more uni-

form resin particles (due to their use in amino acid analysers). They thus pack well and give narrow, chromatographically efficient peaks which are simple to quantify. Although cation-exchange resin columns have given superior results in our laboratory, other workers[13] have demonstrated equally fast and efficient separations of complex mixtures using anion-exchange resins in the sulphate form.

CONCLUSIONS

The most suitable of the organic base counterions for use with a strongly acidic cation-exchange resin for the separation of carbohydrates was trimethylammonium. This resin form gave the optimum compromise between elution volumes, peak widths and analysis times, permitting the rapid separation of the common naturally occurring mono- and disaccharides. The quantitation of the carbohydrate peaks is readily achieved with the modified moving wire/flame ionization detector.

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CHROM. 6120

THE USE OF QUATERNARY AMMONIUM ETHYL CELLULOSE IN THE FRACTIONATION OF TYROSINE-RICH PROTEINS FROM WOOL

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SUMMARY

A group of tyrosine-rich proteins from wool which are only soluble to any extent above pH 10.0, have been chromatographed on quaternary ammonium ethyl cellulose at pH 10.5. The elution profile consisted of two major and nine minor peaks. This fractionation was based largely on differences in the tyrosine content of the proteins which covered the range from 7 to 20 residues %. A combination of chromatography at pH 10.5 followed by gel electrophoresis at pH 8.5 revealed the presence of at least thirty components in this protein preparation, the great majority of which contained 27–33 residues % of glycine, 8–12 residues % of phenylalanine, 9–16 residues % of serine and 3–7 residues % of carboxymethylcysteine.

INTRODUCTION

Small proteins, which contain relatively large proportions of glycine and the aromatic amino acids, particularly tyrosine, appear to be a common constituent of mammalian keratins, occurring in amounts up to an apparent maximum of 30% in *Echidna* quill[1,2]. In spite of the major role which they can play in some keratins, they have received only scant attention, and little is known about them, particularly the extent of their heterogeneity and the limits of their amino acid compositions. One reason for this is that certain components, particularly those low in sulphur, in the reduced and S-carboxymethylated form, are difficult to handle by many conventional techniques, being only slightly soluble below pH 10.0. The anion exchangers based on cellulose, commonly used in protein fractionation are uncharged at these pH values. Although these proteins are soluble in concentrated formic acid and 6 *M* urea solutions, fractionation at acid pH values has had only limited success because they contain few basic groups[3,4], whilst in urea at neutrality, and at slightly alkaline pH values, it has so far proved impossible to prevent carbamylation reactions taking place. This is an important consideration, for the cystine-poor components at least, appear unique amongst keratin proteins in having non-acetylated end groups.

The availability of quaternary ammonium ethyl (QAE) cellulose, which contains anion-exchange groups still ionized at pH values above 10 where the proteins

are soluble, has made it possible to explore the nature and extent of the heterogeneity of these proteins and to isolate one component in a state approaching purity. This paper describes a study made of the group of cystine-poor, high-tyrosine proteins, which have been termed Type I components[1].

EXPERIMENTAL

Preparation of high-tyrosine proteins

Wool was solubilized by alkaline reduction in 6 *M* urea, alkylated with iodoacetate, and the solution dialysed[5]. Both the high-tyrosine and low-sulphur proteins were precipitated from this solution by addition of zinc acetate to 0.02 *M* (pH 6.0). The precipitate was collected by centrifugation, stirred with 0.02 *M* sodium citrate solution for 2 h, and the turbid solution was then dialysed against running deionized water for 2 days.

This heavily turbid solution of zinc-precipitable proteins was centrifuged at 40,000 *g* for 1 h to sediment those high-tyrosine proteins (Type I) which are essentially insoluble below about pH 10. The precipitate was dissolved in 0.1 *M* ammonium hydroxide and the high-tyrosine proteins reprecipitated by dialysing the solution against 0.1 *M* ammonium bicarbonate solution, leaving any contaminating low-sulphur proteins in solution. The precipitate was dissolved in 0.1 *M* ammonium hydroxide and the solution freeze-dried[1].

Chromatography

QAE-cellulose (Schleicher and Schüll, Lot 2282, 0.84 mequiv./g) (20 g) was suspended in 2 l 0.5 *M* NaOH, stirred for 1 h, collected by filtration on a Büchner funnel, and then washed on the funnel with several litres of deionized water. The cellulose was then suspended in 1 l of starting buffer (0.05 *M* β-alanine–0.05 *M* NaOH, pH 10.5) with the aid of a Waring Blendor, stirred for 1 h, collected by filtration, resuspended in 500 ml of starting buffer, deaerated, and used to pack a column immediately. If the suspension stood for more than a few hours before use, the cellulose packed as sheets with pronounced layering. The column dimensions were 50 $\times$ 2 cm, and a packing pressure of 5 lbs/in.^{2} was used.

300 mg of protein was dissolved in 20 ml of starting buffer, the pH adjusted to 10.5 by the dropwise addition of ammonia, centrifuged to remove any insoluble material, and applied to the top of the column. Proteins were eluted by a linear gradient of sodium chloride in the starting buffer to a limiting concentration of 0.5 *M*. The total volume of eluant was 1200 ml with a pumping rate of 1.6 ml/min and a fraction size of about 10 ml. The column was regenerated between runs by passing through 2 l of the starting buffer, and periodically the cellulose was washed with 0.5 *M* NaOH and the equilibration and packing procedure repeated.

Amino acid analysis

The samples of protein were hydrolysed for 22 h *in vacuo* at 108° with 6 *M* HCl and 2 m*M* phenol. Each hydrolysate was freeze-dried, and the amino acid composition estimated with a Beckman-Spinco 120C amino acid analyzer.

Electrophoresis in starch gels

The gel was composed of 13.0 g hydrolysed starch (Connaught), 40 g urea, and 50 ml buffer (pH 8.46), containing citric acid 8.5 m*M*, Tris 75 m*M* and sodium tetraborate 3.7 m*M*. The bridge buffer (pH 8.35) contained boric acid (0.3 *M*) and NaOH (0.06 *M*). The gels were run in a cooled plate apparatus at a voltage gradient of 15 *V*/cm for about 4 h. At the completion of electrophoresis the gels were sliced and stained with nigrosine (0.01%) in water–ethanol–acetic acid (45:45:10)[1].

RESULTS AND DISCUSSION

Type I high-tyrosine proteins are almost completely eluted from QAE-cellulose at pH 10.5 with a salt gradient with a limiting value of 0.5 *M* (Fig. 1). The elution profile consists of two major peaks (C and F) and at least nine minor ones. Ten fractions were prepared for further examination by pooling appropriate fractions as shown on the profile. They were then dialysed against deionized water and freeze-dried. The yields of fractions after dialysis and freeze-drying are shown on Table I. The yield is about 87% which is remarkably good considering the possibility of losses during handling.

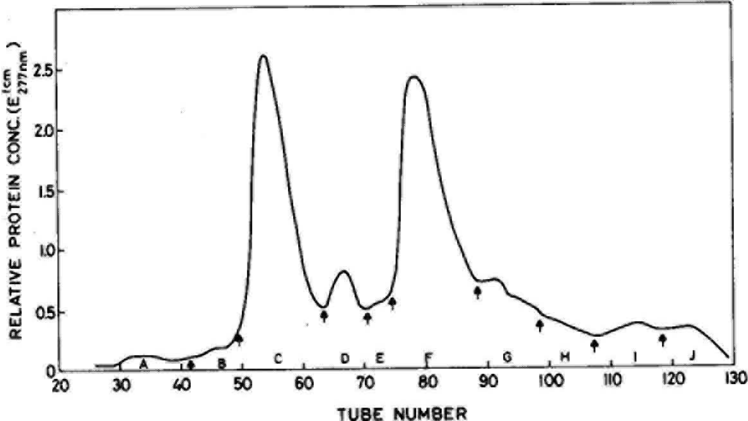

Fig. 1. The chromatography of Type I high-tyrosine proteins on QAE-cellulose at pH 10.5 in β-alanine–NaOH buffer, 0.05 *I*. A linear gradient of sodium chloride rose from 0 to 0.5 *M*. Arrows indicate the pools made for the recovery of the ten fractions referred to in the text, in Fig. 2, and in Table I.

These ten fractions, together with the unfractionated protein, were compared by starch gel electrophoresis. It can be seen (Fig. 2) that there are large differences between the fractions, both in the number and the type of components they contain. The fractionation appears to have been relatively sharp as there is surprisingly little overlap between successive fractions. In the gel patterns of the parent protein a maximum of ten bands can be counted but some of these single bands now appear to contain a number of proteins with very similar mobility but with substantially different chromatographic properties. It is difficult to make an accurate estimate of the total number of components but it is not less than thirty.

TABLE I

PROTEIN YIELDS (mg) AND AMINO ACID COMPOSITIONS (RESIDUES %) OF FRACTIONS OBTAINED FROM TYPE I HIGH-TYROSINE PROTEINS BY CHROMATOGRAPHY ON QAE-CELLULOSE AT pH 10.5

For fraction identification see Fig. 1.

Amino acid		*Amino acid composition*									
		Fraction									
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
	Yield of protein (mg)	*2*	*5*	*100*	*25*	*5*	*70*	*10*	*15*	*25*	*3*
Lys		0.29	0.25	0.17	0.21	0.33	tr[b]	0.21	0.71	0.27	0.27
His		1.76	2.89	1.53	1.43	1.00	0.52	0.85	0.90	0.34	0.36
Arg		7.64	7.72	5.66	7.01	5.58	4.04	4.18	4.72	5.08	4.99
CmCys[a]		5.71	7.27	5.69	6.01	6.25	5.72	4.42	3.68	3.94	3.71
Asp		2.77	3.18	4.40	3.36	3.12	2.57	2.23	4.13	4.35	4.33
Thr		5.95	1.74	4.71	2.07	2.21	2.94	2.23	3.23	3.07	3.17
Ser		15.80	12.40	13.10	12.90	11.90	13.10	12.10	10.90	9.14	9.52
Glu		3.02	0.65	0.44	0.72	0.88	0.74	0.41	1.10	1.60	1.72
Pro		9.87	3.78	6.10	2.15	2.96	5.20	4.08	5.94	6.00	6.08
Gly		15.70	30.20	26.50	33.00	32.90	28.10	31.10	28.00	26.60	27.30
Ala		1.87	0.85	1.16	0.50	0.83	1.89	1.47	1.03	0.60	0.63
Val		3.51	0.90	1.53	tr	1.00	3.15	2.09	2.45	2.95	2.90
Met		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ile		1.14	0.25	tr	tr	tr	tr	tr	0.71	1.12	1.09
Leu		7.83	6.47	6.10	5.08	6.09	4.99	6.10	6.65	4.56	4.35
Tyr		7.75	10.00	12.10	13.20	16.20	17.60	19.70	17.50	19.80	20.20
Phe		9.22	11.60	10.70	12.40	8.67	9.34	8.58	8.33	9.25	9.34

[a] CmCys = S-carboxymethylcysteine.
[b] tr = trace.

Fraction F appears to be substantially (>90%) a single component and this material, after further purification, is being used for physico-chemical and amino acid sequence studies. The amino acid compositions of the ten fractions are shown in Table I. All except fraction A fall into the class of Type I high-tyrosine proteins as they contain between 27 and 33 residues % of glycine, between 20 and 30 residues % of the aromatic amino acids, between 9 and 13 residues % of serine, and no more than 7 residues % of CmCys*[2]. There are other familial resemblances between the fractions, notably their comparatively small content of lysine, histidine, glutamic acid, and isoleucine, and their moderate content of arginine, proline, and leucine. In spite of these similarities there are substantial differences between them. Tyrosine occurs over an almost 2 to 1 range and there are quite significant differences between fractions in their contents of CmCys, arginine, serine, proline, phenylalanine, and leucine. It is clear therefore, that Type I high-tyrosine proteins consist of a group of related proteins which cover quite a range of amino acid compositions.

Methionine, isoleucine, lysine, histidine, valine, glutamic acid, and alanine may be absent or present to less than 1 residue per 100 residues in a number of fractions. Most components contain 90 residues or less and therefore must be deficient in some or all of these amino acid residues[1]. For example, the major component of fraction F

* CmCys = S-carboxymethylcysteine.

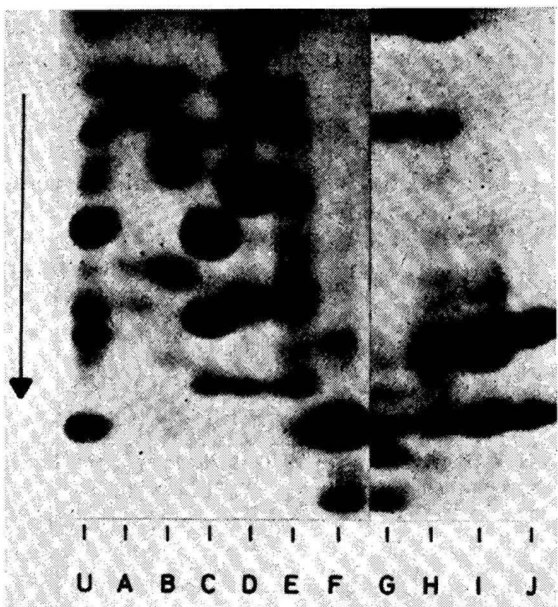

Fig. 2. Starch gel electrophoresis patterns of Type I high-tyrosine fractions isolated by chromatography at pH 10.5. Fraction numbering corresponds to that used in Fig. 1. U is the gel pattern of unfractionated Type I material.

after complete purification by further chromatography was found to be devoid of lysine, histidine, glutamic acid, methionine, and isoleucine.

The actual position in which each fraction elutes is almost certainly dictated by the contribution made to the net charge by ionized tyrosine residues. The relatively minor contribution from other charged groups can be illustrated by reference to fractions C and I which have a large difference in net charge at pH 10.5. However, if their net charge is calculated, excluding tyrosine, it is found to be the same if all carboxyls are free, and to differ by a maximum of two negative charges per mole in the extreme situation that all carboxyls in C are amidated but none of those in I.

Some separations appear to be unrelated to tyrosine content. For example, fractions G, I and J have substantially different elution positions although their tyrosine contents are similar. Other charged residues may in this case provide a secondary separation, although the possibility cannot be excluded that the relative proportions of tyrosine residues which are ionized may differ in the three fractions. Fraction A contains somewhat less tyrosine and glycine than the other fractions but significantly more threonine, serine, proline, leucine, and glutamic acid. Its higher glutamic acid content suggests that it may contain the glutamic acid-rich cell membrane proteins which are only moderately rich in glycine and the aromatic amino acids[6].

QAE-cellulose chromatography has thus proved a useful fractionation procedure for a protein system which is insoluble at pH values below 10 and where contact with urea has to be avoided. No evidence was found for protein damage at pH 10.5, for when a sample of high-tyrosine protein was chromatographed and then wholly recovered, without collecting separate fractions, the starch gel pattern obtained had the same number of bands with the same relative intensities. With more easily damag-

ed proteins it might be necessary to lower the temperature and to place a neutralizing buffer in each collector tube to minimize the time the proteins are at the high pH.

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CHROM. 6175

SEPARATION AND IDENTIFICATION OF FOOD COLOURS

IV. EXTRACTION OF SYNTHETIC WATER-SOLUBLE FOOD COLOURS

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SUMMARY

A method of extraction of synthetic water-soluble food colours using polyamide columns for the purification of the dye extracts is described. An attempt has been made to produce a method for the quantitative determination of the colour content of foodstuffs. The method has been applied to jellies, jams, sweets, cakes, canned meats and sausages.

INTRODUCTION

The direct identification of the synthetic food colours is generally impossible. The colour must be extracted from the foodstuff and purified, as co-extractives interfere with the identification of the colour by thin-layer chromatography and spectrophotometry. The extract must also be concentrated because many food colours are used at very low concentrations.

The wool dyeing technique[1] that is commonly used has several disadvantages. A number of dyes are taken up slowly from the hot acidic solution and the adsorbed dyes often undergo changes during the stripping from the wool with ammonia solution. This technique, although useful qualitatively, does not lend itself to the quantitative determination of the colour in a foodstuff. Various other procedures have been suggested for the extraction and purification of food colours, including solvent extraction[2], column chromatography[3], use of liquid ion exchangers[4] and extraction with quinoline[5] and quaternary ammonium compounds[6]. Of these, a method described by LEHMANN *et al.*[7] using polyamide powder for the purification of the food colours appeared to be the most promising, as the organic colours can be quantitatively absorbed and desorbed from the polyamide powder.

Modification of the LEHMANN *et al.*[7] extraction and clean-up technique enabled the extract to be applied to the thin-layer chromatographic identification scheme[8] and made the quantitative determination of the food colour possible.

MATERIALS AND METHODS

Apparatus

The chromatographic columns used were: (a) 300 mm long, 22 mm I.D., fitted with a ground-glass stopcock; (b) 250 mm long, 15 mm I.D., fitted with a ground-glass stopcock; (c) 200 mm long, 10 mm I.D. with an exit tube 40 mm long and 3 mm I.D. A Soxhlet apparatus was used for extraction.

Reagents

All reagents should be of analytical-reagent grade quality. The following reagents were used:

Acetone.

Acetone–ammonia solution: mix 40 ml of acetone, 9 ml of water and 1 ml of ammonia solution (sp. gr. = 0.88). This should be freshly prepared.

Celite 545.

Chloroform.

Ethanol, absolute.

Formic acid, 90%.

Hydrochloric acid, 0.5 *N* and 0.1 *N*.

Methanol.

Methanol–ammonia solution: mix 90 ml of methanol, 5 ml of water and 5 ml of ammonia solution (sp. gr. = 0.88).

Petroleum spirit, boiling range 40–60°.

Polyamide powder for column chromatography: MN CC6 from Macherey, Nagel and Co.

Polyamide staple fibre: Nylon 66, 3.3 g per 10,000 m of fibre.

Polyoxyethylene sorbitan mono-oleate solution: mix 1 ml of polyoxyethylene bitan mono-oleate with 99 ml of water.

Sand: acid-washed.

Tetramethylammonium hydroxide solution, 25% w/w aqueous.

Procedures

Procedure for samples completely soluble in water (jellies, jams, sweets). Weigh about 5 g of the sample into a beaker, add 50 ml of water and warm the beaker on a water-bath until a solution is obtained. Acidify the mixture with acetic acid. Place a plug of polyamide staple fibre in the end of a chromatography tube (15 × 250 mm) and add a suspension of polyamide powder in water to the tube to give a column approximately 20 mm high. Rinse the walls of the tube with a small volume of acetone to aid the settling of the polyamide and then place sand on top of the polyamide to form a layer about 6 mm deep.

Pour the hot solution through the column and wash the column with six 10 ml portions of hot water and three times with 5 ml volumes of acetone. (Light air pressure may be used, if necessary.) Elute the colours with the minimum volume of acetone–ammonia solution, rejecting the eluate until the colours are eluted. Remove the ammonia by blowing a current of air over the surface of the liquid and then reduce the volume by about half on a steam-bath. Add an equal volume of water and adjust the pH to 5–6 with hydrochloric acid.

Pour the solution through a column of polyamide in a 10 × 200 mm chromatography tube prepared as above and wash the column five times with 5 ml portions of hot water. Elute the dyes with the minimum volume of acetone–ammonia solution. Remove the ammonia as before and evaporate the solution to near dryness on a steam-bath. Dissolve the residue in a few drops of 0.1 *N* hydrochloric acid and use this solution for thin-layer chromatography. (If erythrosine is suspected, dissolve the residue in water.)

Procedure for bakery products (cakes, cake powders, pastries). Weigh about 5 g of the chopped sample into a glass evaporating basin and place the basin in a drying oven at 100° for 30 min. Add sufficient petroleum spirit to cover the dried sample (about 30 ml) and stir the mixture. Allow the solid to settle and decant off the petroleum spirit. Repeat this procedure twice more and then allow the residual petroleum spirit to evaporate. Grind the sample gently so as not to form too fine a powder, add 4 g of Celite and mix.

Place a plug of polyamide staple fibre in the end of a chromatography tube (250 × 15 mm) and transfer the powdered sample to the tube. Pour 30 ml of acetone on to the top of the column and when the solvent has percolated the whole length of the column apply slight air pressure to aid uniform packing. Discard the eluate. Carefully pour 50 ml of a methanol–water–tetramethylammonium hydroxide (40:9:1) solution through the column. (Light air pressure may be used if necessary.) Adjust the pH of the eluate to approx. 6 by the addition of dilute hydrochloric acid. Add 5 ml of 1% polyoxyethylene sorbitan mono-oleate solution and reduce the volume by about one half on a steam-bath with the aid of a current of air blown over the surface of the liquid. Add an equal volume of water to the solution and allow it to cool.

Place a plug of polyamide staple fibre in a 10 × 200 mm chromatography tube and add a suspension of polyamide powder in water to the tube to give a column approx. 20 mm high. Rinse the walls of the tube with a small volume of acetone to aid the settling of the polyamide and then place sand on top of the polyamide to form a layer about 6 mm deep.

Pour the solution of extracted dye through the column and wash the column three times with 5 ml portions of acetone, five times with 5 ml portions of chloroform–absolute ethanol–water–formic acid (100:90:10:1), three times with 5 ml amounts of acetone and finally three times with 10 ml volumes of water. Elute the dyes with the minimum volume of acetone–ammonia solution, rejecting the eluate until the dyes are eluted. Remove the ammonia by blowing a current of air over the surface of the liquid and then reduce the volume by about half on a steam-bath. Add an equal volume of water and adjust the pH to approx. 6 with hydrochloric acid. Pour the solution through a column of polyamide in a 10 × 200 mm chromatography tube prepared as above and wash the column with the same volumes of solvents in the sequence as described above for the first polyamide column. Elute the dyes with the minimum volume of acetone–ammonia solution. Remove the ammonia by blowing a current of air over the surface of the liquid and then evaporate the solution to near dryness on a steam-bath. Dissolve the residue in a few drops of 0.1 *N* hydrochloric acid and use this solution for thin-layer chromatography. (If erythrosine is suspected, dissolve the residue in water.)

Procedure for meat products. Weigh about 25 g of sample on to a glass plate. Chop up the sample with a knife, add 5 g of acid-washed sand and grind the mixture

to a paste. Add 10 g of Celite and mix with a palette knife until a homogeneous mixture is obtained.

Transfer the mixture to a Soxhlet thimble and extract with chloroform for 2 h. After extraction, remove the sample from the thimble and place it in an evaporating basin to allow the residual chloroform to evaporate.

Place a plug of polyamide staple fibre in the end of a chromatography tube (22 × 300 mm) and add the powdered sample to the tube, tapping the column gently to aid packing. Pass methanol–ammonia solution through the column until all the dyes are eluted. (Light air pressure may be used if necessary.)

Add 5 ml of 1% polyoxyethylene sorbitan mono-oleate solution and evaporate the solution on a steam-bath with the aid of a current of air blown over the surface of the liquid until all the ammonia and methanol are removed. Add an equal volume of water and adjust the pH of the solution to 6 with hydrochloric acid.

Place a plug of polyamide staple fibre in the end of a chromatography tube (15 × 250 mm) and add a suspension of polyamide powder in water to the tube to give a column approx. 20 mm high. Rinse the walls of the tube with a small volume of acetone to aid the settling of the polyamide and then place sand on top of the polyamide to form a layer about 6 mm deep.

Pour the solution of dyes through the column and wash the column three times with 10 ml portions of water, twice with 5 ml volumes of acetone, twice with 5 ml portions of a chloroform–absolute ethanol–water–formic acid (100:90:10:1) mixture and twice with 5 ml portions of acetone. Elute the dyes with the minimum volume of acetone–ammonia solution, rejecting the eluate until the dyes are eluted. Remove the ammonia by blowing a current of air over the surface of the liquid and then reduce the volume by about half on a steam-bath. Add an equal volume of water and adjust the pH to approx. 6 with hydrochloric acid. Pour the solution through a column of polyamide in a 10 × 200 mm chromatography tube prepared as above and wash the column as previously described. Elute the dyes with the minimum volume of acetone–ammonia solution. Remove the ammonia by blowing a current of air over the surface of the liquid and then evaporate the solution to near dryness on a steam-bath. Dissolve the residue in a few drops of 0.1 *N* hydrochloric acid and use this solution for thin-layer chromatography. (If erythrosine is suspected, dissolve the residue in water.)

RESULTS AND DISCUSSION

The products tested were divided into three groups: (a) samples completely soluble in water (jellies, jams and sweets); (b) bakery products (cakes, cake powders and pastries); (c) meat products (canned meat and sausages).

Samples completely soluble in water

No major difficulties were experienced with this group. Good recoveries were obtained when the following food colours (Colour Index Number, 1971, in brackets) were incorporated in jellies: Acid Yellow (13015), Amaranth (16185), Brilliant Blue FCF (42090), Erythrosine (45430), Fast Green FCF (42053), Green S (44090), Ponceau SX (14700), Sunset Yellow FCF (15985) and Tartrazine (19140).

Equally good recoveries were also obtained when the following dyes were added

to home-made blackcurrent or apricot jam: Acid Yellow (13015), Amaranth (16185), Black PN (28440), Green S (44090), Ponceau SX (14700), Red 6B (18055), Sunset Yellow (15985) and Tartrazine (19140).

Difficulties were encountered with Indigo Carmine, which decomposes very easily in alkaline solutions.

In order that the recoveries should be as quantitative as possible, the solutions containing the colour were passed through columns of polyamide so that the dyes were adsorbed as a narrow band on the top of the column instead of adding the polyamide to the dye solution as suggested by LEHMANN *et al.*[7]. It was also shown that methanol impaired the adsorption of the dyes by the polyamide and so the excess of methanol was removed from solutions before passing them through the polyamide columns. Acetone–ammonia was used for eluting the dyes from the polyamide column in place of methanol–sodium hydroxide, as the ammonia and acetone can be removed on a water-bath and on addition of acid no salts are formed that interfere with the adsorption of the dyes by the polyamide and also with some of the thin-layer chromatographic systems used for the identification of the dyes.

Bakery products

The procedure described by LEHMANN *et al.*[7] for pastries and baked products gave low recoveries when applied to the sponge and madeira cakes available in Great Britain. It was thought that this was mainly due to incomplete elution of the dyes from the cake–Celite column although much larger volumes of methanol–ammonia solution were used than were specified in the original method.

Consequently, various changes in the eluting solvent were tried. A known volume of a solution of Sunset Yellow FCF was added to 20 g portions of sponge cake and each portion was dried, mixed with Celite and packed into a chromatography column. Then 50 ml of acetone was passed through each column and each column was treated with a different eluting solvent, as listed in Table I. It can be seen from Table

TABLE I

ELUTING SOLVENTS

Solvent mixture		*Amount of dye recovered (%)*
Components	*Proportions*	
Methanol–0.88 ammonia	95:5	50
	85:15	58
Methanol–0.88 ammonia–water	90:5:5	58
	85:10:5	53
	80:15:5	46
Methanol–diethylamine–water	80:1:19	62
Methanol–tetramethylammonium hydroxide (25% w/w aqueous solution)–water	80:0.5:19.5	72
	90:2:8	66
	80:2:18	80
Methanol–tetrabutylammonium hydroxide (40% w/w aqueous solution)–water	90:2:8	56
	80:2:18	54

TABLE II

RECOVERIES OF DYES ADDED TO CAKES

Dye added	*Colour Index No. 1971*	*Recovery (%)*	
		Dye added after baking	*Dye added before baking*
Amaranth	16185	50–80	50–70
Brilliant Blue FCF	42090	89	69
Carmoisine	14720	88	75
Fast Red E	16045	73	—
Orange G	16230	89	—
Orange I	14600	80	50
Ponceau 4R	16255	88	80
Red 6B	18055	84	—
Sunset Yellow FCF	15935	80	79
Tartrazine	19140	87	61

I that the addition of a small amount of water and an increase in the basicity of the solvent enhanced the eluting power of the solvent. However, these changes also increased the co-extracted material. The addition of a small amount of a surfactant (polyoxyethylene sorbitan mono-oleate) dispersed these co-extractives and prevented them from clogging the polyamide column used for adsorbing the colours. The amount of surfactant must be kept to a minimum otherwise it will interfere with the adsorption of the dyes by the polyamide. Other co-extractives that interfered with the thin-layer chromatography of the dyes and the measurement of extinctions for recovery experiments were washed out of the polyamide column with a series of solvents before elution of the dyes with acetone–ammonia solution.

The recovery of various food colours in cakes was checked by adding a known amount of the food colour to weighed amounts of baked cake. The sample of cake was then treated as described above. The results are shown in Table II. Cakes were also prepared in which the food colour was incorporated before baking. A known amount of food colour was added to the flour used for making each cake and, after baking, a 5 mm layer was removed from the whole of the outside of the cake. The remainder of the cake was dried and mixed to a uniform powder and 10 g portions of this powder were taken for recovery experiments. The results obtained are shown in Table II. The procedure is not applicable to Chocolate Brown FB, Chocolate Brown HT or Indigo Carmine, as the two Chocolate Browns are not completely eluted from the polyamide columns and Indigo Carmine decomposes during the extraction.

Meat products

When the method of LEHMANN *et al.*[7] was applied to canned processed meats or sausages, it was found that some of the colour was removed by the treatment with acetone. In order to avoid recovering this colour from the acetone, the sample mixed with Celite is extracted with chloroform in a Soxhlet extraction apparatus. After evaporation of the residual chloroform, the sample is transferred to a chromatography column and the dyes are eluted with methanol–ammonia solution. The food colours are adsorbed on to a polyamide column and co-extracted material is washed out of

the column by a series of solvents before elution of the dyes with acetone–ammonia solution.

By using this procedure, recoveries of about 70% were obtained when Erythrosine (45430), Red 2G (18050), Red 6B (18055) were added to canned luncheon meat.

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CHROM. 6162

AUSNUTZUNG DER ENZYMHEMMUNG VON PHENOXYALKANCARBONSÄURE-HERBIZIDEN ZU DEREN DÜNNSCHICHTCHROMATOGRAPHISCH-ENZYMATISCHEM NACHWEIS

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SUMMARY

The utilization of the inhibition of some enzymes by phenoxyalkanecarboxylic acid herbicides for their thin-layer chromatographic–enzymatic identification

The thin-layer chromatographic–enzymatic inhibition technique using bovine liver esterase, alkaline and acid phosphatase, trypsine, α- and β-amylase, and urease is studied for the detection of phenoxyalkanecarboxylic acid herbicides. With the exception of amylases, which are only weakly inhibited or not inhibited, all of the above-mentioned enzymes can be used for herbicide detection. Of all the enzymes studied, detection by inhibition of the acid phosphatase is most sensitive. In general, irradiation of the herbicides with UV light does not have much influence on the detection limits.

EINLEITUNG

Über die Wirkung der Phenoxyalkancarbonsäuren, zu deren bekanntesten Vertretern 2,4-Dichlor- (2,4-D) und 2,4,5-Trichlorphenoxyessigsäure (2,4,5-T) gehören, ist mit Ausnahme der beiden genannten recht wenig bekannt. Chlorphenoxysäuren hemmen die Hill-Reaktion, doch konnte keine Korrelation zwischen herbizider Aktivität und der Fähigkeit, die Hill-Reaktion zu hemmen, festgestellt werden[1]. Eine etwas grössere Übereinstimmung scheint in der Auffassung zu herrschen, dass 2,4-D über eine Veränderung des Indol-3-essigsäure-Gehaltes in höheren Pflanzen wirkt[2–5]. Bekannt ist auch die stimulierende Wirkung auf die Nucleinsäure-Polymerasen[6–9]. Intensive Stoffwechseluntersuchungen, in deren Verlauf Photosynthese, Ionenabsorption, Protein-, RNA- und DNA-Synthese gemessen wurden, deuten darauf hin, dass die Pflanzen durch abnormes Wachstum in der Wurzel-Spross-Achse sterben[10]. Auch ein Wurzelverfall der behandelten Pflanzen wurde festgestellt[11,12], wobei dieser vor allem auf einen steilen Anstieg der Cellulase-Aktivität zurückgehen dürfte, der wiederum zu einer Durchlässigkeit der Zellwände und damit zu einem Austritt von Nucleotiden und Enzymen führt[12].

Ein bevorzugtes Gebiet für Untersuchungen zum Wirkungsmechanismus ist der Einfluss auf den Kohlenhydratstoffwechsel, doch finden sich hier eine Reihe von Widersprüchen. Einerseits wird berichtet, dass nach 2,4-D-Behandlung der Glucose-Stoffwechsel über den Pentosephosphat-Cyclus stimuliert wird[13], während andere eine Hemmung von Glycolyse und Pentosephosphat-Cyclus durch höhere 2,4-D-Konzentrationen feststellen, wobei die Glycolyse stärker gehemmt wird[14].

Recht interessant sind die toxikologischen Daten für einige Phenoxysäuren. Die akute und chronische Toxizität von 2,4-D wird für Labortiere als gering angegeben, wobei kaum Unterschiede zwischen den verschiedenen Salzen und Estern gegenüber dem reinen Wirkstoff bestehen[15]. Die Toxizität gegenüber Fischen ist dagegen recht erheblich. Eine Zusammenstellung dieser Daten und Untersuchungen zur Toxizität gegenüber Karpfen findet sich bei SCHULZ[16].

