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# A SIMPLE MOLECULAR MODEL OF ADSORPTION CHROMATOGRAPHY

## III. SOLVENT COMPOSITION EFFECTS IN THIN-LAYER CHROMATOGRAPHY OF ACIDIC SOLUTES IN SYSTEMS OF THE TYPE: ELECTRON DONOR SOLVENT–POLYAMIDE

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SUMMARY

An equation is discussed relating the $R_M$ value of a proton donor solute with the composition of a binary developing solvent system of the type electron donor solvent + neutral diluting solvent. The equation was derived assuming that adsorption of the solute by polyamide is mainly governed by competitive H-bonding between the solute (proton donor) and the solvent and the polyamide (electron donors), the formation of solvation and adsorption complexes being described by the law of mass action. Experimental results for some solvent systems were found to be in agreement with theoretical predictions.

## INTRODUCTION

In the preceding papers of this series[1,2], the adsorption of the solute was considered as the reversible formation of molecular complexes between the solute, the solvent and the surface groups of the adsorbent, the law of mass action being applied to the H-bonding equilibria.

These considerations were applied to adsorption by silica; the surface silanol groups were considered as one of the components of a non-aqueous solution whose composition was expressed in mole fractions; the fundamental assumption was that the strongest non-solvated functional group of the solute is adsorbed by a non-solvated silanol group (neglecting non-specific solvation due to dispersion forces as much weaker than H-bonding). In other words, it was assumed that the probability of adsorption of a solute molecule by a silanol group, which is at the time H-bonded by a solvent molecule, is greatly diminished in comparison to adsorption by a free silanol group; the adsorption affinity is thus dependent on the fraction of time which an average silanol group spends in the free (not H-bonded) form. Applying the law of mass action to the competitive H-bonding equilibria and assuming certain simplifications, an equation was derived which postulated a linear relationship between the $R_M$ value

and logarithm of the mole fraction of the active solvent in the binary developing solvent[1] and a linear relationship between the $R_M$ values of related organic bases (adsorbed by single point adsorption) and their $pK_A$ values in aqueous solutions[2].

Silica belongs to a group of adsorbents with predominately proton donor properties[3-5]; it, therefore, seemed of interest to investigate whether the law of mass action could also be applied to adsorbents with different properties, first of all to polyamide which is an adsorbent commonly used in TLC, particularly in the chromatography of proton donor solutes, such as phenols, amino acids etc.[6].

Polyamide has a structure of linear molecules in which the –CO–NH– groups are separated by alkyl chains; the molecules are held together by a three-dimensional network of $=NH\cdots OC=$ hydrogen bonds[6]. In adsorption of proton donor molecules, the carbonyl groups play the role of active sites as a result of their increased basicity due to a mesomeric effect (*cf.* ref. 7, for formamide); however, the =NH groups can also contribute to adsorption properties, although in a minor degree, according to some reports in the literature[8]. Nitrocompounds and some other types of compounds can be also strongly adsorbed by interaction with free amino groups[6,9]. Certain strong solvents with small molecules ($CHCl_3$, $CH_2Cl_2$, $CH_2Cl$–$CH_2Cl$) can loosen the structure of polyamide forming a gel which can dissolve the molecules of the solute[6]; other solvents, *e.g.*, formic acid, can dissolve the polyamide completely.

From a theoretical viewpoint we shall consider the adsorption of a proton donor solute (class A or AB in the classification proposed by PIMENTEL AND MCCLELLAN[10]) from a mixed solvent of the N + B type (*i.e.*, diluting solvent, *e.g.*, cyclohexane, mixed with an electron donor solvent, *e.g.*, cyclohexanone). It is assumed that the adsorption of the solute is caused by H-bonding with the polyamide carbonyl groups and that the variation of the solvent composition does not entail changes in the polyamide structure. Under these conditions the scheme of molecular interactions involved can be represented by the diagram in Fig. 1a (solid lines represent H-bonding, dashed lines—dipole–dipole interactions, dotted lines—dispersion forces). Thus, as opposed to the adsorption by silica where the decisive process is competition of solute and solvent for active sites of the adsorbent surface (Fig. 1b), in the case of adsorption on polyamide the governing process is competition of the solvent and active sites for the solute (*cf.* BARK AND GRAHAM[11]). Solvation of the less strongly adsorbing part of the solute molecule is similar in the adsorbed and non-adsorbed state and thus is

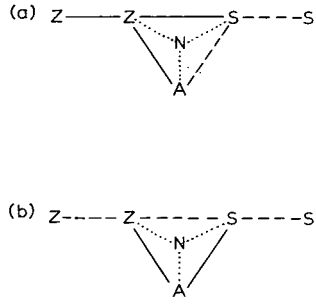



Fig. 1. Diagrammatic representation of interactions involved in adsorption from an electron donor solvent S diluted by an inert solvent N by polyamide (a, proton donor solute) and silica (b, electron donor solute).

largely cancelled (ref. 3 p. 186), association of the solvent S is relatively weak (dipole–dipole interactions) and so is the association of the solute in view of its low concentration and the presence of the electron donor solvent in the solution.

Since in the model considered the solvents are only weakly adsorbed relative to the solute, the Z–S interactions acquire the decisive role, that is to say, the solvation of solute molecules (or rather their active groups); since the same interactions also determine the solubility of the solute in the solvent, the adsorption of the solute can, in these cases, be expected to correlate with its solubility, contrary to the solvent–adsorbent systems in which solvent–solute interactions are of minor importance (see ref. 3, p. 230). In particular, parallelism of adsorption and solubility in a series of binary solvent mixtures could be anticipated (compare ref. 12 for partition and solubility).

As in the preceding papers[1,2] we will assume the components of the non-aqueous system to be the surface carbonyl groups of the polyamide (A); the developing solvent (N + S); the solute (Z); the complexes AZ (solute molecules immobilized at a given moment by H-bonding with the polyamide); and the (mobile) solvation complexes ZS and $ZS_2$ (the latter occur in the case of bifunctional solutes, *e.g.*, dihydroxy compounds). Thus, the composition can be expressed in mole fractions as:

$$X_A + X_S + X_N + X_Z + X_{AZ} + X_{ZS} + X_{ZS_2} = 1$$

As before, we can reasonably assume that

$$X_S + X_N \gg X_A \gg X_{AZ} + X_{ZS} + X_{ZS_2}$$

Applying the law of mass action, we have

$$A + Z \rightleftharpoons AZ \qquad K_{AZ} = X_{AZ}/X_A X_Z$$

the adsorption coefficient of solute Z then being

$$k^0 = X_{AZ}/X_Z = K_{AZ}X_A \tag{1}$$

Assuming that the two proton donor groups of the solute are equivalent, we have

$$Z + S \rightleftharpoons ZS \qquad K_{ZS} = X_{ZS}/X_Z X_S$$
$$ZS + S \rightleftharpoons ZS_2 \qquad K_{ZS_2} = X_{ZS_2}/X_{ZS}X_S$$
$$K_{ZS}K_{ZS_2} = X_{ZS_2}/X_Z X_S^2$$

The overall distribution coefficient, *i.e.*, the ratio of concentrations of the solute in the stationary phase (AZ) and in the mobile phase (Z, ZS and $ZS_2$) is

$$k = X_{AZ}/(X_Z + X_{ZS} + X_{ZS_2}) = X_{AZ}/X_Z(1 + K_{ZS}X_S + K_{ZS}K_{ZS_2}X_S^2)$$
$$k = k^0/(1 + K_{ZS}X_S + K_{ZS}K_{ZS_2}X_S^2) \tag{2}$$

$$R_M = \log k = \log k^0 - \log(1 + K_{ZS}X_S + K_{ZS}K_{ZS_2}X_S^2) \tag{3}$$

The terms in the parentheses belong to MICHAELIS' pH-functions for a biprotic electrolyte, and the last equation is analogous to the theoretical $R_M$ *vs.* pH relationship so that the analysis of the function given in an earlier paper (*cf.* ref. 13 p. 36, 37, eqn. 56) can be applied, assuming that $k^0$, $K_{ZS}$ and $K_{ZS_2}$ are constants. Under certain conditions, linear $R_M$ *vs.* log $X_S$ relationships are obtained, depending on the relative numerical values of the three terms in the parentheses:

$1 \gg K_{ZS}X_S + K_{ZS}K_{ZS_2}X_S^2$ $\quad X_Z \gg X_{ZS} + X_{ZS_2}$ $\quad$ slope = 0

$K_{ZS}X_S \gg 1 + K_{ZS}K_{ZS_2}X_S^2$ $\quad X_{ZS} \gg X_Z + X_{ZS_2}$ $\quad$ slope = 1*

(under these conditions the solute still behaves as a monohydroxy compound)

$K_{ZS}K_{ZS_2}X_S^2 \gg 1 + K_{ZS}X_S$ $\quad X_{ZS_2} \gg X_Z + X_{ZS}$ $\quad$ slope = 2

When double solvates cannot be formed ($K_{ZS_2} = 0$), eqn. 3 simplifies to an equation analogous to the $R_M$ *vs.* pH relationship of a monoprotic electrolyte:

$$R_M = \log k^0 - \log(1 + K_{ZS}X_S) \qquad (4)$$

the $R_M$ *vs.* $X_3$ line then having only two asymptotes: firstly for low $X_S$ values

$1 \gg K_{ZS}X_S$; $\quad X_Z \gg X_{ZS}$, $\quad$ slope = 0

and secondly at higher concentrations of S:

$K_{ZS}X_S \gg 1$, $\quad X_{ZS} \gg X_Z$, $\quad$ slope = 1

Theoretical $R_M$ *vs.* $X_S$ curves are illustrated in Fig. 2 for a monofunctional electrolyte Z′ and a bifunctional electrolyte Z″. The $R_M$ axis is directed downwards so that $R_M$ and $R_F$ both increase.

The above simplified model permits the effect of the molecular structure of the solute, solvent and adsorbent to be expressed as simple physico-chemical parameters, such as the mole fraction of the electron donor solvent, the adsorption coefficient $k^0$ of the non-solvated (not H-bonded) solute, solvation constants etc.

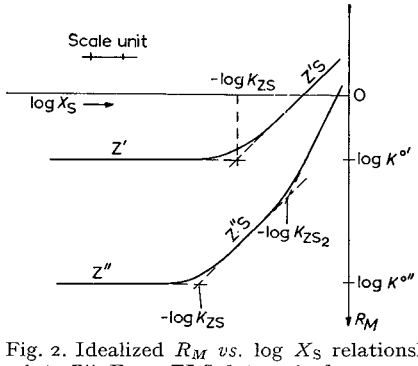



Fig. 2. Idealized $R_M$ *vs.* log $X_S$ relationships for a monohydroxyl solute Z′ and a dihydroxyl solute Z″. From TLC data only fragments of the relationships can be obtained ($R_M$ values in the range −1, +1).

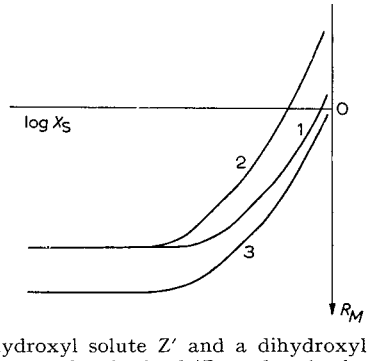



Fig. 3. Idealized $R_M$ *vs.* log $X_S$ relationships of a bifunctional solute adsorbed from three series of binary solvents: N + S (1), N + S′ (2) and N′ + S (3).

Because the intersection points of the asymptotes are determined by the formation constants of solvation complexes, (Fig. 2), a simple interpretation of the effect of the solvent S is possible: its increased basicity should increase the solvation constants $K_{ZS}$ and $K_{ZS_2}$ thus shifting the curve to the left so that the $-R_M$ value of the solute increases at lower concentrations of the active solvent. (Fig. 3, compare curves 1 and 2). Substitution of the diluting solvent influences the position and shape of the

* Absolute values of slopes are given.

curve through the values of $k^0$, $K_{AZ}$ and $K_{ZS_2}$. (Fig. 3, compare curves 1 and 3).

However, the simplifying assumptions introduced in the theoretical considerations may cause deviations from anticipated behaviour in real adsorption systems. Among the possible causes of deformation of the theoretical relationship the following should be mentioned:

(1) Formation of composition gradients (demixion) and variation of the ratio of solvent to adsorbent weight along the chromatogram.

(2) Variation of $k^0 = K_{AZ}X_A$ with the composition of the solvent due to:

(a) Changes of the activity coefficients,

(b) Variation of $X_A$ caused by changes in the structure of the adsorbent (*e.g.*, swelling) and by variations of the molar ratio of the solvent and surface adsorbent groups for a series of mixed solvents (see below).

(3) Not only $K_{AZ}$ but also $K_{ZS}$ and $K_{ZS_2}$ may vary with the composition of the solvent.

(4) The =NH groups can also contribute to adsorption, *e.g.*, by H-bonding with the solvent molecules, thus decreasing their concentration in the solution; however, most authors report only a minor contribution due to imido groups (*e.g.*, ref. 8). In addition, some effect due to desorption of the solvent molecules from the polyamide carbonyl groups can be expected; the effect will differ for the diluting solvent N and the electron donor solvent S, since the former interacts by dispersion forces and the second by dipole–dipole interactions (Fig. 1a) and although these two types of interactions are much weaker than H-bonding, the adsorption of the solute Z is, nevertheless, probably weakened at higher $X_S$ values in view of the competition of the solvent S for active sites on the adsorbent surface. Consequently, increase of $X_S$ may cause increased desorption of the solute not only by stronger solvation of its proton donor groups (formation of ZS and $ZS_2$ complexes) but also by increased competition of the solvent for the active sites. The latter effect results in $k^0$, the adsorption coefficient of non-solvated molecules of the solute, possibly being a decreasing function of $X_S$.

Since the problem of solvent composition effects has been considered from the viewpoint of its application to the theory of thin-layer chromatography, the practical applicability of the theoretical relationships was tested using the TLC technique, although more precise and reliable results could be obtained from column chromatography experiments where at least the evaporation of the solvent and gradient effects can be eliminated[3].

Solvent composition effects have great theoretical and practical significance since most developing liquids employed in liquid chromatography are mixed solvents. Before discussion of our experimental results, it is worthwhile discussing this briefly and comparing other approaches to this problem.

SNYDER[3] derived the following fundamental equation by applying the law of mass action to the displacement of solvent by solute from the adsorbent surface and assuming two-phase partition:

$$R_M = \log (V_a W/V^0) + \alpha(S^0 - A_S\varepsilon^0) \qquad (5)$$

where:

$V_a$ = volume of adsorption layer, proportional to the specific surface area of the adsorbent

$W$ = weight of adsorbent
$V^0$ = volume of solvent
$\alpha$ = adsorbent surface activity function
$S^0$ = adsorption energy of solute on adsorbent of unit activity ($\alpha = 1.00$) from pentane solutions ($\varepsilon^0 = 0$)
$A_S$ = area occupied by absorbed solute molecules (in 8.5 Å$^2$ units)
$\varepsilon^0$ = solvent strength parameter.

In systems of the type silica–electron donor solvent, anomalously large values of $A_S$ are obtained so that the parameter loses its simple physical sense (ref. 3, p. 202).

In the case of binary developing solvents composed of an inert diluting liquid and a strong solvent B($\varepsilon_b^0 \gg \varepsilon_a^0$), and moderately large mole fractions of B, the solvent strength of the mixed solvent is given by the approximate equation (ref. 3, eqn. 8–10a):

$$\varepsilon_{ab}^0 = \varepsilon_b^0 + \frac{\log X_b}{\alpha n_b} \tag{6}$$

where $X_b$ denotes mole fraction of the strong solvent and $n_b$ the area occupied by a single solvent molecule (in 8.5 Å$^2$ units equal to 1/6 of the area of benzene molecule). Combining the last two equations, we have

$$R_M = \log (V_a W/V^0) + \alpha S^0 - \alpha A_S \varepsilon_b^0 - \frac{A_S}{n_b} \log X_b \tag{7}$$

Assuming that the molecules of the solvent and solute displace each other in a 1:1 ratio, the $R_M$ value should be linearly dependent on $\log X_b$, the slope being 1. The remaining terms express various parameters determining the absolute value of $R_M$, *i.e.*, adsorption affinity of the solute ($S^0$) and solvent ($\varepsilon_b^0$) etc. However, the last equations mainly refer to situations where the governing process is the competition between the solute and solvent for the adsorbent surface.

OŚCIK *et al.*[14,15] applied the thermodynamic theory of conformal solutions to the analysis of solvent composition effects deriving the following equation for the adsorption of solutes from binary solvents:

$$R_M = u_1 R_{M_{(1)}} + u_2 R_{M_{(2)}} + (u_1^s - u_1)(\log k_{1,2}^{\infty} + R_{M_{(1)}} - R_{M_{(2)}}) + Y \tag{8}$$

where the subscript 1 denotes the active solvent (S) and 2—the diluting solvent (N), $u$ denotes volume fraction of the solvent, $u_1^s$—volume fraction of the solvent in the surface layer and $k_{1,2}^{\infty}$ is the hypothetical partition coefficient of the solute between solvents 1 and 2 (at a first approximation equal to the ratio of the partition coefficients of the solute in two partition systems: 1/water and 2/water, provided that solvents 1 and 2 are immiscible with water). $Y$ is a function of $u_1$; however, for conformal solutions its maximum value does not exceed 0.075 $R_M$ units and thus its omission does not introduce any significant error in most cases. The effects determining adsorption are expressed here by different parameters, thus, the adsorption affinity of the solute from pure component solvents is expressed by the values of $R_{M_{(1)}}$ and $R_{M_{(2)}}$; differences in solvation of the solute by the two solvents are reflected by the value of $k_{1,2}^{\infty}$; and the difference $u_1^s - u_1$ reflects the effect of the shape of the adsorption isotherm of the solvent S from solutions in the diluting solvent N. For dilute solutions of the active solvent (*i.e.*, $u_1 \to 0$) it may be assumed that $u_1^s \gg u_1$ and since the initial

part of the adsorption isotherm is usually linear ($u_1^s = \text{const} \cdot u_1$), it follows that

$$R_M \doteq u_1 R_{M_{(1)}} + u_2 R_{M_{(2)}} + \text{const} \cdot u_1 \qquad (9)$$

that is to say, $R_M$ is then a linear function of $u_1$. For higher concentrations of the strong solvent the $R_M$ *vs.* $u_1$ relationships were found to be parabolic in shape[15] and tend to straighten in $R_M$ *vs.* log $u_1$ plots (*cf.* ref. 16).

## EXPERIMENTAL

The phenols and their derivatives were chromatographed on thin layers of polyamide (polyamid-pulver, Merck AG, Darmstadt, G.F.R.). The polyamide was mixed with 45 ml of a 3:2 mixture of $CH_3OH$ and $CHCl_3$ and spread over 18 × 19 cm glass plates in layers *ca.* 0.1 mm thick, excess solvent was evaporated and the plates heated for 15 min at 80°[11]. After spotting the samples, the plates were conditioned for 1.5 h in the tanks and developed with binary mixed solvents; the spots were detected

TABLE I

SLOPES OF $R_M$ *vs.* LOG $X_S$ LINES FOR VARIOUS ELECTRON-DONOR SOLVENTS (S) DILUTED WITH CYCLOHEXANE

| *Solute* | | *Electron-donor solvent* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Acetone* | *Methyl ethyl ketone* | *Dioxane* | *Cyclo-hexanone* | *Tetra-hydro-furan* | *Diethyl ketone* |
| Phenol | P | 2.0 | 1.6 | 1.3 | 1.0 | 1.0 | 0.9 |
| *o*-Cresol | 2MP | 2.0 | 1.6 | 1.4 | 1.0 | 0.8 | — |
| *m*-Cresol | 3MP | 2.0 | 1.6 | 1.4 | 1.0 | 0.8 | — |
| *p*-Cresol | 4MP | 2.0 | 1.6 | 1.7 | 1.0 | 0.9 | — |
| 2,3-Xylenol | 23MP | 2.0 | 1.6 | 1.2 | 1.0 | 0.8 | 0.8 |
| 2,4-Xylenol | 24MP | 2.0 | 1.6 | 1.2 | 1.0 | 0.8 | 0.8 |
| 2,5-Xylenol | 25MP | 2.0 | 1.6 | 1.2 | 1.0 | 0.8 | 0.9 |
| 2,6-Xylenol | 26MP | — | — | — | — | 0.8 | — |
| 3,4-Xylenol | 34MP | 2.0 | 1.6 | 1.2 | 1.0 | 0.8 | 0.9 |
| 3,5-Xylenol | 35MP | 2.0 | 1.6 | 1.2 | 1.0 | 0.8 | 0.9 |
| Pyrocatechol | 12HB | 3.1 | 2.0 | — | 1.8 | — | 1.3 |
| Resorcinol | 13HB | 3.8 | 2.0 | — | 2.0 | — | — |
| Hydroquinone | 14HB | 3.8 | 2.0 | — | — | — | 1.4 |
| Phloroglucinol | 135HB | 5.4 | — | — | 2.7 | — | — |
| Pyrogallol | 123HB | — | — | — | — | 2.6 | — |
| *p*-Methoxyphenol | 13MOP | 2.0 | 1.4 | — | 0.9 | — | — |
| Orcinol (3,5-dihydroxytoluene) | OR | — | 2.2 | 2.0 | 1.5 | 2.5 | — |
| *o*-Nitrophenol | 2NP | 1.8 | — | — | — | 1.0 | — |
| *p*-Nitrophenol | 4NP | 2.1 | 2.0 | 2.0 | 1.0 | 0.8 | — |
| *m*-Aminophenol | 3AP | 2.7 | — | — | 1.4 | — | — |
| *p*-Aminophenol | 4AP | 3.0 | — | — | 1.6 | — | — |
| *o*-Chlorophenol | 2CP | — | 1.3 | 0.9 | 0.7 | 2.4 | — |
| *p*-Chlorophenol | 4CP | — | 1.5 | 0.9 | 0.7 | 2.4 | — |
| 1-Naphthol | 1HN | 1.9 | 1.4 | 0.9 | 0.7 | 2.4 | — |
| 2-Naphthol | 2HN | 1.9 | 1.4 | 0.9 | 0.7 | 2.4 | — |
| 1,6-Hydroxynaphthalene | 16HN | 3.9 | 2.1 | 2.0 | 2.0 | 4.0 | — |
| 2,7-Hydroxynaphthalene | 27HN | 3.9 | 2.1 | 2.0 | 2.0 | 4.0 | — |

TABLE II

$R_F \times 100$ VALUES FOR VARIOUS MOLE FRACTIONS OF THE ACTIVE SOLVENT

| *Solute* | | *Mole fractions of acetone* | | | | | *Mole fractions of methyl ethyl ketone* | | | | | *Mole fractions of diethyl ketone* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *0.2* | *0.35* | *0.5* | *0.65* | *0.8* | *0.1* | *0.2* | *0.4* | *0.6* | *0.8* | *0.2* | *0.4* | *0.6* | *0.8* |
| Phenol | P | 13 | 30 | 51 | 62 | 70 | 9 | 23 | 48 | 62 | — | 28 | 41 | 50 | 56 |
| *o*-Cresol | 2MP | 19 | 38 | 57 | 68 | 76 | 11 | 25 | 52 | 65 | — | 31 | 42 | 49 | 58 |
| *m*-Cresol | 3MP | 18 | 37 | 54 | 65 | 74 | 12 | 28 | 53 | 67 | — | 31 | 43 | 49 | 58 |
| *p*-Cresol | 4MP | 12 | 29 | 49 | 61 | 70 | 8 | 21 | 47 | 61 | — | 30 | 41 | 48 | 57 |
| 2,3-Xylenol | 23MP | 21 | 43 | 61 | 70 | 80 | 14 | 32 | 55 | 71 | — | 37 | 48 | 57 | 64 |
| 2,4-Xylenol | 24MP | 21 | 43 | 61 | 70 | 80 | 14 | 32 | 55 | 71 | — | 37 | 48 | 57 | 64 |
| 2,5-Xylenol | 25MP | 21 | 43 | 61 | 70 | 80 | 14 | 32 | 55 | 71 | — | 37 | 48 | 57 | 64 |
| 3,4-Xylenol | 34MP | 25 | 47 | 67 | 77 | — | 15 | 30 | 54 | 70 | 75 | 38 | 54 | 60 | 65 |
| 3,5-Xylenol | 35MP | 25 | 47 | 67 | 77 | — | 15 | 30 | 54 | 70 | 75 | 38 | 54 | 60 | 65 |
| Pyrocatechol | 12HB | — | 13 | 31 | 53 | 68 | — | 5 | 18 | 33 | 46 | 8 | 17 | 23 | 33 |
| Resorcinol | 13HB | — | 8 | 25 | 45 | 63 | — | 5 | 15 | 29 | 43 | — | — | — | — |
| Hydroquinone | 14HB | — | 10 | 33 | 56 | — | — | 5 | 17 | 31 | 43 | 7 | 15 | 24 | 35 |
| Phloroglucinol | 135HB | — | — | 10 | 30 | 52 | — | — | — | — | — | — | 3 | 7 | 14 |
| Pyrogallol | 123HB | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| *p*-Methoxyphenol | 13MOP | 14 | 33 | 53 | 66 | — | — | 29 | 48 | 60 | 71 | — | — | — | — |
| Orcinol (3,5-dihydroxytoluene) | OR | — | — | — | — | — | — | 5 | 15 | 31 | 47 | — | — | — | — |
| *o*-Nitrophenol | 2NP | 30 | 53 | 67 | 79 | — | — | — | — | — | — | — | — | — | — |
| *p*-Nitrophenol | 4NP | 7 | 16 | 30 | 45 | — | — | 6 | 21 | 38 | 49 | — | — | — | — |
| *m*-Aminophenol | 3AP | 5 | 16 | 37 | 57 | — | — | — | — | — | — | — | — | — | — |
| *p*-Aminophenol | 4AP | | | | | | — | — | — | — | — | — | — | — | — |
| *o*-Chlorophenol | 2CP | — | — | — | — | — | 14 | 20 | 38 | 50 | 59 | — | — | — | — |
| *p*-Chlorophenol | 4CP | — | — | — | — | — | 12 | 15 | 33 | 49 | 58 | — | — | — | — |
| 1-Naphthol | 1HN | 15 | 33 | 49 | 61 | 70 | 12 | 20 | 39 | 56 | 66 | — | — | — | — |
| 2-Naphthol | 2HN | 13 | 28 | 44 | 56 | 65 | 9 | 18 | 35 | 53 | 63 | — | — | — | — |
| 1,6-Hydroxynaphthalene | 16HN | — | 6 | 16 | 38 | 58 | — | — | 10 | 22 | 33 | — | — | — | — |
| 2,7-Hydroxynaphthalene | 27HN | — | 8 | 21 | 42 | 62 | — | — | 12 | 25 | 38 | — | — | — | — |

by coupling with bis-diazotized benzidine. The experimental results are presented in Figs. 4–9 as $R_M$ *vs.* log $X_S$ plots and are averages from three chromatograms.

## RESULTS AND DISCUSSION

The solutes are denoted by symbols (P = phenol, N = naphthalene, M = methyl, H = hydroxyl etc.; where 1,6 HN = 1,6-dihydroxynaphthalene, see Table I). The $R_M$ value is defined according to BATE-SMITH AND WESTALL. The experimental results are presented in Tables II, III and in Figs. 4–9.

The results obtained for the system cyclohexane + acetone are plotted in Fig. 4. Although linear $R_M$ *vs.* log $X_S$ relationships are observed, the slopes of the lines are not in agreement with theoretical predictions: Thus, for monohydroxyl compounds possessing additional weakly adsorbing groups ($-CH_3$, $-OCH_3$, $-NH_2$, $-NO_2$) the slopes exceed the expected values by 100% ($n = 2$, *cf.* Table I). Also in the case of dihydroxy compounds the slopes are higher than expected ($n = 4$ instead of 2), except for pyrocatechol where $n = 3$, *i.e.*, exceeds the expected value by 50%.

For systems of the type cyclohexane–methyl ethyl ketone (Fig. 5) the slopes

TABLE III

$R_F \times 100$ VALUES FOR VARIOUS MOLE FRACTIONS OF THE ACTIVE SOLVENT

| *Solute* | | *Mole fractions of dioxane* | | | | | *Mole fractions of cyclohexanone* | | | | *Mole fractions of tetrahydrofuran* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *0.1* | *0.2* | *0.4* | *0.6* | *0.8* | *0.2* | *0.4* | *0.6* | *0.8* | *0.1* | *0.2* | *0.3* | *0.4* | *0.45* | *0.6* | *0.7* | *0.8* |
| Phenol | P | 13 | 27 | 47 | 63 | — | 35 | 50 | 60 | 65 | 16 | 27 | 38 | — | 42 | 58 | 63 | 78 |
| *o*-Cresol | 2MP | 10 | 26 | 47 | 66 | — | 38 | 54 | 62 | 68 | 22 | 32 | 41 | — | 44 | 63 | 68 | 83 |
| *m*-Cresol | 3MP | 12 | 26 | 49 | 63 | — | 35 | 50 | 60 | 65 | 19 | 26 | 37 | — | 43 | 62 | 66 | 82 |
| *p*-Cresol | 4MP | 10 | 24 | 50 | 66 | — | 33 | 48 | 56 | 62 | 18 | 24 | 36 | — | 42 | 59 | 63 | 78 |
| 2,3-Xylenol | 23MP | 18 | 35 | 56 | 68 | — | 41 | 59 | 67 | 75 | 25 | 34 | 43 | — | 52 | 65 | 68 | 85 |
| 2,4-Xylenol | 24MP | 18 | 35 | 56 | 68 | — | 41 | 59 | 67 | 75 | 25 | 34 | 43 | — | 52 | 65 | 68 | 85 |
| 2,5-Xylenol | 25MP | 18 | 35 | 56 | 68 | — | 41 | 59 | 67 | 75 | 23 | 31 | 42 | — | 51 | 62 | 66 | 84 |
| 2,6-Xylenol | 26MP | — | — | — | — | — | — | — | — | — | 42 | 52 | 60 | — | 65 | 80 | 80 | 80 |
| 3,4-Xylenol | 34MP | 23 | 43 | 63 | 72 | — | 39 | 55 | 63 | 70 | 23 | 33 | 44 | — | 48 | 60 | 68 | 84 |
| 3,5-Xylenol | 35MP | 23 | 43 | 63 | 72 | — | 39 | 55 | 63 | 70 | 23 | 33 | 44 | — | 48 | 60 | 68 | 84 |
| Pyrocatechol | 12HB | — | — | — | — | — | 8 | 23 | 40 | 51 | — | 8 | — | 21 | — | 59 | — | 80 |
| Resorcinol | 13HB | — | — | — | — | — | 6 | 19 | 35 | 50 | — | 2 | — | 8 | — | 42 | — | 72 |
| Hydroquinone | 14HB | — | — | — | — | — | — | — | — | — | — | 7 | — | 16 | — | 50 | — | 77 |
| Phloroglucinol | 135HB | — | — | — | — | — | 2 | 8 | 20 | 35 | — | — | — | 4 | — | 20 | — | 60 |
| Pyrogallol | 123HB | — | — | — | — | — | — | — | — | — | — | — | 5 | — | 11 | 25 | 32 | 37 |
| *p*-Methoxyphenol | 13MOP | 35 | 50 | 60 | 63 | — | 40 | 55 | 62 | 68 | — | 5 | — | 12 | — | 29 | — | 51 |
| Orcinol (3,5-dihydroxy-toluene) | OR | — | 3 | 15 | 30 | 43 | 9 | 20 | 33 | 42 | — | 5 | 8 | — | 13 | 30 | 38 | 48 |
| *o*-Nitrophenol | 2NP | — | — | — | — | — | — | — | — | — | — | 56 | — | 71 | — | 76 | — | 83 |
| *p*-Nitrophenol | 4NP | — | 2 | 12 | 23 | 36 | 14 | 25 | 35 | 40 | 5 | 8 | 11 | — | 18 | 35 | 38 | 42 |
| *m*-Aminophenol | 3AP | — | — | — | — | — | 24 | 46 | 61 | 68 | — | 5 | — | 19 | — | 39 | — | 65 |
| *p*-Aminophenol | 4AP | — | — | — | — | — | 25 | 53 | 68 | 75 | — | — | — | — | — | — | — | — |
| *o*-Chlorophenol | 2CP | 19 | 31 | 46 | 55 | 62 | 39 | 50 | 58 | 62 | — | 20 | — | 51 | — | 77 | — | 86 |
| *p*-Chlorophenol | 4CP | 18 | 29 | 45 | 54 | 60 | 42 | 55 | 62 | 67 | — | 21 | — | 53 | — | 78 | — | 87 |
| 1-Naphthol | 1HN | 19 | 31 | 46 | 55 | 62 | 43 | 55 | 62 | 67 | 10 | 25 | — | 56 | — | 80 | — | 87 |
| 2-Naphthol | 2HN | 18 | 28 | 45 | 54 | 60 | 42 | 51 | 60 | 64 | 8 | 18 | — | 50 | | 78 | — | 87 |
| 1,6-Hydroxy-naphthalene | 16HN | — | — | 13 | 23 | 36 | 4 | 12 | 26 | 40 | — | — | — | 8 | — | 31 | — | 57 |
| 2,7-Hydroxy-naphthalene | 27HN | — | 6 | 20 | 31 | 46 | 5 | 17 | 33 | 48 | — | 3 | — | 11 | — | 35 | — | 61 |

are lower but still deviate from the expected values. Better results were obtained with diethyl ketone (Fig. 6), and especially with cyclohexanone (Fig. 7) when the monophenols with additional $-CH_3$ or $-OCH_3$ groups have unit slopes; for dihydroxybenzenes the slope is 2 and for the trihydroxy-benzenes 2.7 which approaches the expected value of 3.0. Good agreement with theoretical predictions was obtained with dioxane for dihydroxy-benzenes and some monohydroxy-phenols (Fig. 8). The data are incomplete because some of the dihydroxy compounds gave low $R_F$ values so that their $R_M$ *vs.* log $X_S$ relationships could not be determined.