2,4,5-T ist in jüngster Zeit sehr in die Diskussion geraten, da man eine teratogene Wirkung feststellte[17], die jedoch zunächst auf Verunreinigungen mit 2,3,7,8-Tetrachlordibenzo-*p*-dioxin zurückgeführt wurde[18]. Diesen Ergebnissen stehen Befunde entgegen, wonach sowohl das Dioxin als auch gereinigtes 2,4,5-T zu den Schädigungen führten[19], und es wurde sogar die Ansicht vertreten[20], dass zwischen den beiden Verbindungen eine synergistische Wirkung bestehen könnte. In einer neueren Arbeit wird gezeigt, dass sich 2,4,5-T selektiv im Dottersackepithel anhäuft[21], woraus sich ein ähnlicher teratogener Wirkungsmechanismus wie für Trypanblau ergäbe, das sich dort ebenfalls anhäuft[22]. Für 2,4-D ist schliesslich noch eine Hemmung der Phosphorylase von Muskeln festgestellt worden[23,24].

In vorliegender Arbeit werden die Phenoxyalkancarbonsäuren nach einem Screening-Verfahren auf ihre Wirkung auf eine Reihe von Enzymen und auf die Möglichkeit untersucht, diese Enzymhemmung für einen Nachweis dieser Verbindungen auszunutzen.

MATERIAL UND METHODEN

Reagenzien

Alle verwendeten Lösungsmittel und Chemikalien waren analysenrein und stammten von der Firma Merck, Darmstadt. Zur Plattenbeschichtung wurde Kiesel Gel G nach Stahl mit *ca.* 13% $CaSO_4$ und einer mittleren Korngrösse von 10–40 μ von der gleichen Firma genommen.

Wirkstofflösungen und Dünnschichtchromatographie

Die in Tabelle I aufgeführten Phenoxyalkancarbonsäuren und ihre Ester wurden in Konzentrationen von 10 mg/ml in Aceton gelöst und bei Bedarf mit dem gleichen Lösungsmittel verdünnt. Die Wirkstoffe werden auf handgegossene Kiesel Gel G-Platten[25] aufgetragen und in Cyclohexan–Aceton (10:4) chromatographiert.

Durchführung des enzymatischen Hemmtests

Die Platten werden nach dem Entwickeln sofort oder nach einstündiger Bestrahlung mit UV-Licht der Wellenlängen 254/366 nm einer Fluotest Universal-Lampe (Hanau) bei einem Abstand Strahler–Platte von *ca.* 20 cm zunächst leicht mit Puffer und anschliessend mit Enzymlösung besprüht. Nach einer Inkubation von 30 min bei 25° und 80–90% Luftfeuchte wird mit Substrat nachgesprüht und in

TABELLE I

NAME UND STRUKTUR DER UNTERSUCHTEN PHENOXYALKANCARBONSÄURE-HERBIZIDE

Trivial-Name	*Chemische Bezeichnung*	*Strukturformel*
2,4-D	2,4-Dichlorphenoxyessigsäure	
2,4,5-T	2,4,5-Trichlorphenoxyessigsäure	
MCPA	2-Methyl-4-chlorphenoxyessigsäure	
Dichlorprop α-(2,4-D)-P 2,4-DP	2-(2,4-Dichlorphenoxy)-propionsäure	
Fenoprop 2,4,5-TP α-(2,4,5-T)-P	2-(2,4,5-Trichlorphenoxy)-propionsäure	
Mecoprop α-MCPP CMPP	2-(4-Chlor-2-methylphenoxy)-propionsäure	
2,4-D-isopropyl	2,4-Dichlorphenoxyessigsäure-isopropylester	
2,4-D-butyl	2,4-Dichlorphenoxyessigsäure-*n*-butylester	
2,4,5-T-butyl	2,4,5-Trichlorphenoxyessigsäure-*n*-butylester	
2,4,5-T-hexyl	2,4,5-Trichlorphenoxyessigsäure-*n*-hexylester	

(Fortsetzung auf S. 336)

TABELLE I *(Fortsetzung)*

MCPA-hexyl	2-Methyl-4-chlorphenoxyessigsäure-*n*-hexylester	Cl–C_6H_3(CH_3)–O–CH_2–C(=O)–O–$(CH_2)_5$–CH_3
MCPA-(2-butoxyäthyl)	2-Methyl-4-chlorphenoxyessigsäure-2-butoxyäthylester	Cl–C_6H_3(CH_3)–O–CH_2–C(=O)–O–CH_2–CH_2–O–$(CH_2)_3$–CH_3
Mecoprop-äthyl	2-(4-Chlor-2-methylphenoxy)-propionsäure-äthylester	Cl–C_6H_3(CH_3)–O–CH(CH_3)–C(=O)–O–CH_2–CH_3
Mecoprop-(3-oxybutyl) MCPP-(3-oxybutyl)	2-(4-Chlor-2-methylphenoxy)-propionsäure-3-hydroxy-*n*-butylester	Cl–C_6H_3(CH_3)–O–CH(CH_3)–C(=O)–O–CH_2–CH_2–CH(OH)–CH_3
Mecoprop-(2-butoxyäthyl) MCPP-(2-butoxyäthyl)	2-(4-Chlor-2-methylphenoxy)-propionsäure-2-butoxyäthylester	Cl–C_6H_3(CH_3)–O–CH(CH_3)–C(=O)–O–CH_2–CH_2–O–$(CH_2)_3$–CH_3
Mecoprop-hexyl	2-(4-Chlor-2-methylphenoxy)-propionsäure-*n*-hexylester	Cl–C_6H_3(CH_3)–O–CH(CH_3)–C(=O)–O–$(CH_2)_5$–CH_3
γ-(2,4-D)-B 2,4-DB	4-(2,4-Dichlorphenoxy)-buttersäure	Cl–C_6H_3(Cl)–O–CH_2–CH_2–CH_2–COOH
γ-(2,4,5-T)-B 2,4,5-TB	4-(2,4,5-Trichlorphenoxy)-buttersäure	Cl–C_6H_2(Cl)(Cl)–O–CH_2–CH_2–CH_2–COOH
γ-MCPB	4-(2-Methyl-4-chlorphenoxy)-buttersäure	Cl–C_6H_3(CH_3)–O–CH_2–CH_2–CH_2–COOH

einigen Fällen direkt oder nach weiterer Inkubation unter obigen Bedingungen ausgewertet.

Enzym- und Substratlösungen

Für die Untersuchungen mit Rinderleberesterase erfolgte die Herstellung der

Enzympräparation und Substratlösung in Anlehnung an ACKERMANN[26], doch wurde, wie schon früher beschrieben[25], das Homogenisieren der Leber und die Verdünnung der Enzympräparation mit 0.02 *M* Phosphatpuffer pH 7.0 durchgeführt. Als Substrat diente Naphthylacetat und als Kopplungsreagenz Echtblausalz B. Für das Besprühen der Platte wurde eine etwa 1:60 verdünnte Enzymlösung (w/v) genommen.

Der dünnschichtchromatographisch (DC)–enzymatische Nachweis mit Hilfe der Phosphatase-Hemmung erfolgte mit saurer Phosphatase aus Kartoffeln (EC 3.1.3.2—Boehringer, 2.0 U/mg) und alkalischer Phosphatase aus Kälbermucosa (EC 3.1.3.1—Serva, 1.0 U/mg) unter Verwendung von Nitrophenyl- bzw. Naphthylphosphat als Substrat, wobei im Falle des Naphthylphosphats Echtblausalz B als Kopplungsreagenz zugegeben wird. Die Methode des enzymatischen Hemmtests wurde an anderer Stelle ausführlich beschrieben[25].

Die Trypsin-Untersuchungen wurden mit Trypsin aus Rinderpankreas (EC 3.4.4.4—Merck, 2.0 U/mg) durchgeführt. Als Substrat diente eine Suspension von N^{α}-Benzoyl-DL-arginin-4-nitroanilidhydrochlorid in Puffer. Eine eingehende Beschreibung des Hemmtests findet sich in einer früheren Arbeit[28].

Als Enzymquelle für den Nachweis mit Hilfe der Amylase-Hemmung diente α-Amylase aus *Bacterium subtilis* (EC 3.2.1.1—Merck, 170 U/mg) oder β-Amylase aus Gerste (EC 3.2.1.2—Merck, 28 U/mg) und als Substrat eine Lösung von löslicher Stärke in dem jeweiligen Puffer. Eine ausführliche Beschreibung der Durchführung des enzymatischen Hemmtests erfolgte an anderer Stelle[29].

Für den Urease-Hemmtest wird eine Lösung von Urease (EC 3.5.1.5—Serva, 250 U/mg) in Phosphatpuffer pH 7.0 als Enzymquelle und eine solche von Harnstoff in einer 0.01% igen Bromthymolblau-Lösung als Substrat verwendet. Auch diese Methode wurde ausführlich an anderer Stelle beschrieben[30].

TABELLE II

UNTERE NACHWEISGRENZE (μg) DER UNTERSUCHTEN WIRKSTOFFE INFOLGE HEMMUNG DER RINDERLEBERESTERASE

Laufmittel: Cyclohexan–Aceton (10:4). I = Farbintensivierung.

Wirkstoff	*Ohne Vorbehandlung*	*Nach UV-Bestrahlung*
2,4-D	6	6
2,4,5-T	8	6
MCPA	8	3
Dichlorprop	7	5
Fenoprop	8	5
Mecoprop	7	2
2,4-D-isopropyl	80	60
2,4-D-butyl	60	60
2,4,5-T-butyl	80	50
2,4,5-T-hexyl	80 I	80 I
MCPA-hexyl	90	60
MCPA-(2-butoxyäthyl)	40	30
Mecoprop-äthyl	80	40
MCPP-(3-oxybutyl)	70	60
MCPP-(2-butoxyäthyl)	50	30
Mecoprop-hexyl	80 I	20
2,4-DB	8	8
2,4,5-TB	10	8
MCPB	20	5

TABELLE III

UNTERE NACHWEISGRENZE (μg) DER UNTERSUCHTEN WIRKSTOFFE MIT UND OHNE UV-BESTRAHLUNG INFOLGE HEMMUNG DER ALKALISCHEN UND SAUREN PHOSPHATASE

Substrat: Nitrophenylphosphat bzw. Naphthylphosphat; Laufmittelsystem: Cyclohexan–Aceton (10:4). I = Farbintensivierung.

Wirkstoff	*Ohne Vorbehandlung*				*Nach UV-Bestrahlung*			
	Alkalische Phosphatase		*Saure Phosphatase*		*Alkalische Phosphatase*		*Saure Phosphatase*	
	Nitrophenyl-phosphat	*Naphthyl-phosphat*	*Nitrophenyl-phosphat*	*Naphthyl-phosphat*	*Nitrophenyl-phosphat*	*Naphthyl-phosphat*	*Nitrophenyl-phosphat*	*Naphthyl-phosphat*
2,4-D	30	20	10	40	30	20	10	30
2,4,5-T	30	20	8	20	30	20	10	30 I
MCPA	30	20	8	30	30	30	10	40
Dichlorprop	30	20	10	30	30	30	10	40
Fenoprop	10	20	4	6	10	20	6	6
Mecoprop	10	30	8	40	10	60	10	40
2,4-D-isopropyl	100	80	2	10	100	120	10	50 I
2,4-D-butyl	100	80	4	40 I	100	80	10	50 I
2,4,5-T-butyl	100	80	2	50 I	100	80	8	50 I
2,4,5-T-hexyl	80	80	2	40 I	80	70	4	6
MCPA-hexyl	150	80	8	8	—	70	6	4
MCPA-(2-butoxyäthyl)	—	80	60	90	—	80	90	70 I
Mecoprop-äthyl	—	80	50	80	—	80	70	60
MCPP-(3-oxybutyl)	150	80	80	100	100	80	90	80
MCPP-(2-butoxyäthyl)	150	80	60	80	100	80	70	70
Mecoprop-hexyl	80	100	6	60	100	60	4	50
2,4-DB	40	—	6	40	50	70	8	90 I
2,4,5-TB	40	—	6	40	50	60	8	90 I
MCPB	40	—	4	40	50	60	6	90 I

ERGEBNISSE UND DISKUSSION

Der DC-enzymatische Hemmtest kann wie andere Verfahren zum Nachweis biologisch aktiver Substanzen herangezogen werden, doch besitzt er zusätzlich den Vorteil, erste Hinweise über mögliche Wirkungen auf das jeweilige Enzym zu liefern.

Wie aus Tabelle II hervorgeht, wird die Rinderleberesterase durch alle untersuchten Wirkstoffe beeinflusst, wobei 2,4,5-T-hexyl und Mecoprop-hexyl eigenartigerweise eine Farbintensivierung hervorrufen, die aufgrund der Art der Fleckenausbildung sicher nicht als eine Aktivierung der Esterase anzusehen ist. Die Nachweisgrenzen liegen für die freien Säuren erheblich niedriger als für die Ester, was eine stärkere Enzymhemmung bedeutet. Da dennoch die akute und chronische Toxizität kaum Unterschiede zwischen beiden aufweist[15], könnte dies ein Hinweis dafür sein, dass es im Körper zu einer schnellen Hydrolyse der Ester kommt. Eine UV-Bestrahlung der Wirkstoffe führt in den meisten Fällen zu einer leichten und in manchen Fällen sogar zu einer stärkeren Verbesserung der Nachweisempfindlichkeit.

Auch beide Phosphatasen werden durch die Phenoxysäuren und ihre Ester gehemmt (Tabelle III). Bei der alkalischen Phosphatase gestaltet sich der Nachweis mit Naphthylphosphat als Substrat und Echtblausalz B als Kopplungsreagenz mit einigen Ausnahmen durchweg empfindlicher als der Nachweis mit Nitrophenylphosphat. Auch in Falle der alkalischen Phosphatase bestätigen sich die bei der Leberesterase gemachten Beobachtungen, dass die Phenoxysäure-Ester im Vergleich zu den freien Säuren das Enzym in den meisten Fällen weniger stark hemmen. Eine UV-Bestrahlung hat in der Regel keinen Einfluss auf die Nachweisempfindlichkeit, obwohl in manchen Fällen leichte Veränderungen nach beiden Seiten in der Hemmintensität festzustellen sind.

Ein Nachweis infolge Hemmung der sauren Phosphatase gestaltet sich in den meisten Fällen empfindlicher, wobei auffällt, dass man nicht mehr eine so klare Trennung zwischen freien Säuren und den Estern in der Hemmintensität beobachten kann. Auffällig ist auch, dass der Nachweis mit Nitrophenylphosphat in der Regel empfindlicher ausfällt. Eine UV-Bestrahlung führt vielfach zu einer Nachweisverschlechterung.

Nicht unerwähnt bleiben sollte auch die Farbintensivierung, die bei einigen Wirkstoffen mit Naphthylphosphat/Echtblausalz B auftritt (Tabelle III). Wie schon bei der Leberesterase scheint auch hier keine echte Aktivierung vorzuliegen, da diese Wirkstoffe bei Verwendung von Nitrophenylphosphat als Substrat deutliche Hemmflecke liefern.

Die Ergebnisse über die Beeinflussung der Phosphatase-Aktivität durch 2,4-D, das in diesem Zusammenhang am besten untersucht wurde, sind recht unterschiedlich. Während einerseits 2,4-D einen Anstieg der Phosphatase-Aktivität bewirken soll[31], finden andere Autoren eine deutliche Verminderung[32], wobei die Ergebnisse in beiden Fällen keine direkte Wechselwirkung zwischen Enzym und Wirkstoff wiedergeben, sondern Enzymspiegelmessungen darstellen.

Die Wirkung auf proteolytische Enzyme wird in Form dieses DC-enzymatischen Nachweises erstmals untersucht. Dabei zeigt sich, dass Trypsin von allen Wirkstoffen gehemmt wird (Tabelle IV). Wie schon bei den anderen Enzymen tierischer Herkunft (Esterase, alkalische Phosphatase) erwiesen sich die freien Säuren auch beim Trypsin-Hemmtest im Vergleich zu den Estern als stärkere Inhibitoren. Auch hier hat UV-

TABELLE IV

UNTERE NACHWEISGRENZE (μg) DER UNTERSUCHTEN WIRKSTOFFE INFOLGE HEMMUNG VON TRYPSIN

Laufmittel: Cyclohexan–Aceton (10:4).

Wirkstoff	*Ohne Vorbehandlung*	*Nach UV-Bestrahlung*
2,4-D	10	30
2,4,5-T	8	8
MCPA	10	20
Dichlorprop	10	30
Fenoprop	8	10
Mecoprop	8	30
2,4-D-isopropyl	70	60
2,4-D-butyl	70	70
2,4,5-T-butyl	70	70
2,4,5-T-hexyl	70	70
MCPA-hexyl	80	80
MCPA-(2-butoxyäthyl)	80	80
Mecoprop-äthyl	80	80
MCPP-(3-oxybutyl)	80	80
MCPP-(2-butoxyäthyl)	80	80
Mecoprop-hexyl	90	130
2,4-DB	20	20
2,4,5-TB	20	8
MCPB	20	10

Bestrahlung nur einen recht geringen Einfluss auf die Nachweisempfindlichkeit.

Recht wenig eignen sich die Amylasen zum Nachweis der Phenoxyalkancarbonsäure-Herbizide (Tabelle V). Die α-Amylase wird nur von sechs der insgesamt neunzehn untersuchten Wirkstoffe leicht gehemmt, und durch UV-Bestrahlung kommt nur noch eine weitere Substanz als Inhibitor hinzu. Mit β-Amylase lassen sich die Phenoxysäure-Herbizide etwas empfindlicher als mit α-Amylase nachweisen, wobei UV-Bestrahlung keinen Einfluss auf die Hemmintensität hat. Diese Ergebnisse würden mit denen von NEELEY *et al.*[33] in Einklang stehen, wonach eine Behandlung von Bohnen mit 2,4-D zu einer Verminderung der β-Amylase-Aktivität führt, und sie ständen in nur geringem Gegensatz zu Ergebnissen von WORT UND COWIE[32], wonach die β-Amylase-Aktivität in Weizen nach 2,4-D-Behandlung zunächst ansteigt, um am 8. Tag nach Applikation deutlich unter die Aktivität der Kontrollen abzusinken.

Die Urease wiederum wird von allen untersuchten Phenoxyalkancarbonsäure-Herbiziden gehemmt (Tabelle VI), doch gestaltet sich der Nachweis nur mit wenigen Substanzen einigermassen empfindlich. UV-Bestrahlung führt bei zwölf Wirkstoffen zu einer leichten Nachweisverbesserung, während es bei sechs Wirkstoffen zu einer Verschlechterung kommt. Deutliche Unterschiede zwischen den freien Säuren und den Estern sind nicht festzustellen.

Die Ergebnisse zeigen, dass sich mit Ausnahme der Amylasen alle Enzyme zum DC-enzymatischen Nachweis dieser Herbizid-Gruppe eignen. Weiter zeigen sich gewisse Unterschiede im Verhalten der Enzyme tierischer Herkunft zu denen pflanzlicher Herkunft.

TABELLE V

UNTERE NACHWEISGRENZE (μg) DER UNTERSUCHTEN WIRKSTOFFE INFOLGE HEMMUNG VON AMYLASE

Laufmittel: Cyclohexan–Aceton (10:4).

Wirkstoff	*Ohne Vorbehandlung*		*Nach UV-Bestrahlung*	
	α-Amylase	*β-Amylase*	*α-Amylase*	*β-Amylase*
2,4-D	—	70	80	70
2,4,5-T	80	70	60	70
MCPA	80	70	80	70
Dichlorprop	—	70	—	70
Fenoprop	60	70	60	70
Mecoprop	80	70	80	70
2,4-D-isopropyl	—	120	—	100
2,4-D-butyl	—	120	—	100
2,4,5-T-butyl	—	120	—	100
2,4,5-T-hexyl	—	100	—	80
MCPA-hexyl	—	100	—	100
MCPA-(2-butoxyäthyl)	—	—	—	—
Mecoprop-äthyl	80	100	80	—
MCPP-(3-oxybutyl)	—	—	—	—
MCPP-(2-butoxyäthyl)	—	100	—	100
Mecoprop-hexyl	60	70	60	100
2,4-DB	—	70	—	70
2,4,5-TB	—	70	—	70
MCPB	—	70	—	70

TABELLE VI

UNTERE NACHWEISGRENZE (μg) DER UNTERSUCHTEN WIRKSTOFFE INFOLGE HEMMUNG DER UREASE

Laufmittel: Cyclohexan–Aceton (10:4).

Wirkstoff	*Ohne Vorbehandlung*	*Nach UV-Bestrahlung*
2,4-D	60	70
2,4,5-T	40	10
MCPA	60	40
Dichlorprop	70	50
Fenoprop	10	20
Mecoprop	90	60
2,4-D-isopropyl	90	60
2,4-D-butyl	90	80
2,4,5-T-butyl	80	60
2,4,5-T-hexyl	50	70
MCPA-hexyl	60	80
MCPA-(2-butoxyäthyl)	100	80
Mecoprop-äthyl	80	50
MCPP-(3-oxybutyl)	80	90
MCPP-(2-butoxyäthyl)	80	80
Mecoprop-hexyl	60	50
2,4-DB	50	80
2,4,5-TB	80	50
MCPB	100	60

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ZUSAMMENFASSUNG

Der dünnschichtchromatographisch–enzymatische Hemmtest mit den Enzymen Rinderleberesterase, alkalische und saure Phosphatase, Trypsin, α- und β-Amylase sowie Urease wird zum Nachweis der Phenoxyalkancarbonsäure-Herbizide herangezogen. Mit Ausnahme der Amylasen, die nur schwach und teilweise überhaupt nicht gehemmt werden, eignen sich alle Enzyme zum Nachweis, wobei dieser mit saurer Phosphatase am empfindlichsten ausfällt. Eine UV-Bestrahlung der Wirkstoffe beeinflusst den Nachweis in den meisten Fällen nur wenig.

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CHROM. 6093

INTERACTION OF TETRACYANOETHYLENE WITH INDOLES ON SILICA GEL AND CELLULOSE THIN-LAYER PLATES*

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SUMMARY

Tetracyanoethylene and indoles readily form dark coloured π-complexes in solution. In most instances the components of such complexes formed from indoles unsubstituted in the 3-position, interact on standing with the formation of 3-tricyanovinylindole derivatives. However the use of tetracyanoethylene as a spray reagent for detecting indoles on thin-layer plates leads exclusively to the formation of these 3-tricyanovinyl substituted products. The structures of some of these compounds have been confirmed using physical and chemical methods.

INTRODUCTION

Electron acceptor reagents have proved to be of considerable value in thin-layer chromatographic (TLC) procedures for the detection and subsequent identification by mass spectrometry of many electron-rich aromatic and heterocyclic compounds, including a number of biologically important indole derivatives, such as 3-indolylacetic acid and serotonin[1-5].

Previous work carried out in these laboratories and elsewhere has shown that a number of different electron acceptor reagents are suitable for this purpose[1-5]. Tetracyanoethylene (TCNE) is one example of a reagent of this type which has been widely used in this manner[1]. However whilst it is generally recognized that TCNE initially forms electron donor–acceptor complexes with indoles (*e.g.* Fig. 1, I) in solution[6], as shown by the rapid formation of deep blue-black coloured products in solution. The components of these complexes (*e.g.* Fig. 1, II) readily interact with each other to form 3-tricyanovinylindole derivatives (*e.g.* Fig. 1, III) by the elimination of hydrogen cyanide[6,7]. This reaction, which has been reported to be base catalyzed[8,9], occurs very readily in solution and in some instances even occurs in the solid state.

SAUSEN *et al.*[8] first reported the preparation of 3-tricyanovinylindole (Fig. 1, IIIc) in 1958, by the interaction of indole and TCNE in pyridine at room temperature using essentially the procedure described by HECKERT[10] for the preparation of some

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(a) R = NO_2
(b) R = Cl
(c) R = H
(d) R = OH

Fig. 1. Complex formation and reaction of TCNE with indoles.

related compounds. Shortly afterwards NOLAND *et al.* improved the overall yield of 3-tricyanovinylindole (Fig. 1, IIIc) to 92% by carrying out the reaction in boiling benzene in the presence of a small amount of pyridine[9].

In cases where the 3-position of the indole nucleus was blocked (*e.g.*, skatole *i.e.* 3-methylindole) tricyanovinylation of the indole nucleus was reported to occur on the nitrogen atom[9].

It had been noted in some preliminary experiments that colours obtained from some indole compounds on thin-layer plates, when sprayed with TCNE, were not the same as those observed in solution. This was particularly true of indole compounds with a free 3-position, but with 3-substituted indoles only dark blue-black colours were obtained both on the plate and in solution suggesting that in these cases complexation only had occurred.

This phenomenon was observed under "neutral" conditions, but in the presence of a small amount of pyridine the dark blue solution obtained by the interaction of a typical 3-substituted indole (3-indolylacetic acid) and TCNE was rapidly discharged, due to the formation of an orange-yellow compound in solution.

We now wish to report the formation of the 3-tricyanovinylindole derivatives from 5-substituted indoles, both in solution and on silica gel or cellulose layers.

MATERIALS AND METHODS

General

The melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. The NMR spectra were obtained on a Varian A-60A instrument using tetramethylsilane as an internal reference. The mass spectra were obtained on a DuPont/C.E.C. 21-491 instrument. The reflectance spectra were obtained with the aid of a Farrand UV-visible chromatogram analyser.

Tetracyanoethylene and all the indole compounds used in this investigation were obtained from the Aldrich Chemical Co.

Chemical procedure (interaction of indoles with TCNE)

Preparation of 3-tricyanovinylindoles, from:

(a) Indole, 5-chloroindole and 5-nitroindole. The 3-tricyanovinylindole compounds (IIIa, IIIb, and IIIc) were prepared by mixing equimolar amounts of the relevant indole compounds (Ia, Ib, and Ic, respectively,) with TCNE in boiling saturated solutions in dichloromethane. The reaction mixtures contained in stoppered flasks were allowed to stand at room temperature for three weeks, after which time the residual solvent was removed *in vacuo* and the dark coloured residues recrystallized from ethyl acetate or ethyl acetate–*n*-hexane mixtures. The resulting crude solid 3-tricyanovinylindoles (IIIa, IIIb, and IIIc, respectively) were finally purified by preparative scale TLC. The physical data for these compounds are given in Tables I and II.

TABLE I

SOME 3-TRICYANOVINYLINDOLES: PHYSICAL PROPERTIES AND MICROANALYSES

3-Tricyanovinylindole derivative	M.p. (°C)	Colour	R_F value[a]	Analysis					
				Found (%)			Calculated (%)		
				C	H	N	C	H	N
IIIa, R = NO_2	269	orange	0.12	58.83	1.85	26.40	59.31	1.91	26.63
IIIb, R = Cl	295	orange-brown	0.26	61.30	1.92	22.22	61.45	1.98	22.47
IIIc, R = H	275[b]	brick-red	—	—	—	—	—	—	—
IIId, R = OH	295	red-brown	0.30	65.85[c]	2.47	24.08	66.66	2.57	23.93

[a] TLC on Silica Gel G; development with benzene–ethyl acetate (10:3).
[b] Literature m.p.: 273–275° (ref. 6); 275–276° (ref. 8); 268–270° (ref. 9).
[c] It was not possible to obtain a completely satisfactory value for carbon in this instance.

(b) 5-Hydroxyindole. All attempts to prepare 5-hydroxy-3-tricyanovinylindole (IIId) by the interaction of 5-hydroxyindole (Id) and TCNE in solution under the conditions described above were unsuccessful. However this product could be obtained by an adaptation of the method of SAUSEN *et al.*[8] or in the following manner.

A solution of 20 mg of 5-hydroxyindole (Id) in 10 ml of benzene was sprayed uniformly onto a Merck precoated Silica Gel G plate (20 × 20 cm; thickness, 0.25 mm). After the plate had been allowed to dry it was repeatedly sprayed with a solution of TCNE in dichloromethane. The silica carrying the product was removed from the plate and the product eluted from the silica with ethyl acetate. The crude 5-hydroxy-

TABLE II

NMR SPECTRAL DATA FOR SOME 3-TRICYANOVINYLINDOLES

The NMR spectra were measured in acetone-d_6 solution using tetramethylsilane as internal reference. (s) = singlet; (d) = doublet; (dd) = doublet of doublets.

3-Tricyanovinyl-indole derivative	H_2	H_4		H_6			H_7	
	δ value[a]	*δ value*[a]	$J_{4,6}$ *value*[b]	*δ value*[a]	$J_{4,6}$ *value*[b]	$J_{6,7}$ *value*[b]	*δ value*[a]	$J_{6,7}$ *value*[b]
IIIa, R = NO_2	8.95 (s)	9.17 (d)	2.0	8.30 (dd)	2.0	9.0	7.97 (d)	9.0
IIIb, R = Cl	8.73 (s)	8.07 (d)	2.0	7.43 (dd)	2.0	9.0	7.70 (d)	9.0
IIId, R = OH	8.73 (s)	7.70 (d)	2.0	7.00 (dd)	2.0	9.0	7.52 (d)	9.0

[a] δ = chemical shift in p.p.m.
[b] J = coupling constant in Hz.

3-tricyanovinylindole (IIId) obtained in this manner was purified by the method described above. Physical data for this compound are given in Tables I and II.

Preparation of complexes

The 3-indolylacetic acid–TCNE and indole–TCNE complexes were obtained as dark blue microcrystalline solids by the method previously described by HUTZINGER AND JAMIESON[4], with the following modification for the 3-indolylacetic acid complex.

A mixture of cold saturated solutions of 3-indolylacetic acid and TCNE in dichloromethane was stored at $-20°$ and the dark blue microcrystalline complex (m.p. 147°) removed by filtration.

Analysis, calculated, for $C_{16}H_9N_5O_2$: C, 63.36; H, 2.99; N, 23.09%. Found: C, 62.78; H, 2.97; N, 23.41%.

Thin-layer chromatography

Plates. Commercially available Merck Silica Gel G or Merck cellulose plates (20 × 20 cm; thickness, 0.25 mm and 0.1 mm, respectively) were used for analytical separations. Preparative scale TLC was carried out on plates (100 × 20 cm; thickness, 0.9 mm) prepared in the laboratory using Merck Silica Gel G as the adsorbant.

Solvent. A benzene–ethyl acetate (10:3) solvent system was used in all cases.

Analytical procedure

The indole compound (10–20 μg) was applied to the plate, from solution in acetone. After development with the solvent system described above, the dried plates were sprayed with a 1% solution of TCNE in dichloromethane, and the colours developed by the spots due to the indole compounds noted directly after spraying. The colours observed are reported in Table I.

Mass spectrometry. Mass spectra were obtained using the standard probe for direct introduction of the sample into the ion source.

Reflectance spectra. Reflectance spectra were recorded by plotting reflectance with reference to background *versus* wavelength at 10-nm intervals between 420 and 600 nm using the single-beam mode of operation. The light source was a xenon lamp and a 1P28 photomultiplier tube was the detector.

The monochromator was removed from the analyzer leg for these measurements, as the monochromator on the exciter leg provided monochromatic light. An auxiliary filter (No. 3-73, sharp-cut at 400 nm) was used in the analyzer leg, with suitable slit aperture reducers appropriate to the phototube response at the various wavelengths (an 0.031-mm reducer was used in the range of optimum response, approx. 450–600 nm, while 0.062- and 0.125-min reducers were used outside this range). The 3/16–11/32 slit set was used for all measurements.

RESULTS AND DISCUSSION

The results of this study confirm the relative ease with which 3-tricyanovinylindoles (*cf.* III) are formed by the interaction of TCNE with indole compounds unsubstituted in the 3-position. However highly coloured electron donor–acceptor complexes are definitely formed initially in solution and these complexes can be isolated in the solid state, if the appropriate precautions are taken. This has now been confirmed in the case of indole itself. The dark blue complex which can be isolated in the solid state by allowing its solutions in dichloromethane to crystallize at $-20°$ is however unstable even in the solid state. On standing at $-20°$ the colour of the solid slowly changes from dark blue to orange indicating the formation of the 3-tricyanovinyl derivative. Nonetheless it was possible to obtain a mass spectrum of a freshly prepared sample of the complex, which clearly shows the presence of both species. Only ions derived from the individual components of the complex can be seen in the spectrum and there is no evidence of peaks at higher m/e values corresponding to 3-tricyanovinylindole (see Fig. 2).

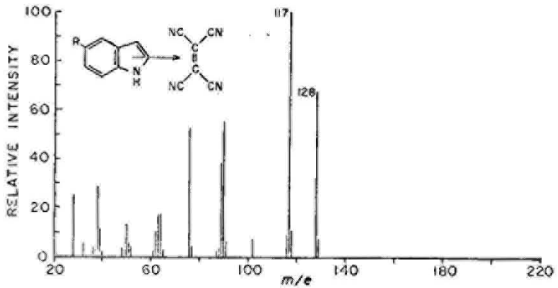

Fig. 2. 70-eV mass spectrum of the indole–TCNE complex at a probe temperature of 40°.

Fig. 3 shows the mass spectra of a few typical 3-tricyanovinylindoles (IIIa, IIIb, IIIc, and IIId). In all examples studied strong molecular ion peaks can be observed, and in no instances are peaks apparent due to the indole compound in question or TCNE.