In the case of pyrocatechol (1,2-dihydroxy-benzene) the slope was lower than for the remaining dihydroxy compounds, presumably due to an *ortho*-effect. ENDRES[17] has reported that in an aqueous system pyrocatechol behaves like a monophenol and explained this by the formation of an internal H-bond. In our experiments (non-aqueous systems) pyrocatechol gave slopes intermediate between those of monohydroxy- and dihydroxy-compounds.

For cyclohexane + tetrahydrofuran mixtures linear relationships of unit

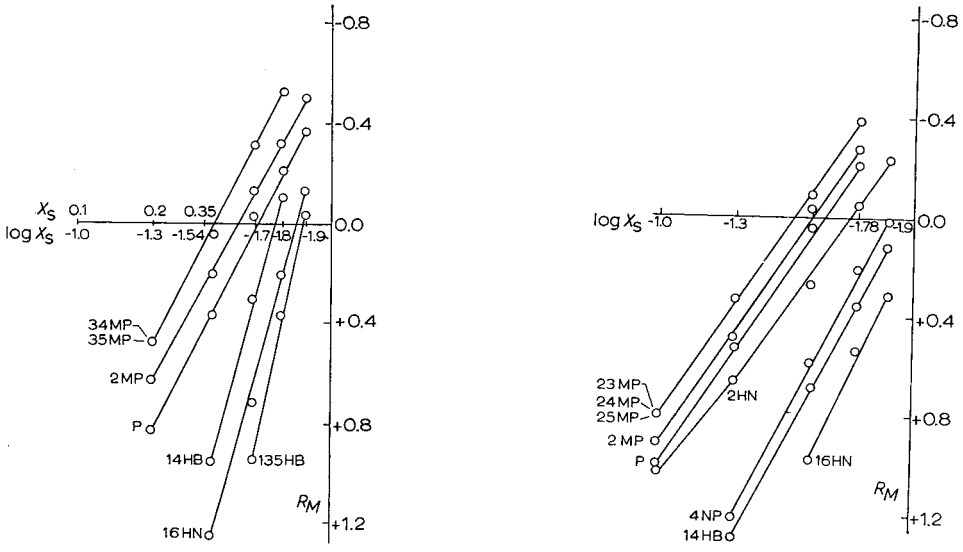



Fig. 4. Experimental $R_M$ *vs.* log $X_S$ relationships for developing solvents composed of cyclohexane and acetone. See Table I for notation of solutes.

Fig. 5. Experimental $R_M$ *vs.* log $X_S$ relationships for developing solvents composed of cyclohexane and methyl ethyl ketone.

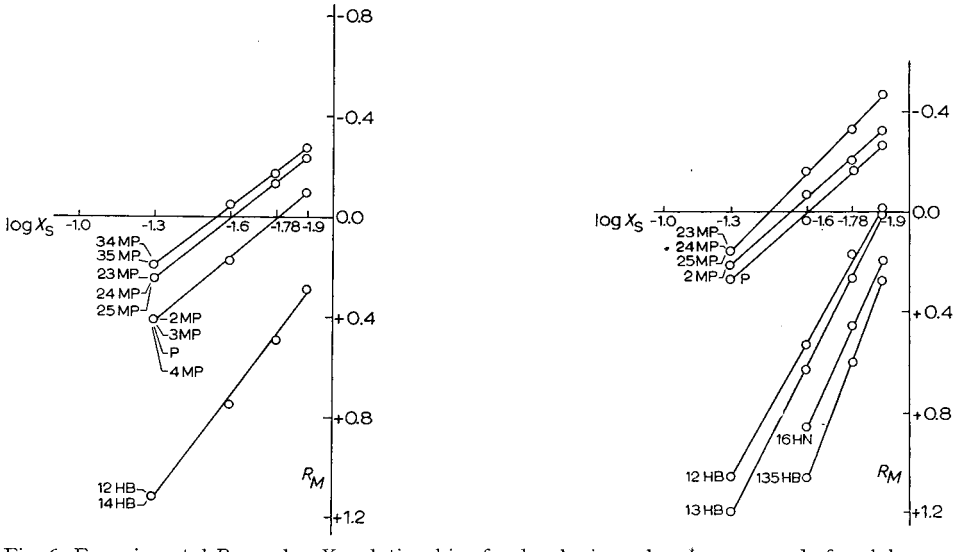
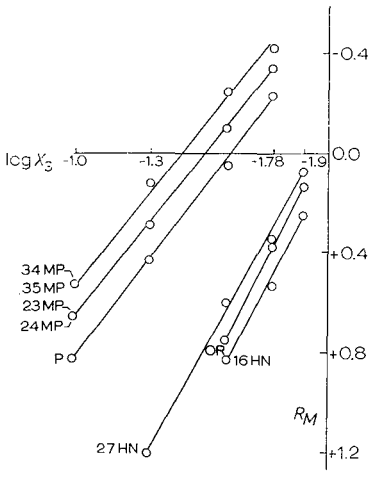



Fig. 6. Experimental $R_M$ *vs.* log $X_S$ relationships for developing solvents composed of cyclohexane and diethyl ketone.

Fig. 7. Experimental $R_M$ *vs.* log $X_S$ relationships for developing solvents composed of cyclohexane and cyclohexanone.



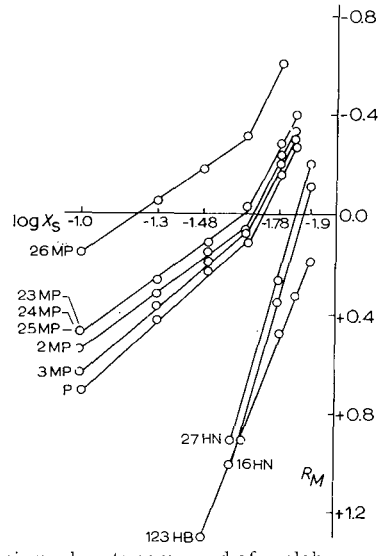




Fig. 8. Experimental $R_M$ *vs.* log $X_S$ relationships for developing solvents composed of cyclohexane and dioxane.

Fig. 9. Experimental $R_M$ *vs.* log $X_S$ relationships for developing solvents composed of cyclohexane and tetrahydrofuran.

slopes are observed up to $X_S = 0.5$, the slope increasing to 2.0 in the range of higher concentrations of tetrahydrofuran (Fig. 9). The dihydroxy-benzenes migrated only at higher concentrations of tetrahydrofuran, the slope being twice that of the expected value as in the acetone systems.

In view of complicating effects it was difficult to demonstrate a clear correlation between the basicity of the solvent and its elution power. Apparently the solvation power increases from acetone ($pK_A = -7.2$) through methyl ethyl ketone ($pK_A = -7.2$), to dioxane ($pK_A = -3.22$) and tetrahydrofuran ($pK_A = -2.08$). The strongest solvation effects were observed in the case of cyclohexanone ($pK_A = -6.8$) presumably due to the good accessibility of the carbonyl group. Steric factors can also play an important role in other solvents, and in the case of dioxane the fact that two ether oxygens are present in a single molecule should be taken into account. Moreover, even with solvents where the steric situation of the active group is similar (*e.g.*, a homologous series of ketones), the chromatographic data are not strictly comparable when the molecular level properties are considered, unless the molar volumes of the solvents are taken into account[2]. Even if it is assumed that the volume of the solvent per 1 g of the adsorbent ($V_{solv}/W_{ads}$) is constant for various solvents, the difference in the molar volumes of two solvents results in the molar ratio of the active groups of the solvent and of the adsorbent not being identical; for a solvent with a lower molar volume its active groups are in a larger excess, so that the mole fraction $X_A$ of the active groups of the adsorbent surface is lower. The same reasoning also applies to a series of mixed solvents where the molar volume of the active solvent (*e.g.* acetone) differs from that of the diluting solvent (*e.g.*, cyclohexane); $X_A$ (and thus also $k^o$, *cf.* eqn. 1) is then a decreasing function of $X_S$ and this effect may contribute to the

abnormally high slopes of the $R_M$ *vs.* log $X_S$ relationships (Fig. 4), the second contribution probably being due to competition of acetone molecules for the adsorption sites resulting from dipole–dipole interactions. For higher homologues, whose molar volumes are comparable to that of cyclohexane, both effects are less pronounced and the slopes are approximately equal to the expected values. The better agreement observed for diethyl ketone and cyclohexanone could thus be attributed to the following circumstances:

(1) Molar volumes comparable to that of the diluting solvent, cyclohexane.

(2) Lower molar volume concentrations of carbonyl groups even at high values of $X_S$ so that the variation of the formation constants with $X_S$ is reduced and the non-specific solvation of the surface carbonyl groups becomes differentiated less at higher and lower values of $X_S$.

(3) Any swelling of the polyamide is reduced in comparison to acetone systems.

(4) Decreased experimental errors due to changes in composition of the solvent caused by evaporation.

For solvents differing in molar volumes it may be advantageous to substitute mole fractions for molar concentrations; analogous $R_M$ *vs.* solvent composition relationships would then be obtained. The formation constants would then have other numerical values and would be more strongly dependent on the concentration of the active solvent, in comparison with the constants expressed in mole fractions.

The authors are aware of the fact that in view of the above mentioned complicating effects the relationships derived require further investigation and improvement, and at the present moment can be only regarded as semi-empirical; nevertheless, it is remarkable that in most cases linear $R_M$ *vs.* log $X_S$ relationships have been obtained, the slopes being different for monohydroxy-, dihydroxy- and trihydroxy-compounds; this partial agreement with theoretical prediction apparently suggests that in certain types of non-aqueous solvents the decisive mechanism is also solvation of the hydroxyl groups of the solute (*cf.* BARK AND GRAHAM, ref. 11). A further stimulus to present the provisional conclusions and experimental results is the importance of solvent composition effects in the theory of optimization of chromatographic solvent systems; moreover, the theoretical relationships suggest that the realization of the simplified model could allow one to determine the solvation constants ($K_{ZS}$,$K_{ZS_2}$) from $R_M$ *vs.* log $X_S$ relationships (see Figs. 2, 3), provided that an appropriately modified adsorbent of the polyamide type is employed and the complicating effects can be eliminated or accounted for.

ACKNOWLEDGEMENT

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CHROM. 4327

# THE RELIABILITY OF PEAK AREA MEASUREMENTS

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SUMMARY

The reliability of various methods of area measurement commonly employed is assessed, for peaks of varying symmetry. Some quantitative data on the use of the new technique of interval programmed integration are presented, and the value of the technique is compared with existing methods. A comparison is also made between the reliability of quantitative data based on peak areas using a differential detector and step heights using an integral detector.

## INTRODUCTION

The majority of gas chromatographic detectors have a differential response, and the composition of a mixture can be estimated either by measuring peak heights or peak areas, after calibration of the detector for the compounds under analysis. The reliability of a quantitative analysis will depend a great deal on the accuracy and precision to which the measurements can be made. A number of papers have been published[1–4] in which various methods of area measurement have been compared. It is not always possible to select the most suitable method for the problem in hand, based on the information presented in these papers. Most comparisons have been carried out on Gaussian or near Gaussian peaks, where several techniques are found to be fairly satisfactory. It is the purpose of this paper to compare several methods of area measurement with a new technique, for the common situations in which all peaks are resolved and fairly symmetrical, and for chromatograms exhibiting drifting baselines. A comparison is also made between the reliability of measurements of peak areas resulting from a differential detector response, and step heights arising from an integral detector.

## DISCUSSION

Using a differential detector the composition of a mixture can be estimated either by measuring peak heights or peak areas. It is more fundamental to measure peak areas in that the total area is proportional to the total amount of material present: this method is normally used where the detector response is a simple function of a stoicheometric property of the components. However, in cases where the detector

response is not predictable it is often adequate to use peak height measurements. The use of peak height which represents the concentration of the component at a given time, demands constant column operating conditions. For example, changes in the operating temperature during an analysis will affect retention times, and hence peak heights are affected, while the corresponding peak areas remain constant.

Commonly used methods of peak area measurement are: (1) cutting out and weighing, (2) planimetry, (3) triangulation, (4) peak height $\times$ width at half height, (5) peak height $\times$ retention distance, and (6) automatic integration. Of these, method 1 has the obvious disadvantages of destroying the chromatogram and of depending on the uniformity of the chart paper. Methods 2, 3, and 4 have been assessed by SCOTT AND GRANT[2], who concluded that method 4 was the most precise for symmetrical peaks. A comparison has been made[3] between methods 1, 2, and 3 for Gaussian peaks of varying height and width at half height ratios. The relative error was greatest for very sharp peaks, broad flat peaks, and those of small area. Minimum error was found for values lying between 1 and 4. It was shown that triangulation offered no advantages, and planimetry was less precise than height/width measurements for small areas but was capable of giving better precision for large areas. It was also found that a significant improvement in precision was obtained when peak width measurements were made much below the half height position. The peaks were not derived from a gas chromatographic system, but were manually constructed.

Method 5 assumes that column performance is independent of the substances under analysis, and that the calibration constants for the detector are proportional to retention volumes.

Method 6, which includes electromechanical and electronic methods of integration, produces results rapidly and demands a minimum of operator time. Electronic methods are costly, particularly when designed to cope with baseline drift.

The precision of an electromechanical and an electronic method of area measurement was compared by EMERY[4]. Most painstaking replicate analyses were carried out, and the resulting peak areas measured using a ball and disc integrator and a relatively expensive electronic integrator. The variation of the areas measured with the electronic integrator was about half that of the corresponding areas measured by the ball and disc integrator. The precision of manual peak height measurements carried out on the same chromatograms lay between the precision of these two methods. The effect of relative component concentration on precision, using only the electronic integrator was measured. Not unexpectedly precision, expressed as the coefficient of variation, improved with increasing proportion of component in the mixture.

DEANS[5] has examined the reliability of using peak height measurements alone to determine the percentage composition of mixtures. The results are compared with those obtained using electronic integrators, on a six-component mixture which included a partially resolved pair of components. All integrators tested, embracing the simplest to those incorporating drift correction devices, gave similar results which were significantly worse than the precision of the height measurements. The measurements were made on peaks of large height/width ratios, and using a system incorporating gas pressure controllers.

It must be recognised that a digital integrator, used conventionally, cannot begin counting until there has been a finite baseline shift, typically 0.2% full-scale deflection (f.s.d.) for a completely noise free signal. As a result the integrator will not

take into account any fraction of the peak area below this value. This is insignificant for symmetrical peaks, but could lead to a substantial error in the case of a peak exhibiting a long tail. For a simple digital integrator it is necessary to maintain a completely stable baseline throughout a run, and any drift in baseline above the 0.2% level will contribute to the peak areas. Recently a method has been described by YEEND[6], in which the integrator is deliberately set above zero when there is zero signal from the gas chromatographic detector. By means of a simple modification, described below, the number of integrator counts at fixed time intervals is recorded. It is thus possible to follow any drift in baseline by observing the change in count rate when no components are being eluted. On elution of a component the count rate of the integrator will change in the normal manner, but it will continue to print out at the same time interval. The peak area is obtained from the sum of the counts, above the zero signal count rate. The method has several advantages over the conventional operating procedure:

(i) detector baseline drift and integrator zero drift can be taken into account in peak area calculations;

(ii) there is no threshold below which the integrator does not count;

(iii) the integrator will count negative peaks without the need for a signal polarity reversal switch, provided that the zero count rate is set sufficiently high. In the conventional operating mode the integrator will not count negative peaks, and a polarity reversing switch can only be used satisfactorily if peaks are well separated;

(iv) the performance of the device should not be significantly affected by peak shape, and should not deteriorate for peaks exhibiting excessive tails.

Some results using this technique (interval programmed integration) are presented below.

Many of the difficulties in the integration of a differential signal would be avoided by the use of an integral detector, but few practical designs are commercially available. The precision and accuracy attainable with the mass detector have been the subject of recent publications[7–9]. For the purposes of comparison some further measurements were made and the results are presented below (Table IV).

## EXPERIMENTAL

Experiments were undertaken to compare the reliability of measurement of peak areas by two methods commonly regarded as fairly satisfactory, namely peak weight, and peak height $\times$ width at half height measurements. In addition a digital and mechanical integrator were used. The initial study was limited to a comparison of the reliability of various methods for completely resolved peaks, and peaks where only a small amount of distortion was observed. It is necessary to separate effects not caused by errors in area measurement. The results must be independent of errors caused by syringe delivery etc. This can be accomplished by injecting nominally the same amount of material of a two-component mixture several times, and expressing peak areas as percentage composition. The true composition of the mixture is not required to calculate the standard deviation of the results (precision), but it is required to find the bias (accuracy). The accuracy and precision of step height measurements, using the mass detector were measured under the same conditions. Mixtures of known

composition were prepared, the details of which are given in Table III. The first series of runs was performed using a Shandon KG2 Chromatograph fitted with a Martin gas density balance and mass detector. The output of the gas density balance was connected to a potentiometric recorder in the usual manner, and in addition to a digital integrator.* A total of 31 runs were performed, each using a nominal sample size of 3 $\mu$l. The peak areas of all runs were calculated from peak height and width measurements, and the results are given in Table IV. Peak heights, and step heights were of the order of 5 cm. The areas of fourteen of the runs were measured by the digital integrator used in the conventional manner. In this mode the integrator cannot begin counting until there is a finite baseline shift, which was set for this experiment at 0.5% f.s.d. Fifteen of the runs were carried out using the integrator in the interval programmed mode. The integrator was set above the zero count position when no signal was received from the gas density balance, and by means of a micro-switch printed out the number of counts at fixed intervals of time. The device used to trip the print-out mechanism of the integrator was made in the laboratory. To the shaft of a synchronous motor, geared to give a speed of rotation of 2 r.p.m. at 50 cycles $sec^{-1}$, was attached a $2\frac{1}{2}$ in. diameter disc. Near the circumference of the disc were attached, at equal distances apart, small protrusions made from 4BA screw heads. A wiping contact was positioned such that each screw head in turn was touched by the wiper as the disc rotated, thus momentarily completing an electrical circuit and causing the integrator to print. With four contacts spaced at intervals of 90°, print out will occur every 7.5 sec. Provision was made for print out at other time intervals by changing the number of contacts, and by using a 1 r.p.m. motor. For the device to be satisfactory the following conditions must be fulfilled: (i) mains frequency must not fluctuate significantly, (ii) the distance between each contact must be identical, and (iii) the wiper must always make contact at the same point on each head.

The performance of the device was checked by timing ten contacts starting at each contact point in turn. The results are given in Table I. The performance was regarded as satisfactory.

The peak areas of fifteen of the runs were obtained by cutting out the peaks and weighing them on an ordinary laboratory four-place balance. The repeatability of weighing a single peak was measured: no variation of results measured to 0.1 mg

TABLE I

VARIATIONS IN PRINT OUT TIMES

| *Contact No.* | *Time for ten contacts (sec)* | *Time per contact (sec)* |
|---|---|---|
| 1 | 74.8 | 7.48 |
| 2 | 74.9 | 7.49 |
| 3 | 75.0 | 7.50 |
| 4 | 75.0 | 7.50 |
| 1 | 75.1 | 7.51 |
| 2 | 75.1 | 7.51 |

* Honeywell Precision Integrator, Honeywell Controls Ltd., Brentford, Middlesex, Great Britain.

TABLE II

VARIATIONS IN CHART PAPER WEIGHT

$\sigma$ = standard deviation; $V$ = coefficient of variation.

| *No. of squares* | *Mean weight (mg)* | *σ (mg)* | *V (%)* |
|---|---|---|---|
| 6 | 12.00 | 0.36 | 3.0 |
| 6 | 11.62 | 0.21 | 1.8 |

(1% of the total weight) was observed. The variation of weight of the chart paper over the length containing the runs was measured by cutting out small squares of equal size, about the weight of a typical peak. The results are given in Table II.

Published data[3] on the coefficient of variation of recorder chart paper weight gives a value of 2.5% for the same area of paper.

Variations in chart speed during a run will affect peak areas obtained by all methods except the digital integrator: no measurable variations in chart speed were observed, and the accuracy of the speed on each setting was quite satisfactory.

The performance of a ball and disc integrator* was assessed. The integrator was attached to a potentiometric recorder, and a similar mixture to that used above was analysed using a Pye 104 chromatograph fitted with a flame ionisation detector. Sample details are given in Table III and results in Table IV.

Data published by SCOTT AND GRANT[2] have been recalculated in the form used in the present work and are given at the foot of Table IV.

TABLE III

SAMPLE DETAILS

GDB = gas density balance; MD = mass detector; FID = flame ionisation detector.

| *Series No.* | *Peak area ($cm^2$)* | *Sample composition* | | *Area correction factors* | | |
|---|---|---|---|---|---|---|
| | | *Compound* | *True % weight* | *GDB* | *MD* | *FID* |
| 1 | 1.7 | *n*-Propyl alcohol | 44.94 | 1.87 | 1.00 | — |
| | 2.8 | Methyl *n*-propyl ketone | 55.06 | 1.48 | 1.00 | — |
| 2 | | *n*-Propyl alcohol | 47.07 | — | — | 1.64 |
| | | Methyl *n*-propyl ketone | 52.93 | — | — | 1.41 |

The most satisfactory peak area measurements were obtained using the digital integrator, but contrary to expectations, better results were obtained using the integrator in the conventional mode. The peak areas under study were "typical" rather than ideal peaks. For a very broad and low peak it would be expected that the results obtained using the integrator conventionally would become poorer, but that the performance of the integrator using the fixed interval print-out technique would

* Disc Series 200 Integrator, Disc Instruments Ltd., Hemel Hempstead, Hertfordshire, Great Britain.

TABLE IV

RELIABILITY OF AREA MEASUREMENTS

$n$ = No. of determinations; $\bar{x}$ = mean % weight of $n$-propyl alcohol; $\sigma$ = standard deviation; $V$ = coefficient of variation.

| *Method of area measurement* | *Series* | $n$ | $\bar{x}$ | $\sigma$ | $V$ | *Bias* | *% bias* |
|---|---|---|---|---|---|---|---|
| Peak height/width | 1 | 31 | 43.41 | 1.4 | 2.9 | −1.53 | 3.4 |
| Peak weight | 1 | 15 | 44.90 | 2.1 | 4.7 | −0.04 | 0.1 |
| Digital integrator:conventional | 1 | 14 | 45.12 | 1.2 | 2.5 | +0.18 | 0.4 |
| fixed interval print out | 1 | 15 | 46.20 | 1.1 | 3.0 | +1.26 | 2.8 |
| Step height (mass detector) | 1 | 8 | 45.36 | 0.9 | 2.0 | +0.43 | 1.0 |
| Ball and disc integrator | 2 | 18 | 45.54 | 1.0 | 2.2 | +2.25 | 4.8 |
| Peak width and height | ref. 2 | 20 | 23.37 | 0.28 | 1.2 | | |
| Triangulation | ref. 2 | 20 | 22.56 | 0.60 | 2.7 | | |
| Planimetry | ref. 2 | 16 | 23.49 | 1.23 | 5.2 | | |

be unaffected. A similar result would occur for a peak with a long tail, irrespective of its height. The precision of the results obtained using the ball and disc integrator was equally satisfactory. Peak weight determinations gave a very accurate result, but the coefficient of variation was high due in part to variations in paper weight (Table II). The mass detector gave results as good as the best peak area results.

The effect of a drifting baseline on the precision and accuracy of several methods of area measurement was measured, using a Pye 104 chromatograph fitted with a katharometer and using helium as carrier gas. Each area was measured manually using the peak height/width method, and by two digital integrators connected in parallel, one used in the conventional mode* and the other in the interval programmed mode**. The interval timer was made from a commercially available timer*** fitted with a synchronous motor geared to give 6 r.p.m. A single wiping contact on the motor shaft tripped a micro-switch once per revolution, giving a print out interval of 10.0 sec.

TABLE V

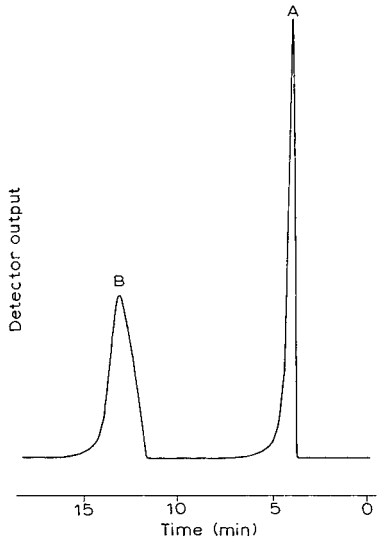
SAMPLE DETAILS

| *Series No.* | *Peak area ($cm^2$)* | *Sample composition* | | *Drift (% f.s.d./min)* | *Area correction factor* |
|---|---|---|---|---|---|
| | | *Compound* | *True % composition* | | |
| 3 | 4.5 | *n*-Propyl alcohol | 42.69 | zero | 0.72 |
| | 5.0 | Isoamyl alcohol | 57.31 | zero | 0.81 |
| 4 | 4.5 | *n*-Propyl alcohol | 42.69 | zero | 0.72 |
| | 5.0 | Isoamyl alcohol | 57.31 | 0.38 | 0.81 |
| 5 | 4.5 | *n*-Propyl alcohol | 42.71 | 1.3 | 0.72 |
| | 5.0 | Isoamyl alcohol | 57.29 | 0.23 | 0.81 |

* Kent Chromalog I Integrator, Kent Instruments Ltd., Luton, Bedfordshire, Great Britain.

** Honeywell Precision Integrator, Honeywell Controls Ltd., Brentford, Middlesex, Great Britain.

*** Constant cycle sequence timer type 221-1, motor type 392, Crouzet (England) Ltd., Brentford, Middlesex, Great Britain.



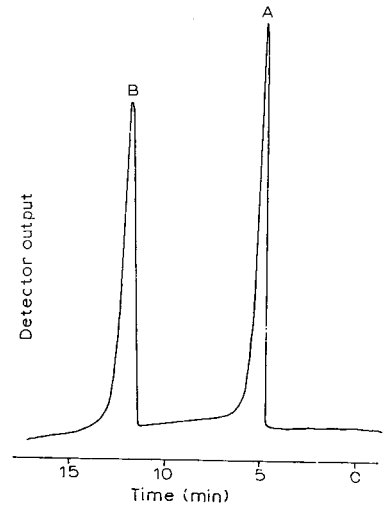




Fig. 1. Chromatogram of a two-component mixture (series 3). A = *n*-propyl alcohol; B = isoamyl alcohol.

Fig. 2. Chromatogram of a two-component mixture (series 4). A = *n*-propyl alcohol; B = isoamyl alcohol.

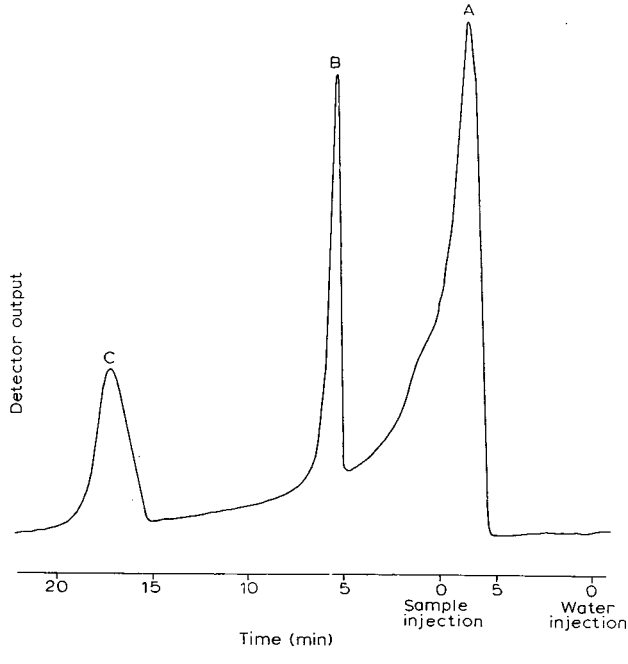



Fig. 3. Chromatogram of a two-component mixture (series 5). A = water; B = *n*-propyl alcohol; C = isoamyl alcohol.

Provision was made for several contacts per revolution and for the interchange of motors of different speeds of rotation. Several sets of runs were performed, each consisting of twenty replicate analyses, the chromatograms of each set exhibiting different degrees of drift (see Figs. 1–3). All peaks exhibited a tail. Reproducibly drifting baselines were induced, in the case of series 4 by temperature programming, and in the case of series 5 by injecting 5 $\mu$l of water 8.0 min before the injection of each sample so that the sample was eluted on the tail of the water peak. A comparison of the reliability of the three methods is given in Table VI. Details of the samples analysed are given in Table V.

TABLE VI

RELIABILITY OF AREA MEASUREMENTS (DRIFTING BASELINE)

| *Method* | *Series* | $\bar{x}$ | $\sigma$ | $V$ | *Bias* | *% bias* |
|---|---|---|---|---|---|---|
| Peak height/width | 3 | 42.41 | 0.95 | 2.18 | −0.27 | 0.63 |
| | 4 | 41.07 | 1.25 | 3.03 | −1.62 | 3.79 |
| | 5 | 43.01 | 0.61 | 1.42 | +0.31 | 0.73 |
| Integrator–conventional mode | 3 | 43.75 | 0.18 | 0.41 | +1.07 | 2.50 |
| | 4 | 45.81 | 1.33 | 2.90 | +3.13 | 7.32 |
| | 5 | 49.52 | 7.17 | 14.48 | +7.82 | 18.4 |
| Integrator–interval programmed mode | 3 | 44.19 | 0.31 | 0.71 | +1.50 | 3.52 |
| | 4 | 42.62 | 0.78 | 1.82 | −0.06 | 0.14 |
| | 5 | 45.34 | 0.46 | 1.01 | +2.64 | 6.17 |

The precision of the manual method was significantly worse than that of the integrators for a straight baseline (series 3), but did not deteriorate as drift increased. The results using the integrator in the conventional mode became progressively worse as baseline drift increased and were excessively poor for series 5 runs. Using the interval programmed technique precision was unaffected by baseline drift and in all cases was satisfactory.

TABLE VII

SAMPLE DETAILS (SERIES 6)

| *Compound* | | *% weight* | *Baseline change (% f.s.d./min)* | *Area correction factor* |
|---|---|---|---|---|
| Solute 4% | *n*-Butyl alcohol | 47.65 | 14.7 | 1.15 |
| | Methyl isobutyl ketone | 52.35 | 2.6 | 0.86 |
| | | 100.00 | | |
| Solvent 96% | Acetone | 20 | | |
| | Water | 80 | | |
| | | 100 | | |

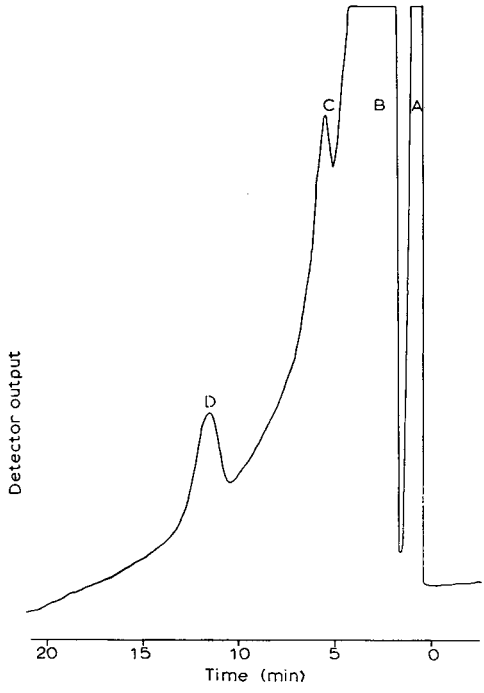



Fig. 4. Chromatogram of a two-component mixture in solvent (series 6). A = acetone; B = water; C = *n*-butyl alcohol; D = methyl isobutyl ketone.