Whilst it was possible to detect the "complex" stage in solution during the interaction of the indole compounds IIIa, IIIb, IIIc, and IIId, the 3-tricyanovinyl-

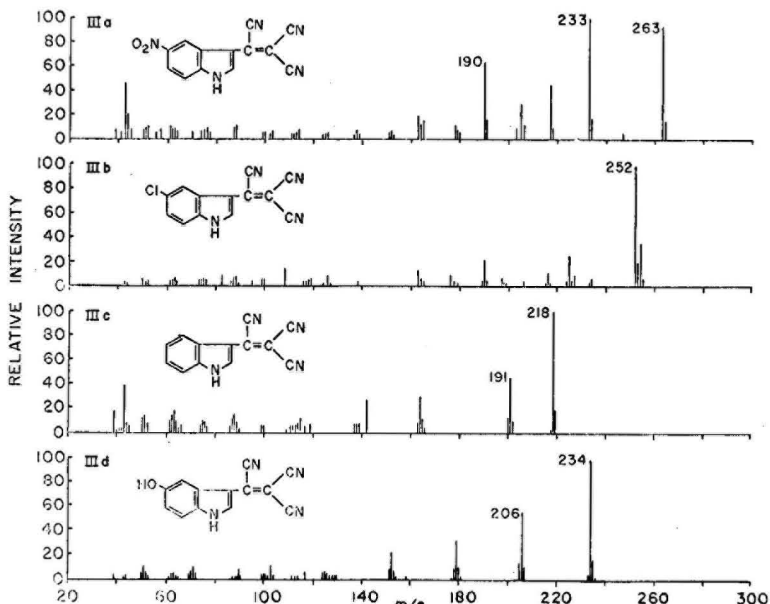

Fig. 3. 70-eV mass spectra of some 3-tricyanovinylindoles; probe temperatures: IIIa, 175°; IIIb, 135°; IIIc, 120°; IIId, 185°.

ation reaction occurred virtually instantaneously on silica or cellulose thin-layer plates. On spraying developed chromatoplates containing spots due to these indole compounds the very dark colours of the complexes were not seen, merely the orange to yellow colours of the 3-tricyanovinyl derivatives.

The NMR spectra of the 5-substituted 3-tricyanovinylindole derivatives confirm that the tricyanovinyl group is in the 3-position of the indole nucleus. The signals due to the H_2, H_4, H_6, and H_7 protons can be clearly seen and show the expected splitting patterns (see Table II). The characteristic signal for the H_3-indole proton, which is usually observed upfield from the H_2 proton and the protons in the 6-membered ring, is not seen in any of the spectra examined (*cf.* JARDINE AND BROWN[11]).

As mentioned earlier the decomposition of the indole–TCNE complexes to form tricyanovinyl derivatives does not occur as readily in the case of 3-substituted indoles, and the 3-indolylacetic acid–TCNE complex has been isolated as a relatively stable dark blue microcrystalline solid. Fig. 4 shows the mass spectrum obtained for this complex at two different temperatures, indicating that differential sublimation of the two components of the complex had occurred and that mass spectra due to both had been clearly obtained. No peaks at higher *m/e* values were observed indicating that no reaction of the two species had occurred. The formation of 1-tricyanovinyl derivatives from 3-substituted indoles has been reported[8] and it appears that this reaction is base catalyzed, since overspraying of blue chromatographic spots obtained from 3-indolylacetic acid with pyridine results in the discharge of the blue colour.

The reflectance spectra of the four 3-tricyanovinylindoles (IIIa, IIIb, IIIc, and IIId) have been measured on cellulose and the results are given in Fig. 5. The results clearly show that a broadening of the reflectance maximum towards longer wave-

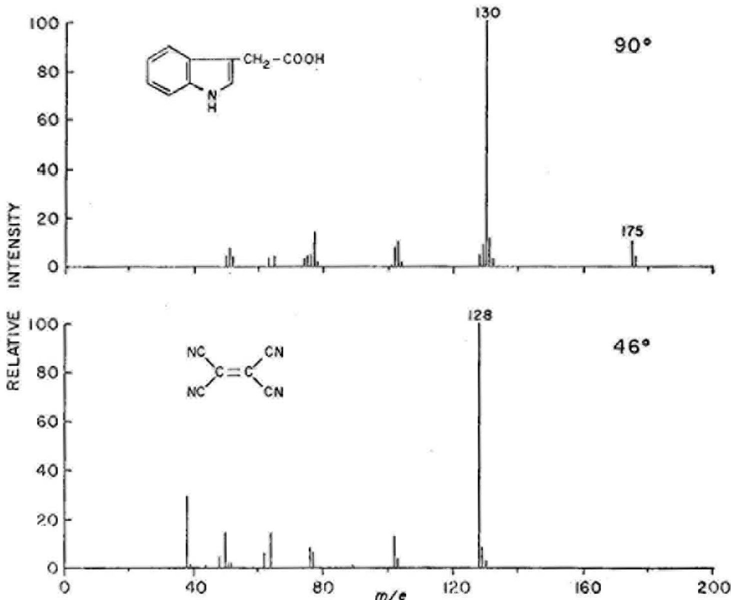

Fig. 4. 70-eV mass spectra of 3-indolylacetic acid–TCNE complex at probe temperatures shown.

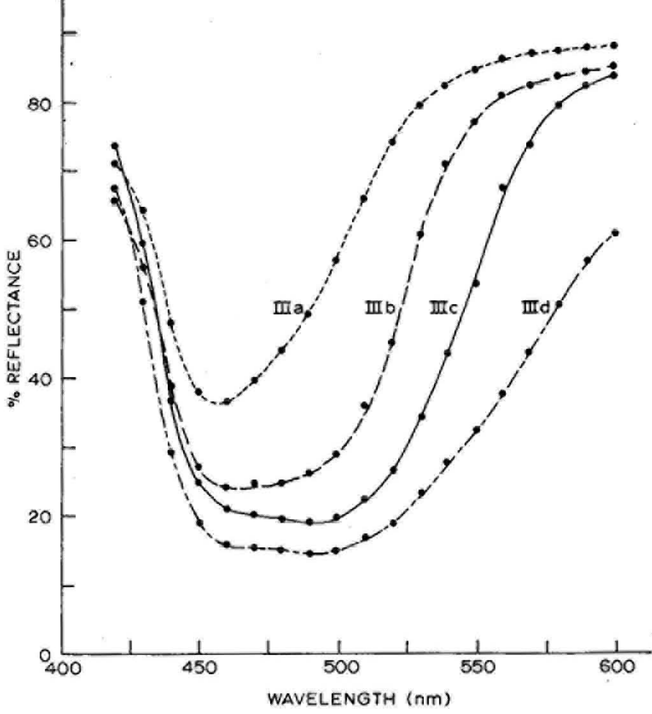

Fig. 5. Reflectance spectra of the 3-tricyanovinyl derivatives of 5-nitroindole, 5-chloroindole, indole, and 5-hydroxyindole (*i.e.* IIIa, IIIb, IIIc, IIId) formed *in situ* on cellulose.

lengths occurs with a decrease in the electron withdrawing properties of the substituent in the 5-position.

The reflectance spectra measurements could possibly form the basis of a quantitative method for the assay of indole compounds. A similar method, based on the formation of complexes between indole compounds and 2,4,7-trinitro-9-fluorenone has already been proposed[5].

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CHROM. 6216

THIN-LAYER CHROMATOGRAPHY OF LYSERGIDE AND OTHER ERGOT ALKALOIDS

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SUMMARY

The chromatographic behaviour of lysergide and fourteen related ergot alkaloids has been investigated using eighteen thin-layer systems. Resolution and correlation between the systems is discussed in relation to the routine identification of lysergide.

INTRODUCTION

Those concerned with the routine identification of lysergide have come to rely principally upon the thin-layer chromatographic (TLC) technique, partly because of its sensitivity and convenience but also because of the limitations of techniques such as ultraviolet and fluorescence spectroscopy, gas chromatography and paper chromatography (PC). In these circumstances it is important that the chromatography system or combination of systems chosen, should adequately resolve lysergide from other chemically similar compounds, and further, since the procedure is to be used routinely, the system(s) should be simple to operate and should give R_F values which are as reproducible as possible.

Many TLC systems have been described which purport to allow identification of lysergide[1-8] but in some cases, compounds related to lysergide have not been run concurrently and the reliance which can be placed upon results obtained, using these systems, is open to some doubt.

In an attempt to assess the value of some TLC systems which are known to have been used for the identification of lysergide, we have run fifteen ergot alkaloids in each of eighteen recommended systems. Our collection of alkaloids, although not comprehensive, represents those which we have been able to obtain from the recognised sources of supply and the likelihood of encountering an illicit ergot preparation not included in our survey would hopefully be small.

EXPERIMENTAL AND RESULTS

For each experiment the reference substances (1–3 μg) were applied to a single

TABLE I

R_F VALUES × 100 FOR ERGOT ALKALOIDS

For description of systems used see text.

Compound		System																
No.	Name	A1	A1[a]	B1	B1[a]	C1	A2	A2[a]	C2	A3	A3[a]	A4	A4[a]	C4[a]	A5	A5[a]	C5	A6
1	D-Lysergic acid	00	00	00	02	00	00	00	00	03	00	00	00	00	00	00	00	02
2	Ergometrine maleate	10	10	26	30	18	07	14	14	44	42	00	00	01	00	00	02	36
3	*Iso*-LSD	11	18	66	55	29	08	07	18	30	26	00	00	03	00	02	08	32
4	Methylergometrine	13	14	31	32	25	09	16	18	51	48	00	00	01	00	01	02	42
5	Methysergide bimaleate	13	13	33	34	25	09	19	19	51	43	00	00	01	00	02	03	54
6	Dihydroergotamine	19	20	40	44	32	12	22	25	54	37	00	00	01	00	01	04	60
7	Lysergide	27	28	70	56	39	23	26	37	54	41	00	00	09	04	06	21	60
8	Ergotamine tartrate	32	31	48	44	39	23	29	33	63	45	00	00	02	02	02	08	62
9	Dihydroergocristine	35	37	64	54	46	27	30	39	64	48	00	00	03	02	02	11	65
10	Ergocristine	51	50	66	56	54	43	42	47	70	52	02	02	15	10	12	32	68
11	Ergocryptine	51	50	68	53	55	43	42	47	69	53	02	02	16	11	12	33	67
12	Ergotoxine ethanesulphonate	51	50	68	54	55	43	43	48	69	52	02	02	15	11	13	32	68
13	Ergosine	31	32	52	44	43	25	30	34	62	44	00	00	03	02	03	11	61
14	Ergocornine	50	50	67	54	53	42	42	45	68	52	03	02	12	11	12	32	67
15	Lysergamide	—	—	27	30	—	—	10	—	—	40	00	00	—	—	01	—	—

[a] Overspotted with NaOH

20 × 20 cm plate as a solution in methanol. The plates were developed in a 4-l, paper-lined tank containing approximately 100 ml of solvent and in each case the solvent was allowed to run 15 cm from the start line. Visualisation of the spots was by examination under 254 nm and 350 nm illumination followed by spraying with a solution of *p*-dimethylaminobenzaldehyde (1 g) in conc. HCl (10 ml) and ethanol (90 ml). Subsequent heating of the plates revealed blue spots.

Generally, commercially available coated glass plates (Merck), or polyester sheets (Eastman Kodak) were used because of the reproducibility which they offer but in a few cases hand-coated plates were prepared.

No special precautions were taken to ensure that the plates were activated before use.

Supports

A Merck Silica Gel F_{254} (0.25 mm pre-coated)
B Merck Aluminium Oxide F_{254} (type E, 0.25 mm pre-coated)
C Eastman Chromagram Sheets 6060 (silica gel with fluorescent indicator)
D Eastman Chromagram Sheets 6063 (alumina with fluorescent indicator)
E Merck Silica Gel F_{254} (0.25 mm hand-coated)
F Merck Cellulose sprayed with 5% sodium dihydrogen citrate and dried

Solvents

1 Acetone
2 Acetone–chloroform (4:1)
3 Acetone–methanol (4:1)
4 Chloroform
5 Chloroform–acetone (6:1)

No.	System A6[a]	A7	A7[a]	A8	A9	A10	A11	A12	A12[a]	B12	B12[a]	B13	B13[a]	D13	B14	B14[a]	D14	B15	B15[a]	C16	F17
1	00	00	00	77	01	00	00	01	02	01	00	00	00	00	00	00	00	00	00	00	17
2	39	10	15	66	52	07	01	09	11	21	14	04	06	03	00	04	00	00	00	18	31
3	33	08	19	66	84	34	21	10	12	59	53	30	39	40	14	22	35	05	08	50	66
4	45	13	20	69	68	09	02	12	14	24	14	05	10	05	00	06	00	00	02	22	42
5	54	26	31	68	57	17	05	21	27	45	34	17	21	23	02	10	13	00	03	40	47
6	59	30	36	68	73	09	02	20	26	54	40	20	26	24	03	10	10	00	04	19	79
7	61	36	42	68	83	36	25	21	27	61	54	36	47	41	19	28	37	12	14	54	59
8	62	35	40	69	73	11	02	20	32	52	40	20	28	23	04	10	10	00	04	30	77
9	66	38	44	72	82	19	05	24	35	63	52	31	40	33	07	16	24	01	05	34	83
10	69	50	55	72	84	29	14	26	36	67	56	36	49	38	13	22	31	04	08	55	83
11	68	49	54	72	85	29	16	27	31	65	55	34	48	37	12	22	31	04	08	58	83
12	68	49	54	72	84	30	17	28	30	65	56	34	48	35	12	22	30	04	08	52	82
13	60	36	45	68	75	12	04	23	24	49	38	20	30	20	03	14	08	00	04	37	75
14	68	49	56	70	83	29	17	28	32	64	54	33	46	27	11	20	29	04	06	54	81
15	38	10	—	—	—	—	—	—	10	—	17	—	08	—	—	04	—	—	00	—	25

6 Chloroform–methanol (4:1)
7 Chloroform–methanol (9:1)
8 Methanol–ammonia (0.88) (100:1.5)
9 Methanol–acetate buffer (pH 4.5) (9:1)
10 Chloroform–cyclohexane–isopropylamine (5:5:1)
11 Chloroform–cyclohexane–diethylamine (5:5:1)
12 1,1,1-Trichloroethane–methanol (9:1)
13 1,1,1-Trichloroethane–methanol (96:4)
14 1,1,1-Trichloroethane–methanol (98:2)
15 1,1,1-Trichloroethane–methanol (99:1)
16 Toluene–morpholine (9:1)
17 *n*-Butanol–citric acid–water (870 ml:4.8 g:130 ml)

The ergot alkaloids used, together with the code numbers given to them for use on the plates, are listed in Table I.

The R_F values obtained for each system (mean of three runs) are also recorded in Table I. For each of the systems being assessed, Merck plates were used and where specifically recommended, Chromagram sheets were run in addition.

In a preliminary series of experiments to determine the effect on the resulting chromatogram of using different procedures for obtaining alkaline conditions on the plate, and indeed, whether alkaline conditions were necessary at all, a series of silica gel plates (0.25 mm) were prepared. Each was then developed in chloroform–methanol (9:1) and the spots were located as described previously. Correlation coefficients between systems were calculated by the method described by SMALLDON[12]. The plates were: (a) hand-coated, using distilled water in the mixing stage, dried (105°), and the reference substances then applied (system E7(a)); (b) hand-coated, using 0.1 *N* NaOH in the mixing stage, dried and the reference substances applied (system E7(b));

(c) hand-coated, using distilled water, dried, and the reference substances subsequently over-spotted with 0.1 N NaOH (system E7(c)); (d) pre-coated Merck F_{254}, sprayed with 0.1 N NaOH, dried and the reference substances applied (system A7(d)); (e) pre-coated Merck F_{254}, and the reference substances subsequently over-spotted with 0.1 N NaOH (system A7*); (f) pre-coated Merck F_{254}, and the reference substances then applied (system A7).

In the resulting chromatograms the "neutral" plates (E7(a) and A7) gave similar resolution to one another and to the basic plates (E7(b), E7(c), A7(d), A7*). Correlation coefficients within the series were also very high (Table II) and it appeared that for these compounds it was not essential to run basic plates although slight tailing of spots was evident under "neutral" conditions. In order to confirm this observation it was considered necessary in the subsequent assessment of the other systerms to run two series of plates in which the conditions were either basic or neutral.

TABLE II

CORRELATION COEFFICIENTS FOR THE SILICA GEL/CHLOROFORM–METHANOL (9:1) SYSTEM USING PLATES PREPARED IN DIFFERENT WAYS

For description of systems used see text.

	E7(a)	*E7(b)*	*E7(c)*	*A7(d)*	*A7**
E7(a)					
E7(b)	0.93				
E7(c)	0.98	0.88			
A7(d)	0.99	0.95	0.98		
*A7**	0.99	0.92	0.98	0.99	
A7	0.99	0.90	0.99	0.98	0.99

A further observation from this preliminary experiment was that the method by which basic conditions were obtained on the plate did not significantly affect the chromatogram, and it was therefore considered that, of the methods available for obtaining basic conditions, the use of the over-spotting technique (A7*), which adequately minimises tailing and which is simple and convenient to perform, was the method of choice. This technique has previously been recommended by PHILLIPS AND GARDINER[5].

Five of the TLC systems being assessed here (A8, A9, A10, A11, C16) required either basic or acidic solvents for development and in these cases the reference materials were not over-spotted with alkali. For the others, using neutral solvents, however, the two series of Merck plates were run in which the reference materials were, or were not over-spotted, and when the original method had specified the use of chromagram sheets, these were run in addition, but using only the conditions stated. Differences in R_F value were observed between the two series of Merck plates but the degree of resolution of lysergide from the other compounds on the plate remained almost unchanged and, again, the correlation coefficients for the two series (given in Table III) were high.

DISCUSSION

Perhaps the most significant observation emerging from our survey is that no

TABLE III

CORRELATION COEFFICIENTS BETWEEN "NEUTRAL" AND NaOH OVER-SPOTTED PLATES
For description of systems used see text.

System	*Correlation coefficient*
A1	0.99
B1	0.98
A2	0.97
A3	0.96
A4	1.00
A5	0.99
A6	0.99
A7	0.99
A12	0.94
B12	0.99
B13	0.99
B14	0.96
B15	0.92

single TLC system which we have tried unequivocally resolves lysergide from the other ergot alkaloids and it follows that a compromise must be found in which either a combination of systems is used or one system is used in combination with an alternative technique. Several pairs of systems tried here could be effective. For example, a number of systems based on a silica gel adsorbent with a single, or mixture of, neutral solvent(s) allow resolution of lysergide from all but ergotamine (8) and ergosine (13). The two dihydro alkaloids (6 and 9) included in our chromatograms run near to lysergide in these cases but these would not be confused with lysergide because they do not fluoresce under 350 nm light, absorb differently to lysergide in 254 nm light and occur as purple spots on spraying (*cf.* lysergide is blue). Either acetone alone or chloroform–methanol (9:1) would suffice (see systems A1, A7 and Figs. 1a and 1b).

The alternative system could be based on either a silica adsorbent together with a basic developing solvent mixture (see systems A10, A11, C16 and Fig. 1c) or on an alumina adsorbent using one of the 1,1,1-trichloroethane–methanol mixtures (see systems B12–B15 and Fig. 1d) or acetone (system B1, Fig. 1e). Each of these latter groups allows resolution of lysergide from all the alkaloids run except *iso*-LSD (3) and the ergotoxine group, ergocristine (10), ergocryptine (11) and ergocornine (14).

Of the several pairs of systems outlined as being suitable, the simplest and most convenient for routine use would in our opinion consist of silica–acetone (A1) and alumina–acetone (B1). This would be advantageous in that: (a) pre-coated silica and alumina plates could be used without activation; (b) a single tank containing acetone could be used for development of both plates; and (c) a single-component developing solvent obviates the danger of incorrect mixing and of changing composition of the developing solvent in the tank due to different vapour pressures of the components.

The correlation coefficient for this combination of systems is fairly high, however, (0.78), and it would consequently be unsuitable for identification of a number of the individual ergot alkaloids, although for lysergide, we feel that it does allow unequivocal identification.

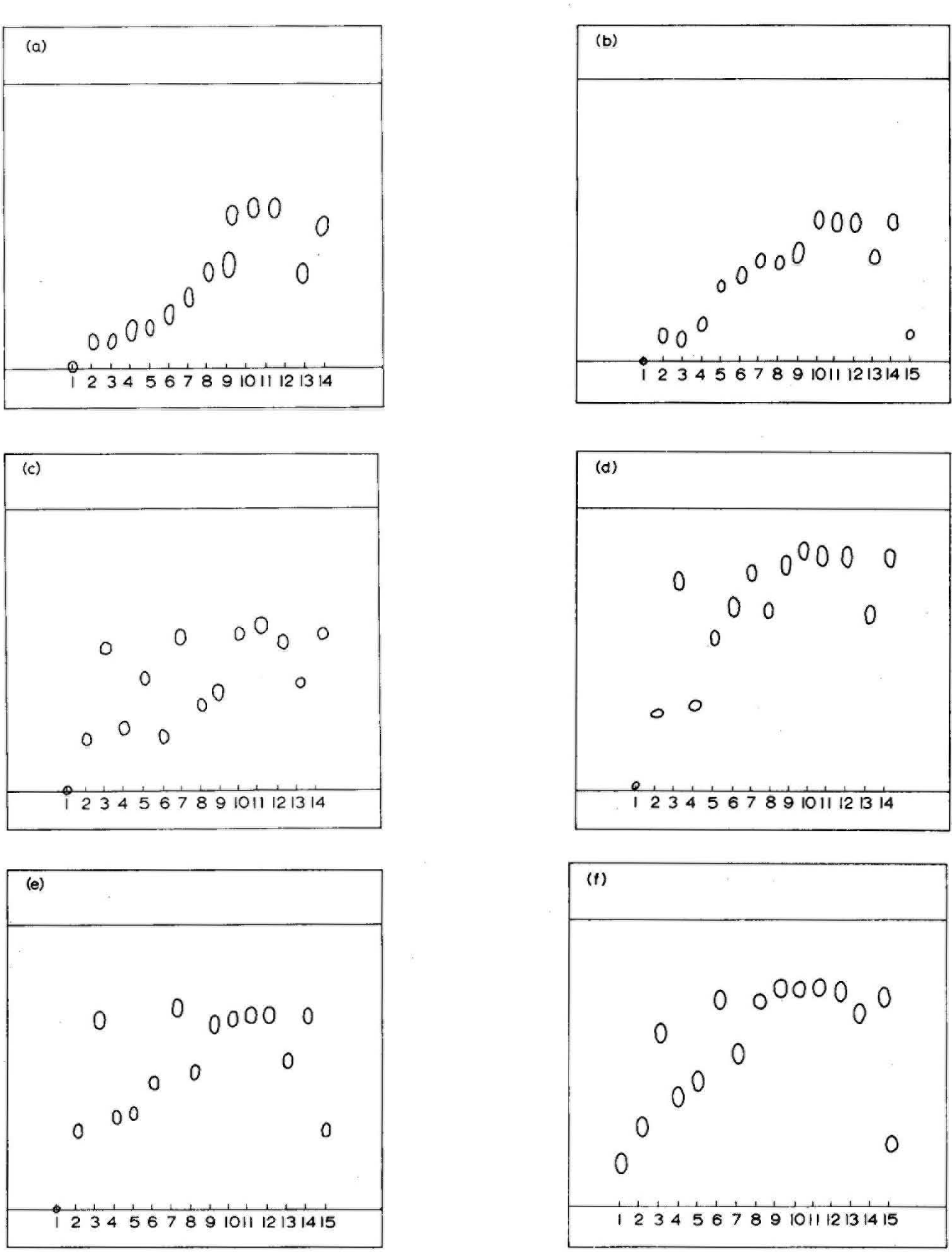

Fig. 1. Chromatograms of ergot alkaloids. (a) System A1; (b) system A7; (c) system C16; (d) system B12; (e) system B1; (f) system F17. See Table I for compound identification.

Where prior indication has not been given that the preparation being analysed may contain lysergide, it is likely that the preliminary stages in the identification will have included PC using the CURRY AND POWELL system[9] or its thin-layer modification[10,11]. This is commonly used as a screening procedure for bases, and R_F values

for over seven hundred basic compounds are recorded. For the ergot alkaloids, using the thin-layer modification, a good spread of R_F values is obtained (system F17, Fig. 1f) and it is interesting to note that lysergide is resolved from the other ergot alkaloids to at least the same extent as the best of the other systems which have been commended previously, although for routine identification of lysergide, the time taken for development (approx. 3 h to run 10 cm) could be considered to be disadvantageous.

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CHROM. 6225

QUANTITATIVE ANALYSIS OF SUGARS BY DENSITOMETRIC INSPECTION OF THIN-LAYER CHROMATOGRAMS: ANALYSIS OF METHOD

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SUMMARY

This paper describes a method for quantifying thin-layer chromatograms, presents data supporting the importance of running separate standards for each sample replicate, discusses possible variation due to spotting errors, and supplies statistical data for comparisons to other studies.

INTRODUCTION

The basic methods used to analyze thin-layer chromatograms quantitatively can be divided into four groups: (1) elution before measurement[1-5]; (2) *in situ* densitometric comparisons[1,6-9]; (3) visual evaluation[1,10]; and (4) measurement of spot area[1,11,12].

The immediate problem is to determine which of these basic methods will yield the required degree of precision. Precision can be estimated by the variance of repeated samples. Also, precision may be expressed in terms of the ratio of the standard deviation to the mean as the coefficient of variation. Unfortunately, many workers[2,9-11] have used the statistics "percent of error" or "percent accurate", which are probably a measure of bias and not of precision. Others[6,12] have made no statistical attempt to test the precision of their method. If all workers had expressed precision in terms of the coefficient of variation, more meaningful comparisons could be made. It is important also to record the number of replications because as replications increase to a certain level, coefficient of variation may become less.

In this report we include a detailed description of *in situ* densitometric quantitation of free sugars on TLC sheets. Also, we present data supporting the importance of cochromatography of standards for each constituent, discuss variation due to spotting

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errors, and supply the basic statistical data so that this method may be compared to others.

EXPERIMENTAL

Materials and methods

Samples of one-year-old needles were collected randomly from the entire crowns of eight 12–16-year-old western white pine trees (*Pinus monticola* Dougl.). Immediately after collection, the needles were submerged in liquid nitrogen and placed in dry ice for transporting to laboratory freezers. A motorized mortar and pestle was used to pulverize the samples which were submerged in liquid nitrogen; then the samples were dried by reduced pressure for 48 h.

Extraction and clearing. All samples were extracted by using chloroform in Soxhlet extractors for 5 h to remove waxes, fats and pigments. Free sugars were extracted using 80% methanol in Soxhlet extractors for 16 h. 80% methanol was added to the extract to equal 100-ml volume. A 25-ml aliquot of each sample was treated with 4 ml of a saturated solution of neutral lead acetate to precipitate substances that interfere with chromatography. 7 ml of saturated sodium phosphate were added to precipitate the excess lead. All precipitated materials were removed by centrifugation. Desalting of the samples was accomplished with ion-exchange columns (Amberlite IR-120 and IRA-400*). After desalting, all samples were concentrated by reduced pressure to 10-ml volume and stored at —10°. At this point the samples were ready for chromatography.

Chromatography. Eastman 20 × 20 cm chromatogram sheets (6060 Silica Gel with fluorescent indicator) were cut into 5 × 20 cm strips and dipped in a 0.1 *M* solution of monobasic potassium phosphate[2,13–15]. The wet chromatograms were dried at 85° for 90 min and then stored over calcium chloride at room temperature. Each chromatographic strip received a standard and sample spot. MARTIN AND WELCH[15] found streaking was a problem when spot concentrations were above 4 μg per sugar**. 2 μg of each sugar standard was therefore considered satisfactory for this experiment. To control spot size[16,17], it was necessary to spot 0.1 μl at a time with a precision microsyringe (Kensington Scientific Co.). Forced air from a hair dryer, without heat, was used to dry between 0.1-μl aliquots.

After spotting, the chromatograms were developed in either solvent system A (ethyl acetate–pyridine–water, 8:2:1) or solvent system B (isoamyl alcohol–pyridine–water, 4:4:1)[15].

Solvent system A was used to separate fructose, glucose, and sucrose (two multiple developments)[15] and solvent system B, raffinose and stachyose (two multiple developments). After development, the sugars were localized by dipping the chromatograms in a solution containing 2 g diphenylamine, 2 ml aniline, 20 ml of 85% phosphoric acid in 200 ml acetone. The chromatograms were then air dried for 15

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** Spot concentrations of 1–3 μg of each sugar resulted in a linear relationship between sugar quantity and integral number.

min, excess phosphoric acid wiped from the chromatograms' backs and then placed in a 100° oven for 5 min.

Densitometer. A Joyce Loebel MK.11 densitometer equipped with a 300–400 nm filter and a 1 × 9 mm slit was adjusted for transmission scanning of the strips. A drive gear ratio of 1:3 was used. This particular densitometer has an automated integrating system which yields an integral number that was used in comparisons of standards to samples.

Procedure

A sample was spotted adjacent to standards on each of sixteen chromatograms (5 × 20 cm); a set of eight chromatograms was developed in solvent system A and the remaining in solvent system B. A given set of eight chromatograms was developed within the same chromatography tank, thus keeping tank effects and development time constant.

RESULTS AND CONCLUSIONS

Fig. 1A illustrates the TLC separation of fructose, glucose, and sucrose, using solvent system A. Separation of raffinose and stachyose, using solvent system B, is depicted in Fig. 1B. The accompanying traceouts with integral numbers for the respective chromatograms are included in these figures.

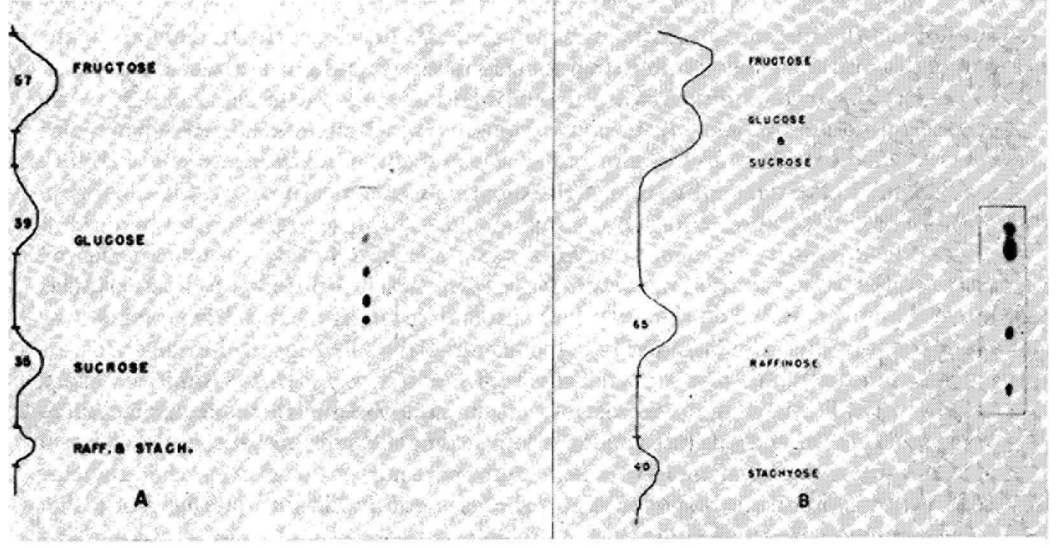

Fig. 1. (A) TLC separation of fructose, glucose, and sucrose with two developments in system A (ethyl acetate–pyridine–water, 8:2:1); (B) TLC separation of raffinose and stachyose with two developments in system B (isoamyl alcohol–pyridine–water, 4:4:1).

The means ($\bar{X}$) and coefficients of variation (CV) for concentrations of each sugar in the eight needle samples are given in Table I. All values were based on eight chromatographic replications. The coefficients of variation ranged from 4.5 to 11.0% for fructose; 5.5 to 14.5% for glucose; 7.2 to 15.1% for sucrose; 7.9 to 12.7% for

TABLE I

MEANS ($\bar{X}$)[a] AND COEFFICIENTS OF VARIATION (CV) OF SUGAR CONCENTRATION BASED ON EIGHT CHROMATOGRAPHIC REPLICATIONS OF EACH NEEDLE SAMPLE

Sugar	*Sample*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Fructose								
$\bar{X}$	16,983	13,584	15,358	18,275	13,327	17,498	15,129	12,850
CV	7.8	10.3	7.6	11.0	6.2	4.9	5.4	4.5
Glucose								
$\bar{X}$	16,553	11,712	12,605	14,761	11,259	14,030	13,166	10,287
CV	7.6	14.5	10.6	10.5	9.1	7.5	10.5	5.5
Sucrose								
$\bar{X}$	5,996	8,907	10,605	9,809	10,153	7,605	7,141	14,731
CV	9.5	10.8	11.3	12.5	14.5	7.2	8.1	15.1
Raffinose								
$\bar{X}$	9,032	10,016	9,672	10,604	9,410	10,470	10,213	11,993
CV	11.1	12.0	12.7	11.7	7.9	8.0	8.8	8.3
Stachyose								
$\bar{X}$	2,096	2,772	2,962	3,282	3,058	2,405	3,134	3,039
CV	14.8	13.8	8.8	22.2	13.2	10.9	12.1	12.8

[a] μg of sugar/g dried needle tissue.

raffinose; and 8.8 to 22.2% for stachyose. It appears that quantitative analyses of those sugars developed in solvent system A (fructose, glucose, sucrose) were less variable as the distance from the origin increased*. Fructose, the lead sugar on the chromatogram, was less variable than its neighboring sugar glucose, which was closer to the origin. Glucose, in turn, was less variable than sucrose, the sugar closest to the origin. These observations could mean two things: (1) each sugar may have a particular spot diffusion that may be optimum for its measurement; or (2) the degree of variability may be due to the inherent nature of the sugar molecule reactivity with localizing reagents.

When quantity of sugar standards was a constant for all chromatograms, we observed a strong relationship between spot lengths, or diffusion, and size of their integral number. Based on the sixty-four chromatograms used in this experiment, the correlation coefficients for individual spot lengths and their integral number for the fructose, glucose, and sucrose standards were $\gamma = 0.879$, $\gamma = 0.708$, and $\gamma = 0.963$, respectively. This strong degree of association could be due to the localizing agent being more efficient on a diffused spot than on a compact spot; perhaps, in the compact spot not all of the sugar molecules have an opportunity to react with the localizing reagent, thus producing a spot that absorbs less light and in turn produces a smaller integral number.

It is evident in the above that differences between spot diffusions, resulting from chromatography of standards and samples in separate runs (developments), may introduce quantitation error. In order to minimize this error standards and samples were cochromatographed in sets of eight replications. Analysis of variance and test of significant difference[18] between means was undertaken on the integral numbers obtained from the eight standard sets of fructose (each set based on eight chromato-

* *i.e.*, as spot size increased.

TABLE II

ANALYSIS OF VARIANCE AND TEST OF SIGNIFICANT DIFFERENCES AMONG THE MEAN INTEGRAL NUMBERS OF THE FRUCTOSE STANDARD SETS

	Analysis of variance							
	Degrees freedom	*Mean squares*	F[a]					
Among sets of standards	7	1,725.06	47.33					
Within sets of standards	56	36.45	—					
	Test of significant differences							
	7	1	4	3	6	2	5	8
Spot length								
$\bar{X}$	38.5	37.1	45.5	45.5	45.6	48.4	50.5	61.1
CV	4.4	7.0	9.1	6.5	9.9	6.8	11.1	7.4
Integral number								
$\bar{X}$	67.3	68.4	78.6	83.1	84.1	89.3	90.0	113.9
CV	4.2	7.9	10.1	6.9	4.6	9.7	6.0	6.4
Significant difference[b]				———	———	———	———	
			———	———	———	———		
		———	———					
	———	———						

[a] $\alpha = 0.01$.

[b] Any two integral means not underscored by the same line are significantly different at the 99% level, Scheffe's test[18].

graphic replications). Fructose was considered typical for all sugars. The results of these tests plus the means and coefficients of variation for spot lengths are given in Table II. Some means were significantly different ($\alpha = 0.01$)[18]. For example, the mean integral number for standard set 7 was 67.3 compared to 113.9 for standard set 8. Therefore *gross error* would occur if the integral numbers of standard set 8 were used to calculate the concentration of 7's samples. This clearly points out the importance of running separate standards for each constituent within a sample set as a means of lowering error. We believe that the major cause of these significant differences between sets was due to the differences in spot diffusions caused by uncontrollable tank and development variations.

A possible source of variation within a chromatographic replication of eight could be due to spotting errors[16,17]. To demonstrate this, a single chromatogram within a sample set would have to contain all the lowest or highest values for each sugar measured. From the analyses of eight sample sets (each set equals eight chromatographic replications) the expected number of single chromatograms having either all the low or high values for each sugar measured would be, in both cases, eight. After examining the sixty-four chromatograms, on a per sample set basis, we found that four sample sets contained single chromatograms having all low values and one sample set contained a single chromatogram having all high values. Therefore, we do not agree with FAIRBAIRN AND RELPH[16] and SAMUELS[17] that spotting error need be a significant source of variation in quantifying thin-layer chromatograms.

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CHROM. 6224

ESTIMATION OF INDIVIDUAL COENZYME Q HOMOLOGUES IN A COENZYME Q MIXTURE BY DENSITOMETRY

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SUMMARY

A method for the densitometric determination of coenzymes Q individual homologues after separation by reversed-phase paper chromatography is described. The parameters associated with the reproducibility and accuracy of the method are discussed. Comparison is made between the total coenzyme Q concentration in liver and spleen of mice obtained by densitometry and by the modified Craven test.

INTRODUCTION

The biosynthetic isoprenylation of the coenzymes Q (co Qs) (I) leads to a series of co Qs with different number of isoprene units in the side chain[1]. Each species biosynthesizes predominantly one co Q, but other homologues with shorter and longer side chains are also formed. When determining the total co Q content of tissues and blood of experimental animals, it is usually necessary to remove interfering substances prior to the actual assay of the coenzymes. This is accomplished by saponification and extraction methods followed by one or several chromatographic steps[2]. The co Q fraction obtained in this manner contains the mixture of all the homologues present which is then quantitatively assayed by means of ultraviolet (UV) spectrophotometry (characteristically by differential UV absorption between the quinone and hydroquinone forms)[2] or by a modified Craven test[3,4]. The results are then usually expressed as the concentration of the predominant co Q. However, the individual homologues present in the extract can be detected by one of several excellent paper (PC) and thin-layer chromatographic (TLC) techniques available, all based on reversed-phase chromatography[2].

In studying the total co Q concentration in tissues or blood, it is at times desirable or even necessary to determine the concentration of the individual homologues in the mixture in a quantitative manner. Although this can be accomplished by extraction of each spot obtained from a reversed-phase chromatogram followed by quantitation by spectrophotometry[5], such elution procedures require, in general, larger amounts of material than is usually available from a single spot. Moreover, in many cases it is necessary to remove, by chromatographic means, the supporting

$$CH_3O,\ CH_3O,\ CH_3,\ O,\ O \quad -\!\!\left[CH_2-CH{=}\overset{CH_3}{\overset{|}{C}}-CH_2\right]_n\!-H$$

material used to impregnate the paper, as its presence usually interferes with the spectrophotometric determination of the coenzymes[6]. Therefore, we have developed a procedure for the direct estimation of the individual spots in the paper chromatogram by means of densitometry. The values of total coenzyme Q concentration obtained by this densitometric technique were compared with those obtained using the modified Craven test of REDALIEU *et al.*[3,4]. Mouse livers and spleens were used for testing and developing the method.

EXPERIMENTAL

Standards

Coenzyme Q_{10} was obtained from Cudahy Laboratories, Omaha, Nebr., U.S.A.; coenzyme Q_9 was isolated from rat liver by the methods described below; coenzymes Q_8, Q_7 and Q_4 were kindly supplied by Dr. KARL FOLKERS, Institute for Biomedical Research, Austin, Texas, U.S.A.; coenzyme Q_6 was obtained from Sigma Chemical Company, Saint Louis, Mo., U.S.A.

All solvents were purified prior to use.

Extraction of the coenzymes Q from tissue

The saponification method of SUGIMURA *et al.*[7] was used to prepare a purified co Q fraction from mouse tissues (liver or spleen). For duplicate determinations 10 g of wet tissue sufficed. The unsaponifiable fraction was purified by two successive thin-layer chromatographies. For development of the first plate (20 × 20 cm, Silica Gel G, 1 mm thick, prewashed in chloroform–methanol (4:1)) hexane–diethyl ether (60:40) was used as solvent. The portion of the silica gel corresponding to a comparison sample of pure "standard" co Q was extracted with diethyl ether. The residue on evaporation of the ether extract was then further purified by TLC using petroleum ether (b.p. 42–60°)–diethyl ether (90:10) as solvent. The residue of eluting with diethyl ether the co Q band was next taken up in ethanol and made up to volume. An aliquot was then used to assay the total co Q content in the tissue by a modified Craven test. The remainder of this residue after evaporation of the ethanol was then taken up in hexane and subjected to PC.

Estimation of the total coenzyme Q content in purified tissue extracts by the modified Craven test

The method described by REDALIEU *et al.* was used[3]. The accuracy of this procedure has been reported to be 97% and its standard error 3.8%[4]. The absorbance measurements were carried out in a Beckman DB spectrophotometer.

The saponification method of SUGIMURA *et al.*[7] was selected for this work because methanol rather than ethanol is used in the saponification medium. It has

been shown that ethoxy derivatives of the coenzymes Q are formed as artifacts during hot alkaline saponification in ethanolic solution[8,9]. When methanol is used as the medium, such alkoxy derivatives are not formed.

This modified Craven test was selected as our standard method for determining the total coenzyme Q concentration on the purified tissue extracts because of its reliability and high specificity. No other methoxy derivative existing in tissue is known which might interfere with this assay[3].

Paper chromatography of the coenzyme Q mixture

The method of LINN *et al.* was used[10]. Whatman No. 1 sheets (24 × 56 cm) were impregnated with petroleum jelly (5%, w/v in petroleum ether) and developed with N,N-dimethylformamide–water (97:3) saturated with petroleum jelly. The ascending technique was used.

After application of the sample, the paper was equilibrated in the vapor phase of the solvent for 0.5 h prior to development. Development time was 42 h. At the end of the chromatography the paper was air-dried in the dark for 6 h at room temperature. The solvent front had advanced approximately 30 cm from the origin.

The R_F values of the coenzymes Q in this system are approximately 0.71, 0.60, 0.49, 0.35, 0.26 and 0.17 for co Q_4, co Q_6, co Q_7, co Q_8, co Q_9 and co Q_{10}, respectively.

The chromatogram was then immersed for 30 sec in a 0.2% solution of $KMnO_4$. Excess permanganate was removed by washing three times with distilled water and the chromatogram then air-dried overnight before using it for densitometry. This treatment gives brown spots against an off-white background.

Densitometric assay

All densitometric measurements were made by transmission, using a Photovolt Densitometer Model 530, equipped with a Varicord variable-response recorder Model 42 with automatic scanning. The light source was an incandescent bulb. The scanning of the spot was carried out perpendicularly to the line of development of the chromatogram and through the center, darker portion of the spot. The recorder response was set to that which corresponds approximately to the logarithm of the light absorption. The densitometric traces obtained were integrated with a planimeter.

In preliminary work it was established that impregnated paper, treated with the $KMnO_4$ solution as described, produced an almost straight base line on the densitometric readings. It was also found that the brown color developed by the spots on permanganate treatment was stable for up to four days, giving reproducible densitometric readings. This is useful for cases where it is not convenient to run the densitometric measurements immediately after treatment.

Only five initial spots were applied on each chromatographic sheet, 4 cm apart. This distance allowed enough separation between adjacent spots in the developed chromatogram to enable the base-line between adjacent densitometric curves to be reestablished.

In the preliminary studies, solutions containing different concentrations of the co Qs were prepared and various volumes of these solutions were spotted on the paper, in order to determine the optimal amount of co Q per spot for densitometry and the optimal volume of solution for each application. Very uniform, small spots were produced by application with a micropipette of between 25–75 λ of solution. After

development of the chromatogram, those spots containing between 5 aud 35 μg of co Q were round and well defined. As will be discussed later, these amounts were the lower and upper limits of accurate detection by densitometry.

RESULTS AND DISCUSSION

Typical calibration curves for some coenzymes Q are shown in Fig. 1. The integrated area under a densitometric peak was plotted against the amount of co Q present on the spot. Spots containing more than 35 μg of co Q reacted incompletely with the $KMnO_4$ solution under the conditions described in EXPERIMENTAL, leading to

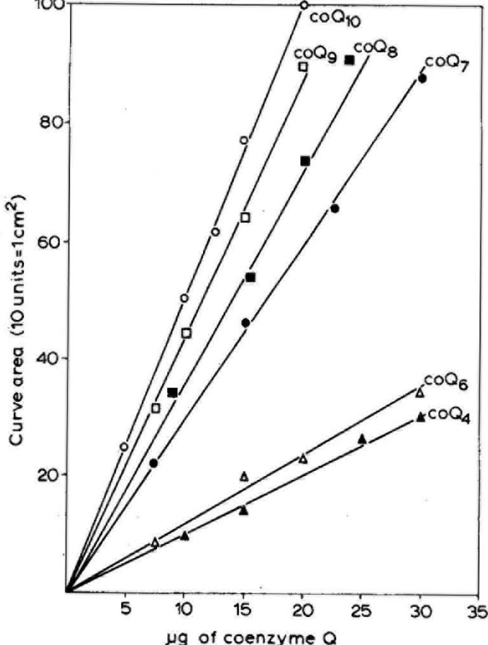

Fig. 1. Calibration curves for a series of coenzymes Q.

deviation of linearity in the densitometric response. In contrast, spots containing less than 5 μg did not develop a sufficiently intense color to be determined with accuracy. Thus the limits of detection were established as between 5 and 35 μg of co Q. It is of interest to note that as the length of the side chain increases in the co Q family, the slope of the response curve increases. This behavior is to be expected since the deposit of MnO_2 should increase as the number of double bonds per molecule increases.

The precision of the densitometric readings (by planimeter) was tested on the same and among different chromatographic sheets by applying a series of five replicates of a coenzymes Q mixture to impregnated paper. Each initial spot was produced from 10 μl of a hexane solution containing 5 μg of co Q_{10}, 5 μg of co Q_9, 12 μg of co Q_7 and 16 μg of co Q_6. Four such sheets were prepared, run chromatographically and stained with the $KMnO_4$ solution. The area under each densitometric peak was integrated and the results analyzed (*cf.* Table I).

TABLE I

REPRODUCIBILITY OF THE ANALYSIS OF A MIXTURE OF COENZYMES Q

Sheet	*Planimeter readings*[a] ±% *coefficient of variation*			
	co Q_{10}	*co* Q_9	*co* Q_7	*co* Q_6
1	26.4 ± 5.7	20.0 ± 6.1	23.8 ± 5.0	29.2 ± 3.6
2	32.0 ± 3.8	25.2 ± 2.4	35.5 ± 0.9	29.8 ± 8.7
3	32.4 ± 6.2	26.8 ± 3.5	30.2 ± 3.1	32.3 ± 5.6
4	27.6 ± 4.7	20.2 ± 5.4	30.8 ± 3.1	26.3 ± 2.9

[a] 10 units = sq. cm.

The fact that area readings for a constant amount of material will vary from sheet to sheet is a well known problem in quantitative paper chromatography[11]. Therefore, in all cases where unknowns were involved a standard solution in two different concentrations was also applied on the same sheet beside the sample and worked up to a calibration curve. The treatment of sample and standards was then identical throughout the chromatography.

A further mixture of co Qs of known concentration was then analyzed for these control purposes. Three aliquots (25, 50 and 75 µl) of a hexane solution containing 156.4 µg of co Q_{10}, 145.6 µg of co Q_9 and 188 µg of co Q_6 per 1 ml of solution were applied to paper, along with two different concentrations of a standard solution. From the calibration curves the amount of co Q in each spot was determined, the concentration of each co Q in the original solution calculated and the values of the three determinations then averaged. Table II shows a comparison between the composition values by known weight and that measured by densitometry, thus giving a measure of the percentage of recovery.

TABLE II

ANALYSIS OF A SYNTHETIC MIXTURE OF THREE COENZYMES Q BY DENSITOMETRY

Coenzyme	*Theoretical values (µg/ml)*	*Experimental values*[a] *(µg/ml)*	*Recovery (%)*
Q_{10}	156.4	149.5 ± 0.3%	95.6
Q_9	145.6	151.3 ± 3.5%	103.8
Q_6	188.0	186.8 ± 6.0%	97.6

[a] Mean ± coefficient of variation of three determinations.

Next the densitometric and the modified Craven tests were compared as to their accuracy for the determination of the total coenzyme Q concentration in a mixture of known composition containing four homologues. Triplicate aliquots (10 µl) of a hexane solution containing 0.5 mg of Q_{10}, 0.5 mg of co Q_9, 1.2 mg of co Q_7 and 1.6 mg of co Q_6 per ml of solution were applied to paper along with two different concentrations of a standard solution. The concentration of each spot was estimated from the standard curves and the mean values and standard deviations calculated. Total co-

TABLE III

COMPARATIVE ANALYSIS OF TOTAL COENZYME Q CONCENTRATION BY DENSITOMETER AND MODIFIED CRAVEN TEST IN A MIXTURE OF FOUR HOMOLOGUES

	Total coenzyme Q content (μmole/ml)	*Individual homologues (μmole (mg)/ml)*			
		co Q_{10}	*co Q_9*	*co Q_7*	*co Q_6*
Theoretical	5.731	0.579 (0.500)	0.628 (0.500)	1.821 (1.200)	2.703 (1.600)
Modified Craven test	5.564 ± 0.1[a]	—	—	—	—
Densitometer	5.827[b]	0.671 (0.580 ± 0.036)[c]	0.6702 (0.533 ± 0.046)[c]	1.997 (1.316 ± 0.035)[c]	2.489 (1.473 ± 0.040)[c]

[a] Mean ± standard deviation of three determinations.
[b] Calculated by adding the results for the individual homologues.
[c] Mean ± standard deviation of three determinations.

enzyme Q concentrations were also determined by the modified Craven test on three aliquots of the solution and mean values and standard deviations calculated again. A comparison of the results obtained by both methods is shown in Table III. It should be noted that the results obtained with the modified Craven test have been expressed as μmole per ml instead of as mg per ml. The molar extinction coefficients of the colored species formed from the reaction of ethyl cyanoacetate and the coenzyme Q individual homologues in basic medium are very similar (*i.e.*, $\varepsilon = 1.3, 1.6, 1.2, 1.2, 1.3 \times 10^3$ for co Q_{10}, co Q_9, co Q_8, co Q_7, co Q_6, respectively, at 630 mμ). In expressing the results as μmole per ml rather than as mg per ml the comparison of the errors is thus standardized.

TABLE IV

COENZYME Q CONCENTRATION IN SPLEEN AND LIVER OF MICE[a]

Tissue	*Total coenzyme Q (μg/g wet tissue)*		*Individual homologues (by densitometry) (μg/g wet tissue)*		
	Modified Craven test[b]	*Densitometer*	*co Q_8*	*co Q_9*	*co Q_{10}*
Liver	51.8 ± 3.4	53.8 ± 5.0	5.2 ± 1.0	48.5 ± 4.3	—
Spleen	12.5 ± 4.9	13.6 ± 4.8	—	10.2 ± 2.3	3.4 ± 0.6

[a] Data are expressed as the mean ± standard error of six determinations.
[b] Expressed as co Q_9.

A comparative study was made to discern whether the concentration of total coenzyme Q determined by densitometry was in agreement with that obtained by the modified Craven test in a natural mixture of individual homologues. Spleen and liver of mice were used in these experiments. These tissues are known to biosynthesize predominantly co Q_9. The results are shown in Table IV.

It can be seen from the figures in the table that a good correlation exists between the two methods. Moreover, the total values found are in excellent agreement with those reported by other workers for these tissues[5,12].

In general tissues do not contain appreciable amounts of more than three or four co Q homologues, with one of them usually being highly predominant. A preliminary qualitative study of the purified coenzyme Q fraction by paper chromatography would be recommended to adjust the amounts to be employed in the present quantitative assay when the nature and relative concentration of the individual homologues is not known.

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PAPIERELEKTROPHORESE VON METALLIONEN IN EDTA-LIGANDENPUFFERN

II. EINIGE ZWEIWERTIGE METALLIONEN UND IONEN DER SELTENERDMETALLE

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SUMMARY

Paper electrophoresis of metal ions in EDTA-ligand buffer solutions. II. Some bivalent metal ions and rare earth metal ions

The electrophoretic mobility curves of alkaline earth ions in Mg^{2+}–MgL^{2-} ligand buffer solutions containing EDTA, and those of bivalent heavy metal ions and of the complete rare earth ions group in Zn^{2+}–ZnL^{2-} ligand buffer solutions at pH 4 and pH 2 were investigated. These migration media were found to be useful for the reproducible electrophoresis of metal ions. Deviations from theory and the levelling effect of ligand buffers on chelate stabilities are examined. Stability constants were estimated from electrophoretic data both numerically and through graphical logarithmic analysis. The applicability for practical separation procedures is briefly discussed.

EINLEITUNG

In der ersten Mitteilung dieser Reihe[1] wurde die theoretische Grundlage der Papierelektrophorese von Kationen in Liganden-gepufferten Lösungen von EDTA behandelt. Der Sinn, die zu erwartenden Vorteile sowie die Grenzen dieser Arbeitsweise wurden ebenfalls besprochen. In der nun vorliegenden Arbeit wird über die experimentellen Untersuchungen des Verhaltens von ausgewählten Kationengruppen berichtet. Als Modellionen wurden einige Kationen von Erdalkalimetallen, einige zweiwertige Schwermetallionen und Kationen aller stabilen Seltenerdmetalle untersucht.

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EXPERIMENTELLES

Ligandenpuffer

Mit Rücksicht auf die thermodynamischen Stabilitäten der EDTA-Chelate der untersuchten Ionen, sowie auf die Möglichkeit einer relativ ungestörten chemischen Sichtbarmachung, wurden die Systeme Mg^{2+}–MgL^{2-} und Zn^{2+}–ZnL^{2-} als Ligandenpuffer gewählt. Diese Puffer sind in den Bereichen (etwa) pL 7.5–9.5 bzw. 15.5–18.5 wirksam. Mit Hilfe einer für Ligandenpuffer dieser Art geltenden Gleichung[1] wurden die pL-Werte beider Systeme bei verschiedenen pH-Werten als Funktion des Quotienten $a = c_M/c_L$ berechnet. Eine nennenswerte Pufferkapazität lässt sich für das berechnete Intervall $0.1 < M:ML < 10$ erwarten (a = 1.1–11). Die Resultate sind in parametrischen Arbeitsdiagrammen Fig. 1 und Fig. 2 dargestellt. Für die Berechnungen wurden folgende Werte der Konstanten benutzt:

Stabilitätskonstanten[2]

$K_{MgL} = 4.9 \cdot 10^8$, $K^{H}_{MgHL} = 8 \cdot 10^3$ und $K_{ZnL} = 3.15 \cdot 10^{16}$, $K^{H}_{ZnHL} = 10^3$.

Brutto-protonierungskonstanten von EDTA[3,4]

$\beta_1 = 1.82 \cdot 10^{10}$, $\beta_2 = 2.64 \cdot 10^{16}$, $\beta_3 = 1.24 \cdot 10^{19}$, $\beta_4 = 1.24 \cdot 10^{21}$, $\beta_5 = 5.20 \cdot 10^{22}$, $\beta_6 = 4.30 \cdot 10^{23}$.

Sämtliche Werte wurden für jene analytische Gesamtkonzentration von EDTA $c_L = 10^{-2}\,M$ berechnet*, welche bei den Versuchen durchwegs verwendet wurde.

Der Verlauf der Kurven bestätigt deutlich, dass in den Aziditätsbereichen, in

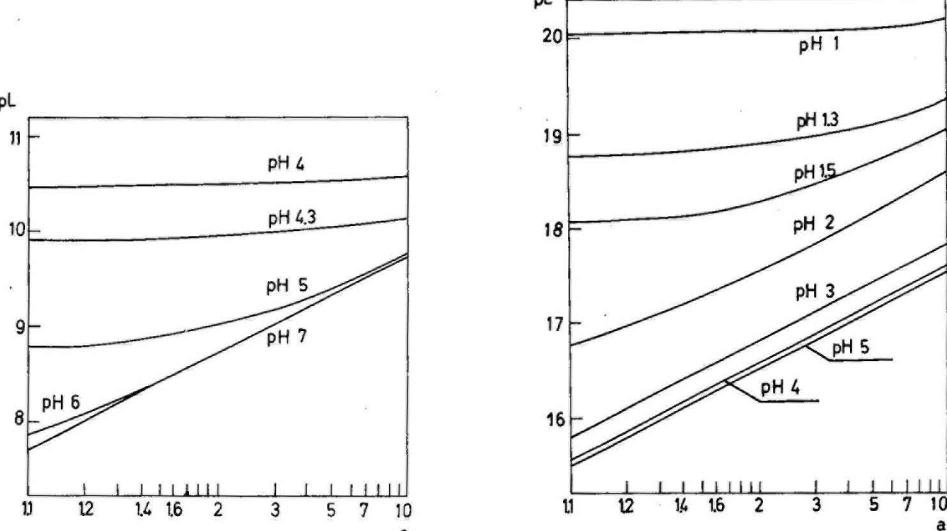


Fig. 1. Abhängigkeit des pL Wertes vom Quotienten $a = c_M/c_L$ in EDTA-Ligandenpuffern Mg^{2+}–MgL^{2-}. $c_{EDTA} = 10^{-2}\,M$.

Fig. 2. Abhängigkeit des pL Wertes vom Quotienten $a = c_M/c_L$ in EDTA-Ligandenpuffern Zn^{2+}–ZnL^{2-}. $c_{EDTA} = 10^{-2}\,M$.

* Bei niedrigeren pH Werten ist nämlich pL auch konzentrationsabhängig.

welchen die Nebenreaktionen praktisch ausbleiben, der pL-Wert nur sehr wenig vom pH abhängt, und entscheidend durch den Quotienten *a* bestimmt wird (z.B. in Fig. 2 die Kurven für pH $\geqslant$ 4). Dagegen ist in stärker sauren Lösungen der Einfluss der Azidität entscheidend, und der pL Wert stimmt mit jenem in einer Lösung des reinen Chelatbildners beinahe überein (Kurve für pH 1, Fig. 2). Dazwischen überlagern sich beide Einflüsse.

Für die Zubereitung der Ligandenpuffer wurden folgende komplexometrisch standardisierte Lösungen benutzt: 0.1 *M* Chelaton 3 (EDTA-Dinatriumsalz), 0.2 *M* $Mg(NO_3)_2$ und 0.2 *M* $Zn(NO_3)_2$. Je nach dem gewünschten pL Wert wurde zu 10 ml Chelatonlösung 5*a* ml der Metallsalzlösung zugefügt und auf etwa 90 ml mit Wasser verdünnt. Der nötige pH Wert wurde unter potentiometrischer Kontrolle mittels 1 *N* HNO_3 oder 1 *N* KOH eingestellt und nachher das Volumen auf 100 ml aufgefüllt.

Materialien

Auftragslösungen waren 0.05 *M* Nitrate der Erdalkalien und der Schwermetalle, bzw. 0.012 *M* Nitrate der Seltenerdmetalle. Als Standard der Beweglichkeit wurde $(C_2H_5)_4N^+$ gebraucht, als Indikatoren der Elektroosmose dienten Antipyrin oder Glukose.

Für die Mittelspannungselektrophorese wurde die Apparatur mit gekühlter Grundplatte OE-205 (Labor, Budapest, Ungarn) und die Stromquelle OE-405 (0–1500 V) benutzt. pH-Messungen erfolgten mit dem pH-Meter GV-52 (Seibold, Wien, Österreich).

Als Papier wurde Whatman Nr. 2 benutzt. Durchwegs wurde ein Volumen von 1–2 μl von den Probelösungen aufgetragen. Detektionslösungen waren: 0.5% Natriumrhodizonat (Ba^{2+}, Sr^{2+}), 5% Ammoniumsulfid (Co^{2+}, Pb^{2+}, Cu^{2+}), Dragendorff-Reagens (Tetraäthylammonium, Antipyrin) und angesäuerte (0.1 *M* an HCl) gesättigte äthanolische Alizarinlösung (Seltenerdmetallionen).

Methodik

Die Elektrophorese erfolgte auf 47 cm langen Papierstreifen bei einem Potentialgefälle von 28–30 V/cm (direkt am Papier gemessen). Das Pherogramm war mittels zweier mit Zellophan umhüllter Papierstreifen mit dem inneren Abteil der geteilten Elektrolytgefässe verbunden, um den Dochteffekt zu vermeiden. In diesem Teil der Gefässe befand sich der Ligandenpuffer, in den eigenen Elektrodenräumen jedoch nur eine 0.01 *M* Chelatonlösung (pH 4.8). Dadurch wurde die elektrolytische Abscheidung des Metalls von dem Ligandenpuffer auf ein Mindestmass herabgesetzt, ohne die Konzentrationsverhältnisse auf dem Pherogramm zu stören. Dauer des einzelnen Versuches bei 20° war 1.5 h.

Sichtbarmachung der seltenen Erden wurde (wegen der Anwesenheit des Chelatbildners) folgendermassen durchgeführt: das trockene Pherogramm wurde mit dem Reagens getränkt, in trockener Ammoniakatmosphäre mindestens 30 min aufbewahrt und an der Luft nachgetrocknet. Die Flecken sind violett und relativ dauerhaft, der Hintergrund wird in einigen Stunden hell.

Alle Beweglichkeiten sind relative Werte. Sie wurden unter Berücksichtigung der Elektroosmose auf das Tetraäthylammonium-Ion ($u = +1.00$) bezogen; sie sind allgemein Mittelwerte von mindestens zwei unabhängigen Parallelversuchen.

ERGEBNISSE UND DISKUSSION

Ionen der Erdalkalimetalle

Mit Schwermetallionen verglichen bilden diese Ionen weniger stabile EDTA-Chelate, s. Tabelle I. Auf Grund der Werte der Stabilitätskonstanten lässt sich die Bildung der Chelate im Bereiche pL 7–9 erwarten; deshalb wurde der Ligandenpuffer Mg^{2+}–MgL^{2-} (pH 6) benutzt (vgl. Fig. 1). Die Bildung der Hydrogenkomplexe ist bei

TABELLE I

STABILITÄTSKONSTANTEN UND RELATIVE ELEKTROPHORETISCHE BEWEGLICHKEITEN DER EDTA-CHELATE EINIGER METALLIONEN (20°)

Ion	*Log* K_{NL}	*Log* K^{H}_{NHL}	u_N	u_{NL}	u_{NHL}
Sr(II)	8.63[a]	3.9[a]	1.65	−0.90	−0.45
Ba(II)	7.76[a]	4.6[a]	1.70	−0.90	−0.45
Co(II)	16.31[b]	3.0[b]	1.60	−1.00	−0.50
Pb(II)	18.04[b]	2.8[b]	1.65	−1.00	−0.50
Cu(II)	18.80[b]	3.0[b]	1.65	−1.00	−0.50

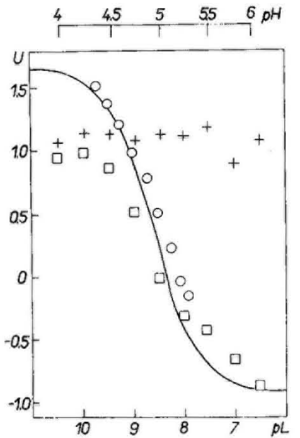
[a] Nach Lit. 5.
[b] Nach Lit. 2.

diesem pH-Wert vernachlässigbar. Die Fig. 3 und 4 enthalten die berechneten Beweglichkeitskurven für Sr(II) und Ba(II) (volle Linien) und die ermittelten Versuchsdaten.

Es wurden drei Serien von Versuchen durchgeführt:

(*i*) Elektrophorese in pH-gepufferten EDTA-Lösungen ($c_{EDTA} = 10^{-2}\ M$) ohne fremde Hilfspuffer, Versuchswerte mit + bezeichnet;

(*ii*) wie (*i*), jedoch mit Zusatz relativ hoher Konzentration des Azetatpuffers (0.1 M), Werte mit □ bezeichnet;



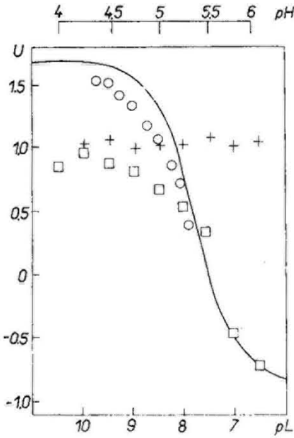


Fig. 3. Berechnete Beweglichkeitskurve und Experimentalwerte für Sr(II). Erläuterung der Zeichen im Text.

Fig. 4. Berechnete Beweglichkeitskurve und Experimentalwerte für Ba(II). Erläuterung der Zeichen im Text.

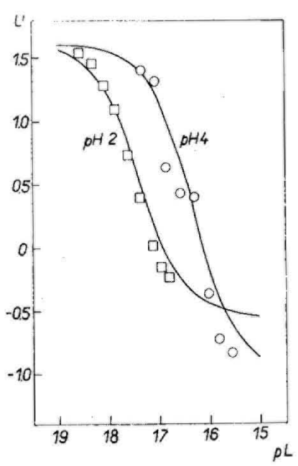
(*iii*) in Ligandenpuffern, Werte mit ○ bezeichnet. Es ist ersichtlich, dass in der Versuchsreihe (*i*) die Chelatbildung an der Beweglichkeit überhaupt nichts geändert hat. In Gegenwart von Hilfspuffern stimmen die Ergebnisse mit der Theorie nur bei niedrigeren pL Werten überein, d.h. bei den Bedingungen, bei denen der überwiegende Anteil von Metallionen im Chelat gebunden ist. Bei der Elektrophorese im Ligandenpuffer ist die Übereinstimmung der experimentellen und berechneten Beweglichkeiten für beide Ionen im ganzen pL Bereiche sehr gut und zeugt eindeutig für dessen Eignung als Migrationsmedium.

Auf Grund dieser Erkenntnisse wurde auf weitere vergleichende Versuche der pH-gepufferten Systeme verzichtet; diese wurden übrigens bereits früher untersucht.

Zweiwertige Schwermetallionen

Als Modellionen wurden nach steigender Stabilität der EDTA-Chelate (s. Tabelle I) Co(II), Pb(II) und Cu(II) Ionen ausgewählt. Hinsichtlich des nötigen pL-Puffergebietes wurde das System Zn^{2+}–ZnL^{2-} als Grundelektrolyt eingesetzt, und zwar bei pH 2 (Puffergebiet pL 16.8–18.6) und bei pH 4 (pL 15.6–17.6), vgl. Fig. 2. Es hat sich als solches voll bewährt. Unter den angegebenen Bedingungen ist bei untersuchten Ionen der Einfluss des gebildeten Hydrogenkomplexes auf den Verlauf der Beweglichkeitskurven bedeutend.