The difficulty of estimating the proportions of components eluted on the tail of a solvent is illustrated in Fig. 4. Clearly it is not possible to use peak height/width measurements since the leading edge of the first peak is only a third of the height of the tailing edge. Peak height alone, or peak weight could be used, bearing in mind the disadvantages previously noted. The new technique of interval programmed integration may offer advantages, and this method was compared with those mentioned above. Sample details are given in Table VII, and results in Table VIII.

Elution of the solutes in the presence of excess solvents will result in a change in response of the detector. Previous results (Table IV) showed that although the peak weight method is not very precise it gives good accuracy. The relative response of the detector was calculated from peak weight measurements assuming zero bias and was

TABLE VIII

RELIABILITY OF MEASUREMENTS (IN THE PRESENCE OF SOLVENT)

| *Method* | $\sigma$ | $V$ | *Bias* | *% bias* |
|---|---|---|---|---|
| Peak weight | 1.58 | 3.33 | — | — |
| Peak height | 0.82 | 1.40 | — | — |
| Interval programmed integration | 4.09 | 11.42 | −2.57 | 5.39 |

used to find the bias of the integrator results. Response factors based on height measurements will be different, so that it was not possible to compare the accuracy of the three methods. However, in view of the nature of the chromatograms and the fact that the solute represented only 4% of the solution, the accuracy of the interval programmed method is acceptable. Although precision in this case is significantly worse than using the other methods, in the long-term interval programmed integration may be preferable since the limitations associated with the other methods are not inherent in interval programming.

## CONCLUSIONS

No single method of area measurement is satisfactory for all types of gas chromatographic peak. Triangulation and planimetry offer no advantages over the other methods. The peak weight method is useful for grossly asymmetric peaks, and gives good accuracy, although precision is rather poor. Quantitative results obtained using the peak height/width method are satisfactory even for chromatograms exhibiting a small degree of baseline drift. The ball and disc, and digital integrators, were satisfactory for symmetrical peaks with a stable baseline. The performance of a digital integrator (without baseline drift correction facilities) used in the conventional mode becomes progressively worse as baseline drift increases. Interval programmed integration was found to give good results not significantly affected by tailing peaks or drifting baselines, and was able to give acceptable results even for an extreme case in which minor amounts of solute were eluted in the presence of a solvent. The technique offers a means of obtaining good precision and accuracy for a variety of peak geometries, by a simple modification to a basic digital integrator. Quantitative analyses based on step height measurements, using an integral detector, were entirely satisfactory.

## ACKNOWLEDGEMENTS

The first set of comparative data is based on work carried out by T.A.G. at Brunel University. T.A.G. wishes to thank R. YEEND with whom discussions on interval programmed integration were held. The help of Dr. P. B. STOCKWELL, who prepared a computer programme for some of the statistical evaluations, is acknowledged.

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CHROM. 4336

# GAS–LIQUID CHROMATOGRAPHY OF PROTEIN AMINO ACID TRIMETHYLSILYL DERIVATIVES*

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SUMMARY

The purpose of the investigation was to make a thorough study of the chemistry of derivatization of the twenty protein amino acids as their N-trimethylsilyl trimethylsilyl (TMS) esters. Major emphasis was directed toward chromatographic separation of the derivatives, precision and accuracy of the method, silylation as a function of reaction temperature and time, molar excess of reactants, stability of the TMS derivatives, quantitative analysis of a synthetic amino acid mixture, and application to biological samples.

The gas–liquid chromatcgraphic separation of the N-trimethylsilyl TMS esters of the twenty protein amino acids was achieved after evaluation of a number of combinations of siloxane liquid phases. The final chromatographic conditions used for the total separation on a single column for all twenty of the amino acids consisted of a mixed liquid phase of 3.0 w/w% OV-7 and 1.5 w/w% OV-22 coated on high-performance 100/120 mesh Chromosorb G in a 1.75 m × 4 mm I.D. U-shaped borosilicate glass column. Phenanthrene was a suitable internal standard as it was completely resolved from the TMS amino acids. The instrumental settings were 75°, initial hold 7 min, program rate 2°/min, and carrier flow ($N_2$) of 42 ml/min for fourteen of the amino acids, and 100° initial column temperature for the other six. Prior to chromatography, it is essential to analyze performance blanks under the same chromatographic and instrumental conditions to establish the purity of all chemical reagents.

The reaction conditions were investigated for the quantitative silylation of the twenty amino acids. Fourteen of the amino acids were reproducibly converted to the respective TMS derivatives in 15 min at 135° in a closed vial using a 30 molar excess of bis(trimethylsilyl)trifluoroacetamide (BSTFA)/total amino acids. A comparison of various silylation temperatures showed that silylation at 135° produced the most

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reproducible relative molar response values. Also, molar excess studies showed that a 30 molar excess of BSTFA was adequate. This molar excess corresponds to 0.24 ml of BSTFA/mg of amino acid. Acetonitrile was used as a solvent in a 1:1 ratio with BSTFA.

For six of the amino acids (glutamic acid, arginine, lysine, histidine, tryptophan, and cystine) the reaction conditions required were 4 h at 135°. For a complete analysis of all twenty of the protein amino acids, the sample is heated for 15 min at 135° in a closed tube with a teflon-lined screw cap, *cooled within* 3 min, then an aliquot is injected into the gas chromatograph. This analysis provides data for the quantitative determination of alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, hydroxyproline, aspartic acid, methionine, cysteine, phenylalanine, and tyrosine. After injection of the sample, the reaction vial is again tightly recapped, placed in a 135° bath for 4 h, again cooled, and an aliquot injected. After the first injection the column was programmed to about 225° and then cooled to the initial holding temperature of 100° prior to the second injection. This gives the analysis for $GLU_2$, $ARG_4$, $LYS_4$, $HIS_3$, $TRY_3$, and $CYS_4$. The subscripts denote the number of TMS groups attached. The chromatographic time is about 50 min for the six amino acids.

A synthetic known mixture at a 1.0 mg level each of selected amino acids was analyzed to determine the quantitation of the total derivatization and chromatographic method. The accuracy was found to be very good, with recoveries ranging from 93.6 to 105.9%, with an average recovery of 100.8%. The average relative standard deviation (RSD) for the relative molar response values of fourteen of the amino acids was 0.80% and 1.25% for the remaining six amino acids.

The N-trimethylsilyl TMS amino acid esters were found to be completely stable for a period of five to seven days when stored at room temperature in a tightly capped vial. Glycine was the only exception, and deteriorated within a period of 3 h. Glycine changed from $GLY_2$ to $GLY_3$. In preliminary experiments, the minimal detectable amount of the TMS derivatives that can be detected in a flame ionization detector at a signal/noise level of 3:1 was found to be 0.3 to 0.5 ng, or $3 \times 10^{-12}$ moles of each amino acid injected. It is most important that the experimental and instrumental conditions as outlined in this manuscript be followed precisely.

The analysis of ribonuclease as the TMS amino acid derivatives established the applicability of this method for the quantitative analysis of biological materials. The data obtained from the GLC analysis of ribonuclease were found to be in agreement with the values obtained by classical ion-exchange chromatography.

The TMS derivatives of urea, ornithine, citrulline, and ammonium chloride were synthesized and silylated at 135° for both 15 min and 4 h. The second peak for urea increased relative to the first with an increasing reaction time; however, an overall decrease in peak size was noted when silylated for 4 h. On silylation at 150° for 4 h, a further decrease in peak size was noted. Citrulline was found to yield three chromatographic peaks when heated at 135° for 15 min. On heating for 4 h at this temperature, only two peaks were observed, with the higher retention temperature peak being the largest. No chromatographic peaks were observed for ammonium chloride when silylated under any of the above experimental conditions. A complete silylation of ornithine required reacting at 135° for 4 h.

## INTRODUCTION

The amino acids have been the subject of intense study by biochemists, nutritionists, medical scientists, biologists, bacteriologists, and investigators in many other areas of science during the past ten years. The literature in this field is immense and provides broad evidence of the striking advances which have been made since the isolation of the first amino acids, asparagine and cystine, about 160 years ago. Since this time, the existence of more than 170 amino acids has been reported, most of them in the last decade. The research on amino acids has been mainly directed toward: (a) a consideration of the amino acids which occur in nature and the forms in which they have been found; (b) observations resulting from nutritional studies; (c) the metabolism of the amino acids, their synthesis, degradation, and the relationships to other metabolites; (d) studies on abnormalities of amino acid metabolism associated with disease states; and (e) protein biosynthesis. In this latter area the scientific advances have been rapid and dramatic during recent years in studies on protein structure. Only some fourteen years separate the reporting by SANGER, Nobel Laureate, of the primary structure of insulin from its complete synthesis almost simultaneously in three different countries.

The increasing activity in this field means that many scientists are faced with the problem of accurate, sensitive, precise, and rapid amino acid analyses.

In the period of 1950 to 1969 the elegant investigations of MOORE, SPACKMAN, STEIN, HAMILTON, PIEZ, and others have developed classical ion-exchange chromatography into a refined method for amino acid analysis. The methods now range from the relatively simple and inexpensive, to the sophisticated, completely automated, and costly.

Also, during the past ten years GLC techniques have reached great sophistication, instruments have become more sensitive and dependable, and there has been a steady flow of reports on the application of GLC to biomedical problems. With such extensive use of GLC for carbohydrates, steroids, lipids, metabolites, and drugs of all kinds, it is at first sight strange that similar methods for the routine analysis of amino acids, compounds of simple structure but of great biochemical interest, have only recently begun to appear. During this same period, research into the functions and behavior of amino acids has formed a fair proportion of the phenomenal research effort that has yielded our present knowledge of the structure of peptides and proteins and of their biosynthesis.

Further, the great upsurge of interest in amino acids and proteins in the last twenty years has led to the development of automated analytical techniques and instrumentation as stated earlier, and their availability has not only taken some of the urgency out of the development of gas chromatographic methods but has also tended to set up resistance to an approach that threatens obsolescence to sophisticated and expensive classical ion-exchange equipment. However, anyone who has used GLC does not need to be convinced of the speed and sensitivity of the method, and any operator of the amino acid analyzer equipment should be further convinced on the grounds of convenience.

The origin of the protein amino acids lies far back in the evolution of life, and chemically they have little in common but their $\alpha$-carbon atom, with its steric L-configuration depending on the attachment of four different groups. Including these,

there are a dozen different organic chemical functional groups, and it is difficult to devise reaction schemes that will deal successfully with all of these groups with their chemical differences and varying reactivities. However, for a satisfactory analysis by GLC a substantially complete derivatization is necessary. GLC of amino acids has been held back by the lack of agreement on the volatile derivative that should be used. Although there are only about twenty amino acids commonly found in proteins, they have varied chemical structures and it would appear that this would permit an easy separation. This has not necessarily been found to be true, and quantitative derivatization of all the functional groups under a single set of experimental conditions has been most difficult.

The research efforts of GEHRKE, co-workers, and graduate students during the period of 1964–1969 have led to the development of a general quantitative GLC method for the twenty natural protein amino acids and of their complete separation. The derivative of final choice was the N-trifluoroacetyl (N-TFA) *n*-butyl ester. Studies were made on yield, volatility, stability, limit of detection, and general applicability. In our research the following criteria were considered most important for a suitable volatile derivative: (a) no rearrangements or structural alteration should occur during formation; (b) derivatization reaction should be 95 to 100% complete; (c) no sample loss on concentration; (d) stability with respect to time; (e) derivative must have increased volatility; and (f) there must be little or no reactivity of the derivative with the substrate and/or support phase. From this research a good quantitative method was developed, and one which met most of the criteria outlined above. However, there is still need of further simplification of the N-TFA *n*-butyl ester derivatization reaction, for a method which will lend itself to automation, and for a derivative that will be simpler in certain of its chromatographic aspects; as well as for entirely new derivatization and chromatographic approaches.

It was the purpose of this investigation to make a thorough study of the chemistry of derivatization of the twenty protein amino acids as their N-trimethylsilyl TMS esters; emphasis was directed toward precision and accuracy of the method, single column separation, substrate–derivative interaction, recovery, stability of derivative, and application to analysis of amino acids in biological samples.

## LITERATURE REVIEW

The trimethylsilyl (TMS) group was introduced by RÜHLMANN AND GIESECKE[1] in 1961 for the GLC analysis of amino acids before BENTLEY *et al.*[2] adopted the TMS group in the carbohydrate field in 1963.

RÜHLMANN AND MICHAEL[3,4] introduced the TMS derivatives into the field of GLC, and reported that after silylation with trimethylsilyldiethylamine (TMSDA) several amino acids could be chromatographed on a column containing 19% silicone oil on Sterchamol. They chromatographed fourteen TMS amino acids, but only valine and phenylalanine were completely resolved, and decomposition was noted for cysteine, lysine, and histidine. Also, RÜHLMANN AND MICHAEL[4] discovered amino acid TMS esters as artifacts in the gas chromatographic analysis of N-TMS amino acid TMS esters, from which they were produced by ammonolysis. The TMS esters (free amino groups) were more volatile than the N-TMS amino acid TMS esters, and a quantitative single-step reaction was devised for their preparation. These authors report quanti-

tative results, but no peaks were obtained for the basic amino acids, presumably because being diamines they would not be sufficiently volatile.

It is well known that gas chromatography of the free bases of amino acid esters is attended by problems associated with the free amino groups. Free amino groups do not lend themselves to GLC and therefore are much better acylated, silylated, or derivatized in some way.

BENTLEY *et al.*[2] dissolved or suspended carbohydrates in pyridine and reacted the mixture with hexamethyldisilazane (HMDS) at room temperature using trimethylchlorosilane (TMCS) as a catalyst, and investigated the analysis of the TMS derivatives of the sugars by GLC.

Until 1965, only a few researchers experimented with the TMS derivatives of the amino acids and with limited success due to the instability of the N-TMS group and the non-availability of an effective silylating reagent. Following the development of some new silylating reagents, studies were renewed on the preparation of the TMS derivatives of the amino acids and it was then considered that the TMS derivatives hold considerable promise as the reaction is complete in one step.

SMITH and coworkers[5,6], in 1965 and 1966, investigated the reaction conditions for the formation of TMS derivatives of amino acids using HMDS and TMCS with different catalysts. They also studied TMSDA, which gave the highest yields (from 89% to 99%, for leucine, serine, and aspartic acid). The boiling points ranged from 76° at 15 mm Hg for alanine, to 147° at 4 mm Hg for lysine. They concluded that TMS diethylamine with some kind of catalyst gave the highest yields of the derivatives of leucine, serine, and aspartic acid. TMS dimethylamine recently has been claimed to react more rapidly and to be more volatile and is recommended for these reasons[7]. BIRKOFER AND DONIKE have suggested the use of N-TMS-N-methyl-acetamide or N-TMS-N-methyl-formamide[8], and KLEBE *et al.* in 1966 reported studies on the synthesis and application of the now widely used N,O-bis(trimethylsilyl)acetamide (BSA)[9] and were able to obtain "sharp single peaks for all the amino acids except arginine, which showed indications of decomposition on the column". Also, the derivatives of glycine and alanine could not be separated from one of the reaction products of BSA, mono(trimethylsilyl)acetamide (MSA), due to its similar chromatographic retention on the SE-30 column they used.

BLAU[10], in a general review of the analysis of amino acids by GLC, cites some further reports on silylation methods by RÜHLMANN AND MICHAEL. They studied the properties of a number of other silylated derivatives[4], *i.e.*, the N-TMS amino acid ethyl esters were not found to be any better for gas chromatography then the N-TMS amino acid TMS esters. Further, an extra step, which was not quantitative, was needed to make them, and this derivative appeared to be less stable and thus was not pursued further. RÜHLMANN AND MICHAEL also tried other trialkylsilyl derivatives, *viz.* N-triethylsilyl amino acid triethylsilyl esters, N-tripropylsilyl amino acid TMS esters, and N-tripropylsilyl amino acid tripropylsilyl esters. These were found to be no longer sufficiently volatile for general gas chromatographic purposes. RÜHLMANN AND MICHAEL also made some N-acetyl amino acid TMS esters and N-TFA amino acid TMS esters that were rapidly prepared and gave sharp symmetrical peaks on gas chromatography.

To obviate the interference due to MSA, lack of complete solubility of BSA in $CH_3CN$, and quantitation of silylation, GEHRKE and coworkers[11,12] in 1968 synthesized

a new chemical reagent, bis(trimethylsilyl)trifluoroacetamide (BSTFA). This new reagent was investigated as to the reaction conditions required for the precise and quantitative formation of the twenty TMS amino acid derivatives. It was found that BSTFA is an active silylating reagent, has increased volatility and appears with the solvent front, and has a lower detector response and greater solubility in some solvents than BSA. The fluorine in BSTFA results in less $SiO_2$ deposits and thus decreased detector noise. Silylation at 125° for 15 min in a closed tube resulted in single reproducible derivatives for eighteen of the twenty protein amino acids, and cystine was derivatized in 30 min at 150°. However, no reproducible chromatographic peak was obtained for arginine. Asp($NH_2$) was converted to the TMS derivative in 30 min at 150°, and Glu($NH_2$) in 30 min at 70°. BSTFA and its reaction product mono(trimethylsilyl)trifluoroacetamide (MSTFA) were found to be more volatile than BSA and did not interfere in the chromatographic separation of alanine and glycine. It was further observed that nonpolar liquid phases must be used in the chromatography of the TMS amino acid derivatives as decomposition occurs on polyester columns. From these studies it was concluded that silylation of amino acids and other biologically important molecules with BSTFA holds considerable promise as the derivatives can be prepared in a single step with little time.

A series of papers has just been published by GEHRKE and coworkers on the experimental conditions for silylation and GLC analysis of some biologically important groups of molecules: nucleic acid components[13,14], iodo-containing amino acids[15], sulfur-containing amino acids[16], and N-acetylneuraminic acid[17]. An extensive series of studies was made on the exact reaction conditions required for quantitative silylation of each organic class. Detailed methods are presented and data reported on the precision, accuracy, recovery, and application of the methods.

## EXPERIMENTAL

In the following sections, the chemical derivatization method, chromatographic and instrumental conditions, and calculations are presented for the analysis of the TMS derivatives of the amino acids.

### *Reagents and materials*

Acetonitrile. Obtained from Mallinckrodt Chemical Works, St. Louis; nanograde purity. Store over anhydrous $CaSO_4$ in a 1-liter screw top bottle with a teflon-lined cap.

Amino acids. Obtained from Mann Research Laboratories, New York, N.Y.; "Mann Assayed" chromatographically pure. Amino acids were also obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and were chromatographically pure.

Methylene chloride (anhydrous). Reflux 1000 ml of ACS reagent grade $CH_2Cl_2$ over 25 g of anhydrous $CaCl_2$ for 30 min. Distill in an all-glass apparatus and store in an all-glass inverted top bottle (Scientific Glass Apparatus Co., JB-1740) to protect from atmospheric moisture.

Bis(trimethylsilyl)trifluoroacetamide. Regis Chemical Co., Chicago. Store in a refrigerator at 4°.

Compressed gases. From Air Products and Chemicals, Inc., Kansas City, Mo. $N_2$, 99.995%; $H_2$, 99.0%; air, water pumped.

Bath oil. Fisher Scientific Thermally Stabilized Bath Oil No. 0-2.

Substrates. OV-7 (phenylmethyl 20/80 siloxane), and OV-22 (phenylmethyl 65/35 siloxane), Applied Science Laboratories, Inc., State College, Pa.

Support material. High-performance 100/120 mesh Chromosorb G. This support material was obtained from Johns-Manville Products Corporation, New York, N.Y.

Silanized spun glass wool. Analabs, Inc., North Haven, Conn. Store in a desiccator over $P_2O_5$.

*Stock solutions*

Standard amino acids solution. An aqueous solution (0.1 *N* HCl) containing the twenty protein amino acids at individual concentrations as given in Table I.

Phenanthrene internal standard solution. An acetonitrile solution of phenanthrene, 1.023 mg/ml (100 ml), $5.74 \times 10^{-3}$ mmole/ml.

TABLE I

GRAMS OF AMINO ACIDS REQUIRED FOR A 2.5 MILLIMOLAR STOCK SOLUTION

Molecular weights of the free amino acids are given. These values must be altered when the amino acid hydrochlorides are used.

| *Amino acid* | *Molecular weight (g)* | *Amount required* | |
|---|---|---|---|
| | | *g* | *μg/10 μl* |
| Alanine | 89.1 | 0.2228 | 2.228 |
| Glycine | 75.1 | 0.1878 | 1.878 |
| Valine | 117.1 | 0.2928 | 2.928 |
| Leucine | 131.2 | 0.3280 | 3.280 |
| Isoleucine | 131.2 | 0.3280 | 3.280 |
| Proline | 115.1 | 0.2878 | 2.878 |
| Serine | 105.1 | 0.2628 | 2.628 |
| Threonine | 119.1 | 0.2978 | 2.978 |
| Hydroxyproline | 131.1 | 0.3278 | 3.278 |
| Aspartic acid | 133.1 | 0.3328 | 3.328 |
| Methionine | 149.2 | 0.3730 | 3.730 |
| Cysteine | 121.2 | 0.3030 | 3.030 |
| Glutamic acid | 147.1 | 0.3678 | 3.678 |
| Phenylalanine | 165.2 | 0.4130 | 4.130 |
| Arginine | 174.2 | 0.4355 | 4.355 |
| Lysine | 146.2 | 0.3655 | 3.655 |
| Tyrosine | 181.2 | 0.4530 | 4.530 |
| Histidine | 155.2 | 0.3880 | 3.880 |
| Tryptophan | 204.2 | 0.5105 | 5.105 |
| Cystine | 240.3 | 0.6008 | 6.008 |

*Apparatus and glassware (macro and semimicro method)*

The oil bath, in which the closed-tube trimethylsilylation reaction was conducted, consisted of a $3\frac{1}{2}$ in. $\times 4$ in. $\times 6$ in. aluminum pan supported on a hot plate to maintain uniform temperature of the oil bath. Temperature control was achieved with two 100-W heaters, and a Variac. A sand bath can be used.

A super D-21-36 safety shield obtained from Instruments for Research and Industry ($I^2R$) was used to provide protection from accidental breakage of the silylation reaction vessel.

An all-teflon rotary evaporator obtained from California Laboratory Equipment

Company (Calif. Lab. Model C rotary evaporator) was used to remove the volatile solvents. The vacuum was produced with a Welch Duo-Seal vacuum pump (W. M. Welch Scientific Co., Chicago, Ill.) having a capacity of 140 l/min. A Calab "cold-finger" condenser containing dry ice in ethylene glycol monomethyl ether was placed between the evaporator and the vacuum pump to prevent volatile compounds from reaching the pump. A sodium hydroxide trap was placed between the condenser and the vacuum pump to protect it from corrosive acidic compounds.

Silylation reaction tube. A standard pyrex glass, Corning Glass Works Co. No. 9826, 16 × 75 mm, screw-cap culture tube with teflon-lined cap was used.

Dry heated bath. Thermolyne Corporation, Dubuque, Iowa. Constant temperature 106°.

Syringes. Hamilton 701 N, 10 μl, Hamilton Co., Whittier, Calif.

Filter-driers for the carrier gas of the gas chromatograph were obtained from MicroTek Instruments, Inc., Baton Rouge, La. (Catalog No. 830041), and contained activated carbon, silica gel, and $CaSO_4$. Filters for the $N_2$ gas used in evaporating the solvents from the samples were obtained from The Koby Corp., Melrose, Mass. (Catalog No. 93975) and contained activated charcoal and $CaSO_4$. Filter-driers can also be prepared by packing 10-in. by 1-in. metal cylinders with silica gel and Linde molecular sieve type 5A. Filters are recommended for the nitrogen, hydrogen, and air lines to remove water and hydrocarbons.

*Instrumental and chromatographic conditions*

*Minimum.* Single-column instrument with temperature programming, single hydrogen flame-ionization detector, and recorder equipped with disc integrator. Instrument should have glass injection ports or glass injector liners which can be inspected for buildup of deposits and readily replaced and cleaned. Early experiments indicated that threonine and arginine were decomposed when injected into a hot metal flash heater. The use of direct on-column injection eliminated problems of derivative thermal breakdown. When glass injectors were used in metal columns at elevated temperatures, no adverse effects on these or other amino acid derivatives were observed. The availability of multiple program rates greatly facilitates the chromatographic examination of samples, since the program rate may be systematically varied. The resolution of derivatives with high retention temperatures is more readily achieved using a more rapid program rate (4 to 6°/min) than the resolution of derivatives with low retention temperatures.

*Instrumental operation and chromatographic conditions used*

*Instruments used.* MicroTek Model MT-220 automatic sequential programmed-temperature instrument equipped with a four-column oven bath, with two dual-flame ionization detectors, Model 73980, and two electrometers, Model 636800. It was also equipped with a linear temperature programmer and a Varian Model 20 dual pen recorder. Each of the four columns could be operated independently. A Packard Instruments Co. Model 7300 dual column gas chromatograph with two flame ionization detectors and equipped with a Honeywell Electronic 16 strip chart recorder was also used. The chromatographs were equipped with an Infotronics magnetic tape recorder Model CRS-42 RSI and a digital readout system Model CRS-11AB/HS/42, and a CRS-104 digital integrator.

*Conditions.* The chromatographic conditions used with the columns of the mixed liquid phase of 3.0 w/w% OV-7 and 1.5 w/w% OV-22 on high-performance (H.P.) 100/120 mesh Chromosorb G in a 1.75 m × 4 mm I.D. U-shaped borosilicate glass column were:

| | |
|---|---|
| Column temperature: | initial 75 and 100°, final 225° |
| Program rate: | 2°/min |
| Detector temperature: | 230° |
| Sensitivity: macro | 10 × 64 |
| semimicro | 10 × 16 |
| Carrier flow, $N_2$: | 42 ml/min |
| Air (to detector): | 475 ml/min |
| Hydrogen (to detector): | 30 ml/min |
| Chart speed: | 0.33 in./min |

*Substrates and supports.* A mixed liquid phase of OV-7 (20/80% phenylmethylsiloxane) and OV-22 (65/35% phenylmethylsiloxane) was used for the separation of the TMS derivatives of the amino acids. Acid-washed (a.w.) H.P. Chromosorb G 100/120 mesh was used with the mixed liquid phase. The liquid phase was coated on the a.w. H.P. Chromosorb G at a concentration of 3.0 w/w% OV-7, and 1.5 w/w% OV-22, then packed in a 1.75 m × 4 mm I.D. glass column.

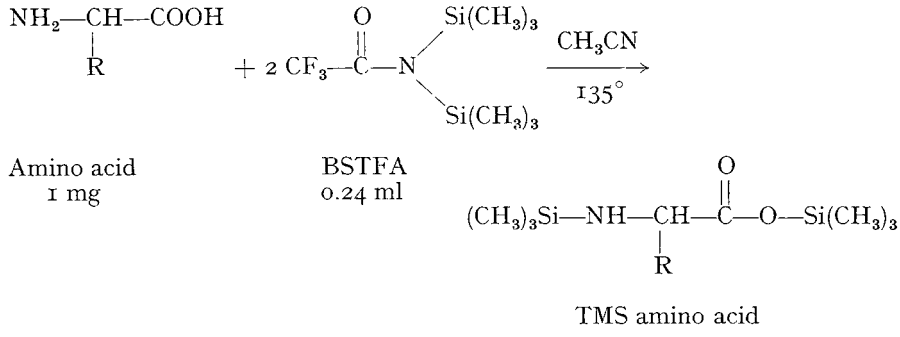
*Column preparation.* To prepare 30.00 g of column packing, weigh 0.90 g of OV-7 into a 50-ml Erlenmeyer flask, and weigh 0.45 g of OV-22 into another Erlenmeyer flask. Dissolve each with anhydrous methylene chloride (25 to 30 ml), respectively. Weigh 28.65 g of 100/120 mesh a.w. H. P. Chromosorb G into a 500-ml ribbed round bottom flask. Add methylene chloride until the liquid level is $\frac{1}{4}$ in. above the Chromosorb G. Then quantitatively transfer the OV-7 and OV-22 solutions to the flask containing the Chromosorb G and methylene chloride. Evaporate the slurry to dryness, *slowly* to achieve uniform coating with a rotary evaporator and a 60° water bath. (30 g of packing is sufficient to pack two 1.75 m × 4 mm I.D. columns.) The column material is now ready for packing in clean, dry glass columns. The column should be gently tapped during filling with the packing to ensure uniform distribution. A plug of silanized spun glass wool is then packed in each end of the column to hold the column packing in place. After placing the column in the oven bath, it is conditioned for at least 24 h at 235° ± 5° with a carrier flow rate of 30 to 35 ml/min of $N_2$ gas.

A properly prepared column gives excellent separation for at least three months, however, it is recommended that a performance standard amino acid mixture be analyzed periodically to evaluate any change in the separation characteristics of the column. *Note.* It is essential that moisture be excluded from the support materials, liquid phases, and solvents during the total preparation procedure.

*Derivatization method—preparation of TMS amino acids*

*General.* The chemical derivatization of amino acids involves the following steps: (1) Removal of water to give the dry amino acid hydrochlorides. (2) Trimethylsilylation of the amino acids.

*Trimethylsilylation of an amino acid*




The reaction is conducted in a closed tube at 135° for 15 minutes *or* 4.0 h. The BSTFA/$CH_3CN$ ratio is 1:1.

*Analytical derivatization methods*

*Macro and semimicro method (20 mg to 100 μg)*

(1) Add an aqueous aliquot containing 0.1–20.0 mg of total amino acids to a No. 9826 culture tube (Note 1).

Evaporate the solution *just to dryness* by placing the tube in a dry, heated sand bath at 106° while directing a regulated stream of *filtered*, dry $N_2$ gas (*ca.* 100 ml/min) into the heated tube, or place tube on a black surface under an IR lamp.

(3) Add 0.5 ml of $CH_2Cl_2$ and evaporate as in Step 2 to ensure complete azeotropic removal of water. *Repeat*, take *just to dryness*.

(4) Add an appropriate *exact* amount of phenanthrene internal standard solution (1–0.1 mg) or an amount equivalent to one of the amino acids in higher concentration.

(5) Add 30 molar excess BSTFA (0.24 ml of BSTFA per 1 mg of amino acids) (Note 2), and the same volume acetonitrile, close tube securely with a teflon-lined cap, and effect solution by manual inversion and ultrasonic mixing.

(6) Trimethylsilylate at 135° in an oil or sand bath for 15 min. Remove the reaction tube after 15 min and *cool immediately* under the tap, dry with tissue paper.

Chromatograph 3–10 μl in the gas chromatograph equipped with a 1.75 m × 4 mm I.D. glass column containing 100/120 mesh a.w. H.P. Chromosorb G which has been coated with a mixture of 3.0 w/w% OV-7 and 1.5 w/w% OV-22 and properly conditioned. After an initial hold for 7 min, program the oven temperature from 75° at 2°/min (Note 3). The detector temperature should be maintained at 255° and the injection port at 300°. The carrier gas ($N_2$) flow rate should be adjusted to achieve maximum separation. This corresponds to a flow rate of 42 ml/min with a pressure of 40 p.s.i.g.

(7) During chromatography, re-silylate the same reaction tube at 135° for 4 h (Note 4). This additional reaction time is necessary for six of the amino acids, *viz.* GLU, ARG, LYS, HIS, TRY, and CYS.

(8) Chromatograph 3–10 μl of the sample (Step 7) on the same column. After an initial hold for 7 min, program the oven temperature from 100° at 2°/min (Note 5).

*Note 1.* All glassware must be thoroughly washed, rinsed repeatedly with double distilled water, and dried.

*Note 2.* Various volumes of BSTFA and acetonitrile were investigated for the complete silylation of 1 mg of amino acid. Also, experiments were made with BSTFA alone, without solvent added. A critical evaluation of all the data showed that the best results for the complete derivatization of the twenty natural protein amino acids was obtained with a solution of 0.24 ml of BSTFA and 0.24 ml of acetonitrile for 1 mg of amino acid.

*Note 3.* About 80 min were required to obtain a total chromatogram from the first peak to the last, cystine.