In Fig. 5–7 sind die berechneten Kurven sowie Versuchsdata für beide pH Werte dargestellt. Beim Co(II) ist die Übereinstimmung gut, für pH 2 sogar sehr gut (hierbei waren die Zonen wesentlich besser abgegrenzt und ihre Lage liess sich voll reproduzierbar bestimmen). Ähnliche Verhältnisse sind auch beim Pb(II) zu beobachten, jedoch ist hier die Streuung der Versuchswerte merklich grösser. Es ist anzunehmen, dass bei sehr stabilen Komplexen bereits der kinetische Faktor ihrer Bildungsreaktionen das elektrophoretische Verhalten zu beeinflussen vermag. Die Theorie der elektrophoretischen Beweglichkeit der schwachen Elektrolyte beruht bekanntlich auf der Mobilität der betreffenden Gleichgewichte; dagegen ist vor allem die Dissoziation



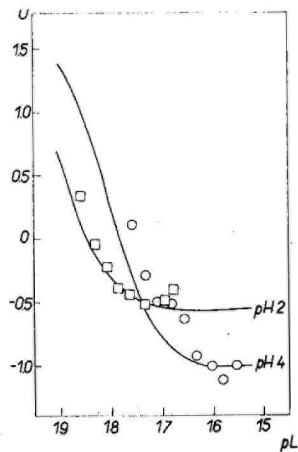


Fig. 5. Berechnete Beweglichkeitskurven für Co(II). ○, pH 4; □, pH 2.

Fig. 6. Berechnete Beweglichkeitskurven für Pb(II). Zeichen wie in Fig. 5.

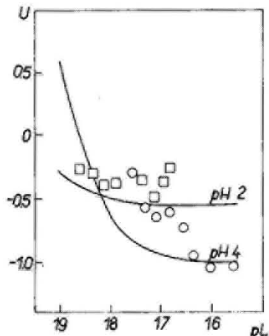


Fig. 7. Berechnete Beweglichkeitskurven für Cu(II). Zeichen wie in Fig. 5.

der stabilen Komplexe manchmal eine relativ langsame Reaktion. Dies tritt beim Kupfer(II)-Chelat noch deutlicher in den Vordergrund. Bei diesem sind ausserdem die Beweglichkeiten bei pH 2 durchwegs und deutlich positiver gegenüber den berechneten Werten. Es ist eine teilweise Bildung des Dihydrogenkomplexes CuH_2L mit der Beweglichkeit $u = 0$ durchaus nicht auszuschliessen. Obwohl die Literatur dessen Bildung in Lösung nicht erwähnt, wurde diese komplexe Säure H_2CuL in festem Zustande isoliert.

Bei dem Pb(II)- und Cu(II)-Chelat ist eine deutliche Verschiebung der experimentellen Beweglichkeitskurven für pH 4 in Richtung der niedrigeren pL Werte bemerkbar. Wir schreiben dies dem sogenannten Nivellierungseffekt zu, den wir im folgenden Abschnitt ausführlicher beschreiben und diskutieren.

Seltene Erdmetallionen

Die ganze Gruppe wurde untersucht (ausgenommen Sc, welches ein extrem stabiles EDTA-Chelat bildet, und Pm). Dies gibt einen Überblick über das elektrophoretische Verhalten in Ligandenpuffern im Rahmen dieser Gruppe sehr nahe

TABELLE II

STABILITÄTSKONSTANTEN DER EDTA-CHELATE DER SELTENERDMETALLIONEN (20°)

Ion	*Log* K_{LnL}	*Lit.*	*Log* K^{H}_{LnHL}	*Lit.*	*Log* $K_{LnL}{}^{*,a}$
Y(III)	18.09	2	1.7	6	17.80
La(III)	15.50	2	—	—	16.45
Ce(III)	15.98	2	—	—	16.60
Pr(III)	16.40	2	—	—	16.95
Nd(III)	16.61	2	2.5	8	17.00
Sm(III)	17.14	2	2.6	8	17.25
Eu(III)	17.35	2	2.6	8	17.35
Gd(III)	17.37	2	2.7	8	17.35
Tb(III)	17.93	2	2.6	8	17.60
Dy(III)	18.30	2	2.8	8	(17.75)
Ho(III)	18.74	7	2.7	8	(18.00)
Er(III)	18.85	2	2.8	8	(18.15)
Tm(III)	19.32	2	2.6	8	—
Yb(III)	19.51	2	2.7	8	—
Lu(III)	19.83	2	2.5	8	—

[a] $K_{LnL}{}^{*}$ elektrophoretisch gefunden, s. Text.

stehender und vom Standpunkt der Trennverfahren interessanter und wichtiger Ionen. Die nötigen Angaben sind in Tabelle II zusammengefasst (Ln = Element der Seltenerdmetallgruppe). Die Beweglichkeiten sind für alle Ionen dieselben: $u_{Ln} = 1.70$, $u_{LnL} = -0.50$. Sie wurden deshalb in die Tabelle nicht einbezogen.

Auch für diese Ionengruppe wurden die Ligandenpuffer Zn^{2+}–ZnL^{2-} mit pH 4 bzw. 2 benutzt. Vom Gesichtspunkt der Stabilitätskonstanten sollte bei pH 4 die Bildung von neutralen Hydrogenkomplexen LnHL in einem merklichen Ausmasse nicht stattfinden. Dagegen bei pH 2 sollte bei den meisten Elementen der Anteil des Hydrogenkomplexes 80% der komplexgebundenen Ionen überschreiten.

In Fig. 8 sind wieder die berechneten Beweglichkeitskurven für beide pH Werte (soweit die Protonierungskonstanten K^{H}_{LnHL} vorhanden waren) dargestellt. Die experimentellen Punkte weichen von den erwarteten in manchen Fällen ab, und die Abweichungen werden nachfolgend diskutiert.

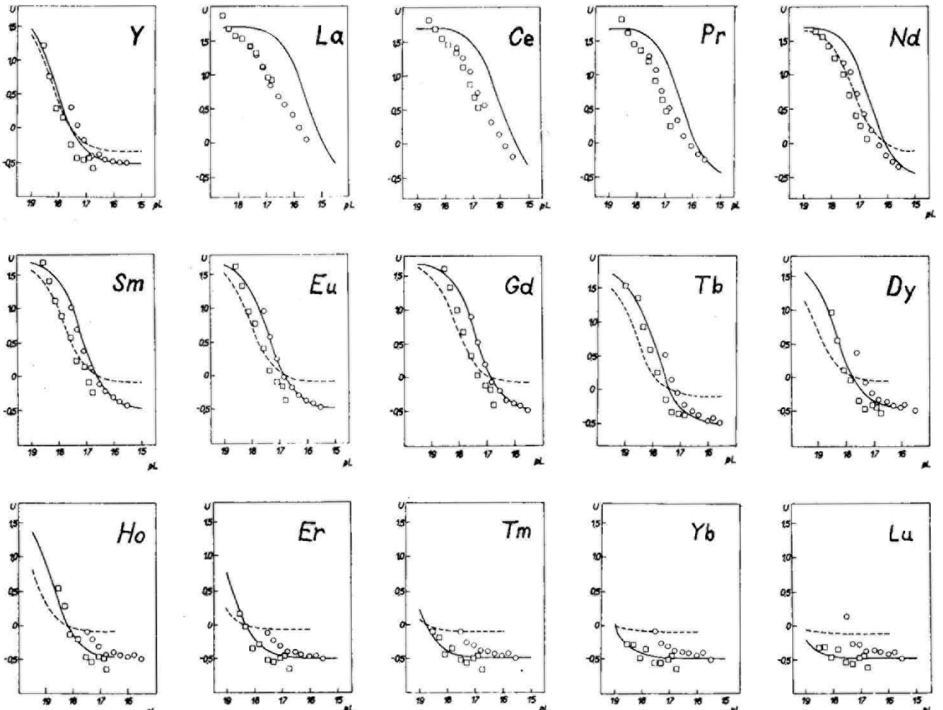


Fig. 8. Berechnete Beweglichkeitskurven für Ionen der Seltenerdmetalle. ———, pH 4; – – –, pH 2; ○, pH 4; □, pH 2.

Die Elektrophorese im Ligandenpuffer bei pH 4 bietet für Sm–Eu Ergebnisse, welche mit der Theorie in vollkommenem Einklang stehen. Die Übereinstimmung ist so gut, dass sich in diesen Fällen die Stabilitätskonstanten K_{LnL} nicht nur graphisch aus dem Werte $U_{1/2}$ bestimmen lassen[9], sondern man kann sie rein rechnerisch aus einzelnen Experimentalpunkten der Beweglichkeitskurve gewinnen. Es wird dabei von der umgeformten Gleichung der Beweglichkeitskurve[1,9] Gebrauch gemacht:

$$\log K_{\mathrm{LnL}} = \log\left(\frac{1.7 - U}{U + 0.5}\right) + \mathrm{pL} \tag{1}$$

(U ist die aktuelle Beweglichkeit zum gegebenen pL Wert). Die Tabelle III fasst die gewonnenen Data zusammen. Es ist eine sehr gute Übereinstimmung mit Literaturangaben (s. Tabelle II) sowie befriedigende Reproduzierbarkeit ersichtlich (mittlere Abweichung etwa $\pm 0.1 \log K$).

Bei den übrigen Ionen treten allerdings grössere Abweichungen auf. Die Chelate der Ionen mit einer niedrigeren Atomzahl verhalten sich stabiler als es der Theorie entspricht. Dies ist einerseits an den Beweglichkeitswerten ersichtlich: diese sind negativer, d.h. im Gleichgewichtsgemisch befindet sich ein höherer Anteil des negativ geladenen Chelates. Dieser Effekt ist beim Lanthan am auffallendsten und vermindert sich allmählich bis zum Samarium. Andererseits kann die Erscheinung auch anhand der graphisch ermittelten[9] Werte von $\log K_{\mathrm{LnL}}$ verfolgt werden: diese sind in Tabelle II mit dem Symbol $K_{\mathrm{LnL}}{}^*$ angeführt. Ihre Differenz mit den Werten der Literatur deutet ebenfalls auf eine scheinbar erhöhte Stabilität der Chelate.

TABELLE III

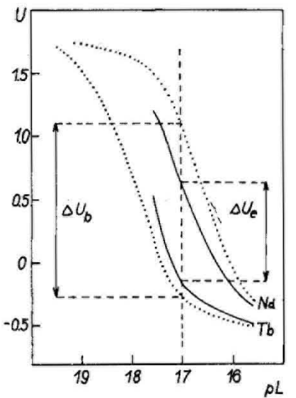
STABILITÄTSKONSTANTEN DER EDTA-CHELATE DES Sm, Eu UND Gd, NACH ELEKTROPHORETISCHEN DATEN BERECHNET (20°)

pL	*Sm*		*Eu*		*Gd*	
	U	*Log K_{SmL}*	*U*	*Log K_{EuL}*	*U*	*Log K_{GdL}*
15.54	−0.43	17.02	−0.48	17.58	−0.49	17.88
15.80	−0.38	17.04	−0.41	17.17	−0.42	17.22
16.02	−0.33	17.10	−0.37	17.22	−0.40	17.34
16.32	−0.24	17.19	−0.30	17.32	−0.35	17.46
16.54	−0.13	17.23	−0.18	17.31	−0.21	17.36
16.84	0.12	17.25	−0.04	17.42	−0.07	17.45
17.09	0.37	17.27	0.24	17.38	0.18	17.44
17.32	0.70	17.24	0.59	17.33	0.52	17.38
17.54	1.00	17.21	0.96	17.25	0.90	17.30
	$\log K_{SmL} = 17.17 \pm 0.08$		$\log K_{EuL} = 17.33 \pm 0.08$		$\log K_{GdL} = 17.42 \pm 0.12$	

Bei Ionen mit höherer Atomzahl als 64 (beim Terbium beginnend) ist ein umgekehrter Effekt zu beobachten. Die Beweglichkeiten sind positiver: Das Chelat verhält sich als ein schwächeres. Ebenfalls die Werte $\log K_{\mathrm{LnL}}{}^*$ weisen diesen Trend auf; er konnte allerdings nur etwa zum Terbium verfolgt werden, wobei die Werte in Klammern bereits extrapoliert (wenn auch höchstwahrscheinlich) sind. Das Yttriumchelat, welches mit seiner Stabilität zwischen Terbium und Dysprosium liegt, reiht sich in dieses Schema in vollem Ausmasse ein.

Zusammenfassend kann diese Tendenz als eine Ausgleichung oder Nivellierung der scheinbaren Stabilitäten der Chelate charakterisiert werden. Infolgedessen vermindern sich sowohl die gegenseitigen Abstände der $K_{\mathrm{LnL}}{}^*$ Werte als auch die Unterschiede zwischen den Beweglichkeitskurven einzelner Chelate. In der analytischen Praxis bedeutet dies eine verschlechterte Separation (siehe schematisch in Fig. 9).

Die Nivellierungstendenz erscheint sehr deutlich bei der graphischen Darstellung der Funktion $\log K_{\mathrm{LnL}}{}^* = \mathrm{f}(\log K_{\mathrm{LnL}})$ (Fig. 10). Die Werte sollten auf einer



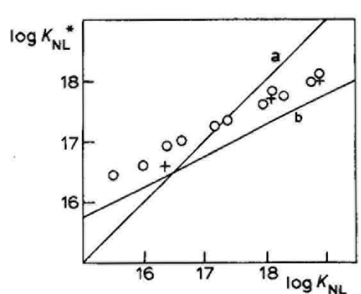


Fig. 9. Beweglichkeitskurven für Tb und Nd, pH 4 (······, berechnet; ———, experimentell) und die zugehörigen Beweglichkeitsdifferenzen ΔU_{ber} und ΔU_{exp}.

Fig. 10. Beziehung zwischen den elektrophoretisch ermittelten Stabilitätskonstanten K_{NL}^* und den in Literatur tabellierten Werten K_{NL}. ○, Lanthanidionen; +, Schwermetallionen. Geraden a, b, siehe Text.

Geraden (a) mit dem Richtungskoeffizienten +1 liegen, in der Tat beträgt dieser jedoch nur etwa +0.5. Unter der Voraussetzung, der ausgleichende Effekt sei durch die Anwesenheit von Zn^{2+} im Grundelektrolyten und dessen Konkurrenzreaktion mit dem Liganden bedingt, haben wir die Mittelwerte $\frac{1}{2}(\log K_{\text{LnL}} + \log K_{\text{ZnL}})$ berechnet und die betreffende Gerade (b) konstruiert. Die Experimentalwerte sind zu dieser Geraden parallel angeordnet, sodass sie rein empirisch aber ziemlich verlässlich mit folgender Beziehung charakterisiert sind:

$$\log K_{\text{LnL}}^* = \frac{\log K_{\text{LnL}} + \log K_{\text{ZnL}}}{2} + 0.4 \qquad (2)$$

Es sei zu bemerken, dass die Abweichungen bei Elektrophorese der zweiwertigen Schwermetallionen ebenfalls durch die Beziehung 2 beschrieben werden können, sodass auch bei diesen der Nivellierungseffekt eintritt.

Zur Bestätigung des Reaktionsmechanismus wurde die graphische logarithmische Analyse der Beweglichkeitskurven der Seltenerdmetallionen verwirklicht. Durch umformen der Gleichung 1 bekommt man die Beziehung

$$-\log\left(\frac{1.7 - U}{U + 0.5}\right) = -\log K_{\text{LnL}} + n \cdot \text{pL} \qquad (3)$$

Die graphische Konstruktion dieser Abhängigkeit für Elemente La–Ho (Fig. 11) zeigt, dass bei höheren pL Werten die Geraden tatsächlich den Richtungskoeffizienten +1 aufweisen, wie es der Bildung einkerniger Chelate von der Zusammensetzung LnL^- entspricht. Lediglich bei niedrigeren pL Werten nähern sich die Richtungskoeffizienten für die leichtesten Elemente dem Werte +0.5, was der Bildung zweikerniger Chelate Ln_2L^{2+} entsprechen würde. Wenn auch solche Chelate bei hohen Konzentrationen des Zentralions evidiert wurden[6], ist ihre Bildung bei Bedingungen der Elektrophorese kaum zu erwarten. Ihre Beweglichkeit müsste dann von der Konzentration der Metallionen abhängig sein. Die Versuche mit veränderlicher Metallionenkonzentration 10^{-2}–10^{-1} M ergaben jedoch konstante Beweglichkeitswerte.

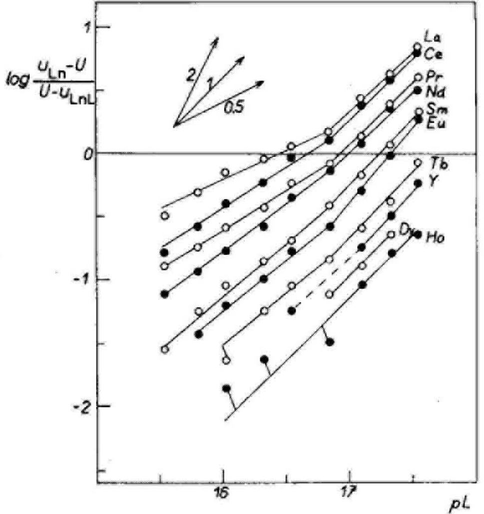


Fig. 11. Logarithmische Analyse der Beweglichkeitskurven von Lanthanidionen in Ligandenpuffern. Links oben die Tangenten für gegebenes Koordinatensystem.

Die Schneidepunkte der Geraden mit der Abszissenachse müssen laut Gl. 3 die Werte von $\log K_{\mathrm{LnL}}^* = \mathrm{pL}$ anzeigen. Diese Stimmen mit den Werten in Tabelle II überein. Für Elemente Er–Lu gestatten die Experimentalangaben keine Auswertung mehr, weil $(1.7 - U) \gg (U + 0.5)$; sie wurden deshalb in das Diagramm nicht einbezogen.

Die experimentellen Beweglichkeitskurven in Ligandenpuffern mit pH 2 gestalten sich unerwartet (Fig. 8). Die Kurven des Sm und der folgenden Elemente sowie des Y weisen bei niedrigeren pL Werten—im Gegensatz zum Erwarteten—eine ausgeprägte anodische Beweglichkeit auf, die sich offenbar von einfachen Komplexen LnL^- (welche bei pH 4 bestehen) nicht unterscheidet. Bei La–Nd wird dies nur deshalb nicht registriert, weil die betreffenden Experimentalpunkte ausserhalb des Puffergebietes des Systems liegen. Es scheint, als ob kein oder nur sehr wenig Hydrogen komplex gebildet würde. Eine Nichtexistenz der Hydrogenkomplexe ist jedoch höchst unwahrscheinlich. Andererseits entspricht der gegenseitige Abstand der Kurven für pH 2 und 4 der Erwartung, bei Sm–Gd decken sich sogar die berechneten und praktischen Werte. (Das Zusammentreffen beider Kurven bei La–Pr ist offensichtlich dem Nivellierungseffekt zuzuschreiben.) Für dieses widerspruchsvolle Verhalten haben wir vorläufig keine befriedigende Erklärung, denn bei zweiwertigen Schwermetallionen ist der Einfluss der Hydrogenkomplexe auf die Beweglichkeiten zweifellos und gesetzmässig.

Es muss betont werden, dass—trotz der diskutierten Abweichungen—die Ligandenpuffer mit pH 2 vom Standpunkt der praktischen Trennungen keine Nachteile besitzen; im Gegenteil, die Zonen sind scharf abgegrenzt und symmetrisch und die Beweglichkeiten sind recht unterschiedlich. Sie wurden sowohl für qualitative als auch quantitative Analysen der Seltenerdgemische verwendet.

ZUSAMMENFASSUNG

Es wurden die elektrophoretischen Beweglichkeitskurven von Erdalkali-ionen in EDTA-haltigen Mg^{2+}–MgL^{2-} Ligandenpuffern, und von zweiwertigen Schwermetallionen sowie von allen Seltenerdmetallionen in Zn^{2+}–ZnL^{2-} Ligandenpuffern (pH 4 und pH 2) ermittelt. Diese Migrationsmedien erwiesen sich als vorteilhaft für reproduzierbare Elektrophorese von Metallionen. Die Abweichungen von der Theorie und der nivellierende Effekt des Ligandenpuffers auf die Stabilität der Chelate wurden diskutiert. Die Stabilitätskonstanten wurden rechnerisch und durch graphische logarithmische Analyse bestimmt. Die Anwendbarkeit für praktische Trennverfahren wurde kurz besprochen.

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Notes

CHROM. 6262

The simultaneous determination of extra-column volumes and flow meter characteristics in gas chromatography

For exacting measurements of column phenomena in gas chromatography (GC) it is necessary to know the volume of the column connections from injection point to column front and from column back to the point of detection. We have developed a novel technique for obtaining this parameter. Simultaneously one obtains from this technique the relationship between the readings of a flow indicator, *e.g.* a rotameter, and the volumetric flow-rate. This method can be employed to determine the effective density of a solid stationary phase in a GC column under actual operating conditions.

Theory

The dependence of a flow meter on the mass flux, F, of a carrier gas in a GC system can be fit to an equation of the form:

$$F = g_0 + g_1R + g_2R^2 + \ldots g_nR^n \tag{1}$$

where R is the reading of a flow indicator, and the g's are best fit coefficients. Since the mass flux in the system is equal to the volumetric flow rate, f, times the density of the flowing gases, ϱ, eqn. 1 can be written:

$$f = b_0 + b_1R + b_2R^2 + \ldots b_nR^n \tag{2}$$

where $b_n = g_n/\varrho$. Now, since, the flow-rate is equal to the total volume, V_t, of the system divided by the time, t_r, required for a pulse of gas to traverse it, then:

$$f = \frac{V_t}{t_r} = \frac{V_c + V_0}{t_r} \tag{3}$$

where V_c and V_0 are the volumes of the column and the end connections, respectively.

The volume of a column is usually an easy measurement to make, *e.g.* empty tubes of known dimensions can be used. Also, t_r can be determined readily by measuring the time for a pulse of a nonadsorbed gas to pass from injection point to detector. Hence, by equating eqs. 2 and 3 and rearranging them we obtain:

$$V_c = -V_0 + b_0t_r + b_1t_rR + b_2t_rR^2 + \ldots b_nt_rR^n \tag{4}$$

which can be solved for V_0 and the coefficients by least square multiple regression with V_c as the dependent variable and t_r and R as independent variables. The retention time, t_r, must be corrected for pressure drop when this effect is significant.

Once the volume of the extra-column connections and the flow meter coefficients are determined in this manner, the open volume of packed columns and the effective

density of packing materials can be calculated. This is achieved by measuring the elution time of a non-adsorbed vapor in a packed column of known dimensions and substituting the values of V_0, t_r, R, and the coefficients into eqn. 4 and thereby finding the open volume, V_c', of the packed column. The effective density of the stationary phase is, therefore, equal to:

$$\varrho_s = \frac{w}{V_c - V_c'} \tag{5}$$

and the average open area in the column is:

$$A_c' = \frac{V_c'}{L_c} \tag{6}$$

where w is the weight of the packing material in the column, L_c and V_c are, respectively, the length and volume of the column when empty, and V_c' is the open volume of the column when packed.

The total porosity of the packed column is now:

$$\varepsilon_c = \frac{V_c'}{V_c} \tag{7}$$

Experimental

All experiments were performed with a modified Hewlett-Packard Series 700 laboratory chromatograph. A thermal conductivity detector was connected to an Infotronics digital readout system (Model CRS-110) and the time-concentration data were automatically punched on paper tape. A circuit board giving 2-sec intervals for the digitized data was used. These data tapes were processed on an IBM 1130 computer.

Helium, purified by a molecular-sieve trap, was used as the carrier gas. Flow-rates were monitored with a Matheson rotameter.

The columns used in this study had volumes of 1.79, 5.52, 6.47, 15.7, 23.3, and 46.3 ml. Experiments were also performed with the end connections joined, *i.e.*, V_c was zero. All of the columns, and the end connections were made of Type 316 stainless steel.

Glass beads, 3 mm in diameter, and Chromsorb P, 45–60 mesh, were used as the stationary phases in the density determination experiments. These supports were packed into stainless-steel columns having volumes of 8.15 ml each. The flow-rates for these experiments ranged from 0.1 to 0.8 ml/sec. The pressure drop across the column was found to be negligible, and therefore, the retention times were not corrected for this effect.

Results and discussion

The column volumes, retention times, and rotameter readings for the empty column experiments were multiply regressed in the form:

$$V_c = C_0 + C_1 t_r + C_2 t_r R + C_3 t_r R^2 \tag{8}$$

where $C_0 = -V_0$, $C_1 = b_0$, $C_2 = b_1$, and $C_3 = b_2$.

TABLE I

COLUMN AND FLOW PARAMETERS

Parameter	*GC method*	*Soap bubble flowmeter*
V_0 (ml)	3.39	
b_0 (ml/sec)	5.81×10^{-2}	3.74×10^{-2}
b_1 (ml/sec)	1.97×10^{-3}	2.18×10^{-3}
b_2 (ml/sec)	2.37×10^{-5}	1.77×10^{-5}

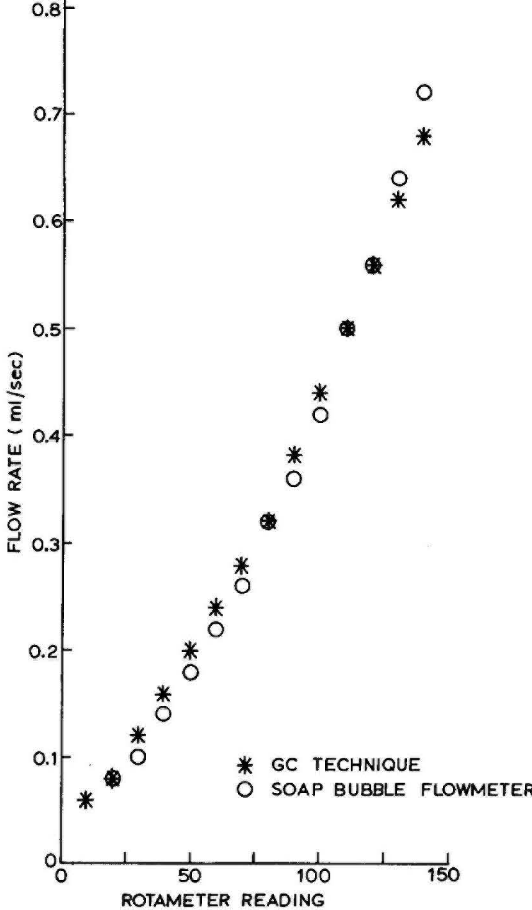

Fig. 1. Rotameter flow calibration curves.

The results of this regression are presented in Table I, along with the best fit coefficients found by calibrating the rotameter with a soap-bubble flow meter. The agreement is quite good, as can be seen from Fig. 1.

The column dead volumes, stationary phase densities, open areas, and total column porosities, as determined from eqns. 4–7 for the packed columns, are presented in Table II.

TABLE II

COLUMN CHARACTERISTICS

	GC method		Classical method	
	Glass beads	Chromosorb P	Glass beads	Chromosorb P
Dead volume of column, V_c' (ml)	3.70	6.66	3.94	6.48
Open area of column, A_c' (cm^2)	0.231	0.416	0.246	0.419
Total column porosity, ε_c	0.454	0.817	0.484	0.795
Packed density of column (g/ml)	1.27	0.389	1.27	0.389
Density of stationary phase, ϱ_s(g/ml)	2.33	2.13	2.46	1.90

Also listed in this table are the values for the same parameters based on the liquid displacement densities of the column packings measured in this laboratory. A 5% difference is noted between the densities of the glass beads determined by the two techniques. The source of this discrepancy may be that not all of the void volume in the column was accessible to the mobile phase. It is conceivable that in densely packed columns there may be some voids which do not contribute to the dead volume available to the flowing gases.

The density of the Chromosorb P determined by the GC method is in excellent agreement with the value of 2.15 g/ml reported by the manufacturer. The value found by liquid displacement, however, is almost 12% lower. This difference is attributable to the incomplete displacement of trapped gases in the porous chromosorb material by the fluid used in the displacement technique.

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CHROM. 6253

An inexpensive pneumatic solvent pump for high-pressure liquid chromatography

A pump to deliver a pulseless flow of solvent at a pressure of 2–3,000 p.s.i. was needed for high-resolution liquid chromatography. The advantages and disadvantages of commercially available solvent pumps have been discussed by HENRY[1]; as suitable pumps are expensive, a pneumatic pump was designed and built in the laboratory.

A serious defect of simple pneumatic pumps is that the actuating gas dissolves in the solvent under pressure and is released as the pressure decreases along the column. BONNELYCKE[2] reviewed methods of preventing this and described a pump in which the gas was separated from the solvent by a metal bellows, which he had used at pressures up to 200 p.s.i. For solvent pressures above the normal gas cylinder pressure, however, pressure amplification is necessary and this is most easily achieved in a piston pump. The pump to be described incorporates two pistons, one under gas pressure and the other exerting pressure on the solvent, which are rigidly connected but separated by a region at atmospheric pressure. The possibility of leakage of gas into the solvent therefore decreases as the pressure increases.

Description

Fig. 1 is a sectional drawing of the pump. It consists of a brass cylinder, 1, 3¼ in. (82.5 mm) O.D. and 2½ in. (63.5 mm) I.D., and cylinder head 2. The piston assembly comprises the gas piston 3 sealed by the chevron packing 4*, the connecting rod 5 and the solvent piston 6. The solvent cylinder is a ground brass or stainless-steel sleeve 7, 2⅛ in. (54 mm) I.D., which is shrunk into 2. Solvent pressure is contained by the PTFE U-seal 8**. The spring 9 returns the piston assembly to the bottom of its stroke when pressure is released by the exhaust valve 10. The valves 11 and 12 control the flow of solvent from the reservoir and to the column. The pin 13, moving in a slot milled in the pump body, indicates the amount of solvent remaining in the cylinder when the piston assembly is approaching the top of its stroke. The slot maintains the region between the gas and solvent pistons at atmospheric pressure. The pump is pivoted in the stand 14 (to allow the solvent cylinder to be completely emptied when changing solvents) and rests against the stop 15. The gas inlet valve 16 is not essential but provides a convenient means of control and enables the pump to be kept under pressure while gas cylinders are being changed.

Operation

To fill the solvent cylinder, the piston assembly is forced to the top of its stroke with valve 11 opened and valves 10 and 12 closed. Valve 16 is then closed and valve 10 opened; solvent is drawn into the cylinder by the action of spring 9. If the pump has been disconnected from the column or solvent reservoir, the solvent cylinder is filled and emptied several times to expel trapped air, but otherwise this is unnecessary. Solvent is pumped through the column by closing valves 10 and 11 and opening valves 12 and 16. The flow-rate is controlled by adjusting the gas pressure.

* James Walker and Co. Ltd., Woking, Surrey, Great Britain, Part No. JW430LNA30.
** Crane Packing Ltd., Slough, Bucks., Great Britain.

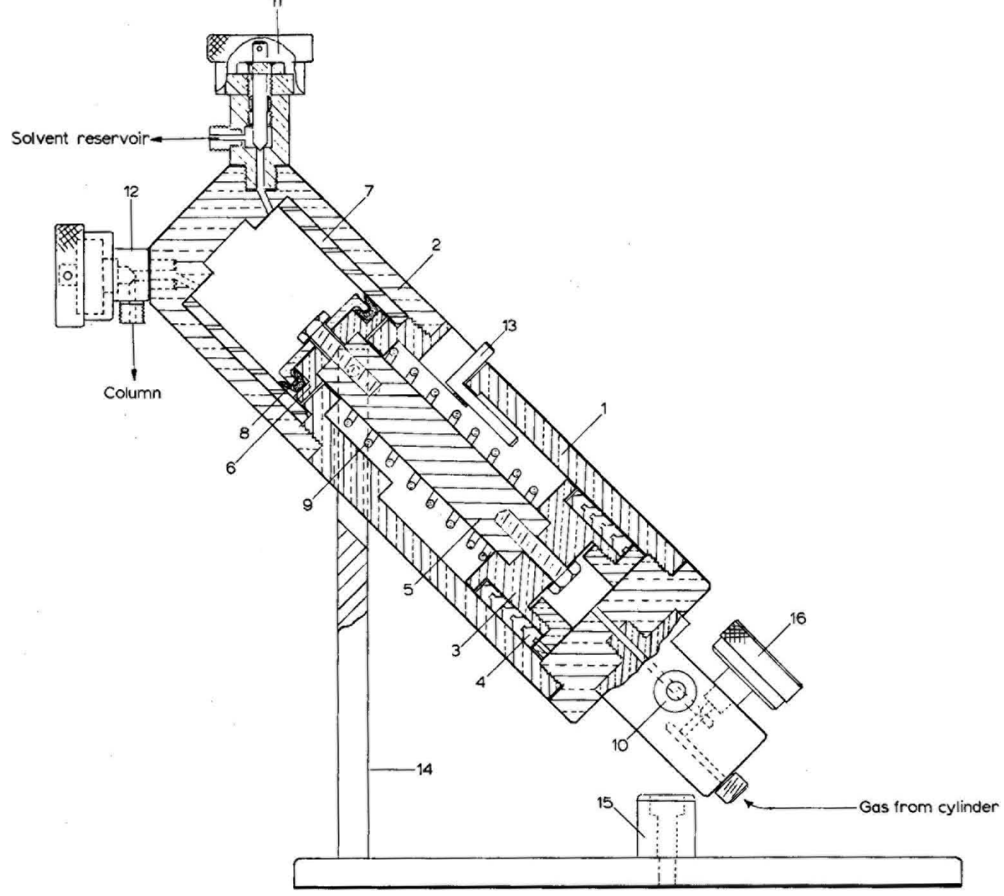

Fig. 1. Sectional drawing of pneumatic solvent pump. Scale: 3:10.

Results and discussion

The construction of the pump is straightforward, although it is important that the cylinder bores are concentric with a high surface finish. The design makes a high-pressure seal between the pump body and the cylinder head unnecessary and hence the only non-metallic components that can make contact with the solvent are the PTFE seals on the solvent piston and valve spindles, and these are not in direct contact with solvent that flows into the column.

The pump has been used continuously for several months at solvent pressures between 2,000 and 3,000 p.s.i. The only trouble during this time has been occasional leakage of solvent past the U-seal caused by small particles becoming trapped between the seal and the cylinder wall. These were easily removed but the fault could probably be cured by fitting a filter in the solvent inlet. It is, of course, important to clean the components of the pump scrupulously before assembly. Although pneumatic pumps have the disadvantage that changes in gas pressure cause changes in the solvent flow, this has caused no inconvenience in practice. Measurement of the flow-rate of the

column eluate during 6 h continuous running with the gas at cylinder pressure showed a steady decrease from 0.75 to 0.70 ml/min owing to decrease in the cylinder pressure. This slow decrease would be avoided at lower pressures by incorporating a reducing valve between the gas cylinder and the pump.

Details of the design can be adapted to suit the required application; in particular, the diameters of the gas and solvent cylinders can be varied according to the solvent pressure required. (In the pump described, the solvent pressure is about 1.4 times the gas pressure.) It is feasible to construct a pump body with interchangeable heads to give a range of maximum pressures and solvent cylinder capacities. If the pump is to be used only for inorganic or alcoholic solvents, the U-seal on the solvent piston could be of a more flexible material and the pump would work satisfactorily with a less highly-finished solvent cylinder bore.

Although pumps based on broadly similar principles are available commercially[1], the simplified design of the pump described makes it much less expensive. The total cost of construction, including time and materials, was less than £100. Full details of the pump are available on request.

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CHROM. 6263

Chromatographic examination of the *p*-nitrobenzoates and α-naphthylurethans of lower aliphatic alcohols

Gas chromatography (GC) has been commonly used for the analysis of the volatile components of alcoholic beverages as well as the derived distillates and fusel oils[1-4]. However, in many instances, the predominant lower aliphatic alcohols have been identified by retention time comparisons alone. In such complex mixtures the separation of individual alcohols on a preparative scale for conclusive identification by chemical and spectroscopic techniques is often difficult and only partially successful. However, complex mixtures of the alcohols can be subjected to further chromatographic examination after conversion to derivatives. Since the *p*-nitrobenzoates and α-naphthylurethans of the lower aliphatic alcohols are relatively stable and simple to prepare, these substances were selected for chromatographic investigation.

Paper chromatography has been used for the examination of various lower aliphatic alcohol derivatives[5-7] although chromatograms usually take relatively long times to obtain. Similarly one-dimensional thin-layer chromatography (TLC) has been investigated also, but only normal alcohol derivatives up to that of butanol were satisfactorily resolved[8]. More recently CHURÁČEK *et al.*[9] have successfully resolved lower aliphatic alcohols, as the coloured N,N-dimethyl-*p*-aminobenzeneazobenzoates, using both paper chromatography and TLC. GALETTO *et al.*[10] have used GC to resolve the lower alcohols as the 3,5-dinitrobenzoates.

Since, as a general rule, superior resolution and relatively rapid speed of analysis have been obtained with TLC and GC, these techniques were used in this investigation.

Experimental

Preparation of derivatives. Reference alcohols were commercial "Analytical Grade" products checked for purity by GC. Derivatives were prepared by the usual synthetic procedures and purified by recrystallisation. With crude mixtures, such as fusel oils, the reaction was performed after removal of water and the product analysed without further treatment.

Thin-layer chromatography. Silica gel (30% by weight; Macherey, Nagel and Co.; MN-Kieselgel G) was made into a slurry with water and spread on glass plates (5 × 20 cm) to a thickness of 0.3 mm using Shandon Unoplan apparatus. The plates were then air-dried at 20° for 16–20 h. The α-naphthylurethans were spotted onto the plates in acetone solution and developed to a distance of 12 cm from the origin in atmosphere saturated tanks at 20°. Developed plates were dried at 100° for 10 min and sprayed with 10% phosphomolybdic acid in ethanol. After 10 min at 100° the α-naphthylurethans appeared as dark blue spots on a yellow background. Two solvent systems were used and between fifteen and thirty replications of each compound made. Solvent 1 was petroleum ether (b.p. 80°–100°)–anhydrous diethyl ether–water (85:15:3), while solvent 2 was benzene–ethyl acetate (9:1). With solvent 1 after one development the plates were dried at room temperature and redeveloped before visualisation.

Gas chromatography. A Varian Model 1400 instrument fitted with 1/8 in. × 5 ft.

stainless-steel columns and a flame ionisation detector was used. Two liquid phases (OV-17 and SE-30) were absorbed (each 3% loading) on AW Chromosorb W (80–100 mesh) and used at temperatures of 130° and 125°, respectively, with N_2 as carrier gas at flow-rates of 25 and 15 ml/min, respectively. The detector and injector were maintained at 230° while the *p*-nitrobenzoates in a 5% (w/v) solution of carbon disulphide were injected (0.7 µl) into the gas chromatograph. Six replications of the R_t determinations for each compound were made.

Results and discussion

Although unsatisfactory for GC, the α-naphthylurethans of normal alcohols up to pentanol were resolved by TLC on silica gel (see Table I). The separation of the isomeric alcohols was limited as has been found in previous work with other derivatives. However, the derivatives used previously present greater difficulties in preparation than the α-naphthylurethans. Crude mixtures of alcohols, such as fusel oils, can be reacted to form the α-naphthylurethans and analysed directly without further purification.

TABLE I

CHROMATOGRAPHIC DATA

Parent alcohol	*TLC R_F values[a] of α-naphthylurethans*		*GC R_t values[b] of p-nitrobenzoates*	
	Solvent 1[c]	*Solvent 2[c]*	*OV-17 (130°)*	*SE-30 (125°)*
Methanol	0.61	0.77	0.45	0.42
Ethanol	0.85	0.91	0.62	0.61
n-Propanol	1.00	1.00	1.00	1.00
n-Butanol	1.11	1.07	1.69	1.67
n-Pentanol	1.15	1.11	2.84	2.63
n-Hexanol	1.21	1.14	—	—
i-Propanol	1.05	1.01	0.66	0.73
i-Butanol	1.09	—	1.26	1.33
i-Pentanol	1.17	—	2.25	2.30
sec.-Butanol	—	—	1.10	1.20
2 Methyl-*n*-butanol	1.17	—	2.14	2.18
Max. coeff. variation (%)[d]	1.6	1.6	1.4	1.9

[a] Relative to *n*-propyl-α-naphthylurethan = 1 (absolute R_F = 0.42 ± 0.08).
[b] Relative to *n*-propyl-*p*-nitrobenzoate = 1.
[c] See *Experimental*.
[d] $\frac{\text{Std. deviation}}{\text{Mean}} \times 100$.

The *p*-nitrobenzoates of the lower alcohols were found to be resolved by GC using packed columns with relatively low liquid phase loadings on the solid support. Reference retention times of the derivatives of a number of common alcohols are included in Table I. Conventional semilog plots of retention time against carbon number for those compounds forming a homologous series (*e.g.* normal and *iso* alcohol derivatives) were found to yield straight lines. Qualitative analyses of various fusel

oils were performed using this GC technique after reaction of the original crude mixture to form the *p*-nitrobenzoates.

It was found that, with comparatively large sample sizes, the use of low liquid phase loadings in this manner readily leads to overloading, *i.e.* production of elution curves exhibiting marked asymmetry. Increasing the size of injections onto the GC column leads to increased asymmetry of the resultant curves and a displacement of the curve maximum towards increased retention times. Thus if the retention time of a compound is measured from the maximum point on its elution curve it will exhibit an apparent increase. A quantitative measurement of the amount of overloading was taken as the difference between the apparent retention time as measured from an asymmetric elution curve and the normal retention time from a normal symmetric curve. To obtain comparative results this difference in retention times was divided by the normal retention time, giving an overloading factor,

$$i.e.\ \text{O/L factor} = \frac{\text{App. } R_t - \text{Norm. } R_t}{\text{Norm. } R_t}.$$

With the *p*-nitrobenzoates the quantity of the substance injected was plotted against the O/L factor exhibited by the resultant elution curve for a range of injection sizes. By extrapolation the maximum size of injection without causing excessive overloading was obtained (see Fig. 1). For a number of compounds this maximum was plotted against molecular weight. This indicated that the maximum decreased with increasing molecular weight in this series of compounds (see Fig. 2).

Therefore, as a general rule, overloading can be expected to be more evident with compounds having longer retention times, since these compounds fall into a

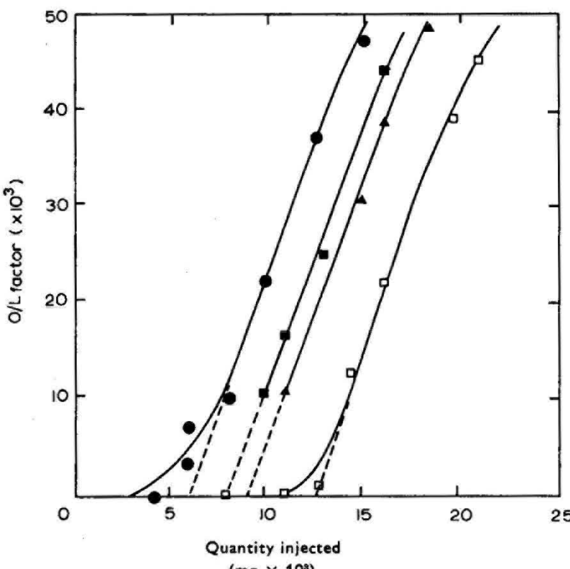

Fig. 1. Plot of the O/L factor against the quantity injected for some *p*-nitrobenzoates of alcohols. ●, *n*-pentanol; ■, *n*-propanol; ▲, ethanol; □, methanol. The broken line indicates extrapolation to obtain the maximum size injection which can be used without causing excessive overloading.

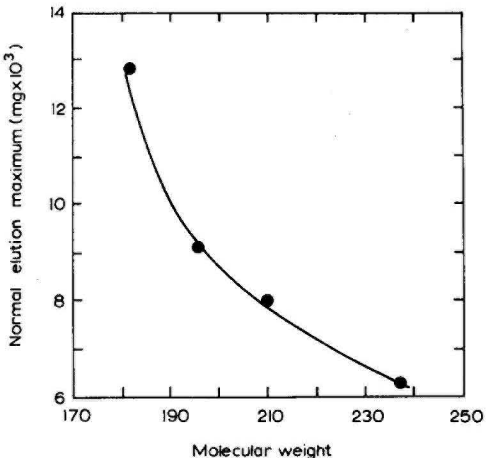

Fig. 2. Plot of the normal elution maximum against molecular weight for some p-nitrobenzoates (see Fig. 1).

high-molecular-weight range (see behaviour of homologous series described above). It is necessary to avoid overloading, since this will lead to retention variations dependent on injection size.

Conclusions

The results indicate that, with the lower aliphatic alcohols, TLC examination of the α-naphthylurethans and gas chromatography of the p-nitrobenzoates can be used to provide confirmatory evidence for identifications made by direct examination of the parent alcohols. The GC technique gives a comparatively high order of resolution and can give detailed information on qualitative composition.

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CHROM. 6233

Dye-complexed bacterial lipopolysaccharide as a void volume marker for permeation chromatography

Permeation chromatography, also commonly termed gel permeation, molecular sieve and exclusion chromatography, is an extensively employed method for the separation, purification and analysis of mixtures of biological materials. The use of agarose gels and controlled-pore glass (CPG) having an exclusion limit above a molecular weight of 2×10^6 daltons presents a problem in accurately determining the void volume (V_0) of the column. Blue Dextran 2000 and tobacco mosaic virus[1] have been employed for this purpose. CAMERON[2] reported the use of a bacterial lipopolysaccharide (LPS) as a V_0 marker for agarose columns with an exclusion limit of 1.5×10^8 but its use is subject to several limitations. The LPS marker is not visible while on the column and is not readily detected spectrophotometrically, necessitating fraction collecting and analysis for carbohydrate content. The attachment of a chromophore to the bacterial LPS permits visualization of the V_0 marker on the column, which provides the investigator with information regarding column properties. In addition, the V_0 marker can be monitored continuously in the eluent spectrophotometrically in both the ultraviolet and visible regions.

Material

Dye-complexed LPS. LPS was prepared from *Salmonella typhimurium* by the phenol–water method as described by LÜDERITZ *et al.*[3] and the trichloroacetic acid method of BOIVIN and MESROBEANU[4]. Commercial LPS preparations were also obtained from Difco Laboratories (Detroit, Mich.). LPS preparations were suspended at a concentration of 0.5% in 0.05 *M* tris(hydroxymethyl)aminomethane (Tris-HCl), pH 10.0 and Procion Scarlet MGS dye (ICI, Stamford, Conn.) was added in the ratio of eight parts dye to one part LPS. The solution was mixed thoroughly and incubated for 1–2 h at 75° with occasional agitation. After cooling, the mixture was centrifuged at 80,000 × *g* for 75 min. The free dye was discarded in the supernatant and the pellet was washed twice with distilled water. The final pellet was resuspended in a minimal amount of water or Tris-HCl buffer, 0.05 *M*, pH 7.2. The uncombined dye could also be removed from the solution after the initial centrifugation by dialysis against water or buffer. Microbial contamination was prevented by heating the dye-complexed LPS at 100° for 15 min and storage in a sterile container at 4° or by the addition of 0.02% sodium azide.

Column matrices. Columns were prepared using spherical beads of agarose obtained from Bio-Rad Laboratories (Richmond, Calif.), agarose (Sepharose) and dextran beads (Sephadex) from Pharmacia Fine Chemicals (Piscataway, N.J.), porous glass beads (Porasil) from Waters Associates (Framingham, Mass.) and controlledpore glass (CPG) from Corning Glass Works (Corning, N.Y.). The specific matrices employed are listed in Table I. Porasil B, D and F were 100–150 mesh and Porasil E was 75–125 mesh. All the surface-treated CPG employed was 100–200 mesh (75–125 μ) particle size with a pore diameter distribution reported by the manufacturer to vary not more than 10% from the average. Polyethylene glycol 20,000 was employed as described by HAWK *et al.*[5] to decrease the surface charge of the CPG for use with biological materials.

TABLE I

ELUTION VOLUMES OF DYE-COMPLEXED LPS FROM *Salmonella typhimurium* ON VARIOUS COLUMN MATRICES

Matrix		*Molecular weight exclusion*[a]	$V_t - V_0$ *(ml)*	*Dye-complexed LPS*		*Blue Dextran 2000*		
				V_e *(ml)*	*Detector (nm)*	V_e *(ml)*	*Detector (nm)*	$K_{av.}$
BioGel								
A-150		150×10^6	102.0	64.0	260	73.0	260	0.09
Sepharose								
2B		20×10^6	107.0	50.0	260	50.0	260	0.00
Sephadex								
G-25		5×10^3	52.0	35.0	260	35.0	260	0.00
G-200		2×10^5	127.0	61.0	260	61.0	260	0.00
Porasil								
Type	APD (Å)							
B	100–200	2.5×10^5	32.0	28.5	260	28.5	260	0.00
D	400–800	1×10^6	34.0	30.0	260	30.0	260	0.00
F	1500	2×10^6	51.0	49.0	RI[b]	58.0	RI[b]	0.09
F	1500	2×10^6	72.0	56.0	510	77.0	650	0.29
Ex	—	4×10^6	27.0	26.8	510	44.5	650	0.66
Treated CPG								
Type	APD (Å)							
10–75	81	3.8×10^4	26.5	33.5	260	33.5	260	0.00
10–370	363	2.7×10^5	29.0	29.5	260	29.5	260	0.00
10–700	693	6.6×10^5	26.5	25.5	260	25.8	260	0.01
10–1250	1195	1.2×10^6	26.3	30.0	260	30.2	260	0.01
10–2000	1915	2.2×10^6	27.0	28.0	260	28.2	260	0.01
10–2000	1915	2.2×10^6	176.0	181.0	RI[b]	194.0	RI[b]	0.07
10–2000	1915	2.2×10^6	180.0	189.0	510	309.0	650	0.67
10–2000	2795	12×10^6	26.0	26.5	260	30.0	260	0.13
10–2000	2795	12×10^6	26.0	26.5	RI[b]	30.0	RI[b]	0.13
10–2000	2795	12×10^6	26.0	26.5	510	45.5	650	0.73

[a] As determined with dextrans or globular proteins by the manufacturer.
[b] Refractive index monitor.

Columns and buffers. Both 1.27- and 2.54-cm-diameter columns were used, which ranged from 30–85 cm in length. The glass columns were fitted with flow adaptors for ascending and descending chromatography and were packed in accordance with the instructions of the manufacturers. A variety of buffers were employed: Tris-HCl, 0.05 *M* pH 7.2; 0.1 *M* pH 8.0; distilled water; 0.02% sodium azide; 1% sodium deoxycholate pH 8.1; and 0.05 *M* sodium chloride. A pressure-regulated gravity elution system was employed with the "soft" gels (via a Mariotte flask) and a piston pump was used to deliver 5–6 ml/min with the rigid glass matrices.

Methods

Samples of 2–10 mg of dye-complexed LPS or Blue Dextran 2000 (Pharmacia) in a volume of 0.5 to 2.0 ml (less than 1% of the bed volume) were applied to the column and eluted with 0.05 *M* Tris-HCl, pH 7.2 unless otherwise noted. The column effluents were monitored continuously with a recording spectrophotometer or a differential refractometer. The dye-complexed LPS was monitored at 260 or 510 nm

and Blue Dextran at 260 or 650 nm. The column V_t (total volume) was experimentally determined with bacitracin and monitored at 280 nm.

Results and discussion

The utility of dye-complexed LPS as a void volume marker is demonstrated in Table I where the leading peak of each column run is reported. On A-150, having a molecular weight exclusion of 150×10^6, the dye-complexed LPS eluted as a single peak at 64 ml while the small leading peak of Blue Dextran eluted at 73 ml and the major peak at 142 ml. The dye-complexed LPS eluted at the same V_e (elution volume) as Blue Dextran on Sepharose 2B, Sephadex G-25 and G-200. Both V_0 markers eluted at the same V_e on Porasil B and D but a significant difference was observed on Porasil F and Ex, where dye-complexed LPS eluted first. On surface-treated CPG with an average pore diameter (APD) of 81, 363 and 1195 Å, the V_e was identical for both V_0 markers. However, on a 1915 Å (APD) glass matrix column the dye-complexed LPS eluted before Blue Dextran. The manufacturer of Blue Dextran reports that it absorbs at both 260 and 650 nm[6]. On the 1915 Å (APD) glass column monitored by refractive index the dye-complexed LPS eluted before the Blue Dextran. On a similar column where the dye-complexed LPS was monitored at 510 nm and Blue Dextran at 650 nm, the LPS eluted at 189 ml and Blue Dextran at 309 ml since the 260 nm absorbing peak was not recorded. The results obtained on the 2795 Å (APD) glass were similar to those observed with the 1915 Å (APD) glass column. The elution profiles of dye-complexed LPS and Blue Dextran are shown in Fig. 1, where the column was monitored by refractive index. Attempts to use dye-complexed LPS on

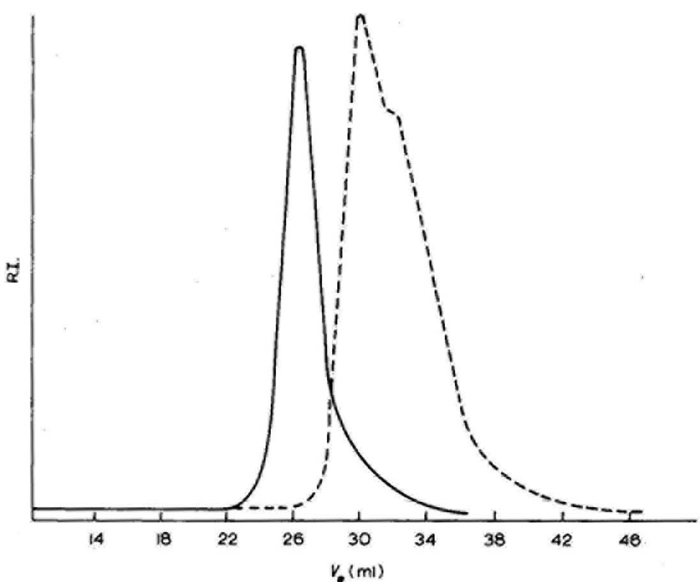

Fig. 1. Elution patterns of dye-complexed LPS (———) and Blue Dextran 2000 (– – –) on surface-treated CPG 10–2000, average pore diameter 2795 Å. The column dimensions were 1.27 × 41.0 cm and the eluent, 0.05 M Tris-HCl, pH 7.2, was monitored continuously with a recording differential refractometer.

the Bio-Gel P matrices (Bio-Rad) resulted in a non-specific attachment of the V_0 marker to the column matrix.

The $K_{av.}$ value[1] is commonly employed to characterize solute migrations.

$$K_{av.} = \frac{V_e - V_0}{V_t - V_0} \tag{1}$$

Thus, for excluded molecules which are incapable of entering the pores of a matrix the $K_{av.} = 0$. The assumption was made that the V_e of the dye-complexed LPS represented the V_0 of the column and the $K_{av.}$ values were calculated for Blue Dextran. Consideration of the $K_{av.}$ value for Blue Dextran on the 2795 Å (APD) glass column clearly indicates that the experimental V_0 marker eluted earlier and also demonstrates the variation in observed elution volume as a function of the method employed for detection. At 260 nm and with refractive index the Blue Dextran elutes with a $K_{av.}$ value of 0.13 while at 650 nm a $K_{av.}$ value of 0.73 was observed.

The use of dye-complexed LPS is of particular value on matrices with a molecular weight exclusion limit of 2×10^6 or greater. The use of Blue Dextran with such matrices will not always give an accurate measurement of the V_0, resulting in incorrect $K_{av.}$ values and incorrect estimates of size. On matrices with an exclusion limit below 2×10^6, Blue Dextran is as useful as dye-complexed LPS for determining the V_0, and, when monitored at 260 nm or by refractive index it is useful on agarose matrices with an exclusion limit up to 20×10^6. However, porous glass matrices with an exclusion limit of 2.2×10^6 show some differences in the V_e of the markers, indicating that some caution is necessary when employing Blue Dextran on large pore size glass columns.

To determine the stability of the dye-complexed LPS in various buffers, it was chromatographed on a 1990 Å (APD) glass column utilizing the buffers listed earlier. In all instances the experimental V_0 marker eluted as a single peak. Also, a dye-complexed LPS preparation was stored at 4° and an aliquot was chromatographed every month for sixteen months on a 1915 Å (APD) glass matrix and the elution pattern remained unchanged.

The dye-complexed LPS is chemically stable and can be stored lyophilized or in solution. If collected after use it can be reconcentrated by dialysis or centrifugation and re-used.

LPS is toxic and although dye-complexed LPS has been found in this laboratory to have lost some of its biological activity, as with other toxic materials, appropriate precautions should be observed in handling.

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The behavior of DNA on basic polyamino acid Kieselguhr columns

The fractionation of DNA on columns of poly-L-lysine supported on Kieselguhr (PLK columns) has been described by AYAD AND BLAMIRE[1], and HELLEINER[2]. Columns of poly-L-arginine (PAK) and poly-L-ornithine (POK) similarly supported, as well as PLK columns have been used by JARVIS *et al.*[3] to separate different kinds of RNA. In this paper the behavior of DNA of different molecular weights and base compositions on PAK and POK columns is compared with that on PLK columns.

Poly-L-arginine hydrochloride (mol. wt. 40,000–80,000) and poly-L-α-ornithine hydrobromide (mol. wt. 100,000), and DNA from *Clostridium perfringens* were obtained from Sigma Chemical Co., St. Louis, Mo. Other kinds of DNA were prepared in the laboratory according to MARMUR[4]. Radioactive *Escherichia coli* DNA was prepared from bacteria grown to the stationary phase in a Tris salts medium[5] containing ^{32}P as KH_2PO_4. *E. coli* DNA of different molecular weights was prepared by treating DNA in a Branson Sonifier for varying periods. The sedimentation constant was determined by means of boundary measurements in the analytical ultracentrifuge, and the molecular weights were estimated according to STUDIER[6].

Columns were prepared as described by AYAD AND BLAMIRE[1], with the substitution of other polyamino acids for poly-L-lysine as indicated. In each experiment, a mixture of 500 μg of some kind of non-radioactive DNA with a tracer amount of radioactive *E. coli* DNA was applied to the column as a solution in 0.4 *M* NaCl. Columns were eluted at room temperature with linear gradients of NaCl buffered with 0.02 *M* potassium phosphate buffer, pH 7.2. For PAK columns the gradient extended from 2.0 to 4.0 *M* NaCl; for POK columns the limits were 1.0 *M* and 3.0 *M* NaCl. Fractions of 12 ml were collected and analyzed for absorbance at 260 nm and radioactivity

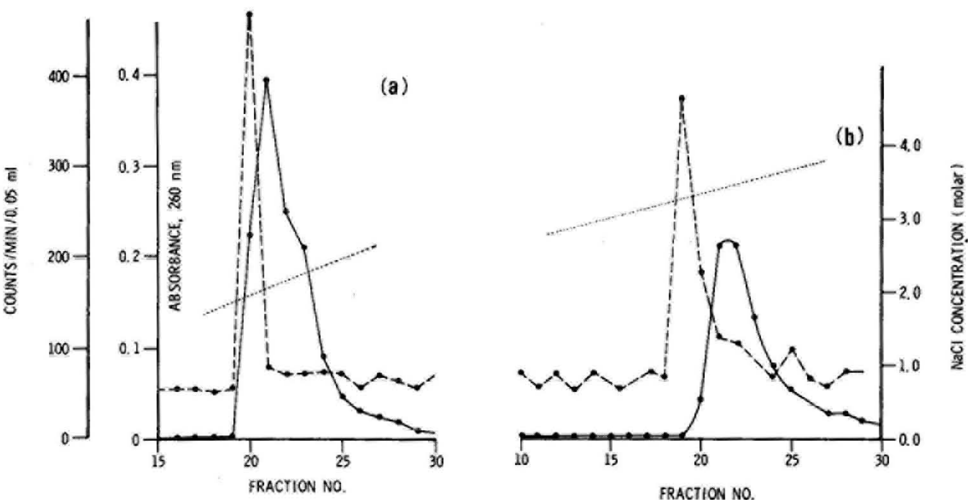

Fig. 1. Elution diagrams of bacterial DNAs from POK columns; (a) 600 μg of *Cl. perfringens* DNA + trace of radioactive *E. coli* DNA; (b) 600 μg of *M. lysodeikticus* DNA + trace of radioactive *E. coli* DNA. ———, Absorbance; — — —, radioactivity; ······, NaCl concentration.

(by spotting samples on filter paper, drying the filters, and counting them, after addition of a PPO-POPOP scintillation fluid, in a scintillation counter).

Fig. 1 shows the elution of *Cl. perfringens* and *Micrococcus lysodeikticus* DNA from a POK column. The *M. lysodeikticus* DNA (about 71 mole per cent guanine + cytosine) precedes the *E. coli* DNA (about 50 mole per cent guanine + cytosine), while the *Cl. perfringens* DNA (about 30 mole per cent guanine + cytosine) follows the *E. coli* DNA. This behavior resembles that on PLK columns. The effect of molecular weight on the position of elution was similar to that seen on PLK columns[2], and also to that on PAK columns (Fig. 3).

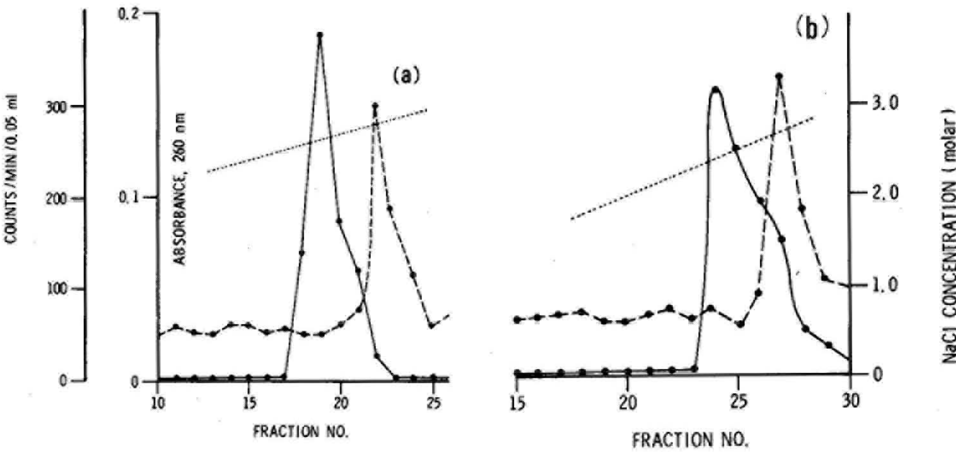

Fig. 2. Elution diagrams of bacterial DNAs from PAK columns; (a) 600 μg of *Cl. perfringens* DNA + trace of radioactive *E. coli* DNA; (b) 600 μg of *M. lysodeikticus* DNA + trace of radioactive *E. coli* DNA. ———, Absorbance; — — —, radioactivity; ······, NaCl concentration.

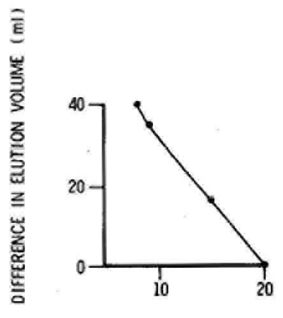

Fig. 3. Effect of sedimentation constant of DNA on elution volume. The difference in elution volume between sonically disrupted *E. coli* DNA of various molecular weights and undegraded *E. coli* DNA is plotted against the sedimentation constant.

Fig. 2 shows the elution of the same kinds of DNA from a PAK column. Here the order of elution is reversed: the DNA with the lowest guanine + cytosine content is eluted first, and that with highest guanine + cytosine content is eluted last. The effect of sedimentation constant on the position of elution of *E. coli* DNA is summa-

rized in Fig. 3. As might be expected, the smaller the molecular weight, the sooner the DNA appears in the elution.

When a preparation of calf thymus DNA was chromatographed on a PAK column, the fractions eluted earliest had the lowest buoyant density in caesium chloride solutions, and successive fractions had higher buoyant densities. This is to be expected from the relationship between buoyant density and base composition[7].

LENG AND FELSENFELD[8] measured the solubility of complexes of DNA and polylysine and polyarginine as a function of salt concentration and base composition, and concluded that while polylysine interacts preferentially with DNA rich in adenine + thymine, polyarginine shows a slight preference for DNA rich in guanine + cytosine. OLINS *et al.*[9] concluded from the hyperchromic dispersion of complexes of DNA and basic polyamino acids that polylysine preferentially stabilizes adenine + thymine-rich regions of DNA, while polyarginine is less discriminating. These results indicate that the binding of DNA to basic polyamino acids is not entirely electrostatic. While they do not explain the behavior of DNA of different base compositions on columns of the type used in this work, they are in accord with our observations.

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A motorized spray device for qualitative and quantitative thin-layer chromatography

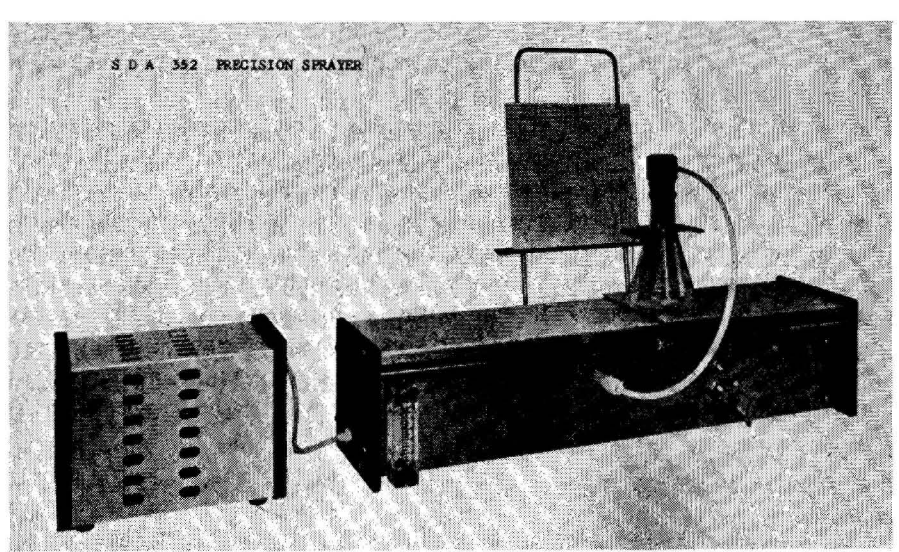
The usual technique for spraying a thin-layer plate that has been subjected to chromatographic analysis is to dissolve the reagent in a solvent and manually spray this reagent solution from an atomizer onto the developed chromatoplate. It is well-known that manual spraying often results in an uneven distribution of the reagent solution on a chromatoplate and therefore produces an inconsistent baseline and non-reproducible spot intensities when scanned by a spectrodensitometer.