*Note 4.* After the initial injection (Step 6), the reaction tube was securely closed and again placed in the oil bath and heated at 135° for 4 h. A reaction temperature of 135° and time of 15 min gave three small peaks for arginine. These are the 2-TMS, 3-TMS, and 4-TMS derivatives ($ARG_2$, $ARG_3$, $ARG_4$). Lysine gave a 3-TMS and a 4-TMS derivative ($LYS_3$, $LYS_4$). Both the 2-TMS and 3-TMS derivatives were obtained for histidine and tryptophan, respectively ($HIS_2$, $TRY_3$). A large 3-TMS derivative was obtained for glutamic acid. However, a small amount of cyclized compound was also obtained for glutamic acid, and is the 2-pyrrolidone-5-carboxylic acid TMS derivative. Cystine gave a small peak. However, when the reaction conditions were changed to 135° for 4 h, arginine, lysine, and histidine were completely converted to the trimethylsilylated derivative, and each gave one peak. Under these conditions, tryptophan gave a large 3-TMS peak and a much smaller 2-TMS peak. The RMR of $TRY_2/TRY_3$ was constant with a value of 0.18, and the RMR of $GLU_2/GLU_3$ was 4.15, and remained constant. Glutamic acid was converted mainly to the 2-pyrrolidone-5-carboxylic acid TMS derivative, with a small amount of the 3-TMS glutamic acid derivative remaining. Many different experimental conditions of temperature and time were investigated. The temperatures studied were: 125°, 135°, 150°, and 170°; reaction times varied from 15 min to 13 h. An evaluation of all the different experimental conditions showed that 135° for 4 h was the best set of conditions.

*Note 5.* A complete chromatogram required about 60 min from the first peak to the last peak, cystine.

*Internal standard method of calculation*

This method was used to calculate the mole%, or w/w%, for each amino acid. The calculation of the absolute amounts of amino acid in a sample is best accomplished by the use of an internal standard (I.S.). Phenanthrene was found suitable as an internal standard because it was well resolved from the amino acids on a column of the mixed liquid phase of 3.0 w/w% OV-7 and 1.5 w/w% OV-22 on 100/120 mesh H.P. Chromosorb G.

$$RMR_{a.a./phen} = \frac{A_{a.a.}/Moles_{a.a.}}{A_{I.S.}/Moles_{I.S.}}$$

In these experiments, electronic and disc integration were used and peak areas were measured as counts. After the molar response of each amino acid has been determined relative to an I.S. (phenanthrene) from at least three independent analyses, these values may then be used to calculate the quantity of amino acids present in a sample.

$$\mathrm{RMR_{a.a./I.S.}} = \frac{\dfrac{A_{\mathrm{a.a.}}}{\dfrac{g_{\mathrm{a.a.}}}{\mathrm{GMW_{a.a.}}}}}{\dfrac{A_{\mathrm{I.S.}}}{\dfrac{g_{\mathrm{I.S.}}}{\mathrm{GMW_{I.S.}}}}}$$

After the addition of an *exact known amount* of the I.S. to the sample and subsequent analysis by GLC, the following formula may be used to calculate the amount of each amino acid present in the sample.

$$g_{\mathrm{a.a.}} = \frac{A_{\mathrm{a.a.}} \times \mathrm{GMW_{a.a.}} \times g_{\mathrm{I.S.}}}{A_{\mathrm{I.S.}} \times \mathrm{GMW_{I.S.}} \times \mathrm{RMR_{a.a./I.S.}}}$$

$$\mathrm{w/w\ \%\ of\ amino\ acid} = \frac{\mathrm{grams\ of\ amino\ acid}}{\mathrm{grams\ sample}} \cdot 100$$

GMW denotes gram molecular weight of amino acid.

*Comments on internal standard method*

(1) Gives exact mole%, or w/w%, of an amino acid with a minimum of instrumental calibration.

(2) Does not require the preparation of calibration curves for amino acids. This requires considerable work if pure reference standards are not available.

(3) Does not require a calibration curve for the internal standard.

(4) Does require information on the *exact amount* of internal standard added to the sample flask.

(5) Does require information on the relationship of $\mathrm{RMR_{a.a./I.S.}}$

(6) Dilution or concentration of sample, after addition of internal standard are of little importance. The area ratio for the unknown and the I.S. remains constant, providing there is no selective substrate–amino acid derivative interaction, or selective loss of I.S. or derivative in some other way.

(7) Amount of derivatized sample injected is not critical as long as a well-sized peak is obtained.

The proposed structures of the N-trimethylsilyl TMS esters of the twenty protein amino acids are given in Table II. Further positive identification of the derivatives will necessitate elemental analysis and other instrumental studies on the pure synthesized derivatives.

## RESULTS AND DISCUSSION

*Chromatographic separation studies*

An evaluation was made of various OV (phenylmethylsiloxane) substrates and combinations for the separation of the twenty protein amino acids. Preliminary experiments with various substrate loadings indicated that difficulties would be encountered in achieving a complete separation.

A fairly acceptable separation was achieved with a 3.0 w/w% 2.0 m column of OV-7 on 100/120 mesh H.P. Chromosorb G (Fig. 1). An extensive series of experiments was then conducted in which the experimental conditions of initial temperature, iso-

thermal hold time, carrier gas flow rate, temperature programming, and column length were evaluated. These experiments were made over a period of about three months, and it was noted that changes in these parameters significantly affected the separation. From this work the best experimental conditions were defined, and these are described in the section EXPERIMENTAL under *Conditions*. For the samples that were derivatized at 135° for 15 min, the initial column temperature was 75°; and for

TABLE II

STRUCTURE OF THE TMS DERIVATIVES OF AMINO ACIDS

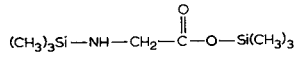
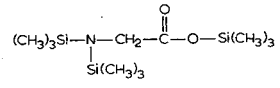
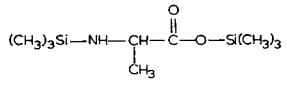
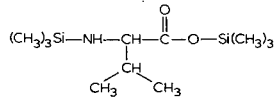
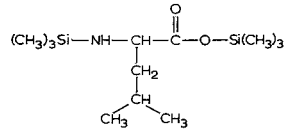
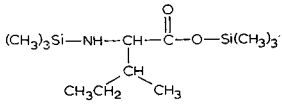
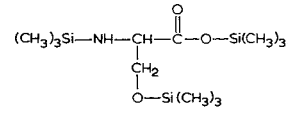
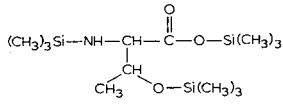
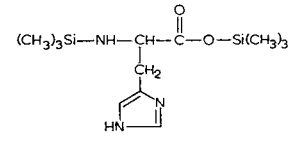
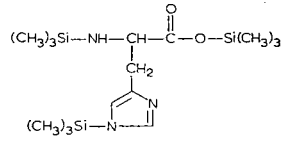
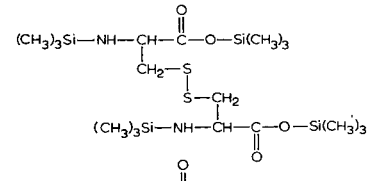
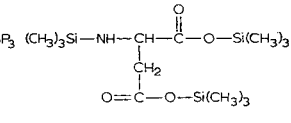
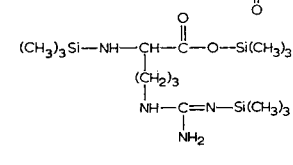
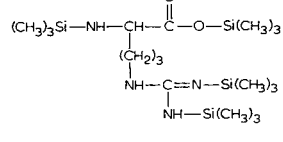
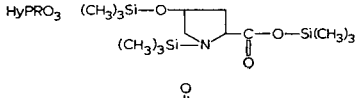
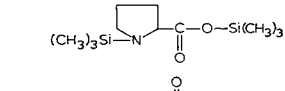
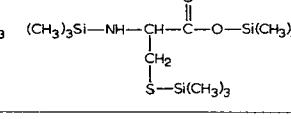
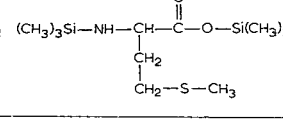
| | |
|---|---|
| Glycine, $GLY_2$  | Glycine, $GLY_3$  |
| Alanine, $ALA_2$  | Valine, $VAL_2$  |
| Leucine, $LEU_2$  | Isoleucine $ILEU_2$  |
| Serine, $SER_3$  | Threonine, $THR_3$  |
| Histidine, $HIS_2$  | Histidine, $HIS_3$  |
| Cystine, $CYS_4$  | Aspartic acid, $ASP_3$  |
| Arginine, $ARG_3$  | Arginine, $ARG_4$  |
| Hydroxyproline, $HyPRO_3$  | Proline, $PRO_2$  |
| Cysteine, $CYSH_3$  | Methionine, $MET_2$  |

TABLE II *(continued)*

| | | | |
|---|---|---|---|
| Lysine, $LYS_3$ | $(CH_3)_3Si-NH-CH(-(CH_2)_4-NH-Si(CH_3)_3)-C(=O)-O-Si(CH_3)_3$ | Lysine, $LYS_4$ | $(CH_3)_3Si-NH-CH(-(CH_2)_4-N(-Si(CH_3)_3)-Si(CH_3)_3)-C(=O)-O-Si(CH_3)_3$ |
| Tyrosine, $TYR_3$ | $(CH_3)_3Si-NH-CH(-CH_2-C_6H_4-O-Si(CH_3)_3)-C(=O)-O-Si(CH_3)_3$ | Phenylalanine, $PHE_2$ | $(CH_3)_3Si-NH-CH(-CH_2-C_6H_5)-C(=O)-O-Si(CH_3)_3$ |
| Tryptophan, $TRY_2$ | $(CH_3)_3Si-NH-CH(-CH_2-C_8H_6N-H)-C(=O)-O-Si(CH_3)_3$ | Tryptophan, $TRY_3$ | $(CH_3)_3Si-NH-CH(-CH_2-C_8H_6N-Si(CH_3)_3)-C(=O)-O-Si(CH_3)_3$ |
| Glutamic acid, $GLU_3$ | $(CH_3)_3Si-NH-CH(-(CH_2)_2-C(=O)-O-Si(CH_3)_3)-C(=O)-O-Si(CH_3)_3$ | 2-Pyrrolidone-5-carboxylic acid, $GLU_2$ | $(CH_3)_3Si-N(-CH(-CH_2-CH_2-C(=O)-)-C(=O))-O-Si(CH_3)_3$ |

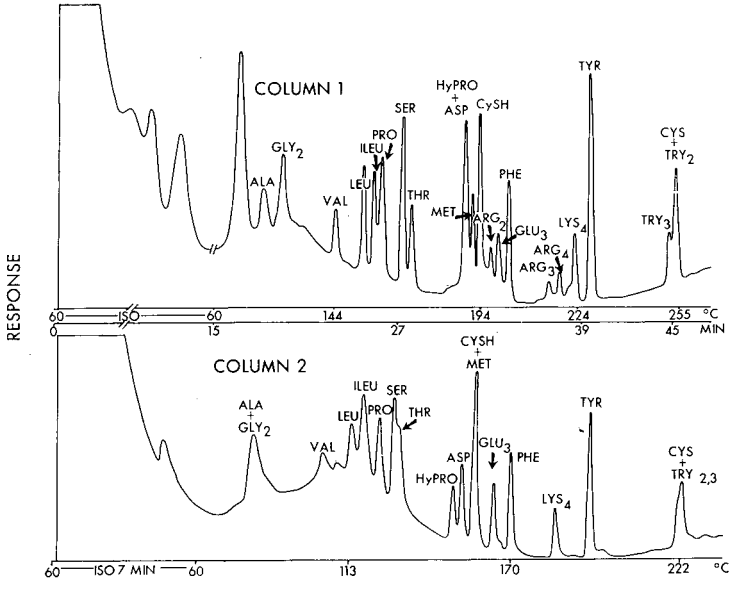



Fig. 1. Separation of TMS amino acids. Column 1: 3.0 w/w % OV-7 on 100/120 mesh H.P. Chromosorb G, 2.0 m × 4 mm I.D., glass; initial temperature 60°, 15 min hold, then 7.5°/min. Column 2: 1.5 w/w % OV-22 on 100/120 mesh H.P. Chromosorb G, 2.0 m × 4 mm I.D., glass; initial temperature, 60°, 7 min hold, then 7.5°/min.

the samples derivatized at 135° for 4 h, 100°. In each case the temperature was programmed to a final temperature of 225°.

Eighteen of the amino acids were separated on a 4.0 w/w%, 1.75 m × 4 mm I.D., column of OV-7 coated on a.w. 100/120 mesh H.P. Chromosorb G. Hydroxyproline and aspartic acid were the only two amino acids that overlapped. The separation of these two TMS amino acid derivatives was achieved on a 1.5 w/w%, 1.75 m × 4 mm I.D., column of OV-22 on a.w. 100/120 mesh H.P. Chromosorb G. However, with this column four other pairs of amino acids had the same retention temperatures. These were alanine and glycine, serine and threonine, methionine and cysteine, and tryptophan and cystine (Fig. 1). From information gained in these experiments a mixed liquid phase of OV-7 and OV-22 was next tried. This resulted in the complete separation of the TMS derivatives of the twenty protein amino acids with phenanthrene as I.S. on a *mixed* liquid phase of 3.0 w/w% OV-7 plus 1.5 w/w% OV-22 on an a.w. 100/120 mesh H.P. Chromosorb G column, 1.75 m × 4 mm I.D.

Sixteen of the amino acids were soluble in BSTFA. Aspartic and glutamic acids, cystine, and histidine were only partially soluble. Thus it was necessary to use a polar solvent with BSTFA in the derivatization reaction. Acetone, tetrahydrofuran, dioxane, and acetonitrile were investigated. The best solvent was acetonitrile. All of the amino acids were soluble with a BSTFA/$CH_3CN$ ratio of 1:1.

In chromatographic experiments it was further found that H.P. Chromosorb G was necessary to obviate the tailing of the acetonitrile solvent peak into alanine and glycine as these amino acids were eluted from the column. Acid-washed Chromosorb G was not satisfactory as a support phase. The silanized H.P. Chromosorb G was entirely satisfactory and apparently all of the hydroxyl groups were masked and thus interaction of the solvent with the support was decreased. With the above recommended *mixed* liquid phase and support material, the life of a column was found to be at least three months. Figs. 2 and 3 represent typical chromatograms for the GLC separation

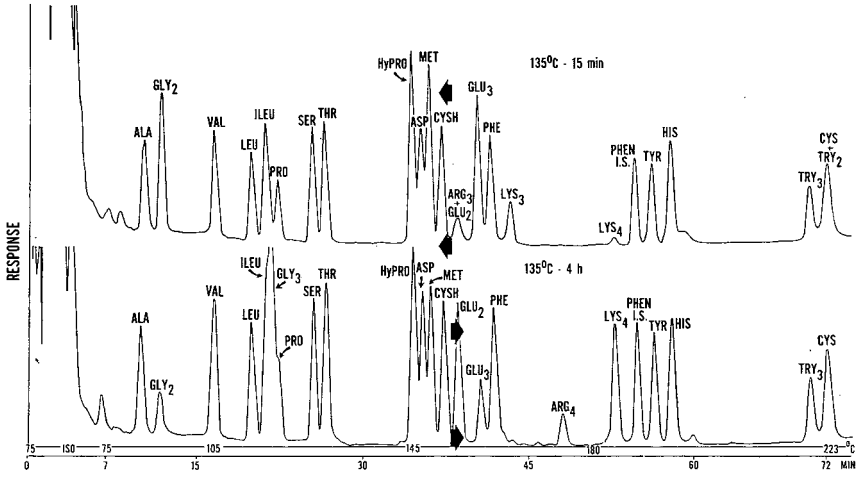



Fig. 2. GLC chromatogram of TMS protein amino acids. Sample: 13.06 mg in 6.2 ml, 21.06 μg total amino acids injected (10 μl). BSTFA/$CH_3CN$ ratio 1:1. Column: mixed liquid phase of 3.0 w/w % OV-7 and 1.5 w/w % OV-22 on 100/120 mesh H.P. Chromosorb G, 1.75 m × 4 mm I.D., glass. Initial temperature 75°, 7 min hold, then 2°/min.

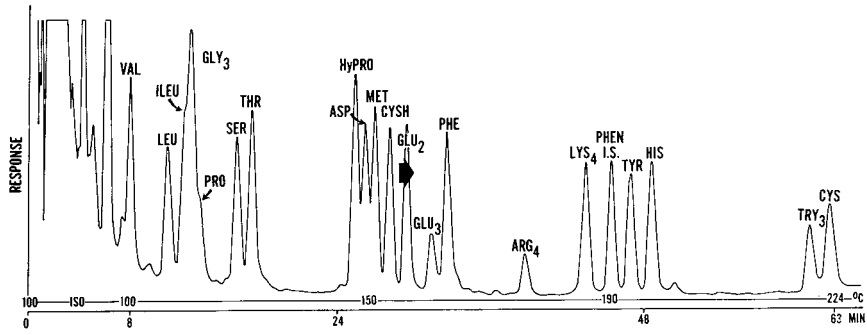



Fig. 3. GLC chromatogram of TMS protein amino acids; 135° for 4 h. Sample: 13.06 mg in 6.2 ml, 21.06 μg total amino acids injected (10 μl). BSTFA/$CH_3CN$ ratio 1:1. Initial temperature 100°, 8 min hold, then 2°/min. Column, same as Fig. 2.

of the TMS protein amino acids. Details for the derivatization, instrumental, and chromatographic conditions are given in the legends to the figures. Fourteen of the amino acids, including phenylalanine and tyrosine (Fig. 2, top), must be derivatized at 135° for 15 min; and for six of the amino acids ($GLU_2$, $ARG_4$, $LYS_4$, $HIS_3$, $TRY_3$, and $CYS_4$) the required derivatization temperature and time were 135° for 4 h (Fig. 2, bottom).

*Injection port temperature.* For the chromatography of the TMS amino acids it was found necessary to maintain the injection port temperature at 250–300°. At lower temperatures incomplete elution of the less volatile derivatives ($HIS_3$, $TRY_2$, $TRY_3$, and $CYS_4$) occurred, resulting in reduced responses and reappearance of these peaks as ghosts or breakdown products in subsequent chromatographic analysis.

*Solvent tailing.* In some instances it was noted that the solvent peak of the reaction mixture (BSTFA, MSTFA, and $CH_3CN$) tailed badly, thus interfering with the quantitation of the most volatile TMS amino acid derivatives ($ALA_2$, $GLY_2$, and $VAL_2$).

Tailing of the solvent peak was apparently a result of the loss of the liquid phase in that portion of the column which was in direct contact with the hot injection port, maintained at 250–300°. The solvent was probably adsorbed on the surface of the altered packing material, and thus was less rapidly eluted than would be the case if the support were coated with liquid phase. This problem can be obviated by removing the packing from that portion of the column which comes in direct contact with the heated injection port.

*Column conditions.* Some important observations and points of emphasis about the chromatographic column and the separations are: the exact w/w% of the OV-7 and OV-22 liquid phases used are not too critical. One of the first observations that a chromatographic column is deteriorating was indicated by the many small peaks between $HIS_3$ and $CYS_4$, and resolution was lost first for phenanthrene (I.S.), $TYR_3$, and $HIS_3$. Other indications were that the base line between BSTFA and $ALA_2$ was not sharp, the $CH_3CN$ solvent peak tailed into the $ALA_2$ peak, and the $ALA_2$ peak appeared early. To achieve a good separation of the twenty amino acids the flow rate of carrier gas ($N_2$) is most critical (40–42 ml/min), and next the temperature program rate (2°/min).

*Performance blank*

Performance blank studies were made on all reagents, and with the chromatographic, and instrumental conditions as presented to determine the existence and source of extraneous peaks and contamination. Chromatograms were made for acetonitrile, BSTFA, BSTFA heated at 135° for 15 min, BSTFA plus $CH_3CN$ heated at 135° for 15 min, and BSTFA heated at 135° for 4 h. A good blank chromatogram was achieved for $CH_3CN$ at the sensitivity settings normally used. However, the commercial BSTFA sometimes showed extraneous peaks. The retention temperatures of these extraneous peaks were at 100°, 115°, and 140°, and a careful distillation removed much of the extraneous material. When the BSTFA was heated at 135° for 15 min, these extraneous peaks became larger; at 135° for 4 h, these three peaks were quite large and other peaks appeared. All of them were found below a retention temperature of 145°. It was most fortunate that these extraneous peaks did not occur at the same retention temperatures for any of the protein amino acids. The commercial BSTFA that is now available does not show these extraneous peaks. In general, these peaks resulted from the silylation of trace impurities present in BSTFA, or from break-down products of impure BSTFA which were then silylated. It is essential that only highly purified BSTFA should be used.

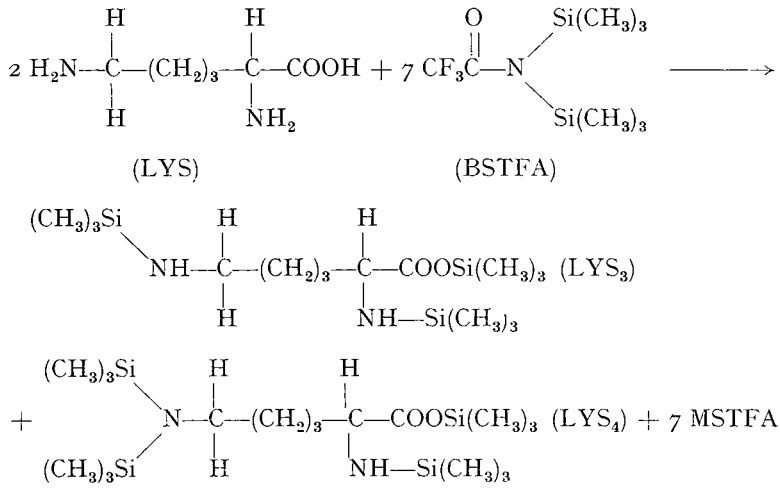
*Derivatization reaction conditions*

Some derivatization experiments were conducted using only BSTFA and no organic solvent. Other experiments were conducted using solvents of different polarities to help dissolve four of the amino acids. Acetonitrile was found to be the best solvent at an equal volume to BSTFA. The following reaction temperatures were investigated: 125°, 135°, 150°, and 170°. Reaction times were: 5, 10, 15, and 30 min, and 1, 2, 3, 4, 5, 6, and 13 h. The molar excess of BSTFA to the total amino acids studied was: 8, 10, 20, 30, 50, and 100. The following amino acids gave multiple peaks under different experimental conditions: glycine, glutamic acid, arginine, lysine, histidine, and tryptophan. Multiple peak formation for arginine and lysine occurs because these amino acids have an $\omega$-amino group which is silylated to different extents; histidine and tryptophan have a heterocyclic nitrogen atom; glutamic acid gives a cyclized compound 2-pyrrolidone-5-carboxylic acid TMS derivative; and the $\alpha$-amino group on glycine can be doubly silylated due to lack of steric hindrance.

*Trimethylsilylation reaction for lysine*

For all the amino acids except glycine, the $\alpha$-amino group is substituted by only one TMS group, whereas the $\omega$-amino group is substituted by one or two TMS groups, depending upon the reaction temperature and time. This is due to the presence of steric hindrance to the $\alpha$-amino group for all of the amino acids except glycine.

The best reaction conditions for the trimethylsilylation of fourteen amino acids is at 135° for 15 min. Phenylalanine and tyrosine are included in the fourteen. Refer to the top chromatogram in Fig. 2. However, to quantitatively convert the remaining six amino acids, it was necessary to heat at 135° for 4 h. These amino acids are $GLU_2$, $ARG_4$, $LYS_4$, $HIS_3$, $TRY_3$, and $CYS_4$. When glycine was derivatized at 135° for 15 min, the ratio of $GLY_2/GLY_3$ was found to be 6.7 and remained constant. Trimethylsilylation at 135° for 4 h gave a ratio of 0.10 for $GLY_2/GLY_3$. Glycine is included with the fourteen amino acids because $GLY_3$ has a retention temperature between that for




isoleucine and proline, and thus interfered in the separation of these two amino acid derivatives (Figs. 2 and 3).

To trimethylsilylate and to chromatographically separate the twenty amino acids, two sets of reaction conditions and two injections were required. The sample was initially heated at 135° for 15 min, then an aliquot was injected onto the column at an initial temperature of 75°. The reaction vessel was closed and again heated at 135° for 4 h, after which time a second injection of the sample was made, with the initial temperature of the column at 100°. After the first injection the column was programmed to about 225° and then cooled to the initial holding temperature of 100° prior to the second injection. An isothermal hold was also used at the beginning of each chromatogram. The amino acids from alanine to cysteine are included in the first chromatogram, plus phenylalanine and tyrosine; and from glutamic acid to cystine in the second chromatogram. Glutamic acid was converted into a cyclized compound 2-pyrrolidone-5-carboxylic acid TMS derivative ($GLU_2$, Table II). At the reaction conditions of 135° for 15 min, $GLU_2$ gave only a small peak; whereas at 135° for 4 h the pyrrolidone derivative was the major peak, and the remaining $GLU_3$ did not interfere in the chromatography. The glutamic acid content of the samples was determined by the summation of the areas of $GLU_2$ and $GLU_3$.

The trimethylsilylation of a number of the amino acids as a function of reaction conditions is presented in Fig. 4. One peak was obtained for $ARG_4$, $LYS_4$, $HIS_3$, and $CYS_4$, respectively. At 135° for 4 h, tryptophan yielded two peaks, $TRY_3$ and $TRY_2$ (Table II). The RMR ratio for $TRY_2/TRY_3$ was found to be 0.18 and remained constant. The GLC chromatogram of the TMS derivatives of tryptophan and cystine is shown in Fig. 5. Although $TRY_2$ and $CYS_4$ have the same retention temperature, the $CYS_4$ content can be measured by subtracting 18% of the total area of $TRY_3$ from the total area for $TRY_2$ plus $CYS_4$ (Fig. 5). From the above experiments on trimethylsilylation conditions, studies on the relative molar response of the amino acid derivatives as a function of temperature (Table III), and molar excess of BSTFA (Table IV), it was concluded that the best reaction conditions for the twenty amino acids were as follows: Take 1 mg total amino acids, add 0.24 ml BSTFA and 0.24 ml $CH_3CN$.

TABLE III

RELATIVE MOLAR RESPONSE OF TMS AMINO ACIDS AS A FUNCTION OF REACTION TEMPERATURE

Molar ratio of BSTFA/Total amino acids = 30. BSTFA/$CH_3CN$ ratio, 1:1. Each value is an average of at least two independent analyses.

| *Amino acid* | *$RMR_{a.a./phen.}$ and silylation conditions* | | | |
|---|---|---|---|---|
| | *125°—15 min* | *135°—15 min* | *135°—4 h* | *150°—2 h* |
| $ALA_2$ | 0.35 | 0.53 | 0.32 | — |
| $GLY_2$ | 0.46 | 0.48 | 0.00 | 0.00 |
| $GLY_3$ | 0.08 | 0.09 | 0.46 | 0.44 |
| $VAL_2$ | 0.68 | 0.87 | 0.51 | — |
| $LEU_2$ | 0.78 | 0.94 | 0.88 | — |
| $ILEU_2$ | 0.95 | 1.06 | 1.04 | — |
| $PRO_2$ | 0.43 | 0.61 | 0.36 | — |
| $SER_3$ | 0.87 | 0.96 | 1.00 | — |
| $THR_3$ | 0.88 | 1.06 | 0.96 | — |
| $HyPRO_3$ | 0.93 | 1.14 | 0.96 | — |
| $ASP_3$ | 0.84 | 0.86 | 0.80 | 0.66 |
| $MET_2$ | 0.48 | 0.56 | 0.52 | — |
| $CYSH_3$ | 0.45 | 0.52 | 0.21 | 0.27 |
| $GLU_2$ | 0.13 | 0.15 | 0.54 | — |
| $GLU_3$ | 0.52 | 0.53 | 0.15 | — |
| $PHE_2$ | 1.02 | 1.08 | 0.93 | 0.85 |
| $ARG_4$ | — | — | 0.51 | 0.24 |
| $LYS_4$ | — | — | 0.89 | 0.52 |
| $TYR_3$ | 1.36 | 1.53 | 1.44 | — |
| $HIS_3$ | — | — | 0.55 | 0.29 |
| $TRY_3$ | — | — | 0.87 | — |
| $TRY_2$ | — | — | 0.16 | — |
| $CYS_4$ | — | — | 0.81 | 0.98 |

TABLE IV

RELATIVE MOLAR RESPONSE OF TMS AMINO ACIDS AS A FUNCTION OF MOLAR RATIO OF BSTFA

Closed tube silylation at 135—°15 min. BSTFA/$CH_3CN$ ratio, 1:1. Molar ratio of BSTFA/Total amino acids = 20, 30 and 50. Each value is an average of at least two independent analyses.

| *Amino acid* | *$RMR_{a.a./phen.}$ and molar ratio* | | |
|---|---|---|---|
| | *20* | *30* | *50* |
| $ALA_2$ | 0.41 | 0.53 | 0.54 |
| $GLY_2$ | 0.49 | 0.48 | 0.36 |
| $GLY_3$ | 0.11 | 0.09 | 0.18 |
| $VAL_2$ | 0.88 | 0.87 | 0.86 |
| $LEU_2$ | 0.96 | 0.94 | 0.90 |
| $ILEU_2$ | 0.97 | 1.06 | 1.12 |
| $PRO_2$ | 0.58 | 0.61 | 0.58 |
| $SER_3$ | 0.93 | 0.96 | 0.86 |
| $THR_3$ | 1.00 | 1.06 | 0.92 |
| $HyPRO_3$ | 1.15 | 1.14 | 0.99 |
| $ASP_3$ | 0.85 | 0.87 | 0.83 |
| $MET_2$ | 0.78 | 0.77 | 0.73 |
| $CYSH_3$ | 0.54 | 0.54 | 0.42 |
| $PHE_2$ | 1.02 | 1.08 | — |
| $TYR_3$ | 1.36 | 1.53 | 1.56 |

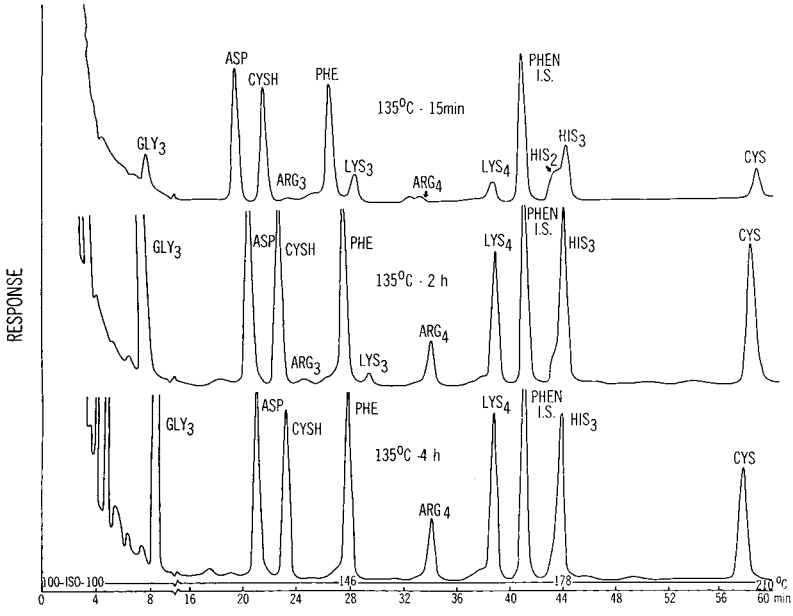



Fig. 4. Silylation of amino acids as a function of reaction time. Sample: 6.3 mg in 4.8 ml, 10.5 $\mu$g total amino acids injected (8 $\mu$l). BSTFA/$CH_3CN$ ratio 1:1. Initial temperature 100°, 4 min hold, then 2°/min. Column, same as Fig. 2.

Heat at 135° ± 2° for 15 min ± 1 min, followed by heating at 135° ± 2° for 4 h ± 10 min.

*Relative molar response of TMS amino acids*

The relative molar response values, $RMR_{a.a./phen.}$, for fourteen of the TMS amino acid derivatives silylated under the optimum conditions of 135° for 15 min are given in Table V. The RMR values for the remaining six amino acids silylated at 135° for 4 h are presented in Table VI. The number of TMS groups attached to each amino acid is denoted as a subscript. Phenanthrene was used as I.S. and was arbitrarily assigned a value of unity. Each solution contained *ca.* 0.5 mg of each amino acid and 0.5 mg of I.S. The amino acids were analyzed singly as well as in groups, and no interactions were observed. All of the chromatographic experiments were made on the mixed liquid phase of OV-7 and OV-22 at a w/w% ratio of 3/1.5. No amino acid derivative–substrate interactions were noted with the siloxane substrate phases. However, it was earlier noted that polyester substrates could not be used for chromatography of the TMS derivatives. Under the experimental conditions used, the RMR values were very reproducible; and the average RSD (%) for the fourteen amino acids (Table V) was found to be 0.80, and the average RSD (%) for the remaining six amino acids (Table VI) was found to be 1.25. It was noted in general that the RMR values for the fourteen amino acids decreased when the silylation time was increased to 4 h (Table V). Data are presented in Table VII giving the RMR values for a number of the TMS amino acid derivatives as a function of reaction time. It was observed that the optimum silylation time for $GLU_2$, $ARG_4$, $LYS_4$, $HIS_3$, $TRY_3$, and $CYS_4$ was 4 h.

TABLE V

RELATIVE MOLAR RESPONSE OF THE TMS AMINO ACIDS

Optimum conditions, 135°—15 min. The number of TMS groups attached to the amino acid is denoted as a subscript.

| *Amino acid* | *$RMR_{a.a./phen.}$ and conditions*[a] | | | | | |
|---|---|---|---|---|---|---|
| | *135°—15 min* | | | *Av.* | *RSD (%)* | *135°—4 h* |
| $ALA_2$ | 0.53 | 0.55 | 0.52 | 0.53 | 0.6 | 0.32 |
| $GLY_2$ | 0.50 | 0.47 | 0.42 | 0.48 | 1.3 | 0.00 |
| $GLY_3$ | 0.09 | 0.11 | 0.08 | 0.09 | 3.3[b] | 0.46 |
| $VAL_2$ | 0.89 | 0.84 | 0.88 | 0.87 | 0.6 | 0.51 |
| $LEU_2$ | 0.94 | 0.93 | 0.95 | 0.94 | 0.1 | 0.88 |
| $ILEU_2$ | 1.07 | 1.06 | 1.04 | 1.06 | 0.2 | 1.04 |
| $PRO_2$ | 0.62 | 0.64 | 0.58 | 0.61 | 1.6 | 0.36 |
| $SER_3$ | 0.98 | 0.94 | 0.95 | 0.96 | 0.3 | 1.00 |
| $THR_3$ | 1.04 | 1.09 | 1.04 | 1.06 | 0.5 | 0.96 |
| $HyPRO_3$ | 1.12 | 1.09 | 1.20 | 1.14 | 1.7 | 0.96 |
| $ASP_3$ | 0.86 | 0.84 | 0.87 | 0.82 | 0.2 | 0.80 |
| $MET_2$ | 0.53 | 0.59 | 0.57 | 0.56 | 1.9 | 0.52 |
| $CYSH_3$ | 0.51 | 0.50 | 0.54 | 0.52 | 1.2 | 0.21 |
| $PHE_2$ | 1.09 | 1.07 | 1.07 | 1.08 | 0.1 | 0.93 |
| $TYR_3$ | 1.52 | 1.51 | 1.55 | 1.53 | 1.3 | 1.44 |

[a] Comparison of silylation in a closed tube at 135° for 15 min, and 135° for 4 h.

$$RMR_{a.a./phen.} = \frac{A_{a.a.}/Moles_{a.a.}}{A_{I.S.}/Moles_{I.S.}}$$

Av. RSD (%) = 0.80.

[b] $GLY_3$ is not included.

TABLE VI

RELATIVE MOLAR RESPONSE OF THE TMS AMINO ACIDS

Optimum conditions, 135°—4 h. The number of TMS groups attached to the amino acid is denoted as a subscript.

| *Amino acid* | *$RMR_{a.a./phen.}$ and conditions*[a] | | | | | |
|---|---|---|---|---|---|---|
| | *135°—4 h* | | | *Av.* | *RSD (%),* | *135°—15 min* |
| $GLU_2$ | 0.52 | 0.56 | 0.53 | 0.54 | 1.1 | 0.15 |
| $GLU_3$ | 0.14 | 0.12 | 0.14 | 0.13 | 6.1[b] | 0.53 |
| $ARG_3$ | 0.04 | 0.00 | 0.00 | 0.01 | — | 0.02 |
| $ARG_4$ | 0.54 | 0.50 | 0.48 | 0.51 | 2.4 | 0.03 |
| $LYS_3$ | 0.00 | 0.00 | 0.00 | 0.00 | — | 0.20 |
| $LYS_4$ | 0.86 | 0.89 | 0.89 | 0.88 | 2.3 | 0.10 |
| $HIS_3$ | 0.55 | 0.54 | 0.55 | 0.55 | 1.8 | 0.31 |
| $TRY_3$ | 0.87 | 0.87 | 0.86 | 0.87 | 1.1 | 0.72 |
| $TRY_2$ | 0.16 | 0.17 | 0.15 | 0.16 | 5.0[b] | 0.67 |
| $CYS_4$ | 0.80 | 0.84 | 0.79 | 0.81 | 0.1 | 0.48 |

[a] Comparison of silylation in a closed tube at 135° for 15 min, and 135° for 4 h.

$$RMR_{a.a./phen.} = \frac{A_{a.a.}/Moles_{a.a.}}{A_{I.S.}/Moles_{I.S.}}.$$

Av. RSD (%) = 1.25.

[b] $GLU_3$ and $TRY_2$ are not included.

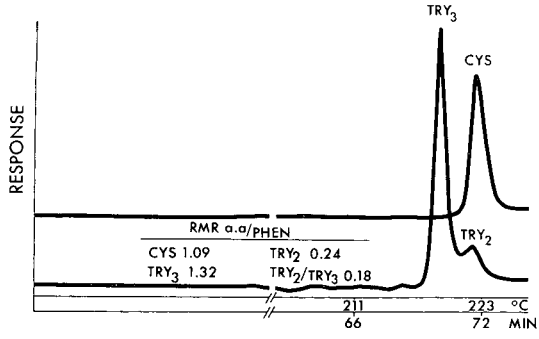



Fig. 5. GLC chromatogram of TMS tryptophan and cystine;. 135° for 4 h. BSTFA/$CH_3CN$ ratio 1:1. Initial temperature 75°, 7 min hold, then 2°/min. Column, same as Fig. 2.

TABLE VII

RELATIVE MOLAR RESPONSE OF TMS AMINO ACID DERIVATIVES AS A FUNCTION OF REACTION TIME

Closed tube silylation at 135°. Molar ratio of BSTFA/Amino acid = 30. BSTFA/$CH_3CN$ ratio, 1:1.

| *Amino acid* | *$RMR_{a.a./phen.}$ and silylation time*[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *15 min* | | | *Av.* | *2 h* | *4 h* | | *Av.* | *6 h* |
| $GLY_2$ | 0.50 | 0.46 | 0.46 | 0.48 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| $GLY_3$ | 0.08 | 0.09 | 0.11 | 0.09 | 0.41 | 0.37 | 0.46 | 0.42 | 0.46 |
| $GLU_2$ | 0.15 | 0.18 | 0.12 | 0.15 | — | 0.52 | 0.56 | 0.54 | — |
| $GLU_3$ | 0.54 | 0.53 | 0.51 | 0.53 | — | 0.14 | 0.12 | 0.13 | — |
| $ARG_3$ | 0.03 | 0.00 | 0.01 | 0.01 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 |
| $ARG_4$ | 0.01 | 0.28 | 0.15 | 0.14 | 0.26 | 0.54 | 0.50 | 0.52 | 0.48 |
| $LYS_3$ | 0.07 | 0.18 | 0.37 | 0.21 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 |
| $LYS_4$ | 0.10 | 0.13 | 0.07 | 0.10 | 0.65 | 0.86 | 0.84 | 0.85 | 0.80 |
| $HIS_3$ | 0.19 | 0.42 | 0.32 | 0.31 | 0.53 | 0.54 | 0.55 | 0.55 | 0.42 |
| $TRY_3$ | 0.88 | 0.80 | 0.47 | 0.72 | — | 0.87 | 0.87 | 0.87 | — |
| $TRY_2$ | 0.46 | 0.64 | 0.96 | 0.67 | — | 0.16 | 0.19 | 0.18 | — |
| $CYS_4$ | 0.36 | 0.51 | 0.51 | 0.57 | 0.86 | 0.78 | 0.84 | 0.82 | 0.78 |

[a] $RMR_{a.a./phen.} = \dfrac{A_{a.a.}/Moles_{a.a.}}{A_{I.S.}/Moles_{I.S.}}$.

At a 6-h reaction time, some of the RMR values decreased. The best value for glycine as $GLY_2$ was at a reaction time of 15 min. On standing, $GLY_2$ changed into $GLY_3$, which had a retention temperature between that of isoleucine and proline. Therefore, it is essential that glycine remains as $GLY_2$. The silylation temperature should be held to 135° ± 2°, and the reaction time to 15 min ± 1 min. The silylated sample must be cooled within 3 min, otherwise glycine changes from $GLY_2$ to $GLY_3$. These conditions are not as critical for the other amino acids in the group of fourteen. At the silylation conditions of 135° for 4 h, the reaction time can be varied ± 10 min. The temperature should again be held to 135° ± 2°.

*Stability of TMS amino acid derivatives*

An important factor considered in this investigation was the stability of the

TMS amino acid derivatives on standing in a closed vial after silylation. The samples were silylated under the optimum conditions as determined earlier, and then analyzed over a seven-day period. Care was exercised to exclude moisture from entering into the reaction tube. Following silylation, and between chromatographic analyses, the silylated samples were held in the teflon-capped reaction vials at room temperature. The vials were opened only long enough to remove the sample for injection into the gas chromatograph. Stability of the TMS amino acid derivatives was evaluated by comparison of the RMR values as a function of standing time. The results are presented in Table VIII. Only glycine showed a lack of stability. It is necessary that the analysis for this amino acid be made within 3 h.

TABLE VIII

STABILITY OF THE TMS AMINO ACID DERIVATIVES AS A FUNCTION OF TIME

Sample held in closed tube at room temperature. Closed tube silylation at 135°—15 min and 135°—4 h. Each value is an average of at least two independent runs. RMR with respect to phenanthrene as internal standard.

| *Amino acid* | $RMR_{a.a./phen.}$ *and time* | | | | | |
|---|---|---|---|---|---|---|
| | *0* | *3 h* | *8 h* | *24 h* | *3 days* | *7 days* |
| *Silylation at 135°—15 min* | | | | | | |
| $ALA_2$ | 0.49 | 0.50 | 0.51 | 0.48 | 0.48 | 0.52 |
| $GLY_2$ | 0.48 | 0.47 | 0.40 | 0.22 | 0.08 | 0.03 |
| $GLY_3$ | 0.09 | 0.12 | 0.29 | 0.48 | 0.54 | 0.59 |
| $VAL_2$ | 0.81 | 0.84 | 0.85 | 0.80 | 0.83 | 0.86 |
| $LEU_2$ | 0.94 | 0.93 | 0.97 | 0.95 | 0.96 | 0.95 |
| $ILEU_2$ | 1.06 | 1.11 | 1.13 | 0.99 | 1.09 | 1.12 |
| $PRO_2$ | 0.56 | 0.58 | 0.54 | 0.60 | 0.58 | 0.56 |
| $SER_3$ | 0.92 | 0.95 | 0.96 | 0.95 | 0.96 | 0.93 |
| $THR_3$ | 0.98 | 1.02 | 0.99 | 1.03 | 0.98 | 0.98 |
| $HyPRO_3$ | 1.07 | 1.09 | 1.04 | 1.08 | 1.12 | 1.02 |
| $ASP_3$ | 0.85 | 0.84 | 0.87 | 0.84 | 0.88 | 0.82 |
| $MET_2$ | 0.82 | 0.86 | 0.83 | 0.87 | 0.81 | 0.81 |
| $CYSH_2$ | 0.52 | 0.53 | 0.55 | 0.55 | 0.54 | 0.56 |
| $PHE_2$ | 1.08 | 1.07 | 1.03 | 1.04 | 1.12 | 1.08 |
| $TYR_3$ | 1.46 | 1.52 | 1.50 | 1.48 | 1.53 | 1.46 |
| *Silylation at 135°—4 h* | | | | | | |
| $GLU_2$ | 0.54 | 0.53 | 0.59 | 0.59 | 0.55 | 0.53 |
| $GLU_3$ | 0.13 | 0.14 | 0.15 | 0.15 | 0.13 | 0.10 |
| $ARG_4$ | 0.55 | 0.53 | 0.60 | 0.56 | 0.61 | 0.58 |
| $LYS_4$ | 0.88 | 0.92 | 0.93 | 0.98 | 1.01 | 1.02 |
| $HIS_3$ | 0.52 | 0.56 | 0.56 | 0.54 | 0.51 | 0.47 |
| $TRY_3$ | 0.87 | 0.86 | 0.87 | 0.89 | 0.86 | 0.82 |
| $TRY_2$ | 0.16 | 0.15 | 0.13 | 0.17 | 0.13 | 0.08 |
| $TRY_2$/ $TRY_3$ | 0.18 | 0.17 | 0.15 | 0.19 | 0.15 | 0.10 |
| $CYS_4$ | 0.81 | 0.81 | 0.88 | 0.91 | 0.93 | 0.83 |

*Quantitative analysis of a synthetic amino acid mixture*

To establish the quantitation of the GLC analysis of the TMS amino acids, a mixture containing known amounts of selected amino acids was analyzed. The syn-

thetic mixture contained the following amino acids and amounts in 100 ml of 0.05 *N* HCl:

| *Amino acid* | *mg* |
|---|---|
| Glutamic acid | 57.6 |
| Arginine | 45.1 |
| Lysine | 41.0 |
| Histidine | 49.2 |
| Tryptophan | 50.6 |
| Cystine | 48.4 |

Three (2.0 ml) aliquots of the solution were placed in three culture tubes, dried, then derivatized, chromatographed, and electronically integrated as described in the section EXPERIMENTAL.

The quantity of each amino acid in the samples was calculated using the relative molar response values, $RMR_{a.a./phen.}$, obtained from the analysis of a standard amino acid solution with phenanthrene as I.S.

The per cent recovery of each amino acid was determined by comparison of the experimental results with the actual, or theoretical, values. The per cent recovery of the six selected amino acids is given in Table IX. The recoveries ranged from 93.6 to 105.9% and were considered good.

The GLC chromatograms for the recovery of the TMS amino acids in a mixture (Table IX) are shown in Figs. 6 and 7.

TABLE IX

GLC ANALYSIS OF TMS AMINO ACIDS IN A MIXTURE

| *Amino acid* | *Milligrams of amino acid* | | | | | |
|---|---|---|---|---|---|---|
| | *Added* | *Recovered by GLC*[a,b] | | | *Av.* | *Recovery (%)* |
| $GLU_2 + GLU_3$ | 1.152 | 1.186 | 1.169 | 1.169 | 1.174 | 101.9 |
| $ARG_4$ | 0.902 | 0.965 | 0.870 | 0.918 | 0.917 | 101.6 |
| $LYS_4$ | 0.820 | 0.838 | 0.773 | 0.783 | 0.798 | 97.3 |
| $HIS_3$ | 0.984 | 1.078 | 1.041 | 1.003 | 1.042 | 105.9 |
| $TRY_3 + TRY_2$ | 1.012 | 0.989 | 0.909 | 0.943 | 0.947 | 93.6 |
| $CYS_4$ | 0.968 | 1.075 | 0.992 | 0.968 | 1.011 | 104.4 |

[a] Phenanthrene as internal standard.
[b] Each value represents an independent analysis.

The per cent recovery of glutamic acid was obtained by summing the peak areas of $GLU_2$ and $GLU_3$. The amount of $TRY_2$ which coincided chromatographically with $CYS_4$ was calculated as being 18% of $TRY_3$, and thus $TRY_3$ and $TRY_2$ were summed to give the total area of tryptophan.

An example of the calculations used to determine the recovery of the amino acids follows. Phenanthrene, as I.S., was assigned the value of unity, and the recovery for histidine in a sample was calculated by the internal standard method:

$$g_{His} = \frac{A_{His} \times GFW_{His} \times g_{I.S.}}{A_{I.S.} \times GFW_{I.S.} \times RMR_{His/I.S.}}$$

$$g_{His} = \frac{20612 \times 155.2 \times 0.000512}{17667 \times 178.22 \times 0.52}$$

$$g_{His} = 1.003 \times 10^{-3} = 1.003 \text{ mg}$$

*TMS derivatives of urea, ornithine, and citrulline*

Urea, ornithine, and citrulline were trimethylsilylated at 135° for 15 min and 135° for 4 h. All of the other chromatographic and instrumental conditions were the same as for the twenty amino acids. The experimental details are given on the respective Figs. 8, 9 and 10. It was observed that urea, ornithine, and citrulline all gave more than one peak. $UREA_3$ was essentially converted to $UREA_4$ at 135° for 4 h, and

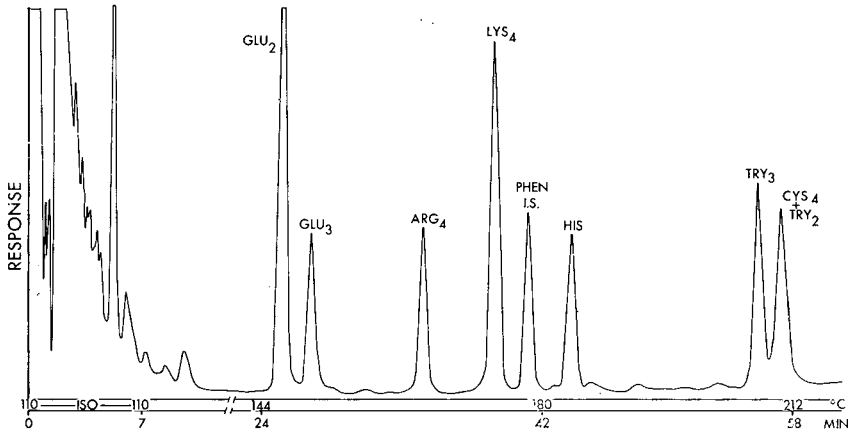



Fig. 6. GLC chromatogram of TMS amino acids; 135° for 4 h. Sample: 6 mg in 3.0 ml, 10 $\mu$g total amino acids injected (5 $\mu$l). BSTFA/$CH_3CN$ ratio 1:1. Initial temperature 110°, 7 min hold, then 2°/min. Column, same as Fig. 2.

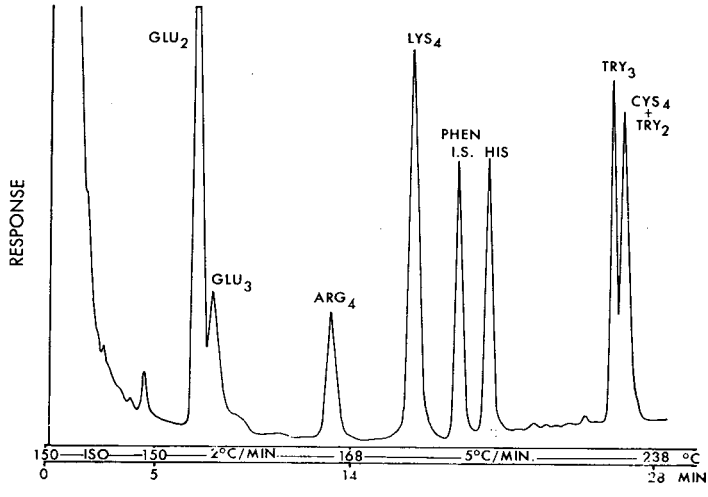



Fig. 7. GLC chromatogram of TMS amino acids; 135° for 4 h. Sample: 6 mg in 3.0 ml, 10 $\mu$g total amino acids injected (5 $\mu$l). BSTFA/$CH_3CN$ ratio 1:1. Initial temperature 150°, 5 min hold, then 2° for 9 min, then 5°/min. Column, same as Fig. 2.

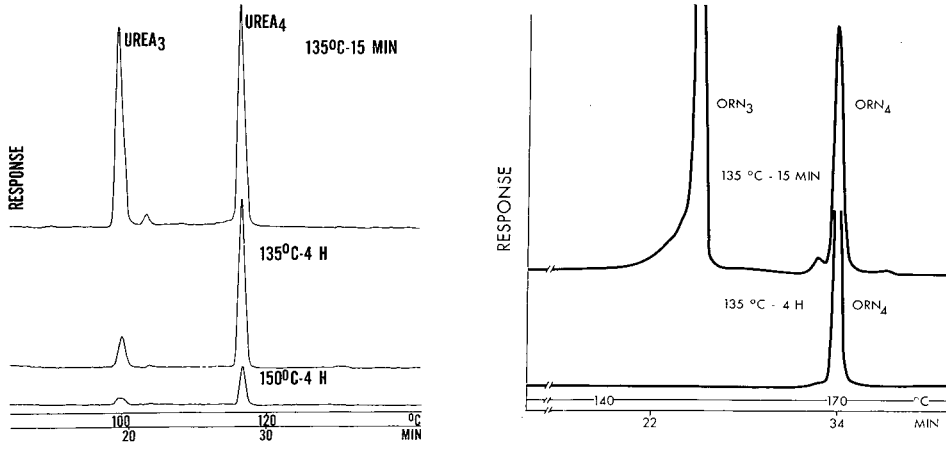



Fig. 8. GLC chromatogram of TMS urea. Sample: 1.0 $\mu$l. BSTFA/$CH_3CN$ ratio 1:1. Initial temperature 75°, 7 min hold, then 2°/min. Column, same as Fig. 2. Attenuation: 10 × 64.

Fig. 9. GLC chromatogram of TMS ornithine. BSTFA/$CH_3CN$ ratio 1:1. Initial temperature 75°, 7 min hold, then 2°/min. Column, same as Fig. 2.

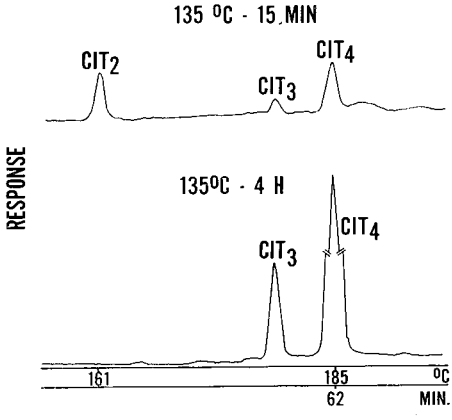



Fig. 10. GLC chromatogram of TMS citrulline. BSTFA/$CH_3CN$ ratio 1:1. Initial temperature 75°, 7 min hold, then 2°/min. Column, same as Fig. 2.

at a temperature of 150° for 4 h the peaks were reduced. Trimethylsilylation of citrulline at 135° for 15 min yielded three peaks, $CIT_2$, $CIT_3$, and $CIT_4$, which upon heating at 135° for 4 h resulted in a loss of the $CIT_2$ peak and an increase in the area for the $CIT_3$ and $CIT_4$ peaks. In a similar manner, ornithine was converted from $ORN_3$ to $ORN_4$ when silylated at 135° for 4 h.

*Analysis of biological materials*

To establish the applicability of the entire TMS derivatization and chromatographic method for the analysis of amino acids in biological materials, ribonuclease was selected as a representative protein. Fifty milligrams of ribonuclease were hydrolyzed with 50 ml of constant boiling HCl (6 *N*) for 18 h at 105° under a nitrogen

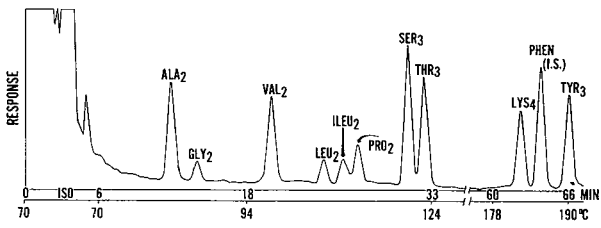



Fig. 11. GLC chromatogram of TMS amino acid derivatives of ribonuclease; 135°—15 min. Sample: 4 mg in 2.0 ml, 8 $\mu$g total amino acids injected (4 $\mu$l). Initial temperature 70°, 6 min hold, then 2°/min. Column, same as Fig. 2.

TABLE X

COMPARISON OF GAS–LIQUID AND ION-EXCHANGE CHROMATOGRAPHIC ANALYSES OF RIBONUCLEASE

Protein hydrolyzed for 18 h at 105° in a closed tube with constant boiling HCl.

| *Amino acid* | *w/w %* | | | | |
|---|---|---|---|---|---|
| | *Gas–liquid chromatography*[a] | | | *Av.*[b] | *Ion-exchange chromatography*[c] |
| Alanine | 6.45 | 6.59 | 6.80 | 6.61 | 6.91 |
| Glycine | 1.56 | 1.71 | 1.62 | 1.63 | 1.78 |
| Valine | 6.43 | 6.60 | 6.28 | 6.44 | 6.33 |
| Leucine | 2.18 | 2.03 | 2.22 | 2.14 | 2.07 |
| Isoleucine | 1.63 | 1.71 | 1.65 | 1.66 | 1.84 |
| Proline | 2.72 | 2.63 | 2.71 | 2.69 | 2.96 |
| Serine | 8.91 | 9.20 | 9.13 | 9.08 | 9.01 |
| Threonine | 6.94 | 6.77 | 6.91 | 6.87 | 7.03 |
| Aspartic acid | 12.72 | 12.89 | 12.63 | 12.75 | 13.06 |
| Methionine | 3.16 | 3.04 | 2.81 | 3.00 | 3.35 |
| Glutamic acid | 11.49 | 11.62 | 11.35 | 11.49 | 11.37 |
| Phenylalanine | 3.47 | 3.41 | 3.21 | 3.36 | 3.24 |
| Arginine | 5.01 | 5.27 | 4.96 | 5.08 | 4.92 |
| Lysine | 10.39 | 10.49 | 10.21 | 10.36 | 10.25 |
| Tyrosine | 6.29 | 6.67 | 6.49 | 6.48 | 6.81 |
| Histidine | 3.49 | 3.42 | 3.21 | 3.40 | 3.69 |
| Cystine | 6.04 | 6.21 | 5.89 | 6.05 | 5.88 |
| | | | | 99.09 | 100.50 |

[a] TMS derivatives.
[b] Average of three independent samples. Phenanthrene as internal standard.
[c] Norleucine as internal standard.

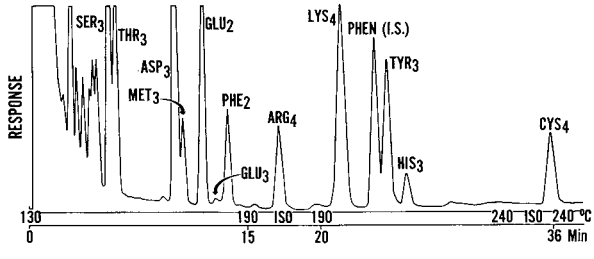



Fig. 12. GLC chromatogram of TMS amino acid derivatives of ribonuclease; 135°—4 h. Sample: 4 mg in 2.0 ml, 8 $\mu$g total amino acids injected (4 $\mu$l). Initial temperature 130°, 4°/min for 15 min, then 5 min hold, then 4°/min to 240°. Column, same as Fig. 2.

atmosphere in a closed tube. After evaporating the sample to dryness at room temperature with a rotary evaporator, the sample was transferred to a 50-ml volumetric flask and brought to volume with 0.1 *N* HCl. Five milliliter aliquots of this stock ribonuclease solution were then transferred to 16 mm × 75 mm glass reaction tubes, dried, and derivatized as described in the section EXPERIMENTAL.

The chromatograms obtained on silylation at 135° for 15 min and 4 h, respectively, are presented in Figs. 11 and 12. The data obtained from three independent GLC analyses are given in Table X, and the results are in good agreement with those from classical ion-exchange analysis.

Preliminary investigations on the GLC analysis of cation and anion-exchange cleaned human urine by the TMS technique have shown that some problems still exist. The urine samples contained a large amount of glycine, and difficulty in obtaining a single peak for glycine was noted. Both the di-trimethylsilyl ($GLY_2$) derivative and the tri-trimethylsilyl ($GLY_3$, *ca.* 10%) derivative were obtained when the samples were derivatized at 135° for both 10 and 15 min. The $GLY_3$ peak interfered with the resolution of TMS leucine and TMS proline due to the large quantity of glycine in the sample. Further investigations are needed to obviate this problem.

## CONCLUSIONS

The experiments conclusively demonstrate that the twenty natural protein amino acids and other nonprotein amino acids can be quantitatively analyzed as their TMS derivatives by GLC. This method offers important advantages in terms of simplicity and speed, as derivatization involves only the addition of reagents with no transfers. The chromatographic separation can be accomplished on a single column. Complex biological materials as urine and blood can be analyzed by this method. Research scientists will find this GLC method of the TMS amino acids valuable in their investigations of biologically important substances which contain many functional groups that can be trimethylsilylated.

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CHROM. 4350

# DETERMINATION OF SEVERAL AMMONIA OR AMINO ACID SAMPLES ON ONE CHROMATOGRAM WITH AN AMINO ACID ANALYZER*

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SUMMARY

A method is described whereby ammonia or an amino acid, produced by the enzyme-catalyzed hydrolysis of an amino acid amide or ester, can be determined in sets of five samples with a single 2-h chromatogram of an amino acid analyzer.

## INTRODUCTION

We have recently examined the different methods of determining ammonia with the object of selecting one which would be suitable for use for a study of the kinetics of the trypsin-catalyzed hydrolysis of some amino acid amides. The direct methods, using reagents such as phenol–hypochlorite[1] or Nessler's reagent[2], were inapplicable because of the known interference on color development by nitrogen-containing compounds[3–9]. The more recently described isocyanurate–salicylate method[10] was considered but also found to be strongly affected by the presence of amino acids and proteins[11]. The virtues and drawbacks of the well known Conway microdiffusion technique[12,13] are apparent from the work of CEDRANGOLO *et al.*[14]. To our disappointment, we came to the conclusion that none of the available methods was entirely satisfactory for our purpose. We therefore resorted to the use of a Beckman amino acid analyzer. This paper describes a procedure, generally suitable for monitoring the enzyme-catalyzed hydrolysis of amino acid amides or esters, by which three to five samples of ammonia or amino acid can be determined on one chromatogram with an amino acid analyzer.

## MATERIALS AND METHODS

Three times crystallized salt-free trypsin (Winley-Morris Diagnostics, Montreal), N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (Calbiochem, Los Angeles), Aminex A-5 spherical resin (Bio-Rad Labs., Richmond), sulfosalicylic acid

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and primary standard ammonium sulfate (Fisher) were from the designated suppliers. α-N-Benzoyl-L-lysinamide was a synthesized product[15]. The amino acid analyzer was a standard Beckman model 120B instrument equipped with an additional intermediate length column (30 cm). The pH 5.28 buffer reservoir was protected from the atmosphere by a citric acid guard tube.

Enzymatic reactions were terminated by the addition of half a volume of 20% sulfosalicylic acid and the tubes were centrifuged after having been cooled for at least 30 min. An aliquot of the supernatant solution was placed on a 0.9 × 15 cm column of Aminex A-5 resin surrounded by a water-jacket kept at 57°. The sample was rinsed into the column as usual with the eluting buffer (3 × 0.2 ml), the column head was filled with buffer, the buffer outlet was secured to the column, and buffer was pumped through at a rate of 68 ml/h. After 10 min, the buffer in the column head was removed, and a second sample was placed on the column. Buffer was pumped through again for 10 min, and this was repeated two or three more times. After the application of the last sample, the ninhydrin pump was turned on (34 ml/h) and the effluent stream was sent to the reaction coil. After use, the column was regenerated with 0.2 *N* sodium hydroxide, and equilibrated with buffer as usual.

## RESULTS AND DISCUSSION

Before proceeding with any experiments, the suitability of the reagents was verified. Some previously unopened bottles of reagent grade sulfosalicylic acid could not be used because they contained too much ammonia. The common buffer tris-(hydroxymethyl)aminomethane was ammonia free, but was deemed unsatisfactory for use because it gave a slight colour with ninhydrin ($C$ = 0.08 relative to 22.0 for lysine) and was eluted from the column with the basic amino acids. Phosphate buffer was incompatible with the calcium ions required for tryptic action assays. A most satisfactory buffer proved to be TES[16], which is completely ninhydrin negative.

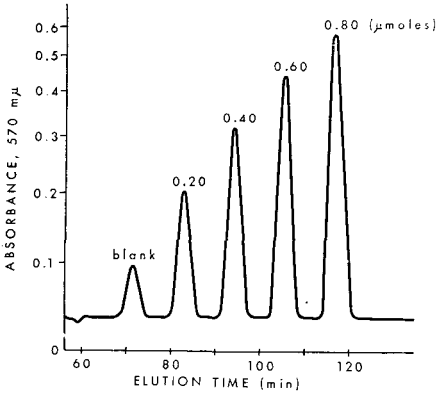


Fig. 1. Chromatography of ammonia on a 0.9 × 15 cm column of Aminex A-5 resin at 57° eluted with 0.35 *N* sodium citrate, pH 5.28, at a flow rate of 68 ml/h. Ammonia solutions consisted of 1 ml of 20% sulfosalicylic acid, 1 ml of 0.02 *M* TES buffer, $x$ ml of a 6 μmole/ml ammonia stock solution, and $1-x$ ml of water. The blank had $x$ = 0. One-milliliter samples were deposited on the column at 10-min intervals.