Variables in spraying such as the droplet size of the reagent solution forming from the atomizer or sprayer, the actual amount of sprayed reagent that comes in contact with the thin-layer plate and the general ability of the operator to manually spray the reagent solution uniformly on the plate has resulted in the hampered growth of quantitative thin-layer chromatography.

A motorized spray device (Fig. 1) has been designed to eliminate the common errors and variables encountered with the traditional spraying techniques.

A synchromous motor produces a constant and even movement which traverses the thin-layer plate. The spray bottle is commercially available and fits snugly in a holder which keeps the bottle in an upright and fixed position. The spray assembly moves along a stationary guide by means of a moving wire.

A switch, actuated by the main drive pulley, will reverse the drive at each end of the 14 in. travel distance. These 14 in. will give 3 in. spaces on each side of a common 8 in. thin-layer plate to prevent nonuniform coating at the reversal point of the sprayer assembly. Also, to prevent nonuniform coating, the sprayer assembly only starts and stops at the left or right reversal point. Therefore, one can make as many traversing passes in either direction as one deems necessary to produce the optimum spray reagent concentration onto the plate.



If the sprayer assembly is switched off during the last pass at any position, it will continue to move until it reaches the next reversal position. There is very little tolerance within the stopping action since a unique dynamic brake stops the synchronous motor. The sturdy stainless-steel case contains all necessary controls and has a moving stage which carries the sprayer assembly, sliding smoothly on the top stainless-steel plate. It also contains within it a filter which removes undesirable material that may come through the airline and clog up the sprayer. Externally, an inlet and outlet tube is provided which allows air (or some other gaseous media) to enter the control box, pass through the filter and then pass into the tube connected to the sprayer.

Two switches control the movement of the sprayer assembly and the sprayer itself. The modes of operation are as follows:

(a) Drive switch—activates the drive as soon as it is moved in the "on" position; in its "off" position it gives the necessary information to the switch logic to activate the dynamic brake and to turn the drive "off" after the sprayer assembly has reached the next extreme left or right position.

(b) Valve switch—activates the sprayer in its "on" position continuously and in its automatic position only simultaneously with the sprayer assembly drive. Pilot lights indicate the activation of the drive as well as the activation of the valve. A flow meter is also incorporated into the central box so as to provide a constant flow of gaseous media to the sprayer.

A new sprayer of unique design has been developed by Schoeffel Instrument Co., Westwood, N.J., to produce a uniform droplet size as many other commercially available sprayers and atomizers produce a wide spectra of particle size. This new sprayer ensemble in conjunction with the motorized spray apparatus should greatly enhance the quantitative aspects of thin-layer chromatography when complemented with commercially available spectrodensitometers and spectrofluorimeters.

It should be mentioned that the original motorized spraying device (since modified and improved by Schoeffel Corporation) has been used routinely in our laboratory for the quantitative determination of fatty acid derivatives employing 50% (w/w) sulfuric acid as the spray reagent. Quantitation of the compounds in question has been accomplished using the charring technique accompanied by a Schoeffel spectrodensitometer Model No. SD3000.

The versatility of the precision sprayer apparatus will be increased by providing two additional accessories. An automatic streaking device will be available which utilizes the traverse motion of the atomizer support. This will enable one to obtain uniform streak densities for quantitative and for preparative thin-layer chromatography. A second accessory will be a spray assembly which can accomodate two sprayers. This will enable the operator to have two different reagent solutions at his disposal. For example, one spray bottle can contain iodine solution or sulfuric acid as a general spray for qualitative analysis or both sprayers may contain different spray reagents which may be required for quantitative analysis of certain compounds.

Quantitative data are being marshalled and they will be the subject of a future paper.

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CHROM. 6250

Fluorometrische Triglyceridbestimmung auf Dünnschicht-chromatogrammen*

Die quantitative Direktauswertung von chromatographisch getrennten Substanzgemischen hat in der letzten Zeit immer mehr an Bedeutung gewonnen. Dieses Verfahren wird allerdings durch eine relativ schlechte Reproduzierbarkeit eingeengt, eine Folge vor allem der Sprayreagenzien beim Sichtbarmachen der Flecken. Aus diesem Grunde scheint es von Vorteil zu sein, wenn das Nachweisreagenz ein Bestandteil der Adsorptionsschicht selbst ist.

Es ist bekannt, dass eine Reihe von lipiden Substanzen, welche mit Schwefelsäure besprüht wurden, beim Erwärmen dann eine charakteristische Farbe bzw. Fluoreszenz zeigen[1,2]. Den gleichen Effekt erhält man auch beim Erhitzen von Platten, welche mit Ammoniumsulfat imprägniert sind[3]. In dieser Arbeit wurde versucht, die erstmals von uns beobachtete Fluoreszenz bei Triglyceriden quantitativ auszuwerten.

Experimentelles

Die in Chromschwefelsäure gereinigten Glasplatten (20 × 20 cm) wurden mit Kieselgel G (Merck), das mit einer 10%igen Ammoniumsulfatlösung im Verhältnis 1:2.3 durchgemischt wird, 250 μm hoch beschichtet. Die Platten wurden luftgetrocknet und zur Reinigung in *n*-Heptan–Diäthyläther (7:3) laufen gelassen[4]. Nach einer einstündigen Aktivierung bei 110° wurden nach Abkühlen der Platten unter Stickstoff die Triglyceride aufgetragen. Beim Auftragen muss darauf geachtet werden, dass die Startflecken gleich gross sind, damit bei der anschliessenden Messung das Verhältnis Messfläche zu Fleckgrösse konstant bleibt. Gleichgrosse Startflecken werden am besten dadurch erhalten, dass gleiche Volumina aus einer Verdünnungsreihe aufgetragen werden. Nach dem Entwickeln bis zu einer Laufhöhe von 10 cm im Fliessmittel *n*-Heptan–Diäthyläther (7:3)[4] bei Kammersättigung wurden die Kammern luftgetrocknet. Je nach dem aufgetragenen Konzentrationsbereich wurden die Platten in einem abgeschlossenen Gefäss im Trockenschrank bei 150° 25, 45 oder 85 min erhitzt. Die Verwendung eines abgeschlossenen Gefässes verhindert die Verschmutzung des Chromatogramms während des Erhitzens.

Die quantitative Auswertung der so entwickelten Fluoreszenz wurde in unserem Labor mit einem Vitatron TLD 100 vorgenommen. Als Anregungslichtquelle diente eine Quecksilberlampe mit einem UVB-Filter. Das gelbgrüne Fluoreszenzlicht wurde unter Vorschaltung einer Kreisblende mit 2 mm Durchmesser und einem Filter bei 450 nm vermessen. Am Schreiber wurde der am stärksten fluoreszierende Fleck auf Vollausschlag, der Untergrund auf 0 eingestellt. Die Messfläche wurde dem Durchmesser des grössten Chromatogrammfleckes angepasst. Zur Errechnung der Peakflächen wurde die Höhe mit der Halbwertsbreite multipliziert. Um Störlicht auszuschalten, musste bei grosser Verstärkung des Photostroms in einem halbdunklen Raum vermessen werden.

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* Herrn Univ. Prof. DDr. Th. Leipert zum 70. Geburtstag gewidmet.

Ergebnisse und Diskussion

Während des Erhitzens steigt die Fluoreszenz auf einen Maximalwert an und sinkt mit fortschreitender Veraschung auf 0 ab. Total veraschte Flecken geben keine Fluoreszenz mehr. Beim Aufstellen der Eichkurve ergab sich, dass zur Erreichung von Eichgeraden die Einhaltung bestimmter Erhitzungszeiten notwendig war. Für einen Konzentrationsbereich von 1–7 μg Triglycerid wurden 45 min (Tabelle I), für einen Konzentrationsbereich von 7–30 μg Triglycerid 25 min (Tabelle II), als optimale

TABELLE I

ERMITTLUNG DER EICHKURVE FÜR DEN KONZENTRATIONSBEREICH VON 1–7 μg TRIOLEIN AUS 15 VERSUCHEN

Erhitzungsdauer 45 min.

Auftragsmenge (μg)	*Peakfläche (mm²)*	*s (%)*
0.58	222 ± 22	10
0.90	342 ± 21	6
1.80	671 ± 15	2
3.60	1290[a]	[a]
5.40	1889 ± 55	3

[a] Die Fläche dieser Konzentration wurde bei jedem Versuch auf 1290 mm² rechnerisch korrigiert.

TABELLE II

ERMITTLUNG DER EICHKURVE FÜR DEN KONZENTRATIONSBEREICH 7–30 μg

Erhitzungsdauer 25 min.

Auftragsmenge (μg)	*Peakfläche (mm²)*	*s (%)*
5.65	385 ± 28	7
11.20	873 ± 36	4
22.40	1787 ± 39	
33.60	2700[a]	[a]

[a] Die Fläche dieser Konzentration wurde bei jedem Versuch auf 2700 mm² rechnerisch korrigiert.

Erhitzungsdauer ermittelt; die Messwerte innerhalb beider Bereiche liegen dann auf Geraden (Fig. 1 und 2). Bei zu langem Erhitzen bog die Eichkurve bei höheren Konzentrationen durch Fluoreszenzlöschung dagegen um (Fig. 3). Es wurde versucht, auch für einen Konzentrationsbereich von 0.1–1.0 μg Triolein eine Eichkurve zu ermitteln. Die Platten wurden dabei 85 min erhitzt. Zu reproduzierbaren Werten gelangt man in diesem Bereich allerdings nur bei Vorreinigung des Adsorptionsmittels und Destillation der Laufmittel, da ansonsten der Untergrund mittels des Vitatron TLD 100 nicht mehr kompensierbar ist. Gleiche Versuche wurden auch mit Tripalmitat durchgeführt. Sie zeigten bei gleichen Bedingungen eine geringere Intensität des Fluoreszenzlichtes im Vergleich zu Triolein, lieferten jedoch ebenfalls eine Eichgerade. Die fluoreszierenden Flecken liessen sich mit Methanol eluieren. Eine Rechromatographie im Laufmittel Chloroform–Methanol (9:1) zeigte, dass der fluoreszierende Fleck aus mindestens vier fluoreszierenden Anteilen besteht.

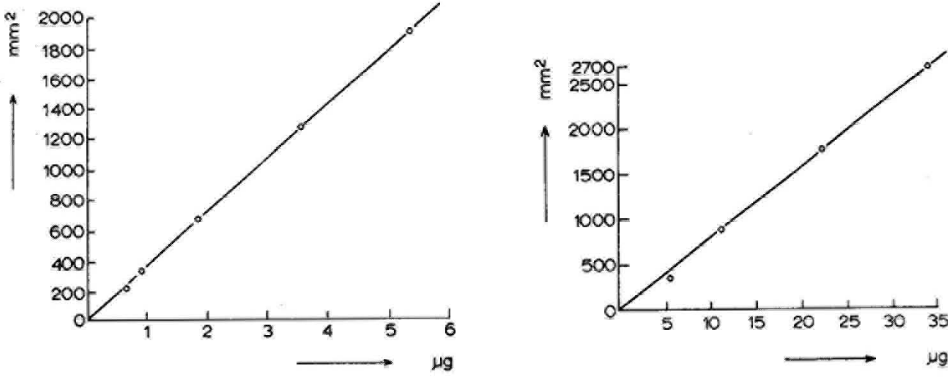

Fig. 1. Eichkurve für den Konzentrationsbereich von 1–7 µg Triolein.
Fig. 2. Eichkurve für den Konzentrationsbereich von 7–30 µg Triolein.

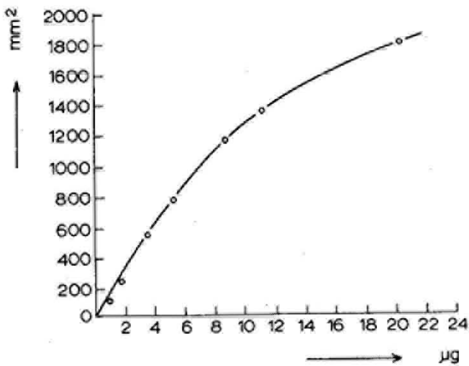

Fig. 3. Abweichung der Eichkurve bei höheren Konzentrationen nach einer Erhitzungsdauer von 45 min.

Eine Anwendung dieser Methode zur Bestimmung von Rattenleber-Triglyceriden ergab eine gute Übereinstimmung mit den enzymatisch gefundenen Werten. Die enzymatische Messung lieferte einen Triglyceridgehalt von 3.1%, während das fluorometrische Verfahren 3.0% (bezogen auf das Frischlebergewicht) Triglyceride ergaben.

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CHROM. 6217

Determination of uric acid in foodstuffs by thin-layer chromatography

When the enzymatic method for the determination of uric acid in foodstuffs[1] could not be used for some reason, the Benedict method has been used, in an attempt to correlate uric acid content with insect infestation[2]. Although the latter method is known not to be strictly specific for uric acid[3], its use in foodstuffs, giving no colour in the extract, has been tolerable. But in certain foodstuffs, such as spices, condiments and pulses, which usually yield some colour in the extract, the method is obviously not suitable because a blank value of fixed magnitude for a particular food cannot be obtained. In this paper a thin-layer chromatographic (TLC) method is reported for the detection and determination of uric acid in foodstuffs.

A paper chromatographic technique for the separation and determination of uric acid was used by TILDEN[4] in infested fruit products, by JOHNSON[5] and DIKSTEIN *et al.*[6] in urine and by VENKATRAO *et al.*[7] in wheat flour. There are some references[8–10] to the use of TLC for separating uric acid, but their aims were different from that of this work. The solvents used by all these previous workers and by a few others for the development of the chromatogram have been tried in the present determination, but one, butanol–5 *N* acetic acid (2:1), has been found to give the best separation and spot formation, the saturation time being 1 h. Cellulose powder (No. 123, Schleicher & Schüll) was found to be a better support than Silica Gel G.

Procedure

Glass plates (20 × 10 cm) with a 250 μm thin layer were air-dried for about 12 h as it is known that the resolving properties of cellulose layers improve on long exposure to air[11].

Standard solutions of uric acid (prepared as described by HAWK *et al.*[12], containing up to 10 μg of uric acid, were spotted onto the plate, which was placed in the solvent chamber and allowed to develop to a height of 10 cm. The run took about 30 min. The plate was then dried in air to remove the solvent completely and sprayed with 5% sodium cyanide solution[3] followed by arsenophosphotungstic acid reagent[3]. Blue spots appeared against a white background with an R_F value of 0.51 and a sensitivity (limit of detection) of 5 μg. The spots of graded concentration then gave the standard curve by colorimetric determination after elution with 5 ml of water or directly by densitometer.

A 2-g amount of a representative sample of food was pulverised and suspended in 20 ml of water at room temperature. The mixture was allowed to stand for 2 h with occasional stirring and then mixed in a blender for 5 min. The whole mixture was then centrifuged and the supernatant was treated with about 5 g of alumina, which adsorbed the natural colours to some extent. Alternatively, the centrifugate can be passed through a small column containing alumina. Thus cleaned-up, the solution was treated as described in the previous paragraph.

A number of samples, both infested and uninfested, of turmeric, coriander, pulses and amchur (a seasoned powder of green mango usually used as a condiment in India) have been investigated. In all instances, the uninfested samples showed some colorimetric reading by the Benedict method but none by the TLC method. As

TABLE I

DETERMINATION OF URIC ACID (mg-%) IN UNINFESTED AND INFESTED FOODSTUFFS

Sample	*Uninfested foodstuff*		*Infested foodstuff*		
	Colorimetric method	*TLC method*	*Colorimetric method*	*Corrected[a] colorimetric value*	*TLC method*
Turmeric	4.2	Nil	10.5	6.3	8.1
	4.3	Nil	21.3	17.0	19.6
	4.4	Nil	12.4	8.0	10.2
	4.2	Nil	11.2	7.0	9.4
Coriander	5.6	Nil	37.9	32.3	35.2
	5.4	Nil	70.6	65.2	66.8
	5.4	Nil	69.4	64.0	66.8
	5.5	Nil	71.3	65.8	67.3
Pulses	4.7	Nil	45.8	41.1	44.5
	4.7	Nil	161.4	156.7	159.0
	4.8	Nil	65.2	60.4	64.6
	4.7	Nil	80.4	75.7	78.6
Amchur	10.8	Nil	112.6	—	105.0
	12.5	Nil	103.8	—	94.5
	11.0	Nil	110.9	—	104.2
	10.8	Nil	113.0	—	106.0

[a] Infested minus uninfested.

expected, the infested samples showed higher contents of uric acid by the colorimetric method than by the TLC method. The recovery of uric acid added to uninfested food has also been found to be satisfactory by the TLC method. The results for a few typical samples are given in Table I. In all the samples except amchur, the uninfested specimen was infested and uric acid was determined after a lapse of a few months. In amchur, stray (unrelated) samples, infested and uninfested, were analysed.

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CHROM. 6279

Separation and determination of some systemic fungicides and their metabolites by thin-layer chromatography

The advantages and usefulness of systemic fungicides for the control of plant diseases have been recognized for a number of years[1,2].

A systemic fungicide is less subject to adverse weather conditions than a contact fungicide and will therefore offer protection from diseases for a longer period of time. The systemic properties of these fungicides allow protection of new plant growth, which otherwise would have to be treated after it develops. For these reasons systemic fungicides offer economical advantages in comparison with contact fungicides.

Benomyl, 1-(butylcarbamoyl)-2-benzimidazolecarbamic acid methyl ester is one fungicide which has received considerable attention during the last few years because of its ability to control a variety of plant diseases[3,4]. Thiophanate methyl, 1,2-bis(3-methoxycarbonyl-2-thioureido)benzene and the chemically closely related thiophanate, 1,2-bis(3-ethoxycarbonyl-2-thioureido)benzene are also of interest because of their broad spectrum of activity[5-7].

Several authors have shown that both benomyl and thiophanate methyl are degraded in aqueous solution to 2-benzimidazolecarbamic acid methyl ester (MBC)[8-10], which is also fungitoxic. It is reported to be the main component found in plants treated with benomyl or thiophanate methyl[8,9]. It is quite stable and persists in the plant for some time[8]. Further chemical degradation of MBC could produce chemical compounds such as 2-aminobenzimidazole and benzimidazole. The presence of these materials in the tissue of treated plants, however, has not been determined. Thin-layer chromatography (TLC) has been used by several workers to separate and determine mixtures of benomyl and MBC and of thiophanate methyl and MBC[8,9]. This paper describes the separation and determination of all three fungicides benomyl, thiophanate methyl and thiophanate with MBC, benzimidazole and 2-aminobenzimidazole. The two last compounds originate from MBC due to loss of the carbamic acid ester group. The separation and determination was achieved by two-dimensional TLC. A sensitive spray reagent for the detection of MBC was also found.

Experimental

Samples of analytical grade quality benomyl and MBC were supplied by the DuPont de Nemours Co. and samples of thiophanate methyl and thiophanate were obtained from Ciba-Geigy Canada Ltd. Benzimidazole and 2-aminobenzimidazole were purchased from chemical supply houses. Eastman Chromagram sheets coated with silica gel (100 μ) and containing a fluorescent indicator were used. The two-dimensional solvent systems used were: Solvent 1, benzene–methanol (9:1) and Solvent 2, ethyl acetate–chloroform (6:4). They were selected as the most efficient after experimenting with a large number of solvents and solvent mixtures. Compounds were detected under 254 nm UV light as dark spots. The chromogenic spray reagent used for the detection of MBC was a 0.5% solution of N-2,6-trichloro-*p*-benzoquinone-imine. This reagent has previously been used for the detection of carbamates[11].

The results of the two-dimensional TLC separation are shown in Fig. 1.

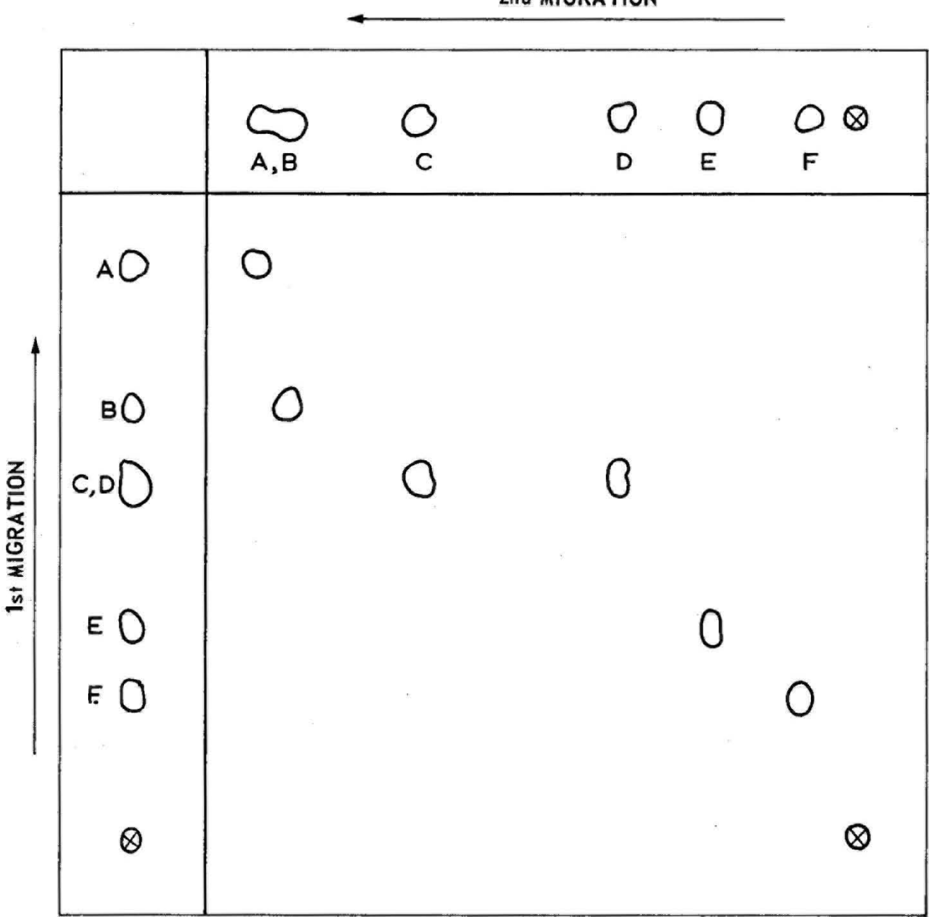

Fig. 1. Two-dimensional thin-layer chromatogram of benomyl (A), thiophanate (B), thiophanate methyl (C), MBC (D), benzimidazole (E), and 2-aminobenzimidazole (F). Solvent systems: (1) benzene–methanol (9:1); (2) ethyl acetate–chloroform (6:4).

Results and discussion

The R_F values of the TLC separation are presented in Table I.

After development of the chromatogram in the two solvent systems, the compounds were visible under short-wave (254 nm) UV light as distinct dark spots on a pink fluorescent background.

It was also found that MBC gave a very sensitive colour reaction when sprayed with a 0.5% solution of N-2,6-trichloro-*p*-benzoquinoneimine in cyclohexane, a reagent which has been used previously for the detection of carbamates[11]. After the spray application the chromatogram was developed at 100° for 10 min to form an intensely blue coloured spot. The colour is not persistent and will fade after a period of several minutes. The other compounds did not give this colour reaction. Amounts as low as 25 ng of MBC can be detected by this method.

TABLE I

APPROXIMATE R_F VALUES

Compounds	*Solvent 1*	*Solvent 2*
Benomyl	0.88	0.91
Thiophanate	0.66	0.87
Thiophanate methyl	0.56	0.68
MBC	0.56	0.37
Benzimidazole	0.34	0.23
2-Aminobenzimidazole	0.25	0.07

It was found that the two-dimensional technique offers excellent accuracy and better separation than the development in one direction only.

Efforts are under way to apply this method to extracts of plant material and soil samples containing these fungicides.

The assistance of G. F. ZAJACZ, technical assistant, is gratefully acknowledged. The author thanks the DuPont de Nemours Co. and Ciba-Geigy Canada Ltd. for the analytical grade samples of benomyl, MBC, thiophanate and thiophanate methyl.

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CHROM. 6210

Thin-layer chromatography of aromatic amines on cadmium acetate impregnated silica gel thin layers*

Extending previous work[1], results of investigations regarding the separation of aromatic amine isomers on silica gel layers impregnated with various cadmium salts, *i.e.* sulphate, acetate, phosphate, etc., are now reported. Better mutual separation of some amines, such as anisidines and xylidines, is achieved on cadmium acetate impregnated layers.

The relationship between R'_M value[2] and basicity of the amine was also studied in more detail. A close correlation, which can be expressed by the following equation, was observed from the data obtained on unimpregnated silica gel layers.

$$R'_M = a + b(\mathrm{p}K_a) + c(\mathrm{p}K_a)^2 \qquad (1)$$

Cadmium sulphate impregnation was found to increase b, the coefficient of the first order term of the above equation, and a linear dependence between the difference of the R'_M values and the pK_a values of the amines, which had been suggested in the previous paper, was also observed in some cases. Cadmium acetate, on the other hand, rather increased the standard deviations between R'_M values and pK_a values.

Experimental

Thin layers, 0.2–0.25 mm thickness, were prepared according to the method described in the previous paper[1], and the amines were spotted on the layers as 0.5% methanolic solutions.

4-Methoxyazobenzene, picric acid, and 4-(2'-hydroxy-1'-naphthylazo)-2,5-dimethoxy-benzanilide** were developed alongside the amines as reference compounds. The R_F values of the amines were then corrected by means of the correction equation proposed by DHONT *et al.*[3] and the R_F values of the reference compounds.

Results

The corrected R_F values of amines are shown in Table I. A few amines gave spots which tailed; R_F data of such spots are marked st(spot length/spot width being between 2 and 4) or t(ratio more than 4).

Mean R_S values (*ortho* isomer = 1.00) of *meta* and *para* isomers were calculated from the data of toluidines, anisidines, chloroanilines, and bromoanilines and are shown in Table II. Judging from these R_S values, cadmium sulphate impregnation seems to give the best separation of isomers. But experimentally better results were sometimes obtained on cadmium acetate impregnated layers. For example, *ortho* and *meta* isomers of anisidines or six isomers of xylidines were separated from each other only on the latter layers.

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* Part of this work was presented at the Kyushu District Meeting of the Japanese Chemical Society, Oct., 1971, Kumamoto, Japan.

** This compound was obtained by coupling diazotized Naphthol Fast Blue RR and 2-naphthol, and purified by the recrystallization from a benzene–pyridine mixture.

TABLE I

$R_F \times 100$ VALUES AND pK_a VALUES OF AROMATIC AMINES ON UNIMPREGNATED AND IMPREGNATED THIN LAYERS

Ref. 1 = 4-Methoxyazobenzene; ref. 2 = picric acid; ref. 3 = 4-(2'-hydroxy-1'-naphthylazo)-2,5-dimethoxy-benzanilide.
Solvent systems: S_1 = benzene–acetic acid (9:1); S_2 = benzene–acetic acid (8:2); S_3 = benzene–ethyl acetate–acetic acid (10:10:1); S_4 = benzene–ethyl acetate–acetic acid (6:3:1). Layers: L_1 = Unimpregnated layer; L_2 = cadmium sulphate impregnated layer; L_3 = cadmium acetate impregnated layer; L_4 = cadmium phosphate impregnated layer.

Amines	*$R_F \times 100$ value*														*pK_a value*
	S_1				S_2				S_3			S_4			
	L_1	L_2	L_3	L_4	L_1	L_2	L_3	L_4	L_1	L_2	L_3	L_1	L_2	L_3	
o-Toluidine	26	13	22	24	28	18t	34st[a]	20	47	41	52	46	22st	41	4.4
m-Toluidine	17	8	12	15	12	11t	26t[a]	12	34	30t	33	37	14st	26	4.7
p-Toluidine	12	4	8	9	17	9t	20t	9	24	17st	23	35st	6st	18st	5.1
o-Anisidine	19	11st	18	20st	28	11t	29t	16st	47	36t	44st	49	22st	31	4.5
m-Anisidine	18	8st	13	16st	26	11t	27t	16st	34	35t	35st	46	20st	26	4.2
p-Anisidine	5	1	6	5	12	4t	19t	3	13	14t	11	13	3	5	5.3
2,3-Xylidine	15	6	26t	18	20	18st	32st	16	36	41st	52	37st	16st	27	4.7
2,4-Xylidine	13	5	23t	15	18	17st	31st	14	33	36st	46	33	13st	23	4.9
2,5-Xylidine	20	10	31t	23	26	22st	38st	19	45	48t	56	47	22	34	4.5
2,6-Xylidine	35	27	49	41	45	41	57	37	62	70	76	61	46	59	4.0
3,4-Xylidine	9	4	12	13	13	12st	22st	9	22	21t	23st	23	6	12	5.2
3,5-Xylidine	15	6	18t	18	17	14st	29st	14	32	36t	34st	36	14st	22	
o-Chloroaniline	62	64	70	60	65	71	70	62	80	87	84	70	72	75	2.6
m-Chloroaniline	44	33	34	44	49	44	44st	44	71	70	63	61	57	50	3.3
p-Chloroaniline	32	15	25	30	37	26st	35st	30	60	45st	48	53	36	39st	3.8
o-Bromoaniline	64	64	73	62	66	73	74	66	81	85	85	74	73	77	2.6
m-Bromoaniline	45	36	36	45	51	49	44st	47	71	71	64	65	60	53	3.5
p-Bromoaniline	34	18	26	34	40	32	35st	30	62	54	51	58	43	41	3.9
1-Naphthylamine	33	27st	35st	37	40	35st	47	49	67	64	67	64st	47st	51	4.0
2-Naphthylamine	24	18	23	27	37	20t	34st	37t	56	28t	52t	55t	24t	36t	4.1
o-Aminobenzoic acid	44	43st	13	41	58	52		55	66		13st				2.1
m-Aminobenzoic acid	14	10	3	14	25	11		24	47		4				3.1
p-Aminobenzoic acid	33	31	12	33	47	42		44	54		17st				2.4
o-Aminobiphenyl	59	51	65	60	61		76		82		86				3.8
p-Aminobiphenyl	27	10t	21	26	33		39		69		50				4.3
o-Phenethidine	26	14t	29	24	30		41		69		61				4.5
p-Phenethidine	9	4	6	12	13		19		21		16				5.3
2,5-Dichloroaniline	71	70	73	65	74	80	75	69	77	78	80	87			1.5
Ref. 1	15	47	4	19	24	65	11	31	23	60	8t	12	54	7	
Ref. 2	74	74	85	72	79	88	91	77	79	80	86	88	90	94	
Ref. 3	45	62	74		60	74	86	63	75	85	90	72	75	83	

TABLE II

MEAN R_s VALUES OF ISOMERS (R_F VALUE OF *ortho*-ISOMER = 1.00)
For solvent systems and thin layers, see Table I.

	S_1				S_2			
	L_1	L_2	L_3	L_4	L_1	L_2	L_3	L_4
meta isomer	0.75	0.61	0.56	0.72	0.80	0.73	0.73	0.76
para isomer	0.44	0.23	0.35	0.42	0.56	0.44	0.56	0.40

	S_3			S_4		
	L_1	L_2	L_3	L_1	L_2	L_3
meta isomer	0.80	0.84	0.73	0.87	0.79	0.71
para isomer	0.58	0.49	0.47	0.58	0.28	0.42

Discussion

The relationship between the R'_M and pK_a values of amines is clearly shown in Figs. 1–6, in which the R'_M values of amines on various layers are plotted against the pK_a values of amines. This shows that the amino group in the amines takes the leading role in the adsorption to the surface of silica gel, and the contribution of the other groups towards the adsorption may be much smaller than that of the amino group, since the adsorption energy[4] of the amino group to the silica gel is much larger than that of the other groups. As is shown in Figs. 1–3, the R'_M values of the amino-

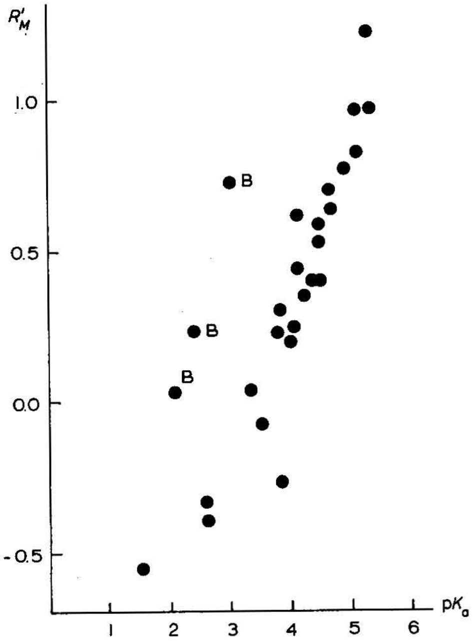

Fig. 1. Relationships between the R'_M and pK_a values of amines on unimpregnated silica gel thin layers. Solvent system: benzene–acetic acid (9:1). B = Aminobenzoic acid.