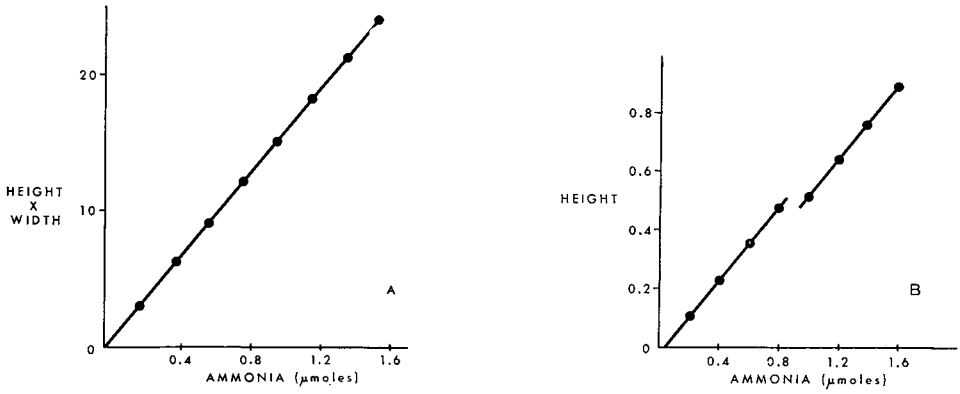


Fig. 2. Standard curves for ammonia from two chromatograms. The blank has been subtracted for each point. Conditions, and solutions prepared, as in Fig. 1.

A chromatogram of different amounts of ammonia, obtained as described above, is illustrated in Fig. 1. A plot of $H \times W$ ($H$ = net height of peak = absorbance; $W$ = width of peak at half net height) *versus* concentration, of data from two chromatograms (one for each half of the curve), gave a straight line going through the origin, as illustrated in Fig. 2A. However, when $H$ was plotted *versus* concentration, two parallel lines, neither going through the origin, were obtained as in Fig. 2B. This shows that $H$ is not a true measure of the concentration of ammonia when the chromatography technique described here is used. This is in contrast to the case where amino acids are determined with an analyzer by the conventional procedure when $H$ is a true measure of the concentration[17]. When doing amino acid analyses, it is also customary to use in the calculations a constant $C$ which is characteristic for each amino acid. When $(H \times W)/C$ was plotted *versus* concentration in our case, a straight line passing through the origin resulted. However, this was not the case when this work was initiated. At that time our instrument was equipped with new high-sensitivity cuvettes*. When data obtained using the high-sensitivity cuvettes were plotted as $(H \times W)/C$ *versus* concentration ($C$ = the constant for a sample giving an absorbance of 0.3), the curve was not linear, but slightly convex due to the variation of the constant with concentration. It transpires that a straight line results from data obtained with the regular cuvettes because the variation of the constant is within the limits of the experimental error. The plot adopted for calculating the results of ammonia determinations by our method was therefore the plot of $H \times W$ *versus* concentration.

Since the ammonia to be determined would be in the presence of large amounts of amino acid amide substrate and an equal amount of amino acid product, as well as some protein, the effect of these substances on the recovery of ammonia and on the applicability of the method was verified. The data recorded in Table I show that the recovery of ammonia from incubation mixtures containing a large excess of a typical substrate and product was within $\pm$ 3% of the amount added. An additional experiment indicated that a 0.40 μmole sample of ammonia could be recovered quantitatively after a 20-min incubation in the presence of 5 mg/ml of trypsin. Since the results in

* These were soon abandoned due to continuous leakage problems.

TABLE I

RECOVERY OF AMMONIA IN THE PRESENCE OF SUBSTRATE AND PRODUCT

Known amounts of ammonia, α-N-benzoyl-L-lysinamide (5 μmoles) and α-N-benzoyl-lysine (5 μmoles) were incubated in 2 ml of 0.01 *M* TES, pH 7.5, containing 0.05 *M* calcium chloride and 0.1 *M* sodium chloride. One milliliter of 20% sulfosalicylic acid was added to each mixture at the time indicated.

| *Time incubated (min)* | *Ammonia added (μmoles)* | *Ammonia recovered (μmoles)* |
|---|---|---|
| 10 | 0.30 | 0.29 |
| 20 | 0.60 | 0.61 |
| 30 | 0.90 | 0.93 |

Table I were obtained from a single chromatogram, they also show that the chromatographic method itself is applicable to the determination of ammonia in the presence of these other substances.

It should be noted that when several samples are successively deposited on a column as in the method described here, the components which are not retarded by the resin emerge with the void volume of effluent of the column shortly after each sample application and have therefore traversed the ammonia peaks of the previously applied samples on their way down the column. This apparently has no effect on the peaks. The components of the last peak applied, however, must emerge before the first ammonia peak to preclude interference with the colour development of the peak. With the Aminex A-5 system described here, the first ammonia peak emerges at 71 min, therefore up to five samples of ammonia, deposited on the column at 10-min intervals, could be determined on one chromatogram. If some component in the mixture other than ammonia is also retarted by the resin, not so many samples can be successfully

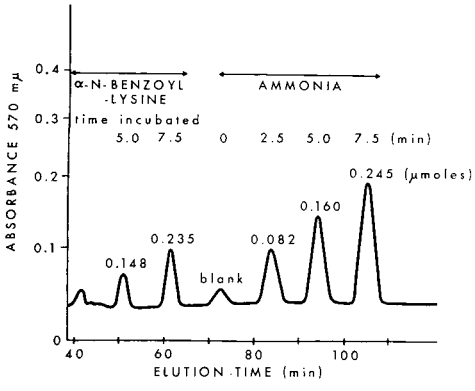


Fig. 3. Chromatography showing the hydrolysis of α-N-benzoyl-L-lysinamide by trypsin at pH 7.5 and 25°. Conditions as in Fig. 1. Trypsin solution was added to equal volumes of 0.02 *M* TES containing 0.1 *M* calcium chloride, 0.2 *M* sodium chloride, and α-*N*-benzoyl-L-lysinamide. Reactions were terminated at the times indicated by the addition of half a volume of 20% sulfosalicylic acid, and the mixtures centrifuged. An aliquot of each supernatant solution was placed on the column at 10-min intervals.

determined on one chromatogram. Such was the case encountered in our kinetic studies when ammonia was determined in the presence of α-N-benzoyl-lysine. The chromatogram from a typical kinetic experiment is represented in Fig. 3. It is seen that four samples could be determined, the first ammonia peak to emerge being preceded by the α-N-benzoyl-lysine from the last sample deposited on the column by only 11 min. This circumstance, however, proved fortuitous since the α-N-benzoyl-lysine was actually the other product of the reaction and it could be determined simultaneously with the ammonia on the same chromatogram. It is seen in Fig. 3 that very good agreement was obtained between the amounts of ammonia and α-N-benzoyl-lysine liberated by the enzymatic reaction after 5 and 7.5 min, respectively. This confirmed the reliability of the chromatographic method and moreover showed that ammonia assay was a reliable criterion as a measure of substrate hydrolysis.

The method described here has been used successfully for studying the kinetics of the trypsin-catalyzed hydrolysis of the α-N-benzoyl amides of ε-N-methyl-L-lysine and DL-homolysine[15], and is presently being used for studying the kinetics of the chymotrypsin-catalyzed hydrolysis of L-phenylalanine esters at basic pH values. For the latter, five samples of phenylalanine are analyzed as a set on one chromatogram using 0.2 *N* sodium citrate, pH 3.80, as the eluent. When the samples are placed on the column at 10-min intervals, the first peak emerges after 64 min, with the remainder following at about 5-min intervals. A similar result is obtained for the analysis of valine samples when the pH 3.28 buffer is used. The method can therefore probably be used for the determination of any amino acid once a buffer of appropriate pH has been found.

We acknowledge that the idea to develop this technique came from the work of JURASEK AND WHITAKER, who determined ammonia in sets of five samples with a single 6 to 7-h chromatogram of an amino acid analyzer after first having separated ammonia from protein using Conway cells[18].

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CHROM. 4338

# SORPTIONSWÄRMEN VON FLÜCHTIGEN STOFFEN AN EINIGEN LEBENSMITTELBESTANDTEILEN

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(Eingegangen am 29. Juli 1969)

SUMMARY

*Sorption heats of volatiles on some components of foodstuffs*

By means of gas chromatography the sorption heats of *n*-pentane, acetone, ethyl acetate and ethanol on glucose, lactose, sucrose, and sodium chloride have been determined (with some exceptions) in the presence of dry helium, wet helium, and dry air as carrier gases. For the sugars, values of 5–19 kcal/mole have been found. They are attributed to physical adsorption. For acetone on sodium chloride, values of 17–20 kcal/mole (dry) and 39 kcal/mole (wet) have been found, on potassium bromide 27 and 31 kcal/mole, resp. They are attributed to another type of sorption, possibly chemisorption.

## EINLEITUNG

Im Verlauf von Arbeiten über die Bindung von flüchtigen Aromastoffen an Lebensmittel, welche sich in festem Aggregatzustand befanden, interessierte auch die Ermittlung der Sorptionswärmen, denn aus deren Grösse lassen sich Rückschlüsse auf die Art und die Stärke der Bindung ziehen. Die Sorptionswärmen wurden mit Hilfe der Elutions-Gaschromatographie bestimmt. Als Aromastoffe wurden vier leichtflüchtige Stoffe ausgewählt, welche sehr unterschiedliche Polaritäten und funktionelle Gruppen besitzen. Als Lebensmittel wurden einige solche ausgewählt, welche bei andersartigen Sorptionsversuchen[1] keine Quellung in den verwendeten Aromastoffen zeigten. Es war nämlich zu vermuten, dass sich zwischen den Aromastoffen und quellbaren Lebensmitteln kein Sorptionsgleichgewicht in der Säule einstellt. Ein solches ist aber Voraussetzung für die angewandten Bestimmungsmethoden.

## EXPERIMENTELLER TEIL

### *Material*

Die Aromastoffe und Festkörper wurden in reinster Form aus dem Handel bezogen. Die Festkörper sind an anderer Stelle[2] näher charakterisiert. Als Trägergase wurden verwendet: Helium 99.99% und besonders reine synthetische Luft (Firma Linde, Höllriegelskreuth).

*Gaschromatographie*

Es wurde das Fraktometer 116 E der Firma Bodenseewerk Perkin-Elmer benützt, welches mit einer Einrichtung zum Anfeuchten des Trägergasstroms (gegebenenfalls mit 0.0136 mg/ml Wasserdampf) und einem Seifenblasenströmungsmesser versehen war. Die genauere Beschreibung findet sich an anderer Stelle[3]. Als Säulen wurden 2 m lange Glassäulen (4 mm innerer Durchmesser) verwendet. Sie wurden mit Festkörpern von der Korngrösse 0.15–0.30 mm gefüllt und vor den Versuchen einige Tage im Trägergasstrom bei 60–80°C ausgeheizt. Wurde feuchtes Trägergas benützt, so wurde dieses bei der Versuchstemperatur einige Tage lang durch die Säule geleitet. Die Aromastoffe wurden zu je $13.6 \cdot 10^{-3}$ mmol eingespritzt. Die gemessenen Daten dienten zur Berechnung des auf die Säulentemperatur korrigierten Retentionsvolumens

$$V_g{}^T = \frac{\lambda_r \cdot f_k \cdot F_m \cdot T_S}{\dot{\lambda} \cdot W \cdot T_m}$$

Dabei bedeuten:

$\lambda_r$ = die Retentionszeit des Aromastoffs, gemessen in cm Papiervorschub des Schreibers,
$f_k$ = den Martinfaktor zur Korrektur der Kompressibilität des Trägergases,
$F_m$ = Volumengeschwindigkeit des Trägergasstroms (ml/min),
$T_S$ = Temperatur der Säule (°K),
$\dot{\lambda}$ = Papiervorschubgeschwindigkeit des Schreibers (cm/min),
$W$ = Gewicht der Säulenfüllung (g),
$T_m$ = Temperatur des Strömungsmessers (°K).

Es wurden stets Dreifach- bis Sechsfach-Bestimmungen ausgeführt.

ERGEBNISSE

Die gefundenen Sorptionswärmen zwischen 30 und 60°C sind in den Tabellen I–III zusammengestellt. Sie wurden aus der Temperaturabhängigkeit des Retentionsvolumens erhalten[4]. Streuten die einzelnen Werte im lg $V_g{}^T/^1/_T$-Diagramm längs einer Geraden, so wurde diese durch Regressionsrechnung genau ermittelt. Dabei wurde die Temperatur als die am wenigsten fehlerbehaftete Grösse betrachtet. Aus der Steigung der Geraden wurde die Sorptionswärme erhalten. Wurde im lg $V_g{}^T/^1/_T$-

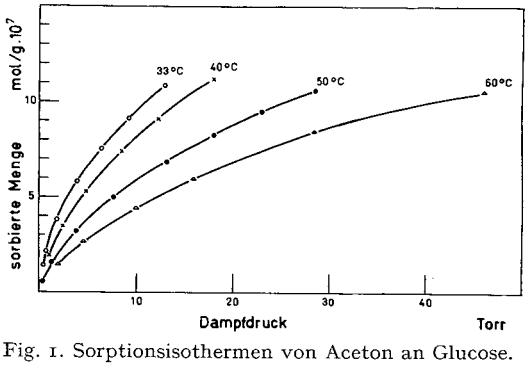


Fig. 1. Sorptionsisothermen von Aceton an Glucose.

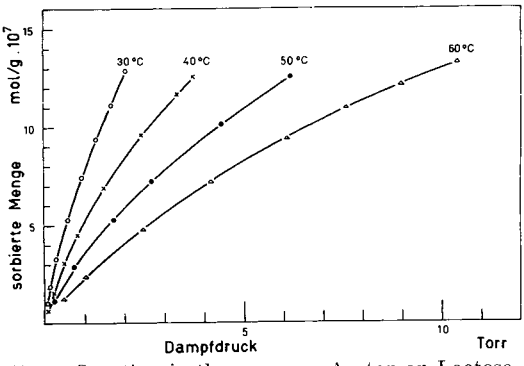


Fig. 2. Sorptionsisothermen von Aceton an Lactose.

Diagramm keine Gerade erhalten, so wurde der Wert nicht angegeben. Die Werte für Äthanol an Glucose und Lactose in Luft wurden deshalb nicht angegeben, weil kein Äthanol-Peak erschien, dafür aber der Peak eines anderen, offensichtlich von Äthanol desorbierten Gases ($O_2$?).

In den Tabellen sind ausserdem angegeben die Vertrauensbereiche der Reproduzierbarkeit für 99% statistische Sicherheit. Sie wurden nach DOERFFEL[5] aus den Streuungen der Punkte längs der Regressionsgeraden berechnet.

Zur Überprüfung der erhaltenen Werte und zur Ermittlung der Abhängigkeit der Sorptionswärmen von der Oberflächenkonzentration wurden diese bei Aceton und

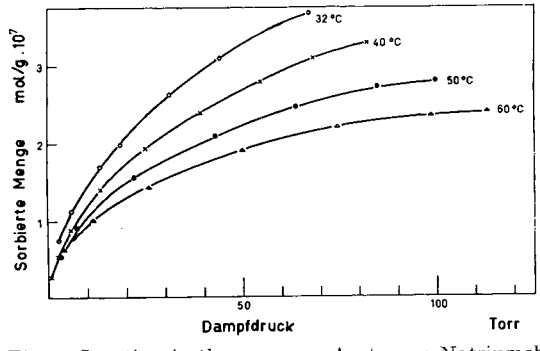


Fig. 3. Sorptionsisothermen von Aceton an Natriumchlorid.

Glucose, Lactose sowie Natriumchlorid in trockenem Helium aus der Rückfront der Peaks nach KNÖZINGER UND SPANNHEIMER[6] ermittelt. Die erhaltenen Sorptionsisothermen sind in den Fig. 1–3 dargestellt. Fig. 4 zeigt die Abhängigkeit der entsprechenden Sorptionswärmen, welche aus den Isosteren berechnet wurden, von der sorbierten Menge.

## DISKUSSION

Um die nach KNÖZINGER UND SPANNHEIMER erhaltenen Sorptionswärmen mit denjenigen aus den lg $V_g^T/^1/_T$-Diagrammen erhaltenen vergleichen zu können, müssen

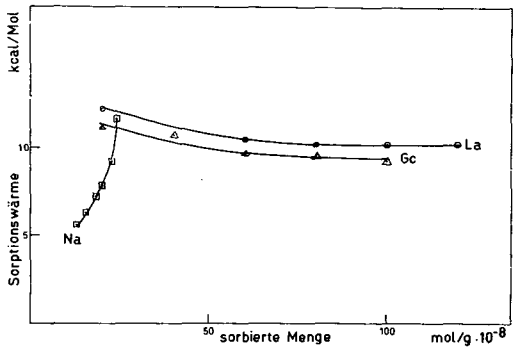


Fig. 4. Abhängigkeit der Sorptionswärmen, berechnet nach KNÖZINGER UND SPANNHEIMER, von der sorbierten Menge. Gc = Glucose, La = Lactose, Na = Natriumchlorid.

die ersteren bei derjenigen sorbierten Menge entnommen werden, welche dem Peak-Maximum entspricht. Dies ist der grösste ermittelte Wert für die sorbierte Menge. Die nach KNÖZINGER UND SPANNHEIMER erhaltenen Werte sind dann grösser als die anderen (bei Glucose und Lactose um 1–2 kcal/mol), stimmen aber doch einigermassen überein. Die Berechnung aus der Temperaturabhängigkeit des Retentionsvolumens gilt als die genauere Methode.

Wie die Tabelle I zeigt, liegen die in trockenem Helium gefundenen Sorptionswärmen in der Grössenordnung der Verdampfungswärmen der Aromastoffe (*n*-Pentan 6.2 kcal/mol, Aceton 7.7 kcal/mol, Essigsäureäthylester 8.7 kcal/mol, Äthanol 10.3 kcal/mol). Sie sind bei den Zuckern an demselben Festkörper normalerweise um so grösser, je grösser die Verdampfungswärme des Aromastoffs ist. Die Sorptionswärmen an Natriumchlorid verhalten sich umgekehrt und erreichen mit 20 kcal/mol bei Aceton

TABELLE I

SORPTIONSWÄRMEN IN TROCKENEM HELIUM (IN KCAL/MOL)

| *Aromastoff* | *Glucose* | *Lactose* | *Saccharose* | *Natriumchlorid* |
|---|---|---|---|---|
| *n*-Pentan | 5.65 ± 0.29 | 7.04 ± 0.01 | — | — |
| Aceton | 8.79 ± 0.06 | 8.40 ± 0.04 | 9.87 ± 0.02 | 19.95 ± 0.19 |
| Essigsäureäthylester | 9.57 ± 0.09 | 9.53 ± 0.05 | 12.95 ± 0.02 | 11.50 ± 0.03 |
| Äthanol | 11.33 ± 0.08 | 10.20 ± 0.06 | 12.35 ± 0.01 | 9.25 ± 0.04 |

eine Grösse, welche eine andere als physikalische Sorption vermuten lässt. An den Zuckern hingegen dürfte stets physikalische Sorption erfolgen.

Dasselbe gilt für die Sorption in Gegenwart von trockener Luft (Tabelle III). Die gefundenen Werte sind aber stets etwas grösser, mit Ausnahme desjenigen von Aceton an Natriumchlorid. Daraus ist zu schliessen, dass die Ergebnisse von Versuchen in Helium nicht unbedingt auf die lebensmitteltechnologische Praxis übertragen werden können, denn hier finden Sorptionen meist in Gegenwart von Luft statt.

In Gegenwart von Wasser (Tabelle II) wurden durchweg noch grössere Sorptionswärmen gefunden, und zwar besonders grosse bei solchen Aromastoffen, welche gut in Wasser löslich sind. Es scheint so, als ob hydrophile Aromastoffe an einer in Gegen-

TABELLE II

SORPTIONSWÄRMEN IN FEUCHTEM HELIUM (IN KCAL/MOL)

| *Aromastoff* | *Glucose* | *Lactose* | *Saccharose* | *Natriumchlorid* |
|---|---|---|---|---|
| *n*-Pentan | — | 4.89 ± 0.03 | — | — |
| Aceton | 11.50 ± 0.13 | 13.45 ± 0.01 | 18.80 ± 0.01 | 38.60 ± 0.17 |
| Essigsäureäthylester | 10.64 ± 0.03 | 12.20 ± 0.03 | 13.29 ± 0.02 | 29.20 ± 0.30 |
| Äthanol | — | 14.02 ± 0.09 | 17.75 ± 0.03 | — |

wart von Wasser hydrophileren Oberfläche stärker gebunden werden, das hydrophobe Pentan hingegen schwächer. Dies würde mit den Beobachtungen von PERRY[7] hinsichtlich der Retentionsvolumina von *n*-Alkanen an verschiedenen polaren und apolaren Säulenfüllungen übereinstimmen.

Bemerkenswert ist der grosse Wert für Aceton an Natriumchlorid. Er ist verbunden mit relativ geringen sorbierten Mengen, was bei einer Chemisorption oft vorkommt, und liegt ausserhalb des Bereichs der physikalischen Sorption. Auch bei Kaliumbromid fanden wir ähnlich grosse Werte, nämlich 27.47 ± 0.10 kcal/mol in trockenem, 30.90 ± 0.02 kcal/mol in feuchtem Helium. Obwohl damit noch keine Chemisorption bewiesen ist, kann doch vermutet werden, dass hier ein anderer Bin-

TABELLE III

SORPTIONSWÄRMEN IN TROCKENER LUFT (IN KCAL/MOL)

| *Aromastoff* | *Glucose* | *Lactose* | *Natriumchlorid* |
|---|---|---|---|
| *n*-Pentan | 6.99 ± 0.01 | 8.13 ± 0.01 | — |
| Aceton | 9.60 ± 0.04 | 9.47 ± 0.01 | 16.70 ± 0.07 |
| Essigsäureäthylester | 10.11 ± 0.03 | 10.70 ± 0.02 | 17.41 ± 0.04 |
| Äthanol | — | — | 18.43 ± 0.08 |

dungstyp vorliegt. Auch bezüglich der Abhängigkeit der Sorptionswärme von der sorbierten Menge wich Natriumchlorid von den anderen Festkörpern ab: die Sorptionswärme nahm mit der sorbierten Menge zu. Vielleicht tritt eine ähnliche Bindung ein, wie sie bei anderen Salzen (z.B. NaI, $NaClO_4$, $ZnCl_2$, $HgCl_2$, $ZnBr_2$) schon untersucht wurde. SLOVOKHOTOVA[8] schloss auf Grund der Veränderungen in den IR- und Raman-Spektren der gesättigten Lösungen solcher Salze in Aceton auf dessen Enolisierung. GULIK-KRZYWICKI UND KECKI[9] stellten fest, dass erst Spuren von Wasser eine Enolisierung hervorrufen.

DANK

Frl. CHRISTA VON STOSCH danke ich für die gewissenhafte Ausführung der Versuche, Herrn Professor Dr. I. HALÁSZ für bereitwillige Diskussion.

ZUSAMMENFASSUNG

Die Sorptionswärmen von *n*-Pentan, Aceton, Essigsäureäthylester und Äthanol

an Glucose, Lactose, Saccharose und Natriumchlorid wurden, mit einzelnen Ausnahmen, in Gegenwart von trockenem Helium, feuchtem Helium und trockener Luft als Trägergase bestimmt. Für die Zucker wurden Werte von 5–19 kcal/mol gefunden und physikalischer Sorption zugeschrieben. Für Aceton an Natriumchlorid wurden Werte von 17–20 kcal/mol (trocken) und 39 kcal/mol (feucht) gefunden, an Kaliumbromid entsprechend 27 und 31 kcal/mol. Sie werden einem anderen Sorptionstyp zugeschrieben, möglicherweise einer Chemisorption.

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CHROM. 4332

# DETERMINATION OF HYDANTOINS IN PHARMACEUTICAL PREPARATIONS BY GAS CHROMATOGRAPHY

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SUMMARY

The gas chromatographic method described is considered a quick routine analytical control method both for the assay of pure hydantoins and of those in drug form.

## INTRODUCTION

Hydantoins are cyclic ureides related in structure to barbiturates. The hydantoins, *e.g.* Diphedan (5,5-diphenylhydantoin) and Sacerno (5-ethyl-3-methyl-5-phenylhydantoin), are anticonvulsants most effective against "grand mal" and psychomotor seizures[1,2]. Diphedan and Sacerno are chosen for this investigation because they are good representative examples of the hydantoins and because they are available, have a wide application and acceptance and are most commonly used in pharmacy and medicine.

The official method of assaying the hydantoins is through the determination of their nitrogen content, based on the principle of the Kjeldahl method. The disadvantage of this method is that it does not differentiate between hydantoins and that it determines only the total nitrogen content. In addition, this long, tedious method has a wide range of error. The added ingredients, usually prescribed with the active constituent, may interfere with the method leading to inaccurate results. Also this assay is not applicable in the presence of biological fluids.

From the above discussion, it is evident that an improved accurate analytical method for the determination of hydantoins, especially those in drug form, is needed.

Gas chromatography is considered a desirable technique and has been found very suitable for analysis. Few such methods have been reported in the literature[3–5].

The purpose of this study was to develop a rapid specific gas chromatographic procedure for hydantoins. Such a method would not be subject to the limitations of the official method and would be suitable for determining and assaying samples both in pure and in drug form.

## EXPERIMENTAL

A Carlo Erba Fractovap, Model D, gas chromatographic apparatus with a Carlo Erba integrator, Model 75, equipped with a flame ionization detector and a Kienzle-type printer were used. The optimum values of gas chromatographic parameters found are shown in Table I.

TABLE I

THE OPTIMUM VALUES OF GAS CHROMATOGRAPHIC PARAMETERS

| | |
|---|---|
| Detector: | FID |
| Attenuation: | 64 × 100 for dioxan and Sacerno<br>8 × 100 for Diphedan |
| Carrier gas: | nitrogen |
| Carrier gas inlet pressure: | 1.65 kp/cm² |
| Carrier gas flow rate: | 6.52 ml/min, measured at 765 torr and 22.8° |
| Auxiliary gases: | oxygen, inlet pressure: 1.75 kp/cm²<br>hydrogen, inlet pressure: 1.25 kp/cm² |
| Column: | spiral of stainless steel, 3.0 m × 4.0 mm I.D.; packing: 12.5 w/w % SE-30 on 60/80 mesh Chromosorb W |
| Column temperature: | 256.0 ± 0.1 ° |
| Temperature of the evaporator: | 369.0 ± 1.0 ° |
| Recorder: | Speedomax G; 2.5 mV; 1 sec |
| Paper speed: | 1.27 cm/min |

Fig. 1 shows the typical chromatogram, under optimum conditions for analysis, of Diphedan and Sacerno.

The pure active constituents, Diphedan and Sacerno, were first quantitatively analyzed, using 1–5 w/w% solutions in dioxan. Certain known aliquots were taken for analysis. Fig. 2 shows the calibration curve of the pure active constituents, with the corrected integrator values. Values were corrected from the integrator-measured area,

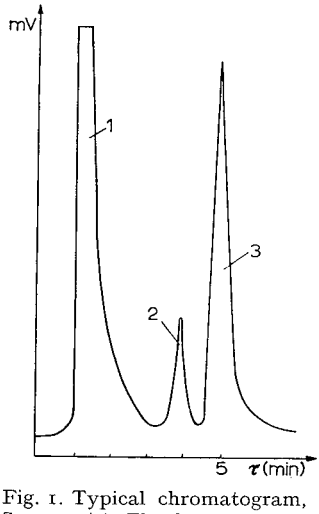



Fig. 1. Typical chromatogram, under optimum conditions for analysis, of Diphedan (2) and Sacerno (3). The first peak on the chromatogram represents dioxan.

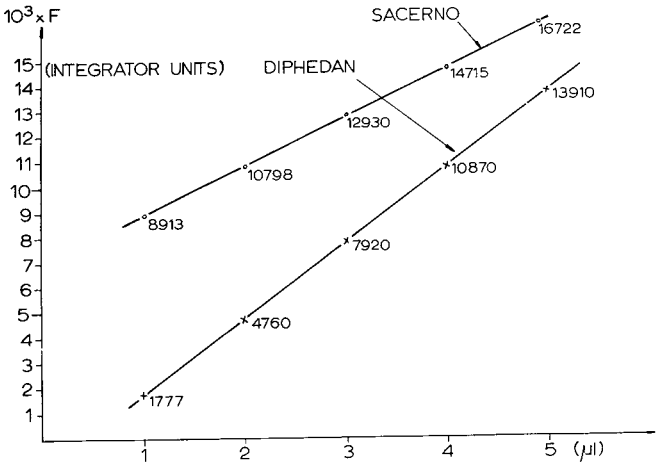



Fig. 2. Calibration curves of the pure active constituents.

while in practice there was always a difference between the base line of the apparatus and the work line of the integrator.

Consecutively, Diphedan and Sacerno in drug form were quantitatively analyzed. The tablets were prepared for analysis by the usual standard pharmacopoeia methods, using dioxan as the solvent. A solution of 1–10 w/w% of each was prepared. Certain known aliquots were taken for analysis. The peak areas were measured with the Carlo Erba integrator, Model 75.

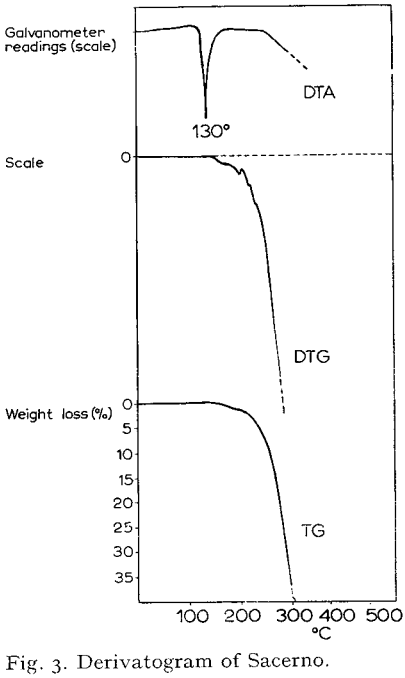
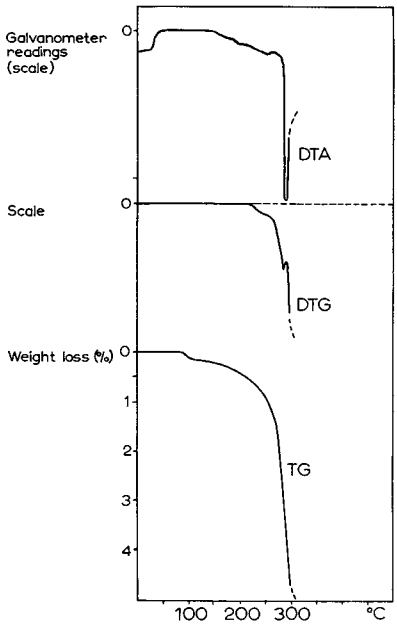



Fig. 3. Derivatogram of Sacerno.



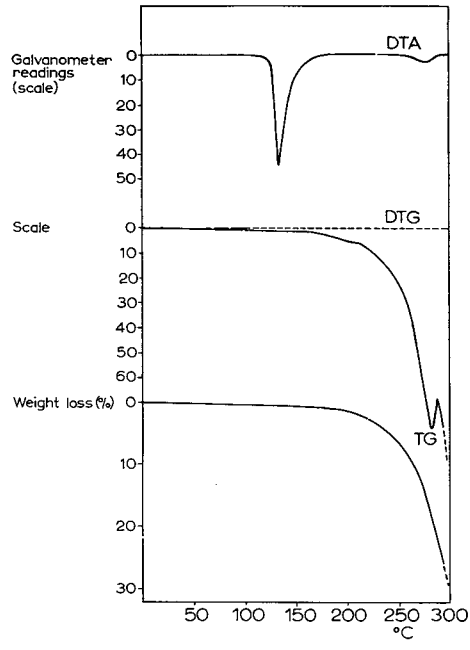




Fig. 4. Derivatogram of Diphedan.

Fig. 5. Derivatogram of the Sacerno–Diphedan (1:1, w/w) mixture.

This corrected improved method was then applied for the final quantitative determination of Diphedan and Sacerno, as pure active constituents as well as in drug form.

For completing the gas chromatographic examinations, derivatograms of Diphedan and Sacerno were made using a Derivatograph instrument (MOM, Budapest, Hungary)[6,7]. The derivatograms are shown in Figs. 3–5.

Fig. 3 shows that up to 150° Sacerno contains neither volatile components nor, in addition, any adsorbed moisture (DTG and TG curves). The DTA curve shows that Sacerno melts at 130° (endothermic peak maximum); above this temperature, at 150°, decomposition takes place in the melted phase and, up to 300°, about 40 w/w% of the sample decomposes.

Fig. 4 shows that Diphedan is practically thermostable up to 100°. Above 100° a slow decomposition process begins, reaching its maximum speed at 290°.

Fig. 5 shows the DTA curve at 130° with the endothermic peak maximum, characteristic for Sacerno. The maximum which appears at 290° on the DTG curve indicates the maximum decomposition speed of Diphedan. An evaluation of the derivatogram of the mixture proved that Sacerno melts at 130° and that, in its melted phase, Diphedan is slowly dissolved. Thus, the viscosity of the melted phase becomes greater, and the loss in weight becomes smaller in relation to what was mentioned before.

Finally we should point out that there is a possible contradiction between the temperature used in gas chromatography and the one used in derivatography. The

reason for this contradiction is that the materials decompose at a lower temperature in derivatography than at the temperature used in gas chromatography. The conditions of the two procedures have been quite different. For derivatographic analysis, the work is carried out in a static air atmosphere in the presence of oxygen, which is contrary to the conditions for gas chromatography, where a dynamic nitrogen atmosphere exists in the absence of oxygen. Under the latter conditions, dioxan also does not decompose[8]. This phenomenon had already been observed[8], but an exact explanation could not be given.

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CHROM. 4345

# AN IMPROVED METHOD FOR THE GAS CHROMATOGRAPHIC IDENTIFICATION OF *DIGITALIS* CARDENOLIDES

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SUMMARY

A simple and efficient method for the identification of steroid cardenolides as their "$\beta$"-anhydro derivatives has been developed, resulting in greatly reduced retention times and enhanced resolution. Retention data of eighteen cardenolides on three liquid phases are reported. Spectral evidence is presented showing that the tertiairy 14$\beta$-hydroxy group is neither affected by esterification nor etherification.

## INTRODUCTION

In connection with biogenetic studies of physiologically important natural products, the need arose for developing a reliable method for the detection of *Digitalis* cardenolides on a microgram scale, which may also be applied to radioactively labelled metabolites. Several colour reactions with 3,5-dinitrobenzoic acid[1–3], picric acid[4–8], *m*-dinitrobenzene[9] and 2,4,2',4'-tetranitrodiphenyl[10] are known for the characterisation of the free genins or their glycosides on thin-layer and paper chromatography[11]. However, none of these reactions can be regarded as specific. The criteria for a specific chemical reaction must be based upon the salient features of the compounds to be investigated.

The socalled cardioactive steroids are distinguished by the presence of a tertiairy hydroxy group at the C-14 ring junction. The pseudoaxial configuration of the alcohol group makes this class of naturally occurring compounds unique among the steroids. Together with the conjugated five membered lactone ring in the case of the cardenolides, or the doubly conjugated six membered pyrone ring in the case of the bufadienolides, this oxygenated substituent defines the strong biological activity of the cardiotonic steroids. We have thus focussed our attention to the 14$\beta$-hydroxy group. In particular its lability caused by 1,3-diaxial interaction with the 17$\beta$-butenolide moiety, and its tertiairy nature. Our approach consisted in utilising this very instability as a point of attack.

Because of its high sensitivity, reproducibility and resolution, as well as its

applicability for the measurement of labelled steroids, gas–liquid chromatography[12] appealed to us as the analytical method of choice.

## EXPERIMENTAL

### *Materials and methods*

Digitoxigenin, gitoxigenin and digoxigenin were obtained from K & K Laboratories, Plainview, N.Y. Their purity was verified by TLC with two solvent systems, ethyl acetate and chloroform–methanol (9:1). All solvents were analytical grade and redistilled before use. Thionyl chloride was freshly distilled shortly before use. Bis-trimethylsilylacetamide, hexamethyldisilazane and trimethylchlorosilane were purchased from Pierce Chemical Company, Rockford, Ill. All silylation reactions were conducted in glass vials under nitrogen atmosphere. Melting points were determined on a Kofler hot stage under microscopic magnification and were not corrected.

### *Thin-layer chromatography*

TLC was performed on 4 × 20 or 20 × 20 cm glass plates, coated with 0.2 mm of Silica Gel $HF_{254}$ (E. Merck, Darmstadt). The spots were visualized either by spraying with KEDDE reagent[13] at room temperature, or with 50% aqueous sulphuric acid and heating at 110° for 2 min.

### *Infrared spectroscopy*

IR spectra were recorded on a Perkin Elmer 457 double beam grating spectrometer equipped with a beam condensor. Micro disks of 1.5 mm diameter were used. The potassium bromide pellets were dried under an IR lamp before measurement. Cardenolide–potassium bromide weight ratios were maintained at 1:100.

### *Gas–liquid chromatography*

A Hewlett-Packard F & M, Model 402 high efficiency gas chromatograph, equipped with dual hydrogen flame ionisation detectors, was used. Column support consisted of 100–120 mesh silanized Gas-Chrom Q (Applied Science Laboratories, State College, Penn.). Three types of glass columns were used: column A, 90 × 0.4 cm, coated with 3% SE-30; columns B and C, 180 × 0.3 cm, coated respectively with 3% OV-1 and 3% QF-1. Operating conditions were: oven temperature 250°, injection port temperature 275°, detector temperature 285°, input attenuation 10, output attenuation 16. Helium carrier gas flow was held at 23 ml/min for column A and 33 ml/min for columns B and C at 40 p.s.i. inlet pressure. A Hamilton 10 $\mu$l syringe (Hamilton Company, Whittier, Calif.) was used for all injections.

### *Preparation of "β"-anhydrodigitoxigenin (3β-hydroxy-5β-carda-14,20; 22-dienolide) IIa*

A solution of 20 mg of digitoxigenin (*Ia*) in 0.6 ml of anhydrous pyridine was cooled in an ice bath. Under nitrogen atmosphere was added 0.2 ml of trifluoroacetic anhydride and the esterification allowed to proceed at room temperature for 2 h. The yellow coloured reaction mixture was then cooled again to 0° and a solution of 0.04 ml of thionyl chloride in 0.2 ml of anhydrous chloroform added. After 16 h at 4°, 2 ml of methanol was added, the crude reaction product taken up in 150 ml of a mixture of chloroform–ether (2:1). The organic extract was washed successively with dilute

hydrochloric acid, aqueous sodium hydrogen carbonate and water, dried over sodium sulphate and evaporated in vacuum to give 16 mg of crude *IIa*. Recrystallisation from acetone–hexane gave 11 mg of colourless needles, m.p. 199–201°. $\nu_{max(KBr)}$: 3500 and 1030 $cm^{-1}$ (–OH); 1775, 1735 and 1620 $cm^{-1}$ (–butenolide ring). A mixed melting point with authentic material* gave no depression and their IR spectra were superimposable.

*Preparation of "β"-anhydrodigitoxigenintrimethylsilylether (IId)*

*(A) From "β"-anhydrodigitoxigenin (IIa)*

*Procedure 1.* 2 mg of *IIa* was dissolved in 0.2 ml of anhydrous pyridine. Under nitrogen atmosphere was added 0.2 ml of N,O-bis-(trimethylsilyl)-acetamide (BSA), followed by two drops of trimethylchlorosilane (TMCS). After 2 h at room temperature, the mixture was blown dry with a stream of nitrogen, the residue dissolved in 1 ml of anhydrous dichloromethane and filtered through a 2 ml syringe, equipped with a Swinny adapter and a membrane filter. 1 to 2 μl of the clear filtrate was then used for each gas chromatographic analysis. Evaporation of the solvent with a stream of dry nitrogen gas gave colourless crystals of *IId*. $\nu_{max(KBr)}$: 1780, 1745 $cm^{-1}$; no –OH absorption bands.

*Procedure 2.* The same as procedure 1, except that the BSA reagent was replaced by an equal volume of hexamethyldisilazane (HMDS).

*(B) Directly in situ from digitoxigenin (Ia)*

2 mg of *Ia* was silylated as described above. The crystalline material obtained was redissolved in 0.2 ml of anhydrous pyridine and cooled to 4°. To the cooled solution was then added 0.4 ml of a freshly prepared mixture of thionylchloride–benzene–pyridine (1:15:5). Dehydration occurred within 15 min and the reaction mixture gave after injection a peak with the same retention time as *IId*, prepared by silylation of *IIa*.

*Preparation of digitoxigeninacetate (Ig)*

2 mg of *Ia* was acetylated overnight with equal (0.2 ml) volumes of anhydrous pyridine and acetic anhydride at 35°. Excess of anhydride was then destroyed by addition of 0.4 ml of methanol. The organic solvents were evaporated over a stream of nitrogen to give slightly coloured crystalls, m.p. 218–224° (ref. 14, reported m.p. 222–225°); $\nu_{max(KBr)}$: 3400, 1025 (OH), 1780, 1620 (lactone), and 1730, 1260 (acetate) $cm^{-1}$.

In analogous manner were prepared gitoxigeninacetate (*Ih*) and digoxigeninacetate (*Ii*).

*General procedure for the preparation of "β"-anhydro derivatives (IIa–i)*

For gas chromatographic analysis, compounds *IId–i* were prepared *in situ* directly from the corresponding 14β-hydroxylated parent cardenolides *Id–i*, by addition of the thionylchloride–benzene–pyridine reagent[15] at 4°, as described previously under procedure (B).

The cardadienolides *IIa*, *IIb* and *IIc* were prepared respectively from *Ia*, *Ib* and *Ic* via their TMS-derivatives, followed by dehydration with the thionylchloride–

* Kindly furnished by Prof. K. MEYER, University Basle, Switzerland.

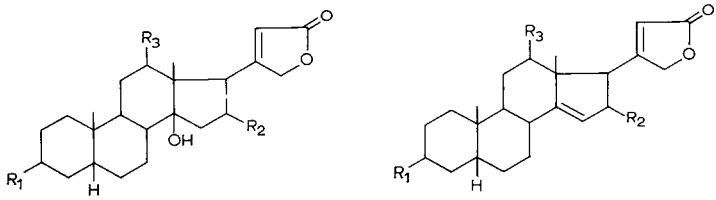



Fig. 1. Structural formula of the cardenolides studied.

| *No.* | *Name of parent cardenolide (I)* | *Substituents* |
|---|---|---|
| *a* | Digitoxigenin | $R_1 = \beta$-OH; $R_2 = R_3 = H$ |
| *b* | Gitoxigenin | $R_1 = R_2 = \beta$-OH; $R_3 = H$ |
| *c* | Digoxigenin | $R_1 = R_3 = \beta$-OH; $R_2 = H$ |
| *d* | Digitoxigenin-TMS | $R_1 = \beta$-OTMS; $R_2 = R_3 = H$ |
| *e* | Gitoxigenin-TMS | $R_1 = R_2 = \beta$-OTMS; $R_3 = H$ |
| *f* | Digoxigenin-TMS | $R_1 = R_3 = \beta$-OTMS; $R_2 = H$ |
| *g* | Digitoxigeninacetate | $R_1 = \beta$-OAc; $R_2 = R_3 = H$ |
| *h* | Gitoxigeninacetate | $R_1 = R_2 = \beta$-OAc; $R_3 = H$ |
| *i* | Digoxigeninacetate | $R_2 = R_3 = \beta$-OAc; $R_2 = H$ |

benzene–pyridine reagent in the cold and subsequent hydrolysis of the TMS-group by treatment with 90% methanol at room temperature.

## RESULTS AND DISCUSSION

The gas chromatographic analysis of cardenolides and bufadienolides on a non-selective phase has been reported previously with more or less success[16–18]. Thus, on a 360 × 0.4 cm SE-30 column, JELLIFFE AND BLANKENHORN[16] found the trimethylsilyl (TMS) ethers of digitoxigenin (*Id*) and of digoxigenin (*If*) to exhibit retention times of 37.5 and 47.5 min respectively. Silylation of the 14$\beta$-hydroxy group in both compounds was thereby assumed. The validity of this critical postulate was not

TABLE I

IR ABSORPTION DATA OF SEVERAL CARDENOLIDES

| *Cardenolide* | $\nu_{max(KBr)}$ *in* $cm^{-1}$ | |
|---|---|---|
| | *OH-group* | *Butenolide and acetate groups* |
| *Ia* | 3400–3510, 1025 | 1780, 1725, 1620 |
| *Ib* | 3320–3480, 1030 | 1780, 1750, 1730, 1620 |
| *Ic* | 3400, 1030 | 1775, 1725, 1620 |
| *Id* | 3350, 1070, 1030 | 1780, 1750, 1650, 1620 |
| *Ie* | 3400, 1030 | 1780, 1720, 1655, 1620 |
| *If* | 3350, 1025 | 1730, 1660, 1620 |
| *Ig* | 3400, 1025 | 1780, 1730, 1620, 1260 |
| *Ih* | 3450, 1030 | 1775, 1730, 1620, 1260 |
| *Ii* | 3480, 1025 | 1780, 1725, 1620, 1245 |
| *IIa* | 3500, 1030 | 1775, 1735, 1620 |
| *IId* | no absorption | 1780, 1745 |
| *IIg* | no absorption | 1780, 1750, 1725, 1250 |

further clarified by the paper of WILSON, *et al.*[17]. Our spectral analysis (Table I) indicate beyond doubt that neither the bulky trimethylsilyl ether group, nor the smaller acetyl cation are able to attack the tertiairy C-14 alcohol substituent. All acetylated and silylated 14$\beta$-hydroxy cardenolides studied (*Id–i*), showed hydroxyl absorbtion. However, no hydroxyl absorbtion bands were observed with the spectra of those compounds, in which the 14$\beta$-OH group has been removed, *i.e. IId* and *IIg*. Although under more drastic conditions in the presence of a strong acidic catalyst, angular hydroxy groups at the steroid nucleus are known to be acetylated, for instance at position 17$\beta$ in 17$\alpha$-methyltestosterone[19] and 17$\alpha$-ethinyl-19-nortestosterone[20], and at the epimeric $\alpha$-position in 17$\alpha$-hydroxyprogesterone[21,22], in the case of the steroid cardenolides, approach to C-14 from the $\beta$-side of the molecule is severely hindered by the bulky 17$\beta$-butenolide moiety. Thus, in the presence of the strong reagent thionyl chloride, nucleophilic attack at the 15$\alpha$-hydrogen atom from the rear is favoured and the cardenolides undergo an elimination reaction with release of steric strain to yield derivatives containing a planar trigonal C-14 atom.

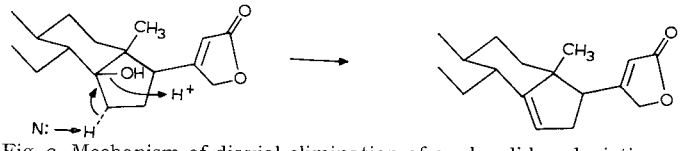



Fig. 2. Mechanism of diaxial elimination of cardenolides, depicting release of steric compression. N = nucleophilic species.

In our approach for a sensitive and specific identification method of *Digitalis*

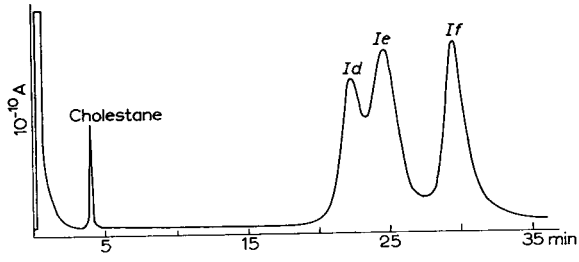
TABLE II

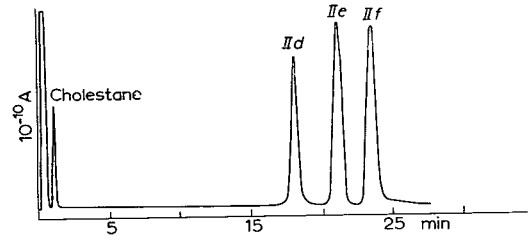
RETENTION TIMES, RELATIVE TO CHOLESTANE, CO-INJECTED AS AN INTERNAL STANDARD

| *Compound* | *Relative retention* | | |
|---|---|---|---|
| | *Column A (SE-30)* | *Column B (OV-1)* | *Column C (QF-1)* |
| Cholestane | 1.00 (2.56 min)[a] | 1.00 (4.35 min)[a] | 1.00 (1.08 min)[a] |
| *Ia* | 5.04 | 5.22 | 49.43 |
| *Ib* | 4.56 | 4.98 | 61.59 |
| *Ic* | 8.78 | 6.83 | 47.96 |
| *Id* | 5.02 | 5.19 | 39.85 |
| *Ie* | 5.84 | 5.69 | 46.52 |
| *If* | 6.12 | 6.69 | 42.69 |
| *Ig* | 6.39 | 6.61 | 102.00 |
| *Ih* | 6.03 | 6.26 | 85.71 |
| *Ii* | 9.24 | 9.00 | 138.76 |
| *IIa* | 3.13 | 3.18 | 18.64 |
| *IIb* | 1.92 | 2.02 | 12.07 |
| *IIc* | 2.51 | 2.66 | 9.66 |
| *IId* | 3.14 | 3.29 | 16.60 |
| *IIe* | 3.66 | 3.75 | 20.00 |
| *IIf* | 4.33 | 4.45 | 21.34 |
| *IIg* | 4.03 | 4.11 | 41.66 |
| *IIh* | 4.89 | 5.05 | 50.38 |
| *IIi* | 6.19 | 6.46 | 39.69 |

[a] Absolute retention time.

cardenolides, we have made use of the inherent instability, offered by the unique combination of a tertiairy C-14 hydroxy group and a C-17 lactone ring, present in a 1,3-pseudodiaxial steric relationship. The possibility of selectively acetylating or silylating only the secondary hydroxy groups at positions 3$\beta$, 12$\beta$ or 16$\beta$, while leaving the 14$\beta$-OH group intact, provided us with a facile method for preparing *in situ*, the desired $\Delta^{14(15)}$-cardadienolides *IIa–i*, suitable for gas chromatographic analysis.



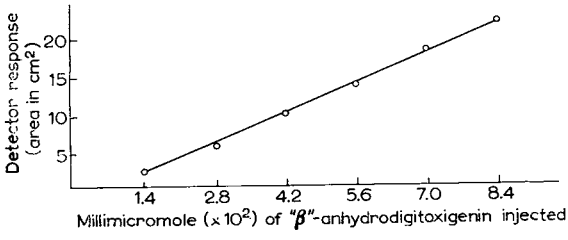




Fig. 3. Incomplete separation of the TMS-ethers of digitoxigenin, gitoxigenin and digoxigenin on OV-1 at 250°. He: 33 ml/min. Glass column, 180 × 0.3 cm.

Fig. 4. Complete separation of the "$\beta$"-anhydro TMS-ethers of digitoxigenin, gitoxigenin and digoxigenin on QF-1 at 250°. He: 33 ml/min. Glass column, 180 × 0.3 cm.

Fig. 5. Relative detector response to "$\beta$"-anhydrodigitoxigenin in the 1.4–8.4 m$\mu$mole range on OV-1 at 250°. Glass column, 180 × 0.3 cm.

Of the free genins, digitoxigenin (*Ia*) has two hydroxy groups, whereas gitoxigenin (*Ib*) and digoxigenin (*Ic*) have each three. Since *Ia* has the smaller molecular weight, it was to be expected that on the non-polar phases SE-30 and OV-1, *Ia* would be eluted before *Ib* and *Ic*. On the other hand, based on polarity considerations, *Ib* and *Ic* should have longer retention times on the selective QF-1 phase. Table II shows that this prediction is indeed true for *Ia* and *Ic*, as well as for the derivatives *Id*, *If*, *Ig* and *Ii*. However, *Ib* and *Ih* exhibited an anomalous pattern on both columns A

and B; they were eluted before *Ia* and *Ig* respectively. The overall behaviour of the cardenolides on the QF-1 phase was even more unpredictable. *Ia* and *Id* were eluted before *Ib* and *Ie* respectively, and *Ic* before *Ia*, but *Ig* was retained longer than *Ih* by the fluorosilicone polymer. It should further be noted that *Ia* and its TMS-ether *Id* displayed nearly identical retention times, both on the SE-30* and OV-1 columns. This was also the case for the "$\beta$"-anhydro compounds *IIa* and its TMS-ether *IId*. The slight difference in molecular weight was apparently not sufficient to cause their separation on a non-selective phase;

All the "$\beta$"-anhydro cardadienolides obtained showed greatly reduced retention times, and were well resolved, when compared with the 14$\beta$-hydroxylated parent cardenolides. The peaks obtained on all three columns were symmetrical; a prerequisite for quantitative measurements.

TABLE III

CALCULATED RETENTION TIME RATIOS CARDENOLIDE/CARDADIENOLIDE

| *Compounds* | *Retention time ratio* | | |
|---|---|---|---|
| | *Column A (SE-30)* | *Column B (OV-1)* | *Column C (QF-1)* |
| *Ia/IIa* | 1.61 | 1.64 | 2.65 |
| *Ib/IIb* | 2.37 | 2.46 | 5.10 |
| *Ic/IIc* | 3.30 | 2.05 | 4.96 |
| *Id/IId* | 1.59 | 1.57 | 2.40 |
| *Ie/IIe* | 1.59 | 1.51 | 2.32 |
| *If/IIf* | 1.41 | 1.50 | 2.00 |
| *Ig/IIg* | 1.58 | 1.60 | 2.44 |
| *Ih/IIh* | 1.23 | 1.24 | 1.70 |
| *Ii/IIi* | 1.49 | 1.39 | 3.49 |

Table III demonstrates the remarkable similar behaviour of the steroids investigated on the SE-30 and OV-1 columns, the only exception being digoxigenin (*Ic*). The greatest reduction in retention times were obtained with column C, which is the better column for the separation of the free genins *Ia*, *Ib* and *Ic*.

The response of a flame ionisation detector to the jet stream of a gaseous organic substance is, when operated under the same parameters of temperature, carrier gas flow and concentration of sample, essentially dependent only on the number of carbon atoms present in the molecule. Since by the dehydration reaction no carbon atoms are lost, our method is suitable for activity measurements of labelled cardenolides as well; with the exception of 15$\alpha$-tritiated compounds. To our knowledge, however, tritiated cardenolides, specifically labelled at position 15$\alpha$, have not been described so far.

We conclude that while no satisfactory separation could be achieved between the cardenolides digitoxigenin and gitoxigenin on a SE-30 or OV-1 column, and between gitoxigenin and digoxigenin on a QF-1 column, after dehydration, in the form of the "$\beta$"-anhydro derivatives, the cardadienolides thus obtained were easily separated on a selective, as well as a non-selective phase.

* We confirm in this respect the findings of WILSON *et al.*[17] with the same two compounds on a SE-30 column.

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CHROM. 4334

# A CHROMATOGRAPHIC AND FLUORIMETRIC METHOD FOR THE DETERMINATION OF OESTRIOL IN PREGNANCY URINE

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SUMMARY

A method is described for the measurement of oestriol in pregnancy urine. After hydrolysis of the urine, oestrogens are reacted with 1-dimethylaminonaphthalene-5-sulphonyl chloride (DANSYL-chloride) to give fluorescent derivatives. The oestriol derivative is separated by thin-layer chromatography on Kieselgel G in a solvent of ethanol–chloroform (5:95). It is located by viewing under UV light, eluted and measured in a spectrofluorimeter, using wavelengths of 346 m$\mu$ and 525 m$\mu$, respectively, for activation and emission.

## INTRODUCTION

Urinary oestriol is generally considered to be a valuable index of foetal viability. However, since the clinician requires results as soon as possible, most methods for determination of oestriol measure only total oestrogens and rely on the observation that oestriol is usually by far the major component in the later stages of pregnancy.

In order to measure oestriol specifically, BROWN AND COYLE[1] developed a method involving methylation and column chromatography. This procedure has become widely adopted but is still longer and more complicated than the less specific methods.

More recently, a note appeared[2] (without description of procedure) on thin-layer chromatographic separation of the fluorescent dimethylaminonaphthalene sulphonyl (DANSYL) derivative of oestrogens, followed by measurement of the fluorescence intensity. This procedure was applied to the determination of oestrogens in the plasma of the domestic fowl, where, however, there was much interference from other phenolic constituents.

In human pregnancy urine, the content of oestriol is considerably elevated, and we considered that the preparation, separation and measurement of the DANSYL derivative in this case should be a relatively simple matter.

## MATERIALS AND METHODS

1-Dimethylaminonaphthalene-5-sulphonyl chloride (DANSYL-chloride) was

obtained from the Aldrich Chemical Company, Inc. Material obtained from three other sources was unsuitable as judged by melting point determination and solubility in acetone. The DANSYL-ation reagent was prepared as a solution of 2 mg/ml of DANSYL-chloride in acetone. The solution was kept in the refrigerator and was stable for at least one month. In practice, however, fresh solutions were prepared every week.

Acetone and ethanol were analytical-grade reagents, and the latter was redistilled. Diethyl ether was washed with ferrous sulphate solution and redistilled. Oestriol was obtained from Ikapharm, Ltd.

Buffer solution (pH 10.5) was prepared by mixing 100 ml of 8% sodium bicarbonate solution with 15 ml of 5 *N* sodium hydroxide, and by adjusting the pH exactly with the aid of a pH meter with an addition either of more sodium hydroxide or bicarbonate.

*Final procedure for determination of oestriol*

Samples of 1 ml of a 24-h urine collection were diluted to 10 ml with distilled water in a 100-ml round-bottomed flask fitted with a reflux condenser. Concentrated hydrochloric acid was added (1.5 ml), and the solution was boiled under reflux for 30 min, then 1.5 g of sodium chloride were added to prevent emulsification. The cooled solution was extracted with 20 ml of ether, and the ether layer was washed with 8 ml of 8% sodium bicarbonate.

The ether extract in a 25-ml conical centrifuge tube fitted with a ground-glass joint was evaporated to dryness by warming in a water bath at 40–45° under a stream of air. In order to aid evaporation of residual water, 0.2 ml of alcohol was added, and the evaporation was repeated. To the evaporated extract were added 0.2 ml of buffer solution (pH 10.5) and 0.2 ml of DANSYL-chloride reagent. The mixture was allowed to stand at room temperature for 15 min and then was heated in a beaker of water. The heating was continued until the water had reached boiling point, and for a subsequent period of 20 min. The mixture was cooled, 3 ml of 0.5 *N* aqueous sodium hydroxide solution were added and the DANSYL-oestriol was extracted into 6 ml of ether by shaking in the stoppered tube. The lower, aqueous layer was removed by suction and the ether layer was evaporated to dryness, as previously, in the same tube.

To the evaporated extract was added 0.5 ml of chloroform, and 0.1 ml of this solution was applied to a thin-layer chromatography plate of Kieselgel G (Merck), 250 $\mu$ thick, which had been activated at 120° for 30 min. Chromatography was performed in a solvent of ethanol–chloroform (5:95) and, after allowing the solvent to evaporate, the separated DANSYL-oestriol was identified by viewing the plate under a UV-lamp giving maximum transmission at about 366 m$\mu$. The spot, having an $R_F$ value of about 0.3, was scraped into a centrifuge tube and extracted by standing for 3 min in 3 ml of ethanol (after initial vibration on a vortex mixer). The centrifuged solution was decanted into a 1-cm-square quartz fluorimeter cuvette, and the fluorescence intensity was measured in a Farrand MK-1 spectrofluorimeter. Wavelength settings were 346 m$\mu$ for activation and 525 m$\mu$ for emission. Slit system number 20 was used and the sensitivity setting was 0.1.

A standard sample of 10 $\mu$g oestriol was run with each batch of urine samples, and oestriol values were estimated in terms of fluorescence intensity of the standard.

*Variations of the method*

*Formation of DANSYL derivative.* Variables in the DANSYL-ation procedure include:

(1) Solvent for preparing the solution of DANSYL-chloride and concentration of the solution.

(2) Composition and pH of the medium in which DANSYL-ation is carried out.

(3) Time and temperature of the reaction.

In the present study, methyl ethyl ketone was tried as a substitute for acetone in order to use a higher-boiling solvent. Concentrations of DANSYL-chloride were in the range of 1–5 mg/ml. In all cases, 0.2 ml of the DANSYL-chloride reagent was treated with 0.2 ml of an alkaline solution. For the latter, three different solutions were tried: 8% $NaHCO_3$, buffer (pH 10.5) and 0.5 *N* NaOH. Temperature was varied from ambient to 100°, and time of reaction from 1 min at 100° to several hours at room temperature.

*Chromatography.* Kieselgel G plates were used as described above. Although the DANSYL-oestriol is soluble in a number of solvents, chloroform was found to be most suitable for applying a compact spot.

Suitable solvents for chromatography were ethanol–benzene (5:95), ethanol–chloroform (5:95) and dioxan–chloroform (5:95). In these solvents, DANSYL-oestriol gave spots of low $R_F$, and DANSYL-oestradiol gave high $R_F$ values. DANSYL-oestrone ran almost with the solvent front.

Cyclohexane–ethyl acetate (3:2) was also tried in order to lower the $R_F$ value of DANSYL-oestrone, but with this solvent the $R_F$ value of DANSYL-oestriol was too low.

## RESULTS

*Formation of DANSYL-oestriol*

Change of concentration of DANSYL-chloride in acetone from 1–5 mg per ml gave no changes in results. However, in order to allow for a safety margin in the event of deterioration of the reagent, it was decided to adopt a concentration of 2 mg/ml.

Of the various alkaline solutions tried, there was no appreciable difference between them, but buffer solution (pH 10.5) gave the most constant results. Linear calibration graphs were obtained when the reaction mixture was left overnight at room temperature, but quantitative formation of DANSYL-oestriol could be attained much more quickly by heating in a boiling water bath. The time of heating was relatively unimportant within the range of 1–30 min, and a 20-min period was finally adopted.

Although, in practice, the results were reproducible, there appeared to be a disadvantage in using so volatile a solvent as acetone and heating it at 100°. Under these conditions, the acetone is volatilised very quickly, with evolution of large bubbles. This brings about two problems: firstly, the reaction no longer takes place in a medium that is 50% aqueous acetone and, secondly, some of the solute tends to be carried onto the sides of the tube above the bulk of the reaction mixture.

In an attempt to lessen the volatility of the solvent, the next higher homologue, methyl ethyl ketone, was tried. This is not entirely miscible with water and did not

give reproducible results. An additional disadvantage was the need for more vigorous conditions for volatilising the solvent at a later stage.

Finally, as a compromise measure, acetone was again used as solvent, but the reaction mixture was allowed to stand at room temperature for 15 min, and was then brought gradually to 100° by standing in a water bath that was heated from room temperature to boiling. The tube was kept in the water bath for a further 20 min. Under these conditions, constant results and maximum derivative formation were obtained.

*Chromatography*

Thin-layer chromatography of standard DANSYL-oestrogens and of DANSYL-ated urine extracts is shown in Fig. 1. The solvent was ethanol–chloroform (5:95).

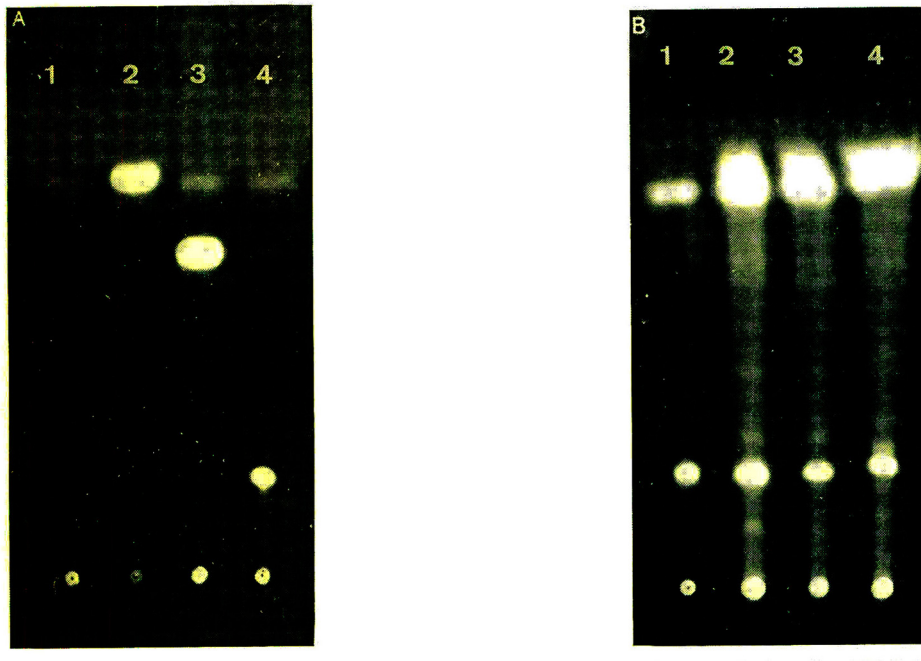


Fig. 1. Thin-layer chromatograms of DANSYL-ated oestrogens, photographed under UV light at 366 mμ. The support is Kieselgel G (Merck) applied to a thickness of 250 μ. Solvent is ethanol–chloroform (5:95). (A) Chromatogram of DANSYL-ated oestrogen standards. 1 = 'blank' (no oestrogen added); 2 = oestrone; 3 = oestradiol; 4 = oestriol. (B) Chromatogram of DANSYL-ated urinary oestrogen extracts. 1 = standard oestriol; 2, 3 and 4 = oestrogen extracts of pregnancy urines.