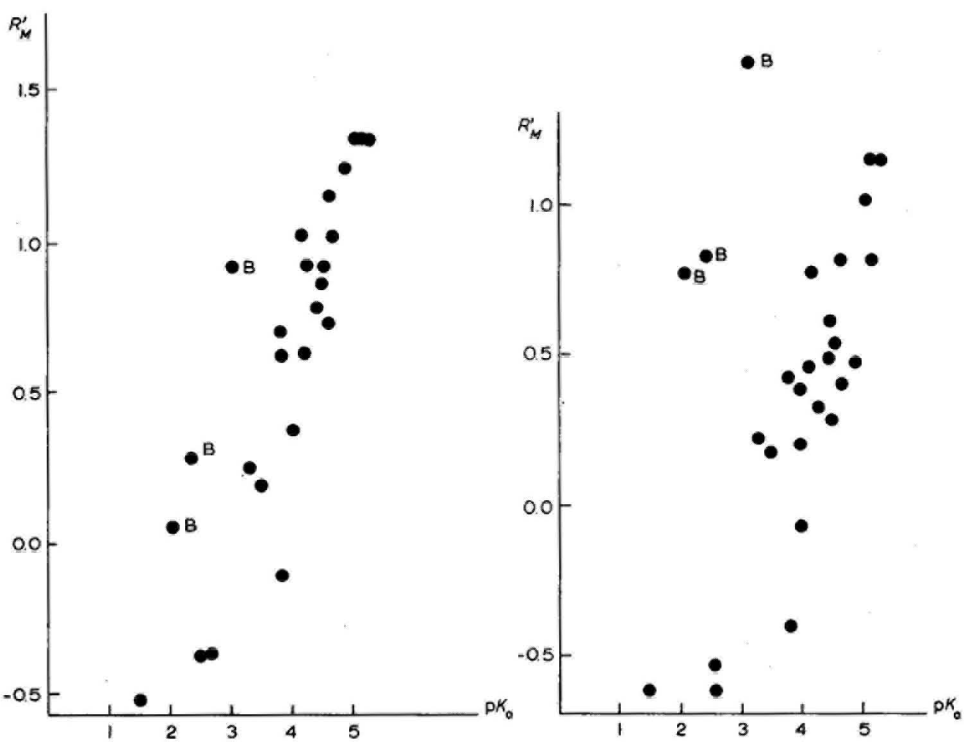

Fig. 2. Relationships between the R'_M and pK_a values of amines on cadmium sulphate impregnated silica gel thin layers. Solvent system: benzene–acetic acid (9:1). B = Aminobenzoic acid.

Fig. 3. Relationships between the R'_M and pK_a values of amines on cadmium acetate impregnated silica gel thin layers. Solvent system: benzene–acetic acid (9:1). B = Aminobenzoic acid.

benzoic acids have properties which differ from those of the other amines. This may be due to the larger adsorption energy of the carboxyl group as opposed to that of the amino group.

The relationship between the R'_M and pK_a values of the amines can be expressed as the general equation (1). The coefficients of each of the terms were calculated and are shown in Table III. The R'_M values of the aminobenzoic acids were, however, omitted from the calculation, since the values were far from normal.

Impregnation with cadmium sulphate increased b, the coefficient of the first order term, and, as is shown in Figs. 7 and 8, the $\Delta R'_M$ values, *i.e.*, the differences between the R'_M values on layers impregnated with salt and on unimpregnated layers, were found to have a clear relationship with the pK_a values of the amines. This may suggest that the amino group of the amines, or more correctly, an unshared electron pair in the amino group, also plays a part in the adsorption of the amino groups on cadmium sulphate. As the pK_a values of aromatic amines generally increase in the order of the *ortho*, *meta* and *para* isomers, good separation can be expected on

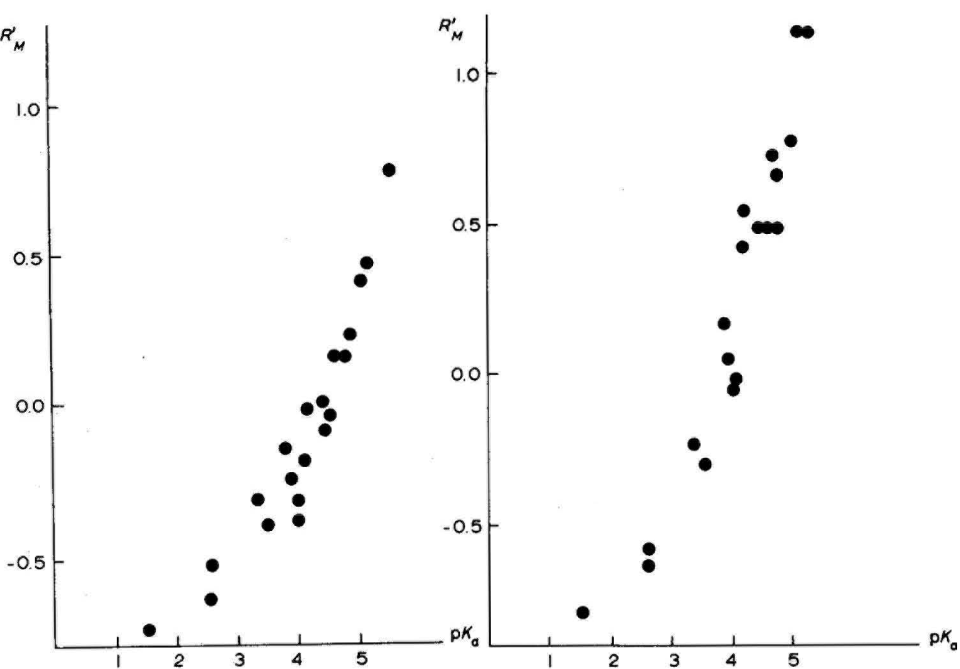

Fig. 4. Relationships between the R'_M and pK_a values of amines on unimpregnated silica gel thin layers. Solvent system: benzene–ethyl acetate–acetic acid (6:3:1).

Fig. 5. Relationships between the R'_M and pK_a values of amines on cadmium sulphate impregnated silica gel thin layers. Solvent system: benzene–ethyl acetate–acetic acid (6:3:1).

TABLE III

COEFFICIENTS OF EQUATION (1) FOR THE FOLLOWING RELATIONSHIP BETWEEN R'_M AND pK_a: $(X = pK_a;\ y = R'_M)\ y = a + b(X-\bar{X}) + c(X-\bar{X})^2$

For solvent systems and their layers, see Table I.

Solvent system	*Thin layer*	$\bar{X}$	*a*	*b*	*c*	*Standard deviation*	*Fo 1st order*[a]	*Fo 2nd order*[a]
S_1	L_1	4.11	0.37	0.47	0.05	0.13	241	12
	L_2	4.11	0.68	0.62	0.06	0.21	181	7.1
	L_3	4.11	0.36	0.48	0.05	0.25	73	1.7
S_3	L_1	4.23	−0.15	0.57	0.14	0.26	87	5.4
	L_2	4.17	−0.08	0.68	0.07	0.26	106	1.0
	L_3	4.23	−0.09	0.36	0.07	0.30	49	0.5
S_4	L_1	4.04	−0.09	0.36	0.06	0.10	217	28
	L_2	4.04	0.31	0.60	0.08	0.13	368	27
	L_3	4.04	0.21	0.49	0.05	0.18	139	5.4

[a] *Fo* means that the F-test was made from observed values.

cadmium sulphate impregnated layers. Unsatisfactory results were, however, sometimes obtained on this layer, especially for the separation of amines with similar pK_a values.

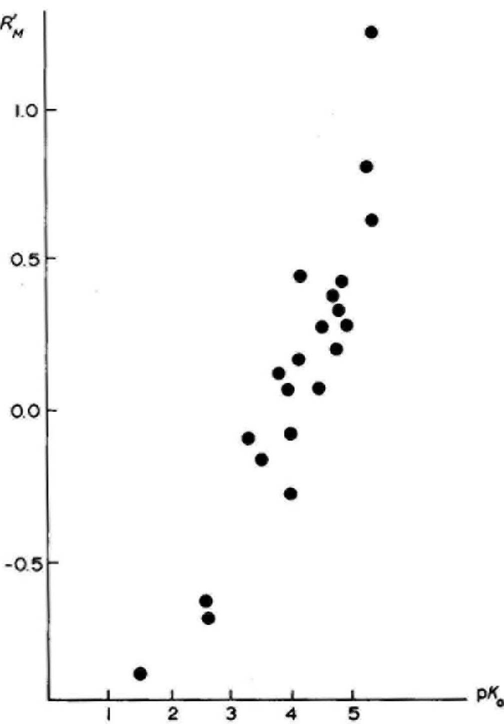

Fig. 6. Relationships between the R'_M and pK_a values of amines on cadmium acetate impregnated silica gel thin layers. Solvent system: benzene–ethyl acetate–acetic acid (6:3:1).

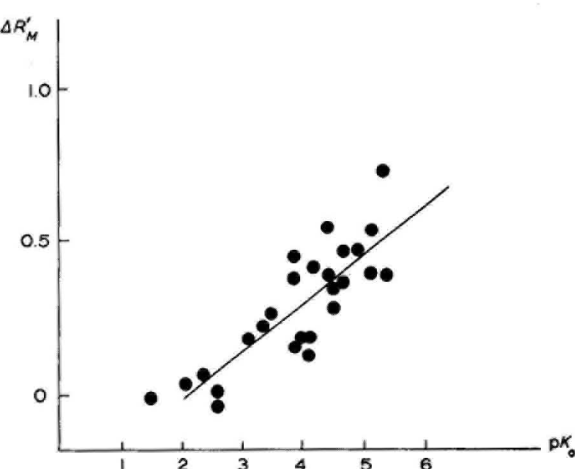

Fig. 7. Relationships between the $\Delta R'_M$ and pK_a values of amines on cadmium sulphate impregnated silica gel thin layers. Solvent system: benzene–acetic acid (9:1). $\Delta R'_M = 0.16\,[pK_a] - 0.34$; $r = 0.84$.

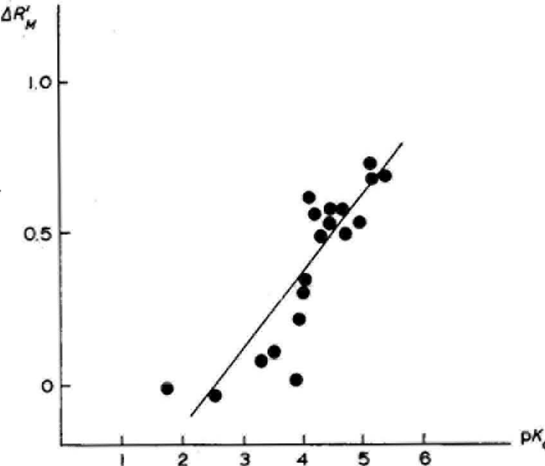

Fig. 8. Relationships between the $\Delta R'_M$ and pK_a values of amines on cadmium sulphate impregnated silica gel thin layers. Solvent system: benzene–ethyl acetate–acetic acid (6:3:1). $\Delta R'_M = 0.25\ [pK_a] - 0.63$; $r = 0.89$.

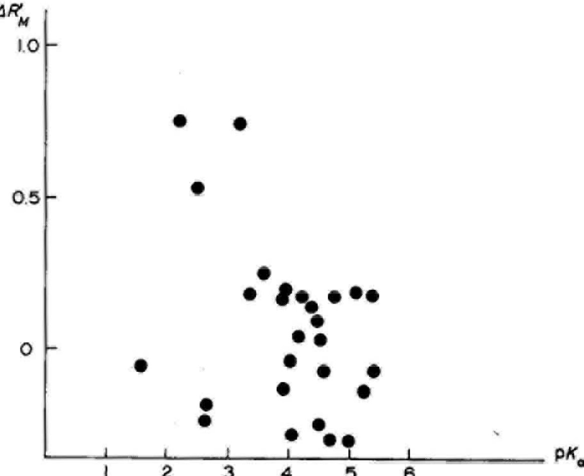

Fig. 9. Relationships between the $\Delta R'_M$ and pK_a values of amines on cadmium acetate impregnated silica gel thin layers. Solvent system: benzene–ethyl acetate–acetic acid (6:3:1).

Cadmium acetate, on the other hand, had the effect of increasing the standard deviations in the equation. As is shown in Fig. 9, the $\Delta R'_M$ values have little correlation with the pK_a values on cadmium acetate impregnated layers. Supposedly, impregnation with the latter salt introduces some new factors which affect the separation mechanism on the layers. A characteristic of the separation of amines on cadmium acetate impregnated layers was the partial reduction of the tailing of spots, but these unknown factors may be considered to be primarily responsible for it.

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CHROM. 6229

Dünnschichtchromatographie der Acridon-Alkaloide

In dem Jahr 1948 entdeckten HUGHES und Mitarb.[1] die Acridon-Alkaloide. Seitdem sind etwa dreissig Alkaloide dieses Typs bekannt geworden[2]; sie wurden bisher ausschliesslich in Rutaceen-Arten angetroffen und konnten vornehmlich aus den Blättern und der Rinde dieser Pflanzen isoliert werden. Die z. T. bemerkenswerten biologischen Eigenschaften dieser Verbindungen lassen es wünschenswert erscheinen, Drogen auszuselektieren, welche diese Alkaloide in grösseren Mengen enthalten. DC-Methoden scheinen hierzu besonders geeignet zu sein[3,4], systematische Untersuchungen fehlen bisher jedoch.

Material und Methoden

Zur Untersuchung des dünnschichtchromatographischen Verhaltens der Acridon-Alkaloide* (Tabelle I) wurden ihre Azeton-Lösungen auf Hand gegossene Kieselgel-G-Platten bzw. Fertigplatten $F_{254+366}$ (Woelm) aufgetragen und in den Fliessmittelsystemen I–III chromatographiert. Nach dem Entwickeln wurden die Substanzflecke bei Tageslicht und im UV-Licht (365 nm) beobachtet, danach mit den in der Tabelle I wiedergegebenen Detektionsmitteln angesprüht. Die Ergebnisse sind in der Tabelle I zusammengefasst.

Reagenzien. Die folgenden Reagenzien wurden verwendet: 2%-ige $FeCl_3$-Lösung in abs. Äthanol; 1%-ige $AlCl_3$-Lösung in Methanol, 10 min, 110°; Dragendorff-Reagenz nach MUNIER UND MACHEBOEUF[5]; 3:1 Gemisch von einer 3%-igen wässrigen Borsäure- und einer 10%-igen wässrigen Oxalsäure-Lösung, 10 min, 110°.

Detektion. Alle Acridon-Alkaloide geben sich auf dem DC bei Tageslicht als gelbgefärbte Flecken zu erkennen. Mit dem sauren Fliessmittel werden die Nor-Derivate rötlich, während die anderen ihre ursprüngliche Farbe behalten.

Auf Kieselgel G zeigen die Nor-Derivate im langwelligen UV-Licht stets eine schwache dunkelbraune oder rotbraune Fluoreszenz, die 1-Methoxy-Derivate fluoreszieren dagegen unterschiedlich (s. Tabelle I). Bei dem vorliegenden Untersuchungsmaterial lassen sich noch keine Regelmässigkeiten feststellen (s. hierzu Lit. 6).

Mit Dragendorff-Reagenz geben alle Proben eine orangebraune Färbung.

Mit Eisenchlorid-Lösung färben sich die Nor-Derivate grün, die übrigen zeigen keine Reaktion. Dieser Test wurde bereits früher von HUGHES und Mitarb.[7] zur Differenzierung der 1-Hydroxy-Derivate von den 2-, 3- und 4-Hydroxy-Derivaten herangezogen. Die Angaben in der Tabelle I machen deutlich, dass sich diese Färbemethode auch zum dünnschichtchromatographischen Nachweis dieser Verbindungs-

* Die Acridon-Alkaloide verdanken wir den folgenden Kollegen: Acronycin, Melicopin und Melicopidin—Dr. F. N. LAHEY, Brisbane, Australia; Acronycin und Melicopicin—Dr. G. H. SVOBODA, Ind., U.S.A.; Melicopicin, 1,3-Dimethoxy-N-methylacridon und Xanthevodin—Dr. J. A. LAMBERTON, Melbourne, Australia; Evoprenin, Xanthoxolin und 1,2,3-Trimethoxy-N-methylacridon—Prof. Dr. E. RITCHIE, Sydney, Australia; Evoxanthin—Dr. R. H. PRAGER, Adelaide, Australia; Atalaphyllin und N-Methylatalaphyllin—Dr. N. VISWANATHAN, Bombay, India; Tecleanthin—Dr. K. H. PEGEL, Durban, South Africa; Evoxanthidin—Prof. Dr. F. DALLACKER, Aachen, B.R.D.

TABELLE I

DC-CHARAKTERISTIKA EINIGER ACRIDON-ALKALOIDE[a]

Fliessmittelsysteme: (I) Benzol–Äthylazetat (6:4); (II) Toluol–Äthylazetat–Ameisensäure (5:4:1); (III) Benzol–Äthylazetat (8:2), konz. NH_3-Atmosphäre.

Nr.	*Name*	*Struktur*						*hR_F-Werte (Kieselgel G)*		
		C-1	*C-2*	*C-3*	*C-4*	*C-5*	*R*	*I*	*II*	*III*
1	N-Methylacridon	H	H	H	H	H	CH_3	58	70	49
2	1-Hydroxy-N-methylacridon	OH	H	H	H	H	CH_3	62	73	62
3	1,3-Dimethoxy-N-methyl-acridon	OCH_3	H	OCH_3	H	H	CH_3	12	11	9
4	Xanthoxolin	OH	OCH_3	OCH_3	H	H	H	28	55	4
5	Arborinin	OH	OCH_3	OCH_3	H	H	CH_3	39	61	27
6	1,2,3-Trimethoxy-N-methylacridon	OCH_3	OCH_3	OCH_3	H	H	CH_3	21	9	17
7	Evoxanthidin	OCH_3	$O-CH_2-O$		H	H	H	16	8	6
8	Evoxanthin	OCH_3	$O-CH_2-O$		H	H	CH_3	23	7	19
9	Tecleanthin	OCH_3	$O-CH_2-O$		H	OCH_3	CH_3	28	16	22
10	Melicopicin	OCH_3	OCH_3	OCH_3	OCH_3	H	CH_3	43	60	37
11	Xanthevodin	OCH_3	$O-CH_2-O$		OCH_3	H	H	21	13	13
12	Melicopidin	OCH_3	$O-CH_2-O$		OCH_3	H	CH_3	36	27	28
13	Melicopin	OCH_3	OCH_3	$O-CH_2-O$		H	CH_3	43	50	34
14	Des-N-methylnoracronycin	OH	H	O		H	H	64	74	37
15	Noracronycin	OH	H	O		H	CH_3	75	82	66
16	Acronycin	OCH_3	H	O		H	CH_3	29	51	21
17	Rutacridon	OH	H	H_2C O C CH_3		H	CH_3	68	81	62
18	Gravacridondiol	OH	H	$HOCH_2$ O HO CH_3		H	CH_3	5	49	0
19	Gravacridondiol-monomethyläther	OH	H	CH_3OCH_2 O HO CH_3		H	CH_3	22	54	15
20	Gravacridonchlorin	OH	H	$HOCH_2$ O Cl CH_3		H	CH_3	45	66	21
21	Gravacridonolchlorin	OH	H	$HOCH_2$ O Cl $HOCH_2$		H	CH_3	14	52	3
22	Evoprenin	OH	OCH_3	O	H	H	CH_3	65	74	48
23	Atalaphyllin	OH		OH		OH	H	67	74	0
24	N-Methylatalaphyllin	OH		OH		OH	CH_3	73	80	20

[a] Lit.-Angaben über Vorkommen, Isolierung, Analytik etc. siehe Lit. 2 und 6.

UV-Licht (Kieselgel G)			*$FeCl_3$*	*Borsäure/Oxalsäure*		*hR_F-Werte (Fertigplatten)*	
I	*II*	*III*		*I*	*II*	*I*	*II*
blau	blau	blauviolett	—	blaugrün	blaugrün	37	56
rotbraun	rotbraun	rotbraun	grün	gelb	gelb	44	54
blau	grüngelb	blau	—	grün	grün	3	2
dunkelbraun	dunkelbraun	dunkel	grün	gelb	dunkelgelb	10	24
dunkelbraun	dunkelbraun	dunkel	grün	gelb	gelb	19	33
blaugrün	braungelb	blaugrün	—	gelbgrün	gelbgrün	6	2
blassblau	orangegelb	blau	—	grün	grün	5	2
blassgrün	orangegelb	blaugrün	—	grün	grün	9	2
blassgrün	goldgelb	blaugrün	—	gelbgrün	gelbgrün	12	2
orangegelb	rot	gelb	—	gelbbraun	gelbbraun	24	32
gelbgrau	rot	gelb	—	dunkelbraun	gelbbraun	8	24
orangegelb	rot	gelb	—	rotbraun	gelbbraun	18	5
orangegelb	rot	gelb	—	rot	rot	21	14
dunkelbraun	dunkelbraun	dunkel	grün	grün	blaugrün	49	62
dunkelbraun	dunkelbraun	dunkel	grün	rotbraun	rotbraun	60	69
gelbgrün	rot	gelb	—	rotbraun	gelbbraun	11	14
rot	rot	rot	grün	gelb	gelb	57	68
rot	rot	rot	grün	gelb	gelb	1	12
rot	rot	rot	grün	gelb	gelb	10	31
rot	rot	rot	grün	gelb	gelb	27	44
rot	rot	rot	grün	gelb	gelb	6	28
dunkelbraun	dunkelbraun	dunkel	grün	gelb	dunkelgelb	46	54
grün	dunkelbraun	grün	grün	gelb	dunkelgelb	56	68
dunkelbraun	dunkelbraun	dunkel	grün	goldgelb	goldgelb	60	73

typen eignet. Die Reaktion beruht auf einer Komplexbildung, wie sie beispielsweise auch bei den Flavonoiden anzutreffen ist.

Mit $AlCl_3$-Reagenz geben alle Acridon-Derivate nach Wärmebehandlung (110°, 10 min) unterschiedliche Fluoreszenzfarben. Die Reaktion beruht ebenfalls auf einer Komplexbildung. Es lässt sich noch keine Abhängigkeit der Farbtöne von bestimmten Strukturmerkmalen erkennen.

Auch mit dem Oxalsäure–Borsäure-Reagenz geben die Acridone charakteristische Fluoreszenzfarben (UV-Licht 365 nm), die auf das Entstehen eines Boroxalsäure-Komplexes (analog den Flavonoiden[8]) zurückzuführen ist.

Ergebnisse und Diskussion

Mit den angeführten Fliessmittelsystemen I–III, welche sich vorteilhaft auch zur Chromatographie anderer Rutaceen-Inhaltsstoffe (Cumarine, Chinolin-Alkaloide etc.) verwenden lassen[9], sind alle untersuchten Acridon-Alkaloide trennbar.

Hinsichtlich der hR_F-Wert–Strukturbeziehungen liessen sich folgende Regelmässigkeiten beobachten:

(1) Die 1-Hydroxy-Derivate (Nor-Verbindungen) weisen im allgemeinen einen höheren hR_F-Wert auf als die 1-Methoxy-Derivate, wie z.B. an den Paaren Arborinin und 1,2,3-Trimethoxy-N-methylacridon sowie Acronycin und Noracronycin zu erkennen ist. Dieser Effekt wird durch die starke Chelatisierung der 1-Hydroxy-Gruppe verursacht.

H
O O
N

(2) Durch ihre stärkere Basizität haben die N-Methyl-Derivate (im neutralen und sauren Fliessmittelsystem) stets einen höheren hR_F-Wert als die entsprechenden NH-Derivate. (Beispiele: Xanthoxolin–Arborinin, Ataläphyllin–N-Methylatalaphyllin, Des-N-methylnoracronycin–Noracronycin, Evoxanthidin–Evoxanthin, Xanthevodin–Melicopidin).

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CHROM. 6206

Méthode de dosage de la chlortétracycline et de l'oxytétracycline en mélange dans un lait réengraissé

Tel qu'il est décrit dans le recueil des méthodes d'analyse de l'Association of Official Agricultural Chemists[1] ou dans les méthodes de la Communauté Économique Européenne[2], le dosage de faibles concentrations de tétracyclines, par diffusion dans un milieu gélosé ensemencé avec *Bacillus cereus*, est attrayant par sa simplicité d'exécution. Mais il se révèle incapable de mesurer, dans un mélange de tétracyclines, l'activité propre à chacun de ces antibiotiques par suite du manque de sélectivité des solvants d'extraction proposés et de la sensibilité du germe à tous les antibiotiques de ce groupe.

Or, les trois substances principales qui le constituent, oxytétracycline (OTC), chlortétracycline (CTC) et tétracycline (TC), peuvent se retrouver associées dans un aliment pour animaux. Leur efficacité biologique pouvant être très variable, selon l'espèce animale considérée et son état physiologique, il est indispensable de disposer de méthodes non seulement de détection mais encore de dosage spécifiques.

Dans ce domaine, la chromatographie de partage sur papier a suscité l'intérêt de plusieurs chercheurs. En 1957, SELZER ET WRIGHT[3], étudiant des produits pour l'alimentation animale contenant un mélange de 1,000 à 12,000 p.p.m. de tétracyclines, ont séparé par cette technique la CTC de l'OTC. Les zones d'activité, localisées en lumière UV, ont été découpées et, après élution, l'antibiotique dosé par la méthode de GROVE ET RANDALL[4]. BLAKELY *et al.*[5], en 1969, ont recherché et identifié par chromatographie les tétracyclines éventuellement présentes dans le poisson conservé et ont procédé, lorsqu'ils n'en mettaient qu'une seule en évidence, à son dosage par la méthode de KRAMER *et al.*[6].

Nous avons, dans notre étude, cherché à assurer non seulement la séparation, mais encore le dosage de la CTC et de l'OTC aux concentrations où elles sont habituellement présentes dans les aliments des animaux (10–160 p.p.m.).

Étude critique de la méthode

Les tétracyclines sont extraites par un mélange méthanol–acétone–acide chlorhydrique (49:49:2). L'extrait est déposé sur papier Schleicher & Schüll 2040b préalablement humidifié par de l'acétone–tampon McIlvaine pH 4.6 (30:70) (BLAKELY *et al.*[5]). Après migration du solvant sur 20 cm, les antibiotiques sont révélés par bioautographie sur gélose ensemencée de *Bacillus cereus* ATCC 11778, selon un principe analogue à celui décrit par MARTEN[7]. La détermination quantitative est effectuée par comparaison de la surface de la zone d'inhibition ou de sa racine carrée à une courbe étalon.

Les caractéristiques particulières de la méthode sont les suivantes:

(1) Chromatographie ascendante—Elle a l'avantage sur la chromatographie descendante citée par BIRD ET PUGH[8] ainsi que par HICKEY ET PHILLIPS[9] d'éviter des traînées lors de la migration.

(2) Humidification du papier par le mélange acétone–tampon pH 4.6—SELZER ET WRIGHT[3] emploient un tampon pH 3.5. Cependant, BLAKELY *et al.*[5] ayant remarqué,

dans ce cas, la formation de zones d'inhibition non spécifiques, nous avons adopté le tampon pH 4.6 que ces auteurs proposent.

(3) Composition de l'éluant — Plusieurs éluants ont été préconisés pour la séparation chromatographique des tétracyclines (BLAKELY *et al.*[5], MARTEN[7]) mais la composition que nous avons retenue (nitrométhane–chloroforme–α-picoline, 20:10:3) donne, après bioautographie, des taches à contours plus nets favorables à un dosage précis.

(4) Utilisation du bleu de méthylène — L'addition de ce colorant au milieu de culture non seulement accentue, par contraste, la mise en évidence des zones d'inhibition mais encore les révèle sur le papier et facilite leur délimitation sur le chromatogramme lui-même, leur mesure et leur conservation ultérieures.

(5) Mesure des zones d'inhibition par planimétrie — Nos essais ont montré que, suivant la quantité d'antibiotique déposée et la sensibilité du germe test, le logarithme de la concentration est relié linéairement soit à la surface de la zone d'inhibition soit à sa racine carrée.

Application et résultats

Cette technique a été appliquée à la détermination du taux de recouvrement de la CTC et de l'OTC:

(1) Dans une solution contenant un mélange de ces deux substances — La Fig. 1 montre que, dans ce cas, la séparation des deux antibiotiques est satisfaisante (R_F

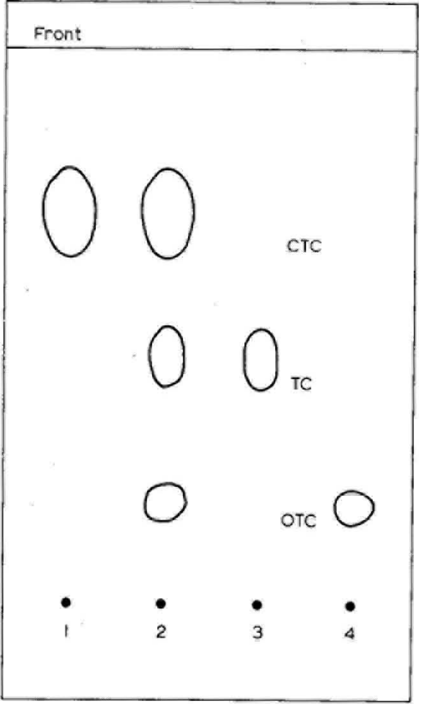

Fig. 1. Séparation des tétracyclines pures. (1) 0.0075 μg deCTC; (2) 0.0075 μg de CTC + 0.020 μg de TC + 0.015 μg de OTC; (3) 0.020 μg de TC; (4) 0.015 μg de OTC.

voisins de 0.70 pour la CTC et 0.20 pour l'OTC) et que, de plus, aucune des deux tétracyclines n'interfère dans le taux de recouvrement de l'autre; ces taux sont respectivement de 106% pour 0.75 μg/ml de CTC et 102% pour 1.5 μg/ml d'OTC.

(2) Ajoutées en solution à un lait réengraissé par 35% de suif (constituant principal des aliments pour veaux) soit isolément, soit en mélange—Les teneurs choisies étaient de 80 p.p.m. pour la CTC et de 160 p.p.m. pour l'OTC.

L'examen des données analytiques permet les conclusions suivantes: (a) dans le cas d'un seul antibiotique, le dosage réalisé par la méthode proposée donne des pourcentages de recouvrement comparables à ceux obtenus par les techniques microbiologiques classiques, soit 116% pour la CTC et 96% pour l'OTC; (b) le dosage quantitatif d'un mélange de CTC et d'OTC (Tableau I) peut être réalisé avec autant de précision que dans le cas de solutions pures, le lait n'ayant par lui-même aucune action interférente.

De la même façon il serait possible de procéder au dosage de la TC qui pourrait se trouver associée aux deux antibiotiques précédents; dans les conditions d'analyse retenues la séparation des trois tétracyclines est, en effet, très nette (Fig. 1).

TABLEAU I

RECOUVREMENT DE CHLORTÉTRACYCLINE (80 p.p.m.) ET D'OXYTÉTRACYCLINE (160 p.p.m.) AJOUTÉES EN MÉLANGE À UN LAIT RÉENGRAISSÉ À 35% DE SUIF

Essai	*Chlortétracycline*		*Oxytétracycline*	
	Quantité trouvée (p.p.m.)	*Recouvrement (%)*	*Quantité trouvée (p.p.m.)*	*Recouvrement (%)*
1	81	101	146	91
2	74	93	177	111
3	92	115	171	107
4	89	111	183	114
5	82	103	172	107
Moyenne	84	105	170	106
S	7		14	
C.V. %	8		8	

Conclusion

La méthode que nous avons décrite trouve une application concrète dans le dosage des aliments d'allaitement pour veaux; la CTC et l'OTC y sont en effet souvent associées dans des proportions voisines de celles citées dans cette note.

Ce mélange d'antibiotiques peut également être présent à des doses plus faibles (10 à 20 p.p.m.) dans des formules très différentes et destinées à d'autres espèces animales. Il sera alors vraisemblablement nécessaire de modifier certains détails du mode opératoire pour rester dans les limites des concentrations de la présente méthode.

Nous remercions THÉRÈSE MERA pour sa collaboration technique.

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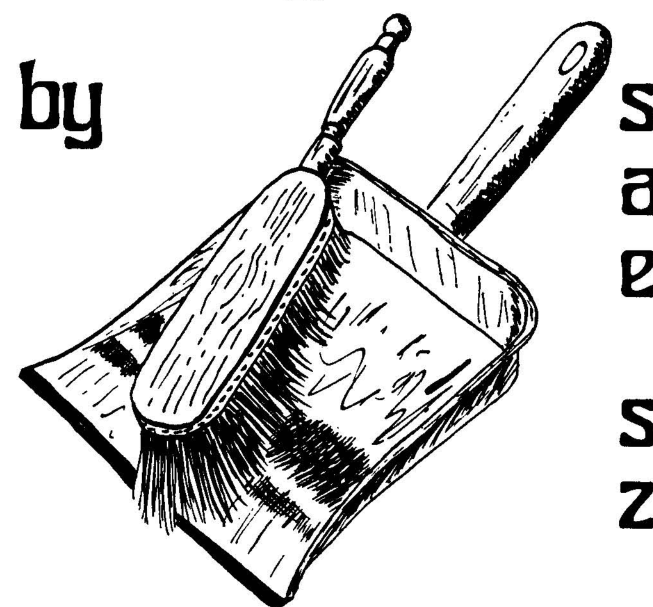

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