The $R_F$ value of the oestriol derivative is about 0.3, and this allows separation both from more polar and less polar materials. This solvent was highly suitable for DANSYL-oestriol but less so for DANSYL-oestradiol; the oestrone derivative ran almost with the solvent front.

*Sensitivity and reproducibility of the method*

Linearity of the fluorescence intensity with quantity of chromatographed spots

of DANSYL-oestriol was obtained over a range of 0.1 to 50 $\mu$g oestriol in the original sample. So it can be said that measurements can be made on 1 ml of a 24-h urine that contains approximately 0.1–50 mg oestriol. The only adjustment required is change in the sensitivity setting of the spectrofluorimeter.

Reproducibility was studied on twenty urine samples. Duplicate samples were measured; then recovery experiments were performed with known amounts of standard oestriol added before and after hydrolysis of the urine. The standard deviation between duplicates was $\pm$ 4.4% of the quantity of oestriol. There was no significant difference between recovery of oestriol added before or after hydrolysis. Thus the average recoveries, with standard deviations, were 92.4 $\pm$ 7.7% and 92.9 $\pm$ 7.2%, respectively, for oestriol added before and after hydrolysis of the urine.

## DISCUSSION

It is by no means certain that oestriol determination is the best oestrogenic measure of foetal distress. Thus COHEN[3,4] has shown that oestrogen fractions other than the three classical ones may be of importance, especially in complicated pregnancies, and that these compounds are acid labile. A further drawback in methods involving acid hydrolysis is the destruction of oestriol in the presence of large quantities of sugar, as may occur in diabetic pregnancy.

Despite these reservations, oestriol measurement is still a valuable aid in obstetric practice, and a need exists for a speedy and specific method for its determination. Not only does TLC satisfy this requirement for specificity, but presumably the method could also be applied to the labile oestrogens if a sufficiently mild, though speedy, procedure could be found for their release from the conjugates.

In the present method, the advantage of fluorescence has been added in order both to permit location of the separated oestriol and to provide great sensitivity. The latter factor allows for the dilution of the urine by a factor of 10 before hydrolysis, which means that any deleterious effect of sugar will be minimised. A further benefit of the fluorimetric method is that oestriol may be measured over the whole concentration range, without the need to dilute the sample or to change the standard.

Since derivative formation is performed on the crude ether extract of the hydrolysed urine and purification is effected by means of TLC, the whole procedure is speedy, and a complete analysis can easily be performed during one working day. It may also be noted that many samples may be analysed simultaneously since the time-consuming steps are hydrolysis, evaporation of solvent, derivative formation and chromatography. There is no theoretical limit to the number of samples that can be put simultaneously through these stages.

As may be seen in Fig. 1, DANSYL-ated urine extracts give rise to slight tailing in the chromatographic system used. In theory, therefore, it should be necessary to measure the background fluorescence and to subtract this 'blank' determination from the oestriol value. This could indeed be important when measuring samples in which the level of oestriol is much less than 1 mg/day. However, in pregnancy urine there is no significance in measuring quantities less than this. Inasmuch as 'blank' values in normal pregnancy excretion of oestriol are less than 1% of the measured value, it is unnecessary to measure the background fluorescence.

Similarly, in theory, it is advisable to use more than one standard sample, but

in practice the linearity of fluorescence with concentration over the entire range is so good that the one oestriol standard is sufficient. Nevertheless, when first introducing the method, it may be advisable to use two or more standards in order to check this linearity.

A final test of any clinical method is the comparison with an existing accepted method. Since most methods for determining oestriol in pregnancy are simply measurements of total oestrogens, we have preferred to compare our results with those obtained by the method of BROWN AND COYLE[1], which is also a chromatographic procedure. In general, results have been similar, but in cases of missed abortion we have occasionally noted complete absence of oestriol by the present method whilst obtaining positive values by the method of BROWN AND COYLE. In this condition, there is excretion of other oestrogenic material, which, however, is not oestriol. The nature of this material will be described in a future publication.

The method described in this communication is economical in terms of quantity of reagents and solvents required and can be performed by relatively unskilled technicians. It has been in routine use in our laboratory for nearly a year, and the results are in good accordance with clinical findings.

ACKNOWLEDGEMENTS

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CHROM. 4335

# SYSTEMATIC STUDIES OF CHROMOGENIC REAGENTS FOR DETECTING ORGANIC COMPOUNDS ON THIN-LAYER CHROMATOGRAMS

## I. PRIMARY, SECONDARY, AND TERTIARY AMINES

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SUMMARY

Seven chromogenic reagents for detecting primary, secondary, and tertiary amines on thin-layer chromatograms have been evaluated systematically. The reagents are *p*-dimethylaminobenzaldehyde, 2,3-dichloro-1,4-naphthoquinone, salicylaldehyde, furfural, copper sulfate, 2-thiobarbituric acid, and *o*-tolidine. All were screened for usefulness on at least three amines; the first four were further evaluated on 28 amines and 24 other organic compounds. 2,3-Dichloro-1,4-naphthoquinone is the most generally useful reagent, although it also reacts with classes of compounds other than amines. *p*-Dimethylaminobenzaldehyde will detect most aromatic amines and a few aliphatic amines and gives the best sensitivity of any of the tests. Salicylaldehyde is useful only for detecting primary amines.

## INTRODUCTION

Because of the increasingly widespread use of thin-layer chromatography for the separation and identification of organic compounds, many classes of chromogenic reagents for detecting these separated compounds must be systematically studied to permit the elucidation of the most satisfactory reagents. This paper is the first in a series intended to fulfil this objective. Because of widespread interest in detection of primary, secondary, and tertiary amines, chromogenic reagents for their detection were selected for our first report.

Aliphatic and aromatic amines must often be detected or determined at trace levels in complex samples or in micro amounts as impurities in natural waters or in body fluids. If the detection follows separation by paper or thin-layer chromatography, the detection reagent should be capable of producing a visible color with microgram amounts of many different amines.

Many chromogenic reactions have been proposed for the detection of amines

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on paper and thin-layer chromatograms and for spot-test and colorimetric analysis. Among the more widely used reactions are those employing Dragendorff's reagent for tertiary and quaternary amines[1,2], diazo salts for aromatic amines[3–6], furfural for aromatic amines[7–10], ninhydrin[11,12], salicylaldehyde for primary arylalkyl amines[13,14], and *p*-dimethylaminobenzaldehyde[5,6,15].

Other reactions of interest employ 2,4-dinitrofluorobenzene[16,17], 2-nitro-1,3-indanedione[18], aconitic anhydride for tertiary and quaternary amines[19,20], piperonal for aromatic amines[21], 2,3-dichloro-1,4-naphthoquinone for *n*-alkylvinylamines[22,23], copper sulfate for secondary aliphatic and cyclic amines[24], 2-thiobarbituric acid for pyrimidines[25], *o*-tolidine for pyridines[26], and nitroprusside for secondary amines[27]. Most other reagents, such as acid–base indicators, will detect too many other functional groups and structures.

Thorough examination of the literature relating to the detection and identification of amines by chromogenic reagents resulted in the selection of seven reagents for systematic study. These were *p*-dimethylaminobenzaldehyde, 2,3-dichloro-1,4-naphthoquinone, salicylaldehyde, furfural, copper sulfate, 2-thiobarbituric acid, and *o*-tolidine. These reagents were selected because (1) they have been reported to selectively detect trace amounts of different types of amines by producing brilliant colors, (2) they are simple to use, and (3) they are relatively specific for amines.

Four of these reagents survived preliminary evaluation and were tested with 28 different amines and compounds containing amino groups, to determine their sensitivity, and with 24 other organic compounds, to determine what other types of compounds might also react and thus interfere with the test under the selected experimental conditions. The ISCC-NBS color name charts[28] were used to classify the observed colors. The 2,4-dichloro-1,4-naphthoquinone reagent appears to be the most widely useful reagent. Both *p*-dimethylaminobenzaldehyde and salicylaldehyde also have areas of utility, and the latter reacts with the greatest variety of amines. Both were reasonably free from interferences caused by other classes of organic compounds.

## EXPERIMENTAL

In order to evaluate as many chromogenic reagents as possible, the experimental program was divided into three series of tests: preliminary screening, general applicability, and evaluation of interferences. In the first series, all of the reagents selected for study were screened for their potential usefulness by studying their reactions with 5, 10, and/or 50 $\mu$g of three amines of the type with which the reagent was reported to react. The compounds selected for the preliminary screening tests are given in Table I. An additional six amines were used to evaluate the copper sulfate reagent because of inconclusive results on the first three.

In the second series, the four reagents that survived the first series were evaluated on twenty-eight amines. These compounds are listed in Table IV. Both simple amines and amines containing other functional groups were included. Heterocyclic nitrogen compounds were omitted unless they also contained an amino group.

In the third series, the same four reagents were evaluated on twenty-four organic compounds representing a variety of functional groups and structures. These compounds are shown later in Table VI. One amino compound, morpholine, was included in this group rather than in the second group because it often undergoes

TABLE I

COMPOUNDS USED IN PRELIMINARY SCREENING TESTS

| *Chromogenic reagent* | *Compound* |
|---|---|
| *p*-Dimethylaminobenzaldehyde | *p*-Nitrosodiphenylamine, furosemide, benzidine |
| 2,3-Dichloro-1,4-naphthoquinone | Allylamine, benzimidazole, *n*-butylamine |
| Salicylaldehyde | Nicotinamide, isonicotinic acid hydrazide, *n*-butylamine |
| Furfural | 2,4-Toluenediamine, phenylenediamine, 2-aminonaphthalene |
| Copper sulfate | Morpholine, diphenylamine, l(−)-proline, dipropylamine, piperidine, adrenaline, caprolactam, diethylamine |
| 2-Thiobarbituric acid | Barbituric acid, folic acid, theobromine |
| *o*-Tolidine | Isonicotinic acid hydrazide, nicotinonitrile, nicotinamide |

different reactions than do amines. Because of our interest in organometallic and organophosphorus compounds in natural waters, four of these compounds were included in the test group.

All of the tests were conducted on Brinkman Catalog No. 5762 precoated silica gel thin-layer chromatoplates. Ten microliters of the test solutions were evaporated on the plates with the aid of a 50-$\mu$l Hamilton syringe, using a technique to produce a spot about 2 mm in diameter. Most evaporations were accomplished by warming the chromatoplate slightly on a hot plate. When the volatile amines were spotted, the chromatoplate was kept at room temperature. These amines are strongly retained by the silica gel, and the solvent was simply removed by directing air across the plate. All chromogenic reagent solutions were sprayed on the plates with MISCO 10-ml tube sprayers selected to produce a fine spray when compressed air at about 10 p.s.i.g. was used as a carrier.

The three sets of test compounds were originally prepared for use as 5 or 2.5 mg/ml solutions, in the solvents shown in Table II, so that 10 $\mu$l would contain the largest amount (50 or 25 $\mu$g) of the compound that was tested. When smaller amounts of compounds were desired for testing, they were obtained by diluting the 5 or 2.5 mg/ml solution with the appropriate solvent to produce a new solution containing the required amount in 10 $\mu$l. All of these test solutions were stored in 2-oz. glass bottles fitted with Poly-Seal caps, which contain a polyethylene conical seal.

*Chromogenic reagents*

*p-Dimethylaminobenzaldehyde.* Spray the plate with a 1% (w/v) solution of *p*-dimethylaminobenzaldehyde in glacial acetic acid.

*2,3-Dichloro-1,4-naphthoquinone.* Prepare a 1% (w/v) solution of 2,3-dichloro-1,4-naphthoquinone in benzene. Use this solution as the chromogenic reagent.

*Salicylaldehyde.* Prepare a 1% (w/v) solution of salicylaldehyde in benzene. Use this solution as the chromogenic reagent.

*Furfural.* Add 500 mg (about ten drops) of furfural to 10 ml of glacial acetic acid and mix the solution thoroughly. Use this solution as the chromogenic reagent.

*Copper sulfate.* Prepare a 5% (w/v) solution of copper sulfate pentahydrate in distilled water. Prepare a 1:3 (w/v) mixture of carbon disulfide and benzene. Spray

TABLE II

SOLVENTS USED FOR TEST SOLUTIONS

| *Solvent* | *Compound* | *Solvent* | *Compound* |
|---|---|---|---|
| Ethyl alcohol, absolute | Adipic acid | Diethyl ether (cont.) | Benzonitrile |
| | Benzenearsonic acid | | Benzyl alcohol |
| | Benzimidazole | | Benzylamine |
| | Caprolactam | | *n*-Butylamine |
| | Carbazole | | 3-Chloroaniline |
| | Diphenylamine | | *p*-Chlorobenzenethiol |
| | Folic acid | | Diethylamine |
| | Isonicotinic acid hydrazide | | N,N-Diethylaniline |
| | Maleic acid hydrazide | | Dipropylamine |
| | N-Methylphenylamine | | *n*-Heptylamine |
| | Nicotinamide | | 2-Hydroxyethylhydrazine |
| | Piperidine | | Isobutylamine |
| | | | Methyl benzoate |
| Acetone | Furosemide | | Morpholine |
| | Hexamethylenetetramine | | 1-Naphthol |
| | | | Nicotinonitrile |
| Benzene | Anthracene | | Nitrobenzene |
| | 1,6-Hexanediamine | | N-Nitrosodimethylamine |
| | Lindane | | *p*-Nitrosodiphenylamine |
| | | | 2,4-Pentanedione |
| Water | Adrenaline | | *o*-Phenylenediamine |
| | Chloramine-T | | *p*-Phenylenediamine |
| | 1(−)-Proline | | Phloroglucinol dihydrate |
| | Theobromine | | Propylamine |
| | | | Pyrrole |
| Diethyl ether | Acetophenone | | 2,4-Toluenediamine |
| | Acridine | | *α*-Toluenethiol |
| | Allylamine | | Tributylamine |
| | 1-Aminonaphthalene | | Triphenylamine |
| | 2-Aminonaphthalene | | |
| | Aniline | Methyl alcohol | Dimethylchlorophosphate |
| | Anisole | | Monoethyl acid ortho-phosphate |
| | Barbituric acid | | |
| | Benzaldehyde | | Phenylphosphonic acid |
| | Benzoic acid | | |

the thin layer plate consecutively with the copper sulfate solution, with ammonium hydroxide, and with the carbon disulfide–benzene mixture.

*2-Thiobarbituric acid.* Dissolve 250 mg of 2-thiobarbituric acid and 0.25 ml of 4 *N* sodium hydroxide in distilled water and dilute the solution to 25 ml with distilled water. Dissolve 1.85 g of sodium citrate dihydrate in 1.6 ml of concentrated hydrochloric acid and dilute this solution to 12.5 ml with distilled water. Mix these two solutions to obtain the chromogenic reagent.

*o-Tolidine.* Two different sets of reagents were tested; in one the order of addition of the reagents was changed so that three different studies were conducted. (1) Prepare a 1% solution of *o*-tolidine in ethyl alcohol. Add saturated bromine water to a 1% (w/v) aqueous solution of potassium cyanide until a permanent yellow color is obtained. Then decolorize the solution by dropwise addition of more of the potassium cyanide solution. A solution of cyanogen bromide is thus produced. Spray the thin layer plate first with the *o*-tolidine solution, then with the cyanogen bromide solution. (2) Prepare a saturated aqueous suspension of *o*-tolidine, a saturated aqueous solution

of bromine, and a 1% aqueous solution of potassium cyanide. Spray these reagents in the order given. (3) Prepare a saturated aqueous solution of bromine, a 1% aqueous solution of potassium cyanide, and a 1% solution of *o*-tolidine in ethyl alcohol. Spray these reagents in the order given.

The sources of the compounds used in these studies are shown in Table III.

TABLE III

SOURCES OF COMPOUNDS, SOLVENTS, AND REAGENTS

| *Compound* | *Source* | *Grade (if any)* |
|---|---|---|
| Acetic acid | Matheson Scientific | ACS Reagent |
| Acetone | Mallinckrodt | Analytical Reagent |
| Acetophenone | Fisher | Certified |
| Acridine | Eastman | |
| Adipic acid | Fisher | Certified |
| Adrenaline | Eastman | |
| Allylamine | Eastman | |
| 1-Aminonaphthalene | MCB | |
| 2-Aminonaphthalene | Aldrich | |
| Ammonium hydroxide | Matheson Scientific | ACS Reagent |
| Aniline | Eastman | |
| Anisole | Fisher | Certified |
| Anthracene | Eastman | |
| 1(+)-Ascorbic acid | MCB | |
| Barbituric acid | Eastman | |
| Benzaldehyde | Fisher | Certified |
| Benzene | Mallinckrodt | Analytical Reagent |
| Benzenearsonic acid | Eastman | |
| Benzidine | E. Merck AG | Reagent grade |
| Benzimidazole | Eastman | |
| Benzoic acid | J. T. Baker | Bakers Analyzed |
| Benzonitrile | Eastman | |
| Benzyl alcohol | Fisher | Certified |
| Benzylamine | Eastman | |
| *n*-Butylamine | Eastman | |
| Caprolactam | National Aniline | Spectranalyzed |
| Carbon disulfide | Fisher | Certified |
| Carbazole | Fisher | Certified |
| Chloramine-T | Eastman | |
| *p*-Chlorobenzenethiol | Eastman | Practical |
| Copper sulfate pentahydrate | Mallinckrodt | Analytical Reagent |
| Chloroaniline | MCB | |
| N,N-Diethylamine | Eastman | |
| N,N-Diethylaniline | Eastman | |
| Diethyl ether | Mallinckrodt | Analytical Reagent |
| Dimethylchlorophosphate | Aldrich | |
| *p*-Dimethylaminobenzaldehyde | E. Merck AG | |
| Diphenylamine | Fisher | Purified |
| Dipropylamine | Eastman | |
| Ethyl alcohol | U.S. Industrial | USP/NF |
| Folic acid | City Chemical | USP |
| Furaldehyde (furfural) | MCB | |
| Furosemide | National Laboratories | |
| *n*-Heptylamine | Eastman | |
| Hexamethylenetetramine | Eastman | |
| Hexanediamine | Eastman | |
| Hydrochloric acid | Matheson Scientific | ACS Reagent |
| 2-Hydroxyethylhydrazine | Aldrich | |
| Isobutylamine | Eastman | |

TABLE III *(continued)*

| | | |
|---|---|---|
| Isonicotinic acid hydrazide | Eastman | |
| Maleic acid hydrazide | Eastman | Practical |
| Methyl alcohol | Mallinckrodt | Analytical Reagent |
| Methyl benzoate | Fisher | Reagent |
| N-Methylphenylamine | Eastman | |
| Monoethyl acid orthophosphate | Victor | |
| Lindane | Diamond-Shamrock | |
| Morpholine | Fisher | Purified |
| 1-Naphthol | Eastman | |
| Nicotinamide | Eastman | |
| Nicotinonitrile | Eastman | |
| Nitrobenzene | Fisher | Certified |
| N-Nitrosodimethylamine | Eastman | |
| *p*-Nitrosodiphenylamine | Eastman | |
| 2,4-Pentanedione | Eastman | |
| *o*-Phenylenediamine | Eastman | Practical |
| *p*-Phenylenediamine | Fisher | Certified |
| Phenylphosphonic acid | MCB | |
| Phloroglucinol dihydrate | MCB | |
| Piperidine | Fisher | Certified |
| Potassium cyanide | Merck | Reagent grade |
| l(−)-Proline | Aldrich | |
| Propylamine | Eastman | |
| Pyrrole | MCB | Practical |
| Salicylaldehyde | MCB | Practical |
| Sodium citrate | Merck | Reagent grade |
| Sodium hydroxide | Mallinckrodt | Analytical Reagent |
| Theobromine | Eastman | |
| α-Toluenethiol | Eastman | |
| 2,4-Toluenediamine | Eastman | Practical |
| Tributylamine | Eastman | |
| Triphenylamine | Eastman | |

## RESULTS AND DISCUSSION

Four reagents—*p*-dimethylaminobenzaldehyde, 2,3-dichloro-1,4-naphthoquinone, salicylaldehyde, and furfural—survived the preliminary evaluation, while the other three tests—copper sulfate, 2-thiobarbituric acid, and *o*-tolidine—failed. The four tests chosen for further study all gave positive tests with a least one amine at the 5-μg level. The copper sulfate test proved not to be reliable because it produced a color only with two of the nine amines that were tested. 2-Thiobarbituric acid was rejected because it produced no color with 50 μg of the three amines and produced rapidly fading colors with 5 μg. *o*-Tolidine suspended in water or dissolved in absolute alcohol was evaluated in several combinations with *in situ* prepared cyanogen bromide. The combination of *o*-tolidine in absolute ethyl alcohol and preprepared cyanogen bromide gave a color with 50 μg of the three amines but not with smaller amounts, and the other combinations gave no positive tests.

Results that were obtained on the 28 amines with the four chromogenic reagents that survived the preliminary screening tests are shown in Table IV. Results obtained on the 24 other organic compounds with the 2,3-dichloro-1,4-naphthoquinone reagent are shown in Table V. Tests on these 24 compounds with *p*-dimethylaminobenzaldehyde

TABLE IV

COLORS PRODUCED BY FOUR CHROMOGENIC REAGENTS FOR AMINES

| | *Color observed for amount tested (μg)* | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *p-Dimethylaminobenzaldehyde* | | | | | *2,3-Dichloro-1,4-naphtho-quinone* | | | | | *Salicylaldehyde* | | | | | *Furfural* | | | | |
| | *50* | *5* | *1* | *0.1* | *0.01* | *50* | *5* | *1* | *0.1* | *0.01* | *50* | *5* | *1* | *0.1* | *0.01* | *50* | *5* | *1* | *0.1* | *0.01* |
| Propylamine | a[a] | a | a | a | — | 4[b] | 52[b] | a | a | — | 83 | 89 | 89 | a | a | a | a | a | a | — |
| *n*-Butylamine | 52 | a | a | a | — | 52 | 52[b] | a | a | — | 89 | 83 | 89 | a | — | a | a | a | a | — |
| Isobutylamine | a | a | a | a | — | a | 52[b] | 52[b] | a | — | 83 | 93 | 89 | a | — | a | a | a | a | — |
| *n*-Heptylamine | a | a | a | — | — | 4 | 52[b] | a | a | — | 83 | 83 | 89 | a | — | a | a | a | — | — |
| Allylamine | 89 | a | a | a | — | 52[b] | 73[b] | a | a | — | 82 | 83 | 92 | a | — | a | a | a | a | — |
| Diethylamine | a | a | — | — | — | a | a | — | — | — | a | a | — | — | — | a | a | — | — | — |
| Dipropylamine | a | — | — | — | — | a | — | — | — | — | a | — | — | — | — | a | — | — | — | — |
| Tributylamine | a | — | — | — | — | a | — | — | — | — | a | — | — | — | — | a | — | — | — | — |
| N-Nitrosodimethylamine | a | — | — | — | — | a | — | — | — | — | a | — | — | — | — | a | — | — | — | — |
| Chloramine-T | a | a | a | — | — | a | a | a | a | — | 86[b] | 89[b] | a | — | — | a | a | a | — | — |
| 1,6-Hexanediamine | 89 | a | a | a | — | 52 | 52[b] | a | a | — | 82 | 83 | 89 | a | — | a | a | a | a | — |
| Haxmethyleenetetramine | a | — | — | — | — | a | — | — | — | — | a | — | — | — | — | a | — | — | — | — |
| Maleic acid hydrazide | a | — | — | — | — | a | — | — | — | — | a | — | — | — | — | a | — | — | — | — |
| Polic acid | a | | — | — | — | a | | — | — | — | a | | — | — | — | a | | — | — | — |
| Diphenylamine | 86[b] | a | a | a | — | 223 | 221 | a | a | — | a | a | a | a | — | a | a | a | a | — |
| N-Methyldiphenylamine | a | a | a | a | — | 223 | 221 | a | a | — | a | a | a | a | — | a | a | a | a | — |
| *p*-Nitrosodiphenylamine | a | — | — | — | — | a | — | — | — | — | a | — | — | — | — | a | — | — | — | — |
| Triphenylamine | 89 | a | a | — | — | 221 | 221 | a | — | — | a | a | a | — | — | 226[b] | a | a | — | — |
| Benzylamine | 89 | a | a | a | — | 52 | 52[b] | a | a | — | 82 | 83 | 89 | a | — | a | a | a | a | — |
| Aniline | 82 | 82 | 83 | 89 | 92 | 26 | 52[b] | a | a | — | 83 | 89 | a | a | — | 248[c] | 4 | a | a | — |
| 3-Chloroaniline | 82 | 82 | 83 | 89 | 92 | 52 | 52[b] | a | a | — | 83 | 89 | a | a | — | 248[c] | 4 | a | a | — |
| N,N-Diethylaniline | 226[b] | a | a | — | — | 224/ 18[d] | 221/ 5[d] | a | — | — | a | a | a | — | — | 226[b] | a | a | — | — |
| 2,4-Toluenediamine | 67 | 70 | 83 | 86 | 89 | 230 | 224/ 201[d] | 231 | 9 | — | 82 | 83 | 90 | a | — | 257 | 256 | 70 | 89 | a |
| *p*-Phenylenediamine | 37 | 52 | 27 | 31 | 9 | 213 | 256 | 231 | a | — | 84 | 83 | 83 | 89 | 92 | 257 | 256 | 90 | 89 | 92 |
| *o*-Phenylenediamine | 82 | 70 | 67 | 73 | 73 | 221 | 52 | a | a | — | 83 | 89 | 92 | a | — | 256/ 87[d] | 89 | 92 | a | — |
| 1-Aminonaphthalene | 82 | 83 | 83 | 89 | 92 | 221 | 221 | 9 | a | — | 83 | 70 | 73 | a | — | 250 | 73 | 79 | a | — |
| 2-Aminonaphthalene | 50 | 70 | 83 | 73 | 89 | 43 | 52[b] | 9 | a | — | 83 | 83 | 73 | a | — | 224 | 256 | 9 | 89 | a |
| 2-Hydroxyethylhydrazine | 52[b] | 89/ 86[d] | a | a | — | 67 | 52[b] | a | a | — | a | | a | a | — | 83 | a | a | a | — |

[a] a = No color observed.
[b] Color developed on standing.
[c] Fluorescent color.
[d] Color changed on standing.

TABLE V

COLORS PRODUCED BY ORGANIC COMPOUNDS WITH 2,3-DICHLORO-1,4-NAPHTHOQUINONE

| *Compound* | *Color observed for amount tested (μg)* | | |
|---|---|---|---|
| | *25* | *5* | *1* |
| Benzoic acid | a[a] | a | a |
| Adipic acid | a | a | a |
| Anisole | a | a | a |
| Benzaldehyde | a | 28 | 28 |
| Acetophenone | a | 28 | 28 |
| 2,4-Pentanedione | a | a | a |
| Methyl benzoate | a | a | a |
| Benzonitrile | a | a | a |
| *p*-Tolyl disulfide | 86 | a | 31 |
| Morpholine | 34 | 34 | 34 |
| Carbazole | 29 | 2 | 4 |
| Acridine | 98/97[b] | 98/97[b] | 98/101[b] |
| Benzylalcohol | a | a | a |
| Nitrobenzene | a | a | a |
| 1-Naphthol | 15/55[b] | 5/55[b] | 7/54[b] |
| Phloroglucinol dihydrate | 68 | 66 | 76 |
| *α*-Toluenethiol | a | 86 | 86 |
| Anthracene | 221 | 221 | 226 |
| Benzenearsonic acid | a | a | a |
| Lindane | a | a | a |
| *p*-Chlorobenzenethiol | 83 | 101 | 28 |
| Dimethylchlorophosphate | a | a | a |
| Monoethyl acid ortho-phosphate | a | a | a |
| Phenyl phosphonic acid | a | a | a |

[a] a = No color observed.
[b] First color listed is color observed before spraying.

gave only one positive reaction, color number 84, with acridine at 25, 5 and 1 μg. Tests with salicylaldehyde and furfural gave the colors listed in Table VI.

For convenience in comparing the colors, a list of names for the color numbers, as designated by the National Bureau of Standards, is shown in Table VII.

TABLE VI

COLOURS PRODUCED WITH SALICYLALDEHYDE AND FURFURAL

| *Compound* | *Color observed for amount tested (g)* | | | | | |
|---|---|---|---|---|---|---|
| | *Salicylaldehyde* | | | *Furfural* | | |
| | *25* | *5* | *1* | *25* | *5* | *1* |
| Acridine | 98/104[b] | 98/101[b] | 98/104[b] | 97 | 97 | 97 |
| 1-Naphthol | 42/15[b] | 33/29[b] | 31/32[b] | 9 | 8 | 9 |
| Phloroglucinol dihydrate | 73 | 73 | [a] | [a] | [a] | [a] |

[a] No color observed.
[b] First color is color before spraying.

TABLE VII

COLOR NUMBERS AND COLOR NAMES

| *Number* | *Name* | *Number* | *Name* |
|---|---|---|---|
| 2 | Strong pink | 68 | Strong orange yellow |
| 4 | Light pink | 70 | Light orange yellow |
| 5 | Medium pink | 73 | Pale orange yellow |
| 7 | Pale pink | 76 | Light yellowish brown |
| 8 | Grayish pink | 79 | Light grayish yellowish brown |
| 9 | Pinkish white | 82 | Vivid yellow |
| 15 | Medium red | 83 | Brilliant yellow |
| 18 | Light grayish red | 84 | Strong yellow |
| 26 | Strong yellowish pink | 86 | Light yellow |
| 27 | Deep yellowish pink | 87 | Medium yellow |
| 28 | Light yellowish pink | 89 | Pale yellow |
| 29 | Medium yellowish pink | 90 | Grayish yellow |
| 31 | Pale yellowish pink | 92 | Yellowish white |
| 32 | Grayish yellowish pink | 97 | Vivid greenish yellow |
| 33 | Brownish pink | 98 | Brilliant greenish yellow |
| 34 | Vivid reddish orange | 101 | Light greenish yellow |
| 37 | Medium reddish orange | 104 | Pale greenish yellow |
| 42 | Light reddish brown | 201 | Dark purplish blue |
| 43 | Medium reddish brown | 213 | Vibid pale violet |
| 50 | Strong orange | 221 | Vivid light purple |
| 52 | Light orange | 223 | Moderate purple |
| 54 | Brownish orange | 224 | Dark purple |
| 55 | Strong brown | 226 | Vivid pale purple |
| 66 | Vivid orange yellow | 230 | Blackish purple |
| 67 | Brilliant orange yellow | 231 | Purplish white |
| | | 248 | Deep purplish pink |
| | | 250 | Moderate purplish pink |
| | | 256 | Deep purplish red |
| | | 257 | Very deep purplish red |

*p-Dimethylaminobenzaldehyde test*

The *p*-dimethylaminobenzaldehyde test used in this study was developed for use in detecting aromatic amines[15]. The compound will form Schiff-type bases with primary aromatic amines[5]. It has also been used for detecting carboxylic acids[29] and pyrroles[30]. Table IV shows that *p*-dimethylaminobenzaldehyde gave a color primarily with aromatic amines. It failed to give a color with only three of the ones tested, *viz.* folic acid, N-methyldiphenylamine, and *p*-nitrosodiphenylamine. It gave colors with five aliphatic amines, *viz.* *n*-butylamine, allylamine, 1,6-hexanediamine, 2-hydroxyethylhydrazine, and benzylamine.

No clear pattern emerges regarding the classes of amines that did or did not give a color with *p*-dimethylaminobenzaldehyde. The differences in color formation observed for similar compounds, such as *n*-butylamine and isobutylamine, or diphenylamine and *p*-nitrosodiphenylamine, may have arisen because of the arbitrary rules selected for determining if a color formed, *i.e.*, more colors may have been observed if higher levels of some amines were tested or if longer times were allowed for color formation.

As previously noted, *p*-dimethylaminobenzaldehyde gave a color with only one of the other types of compounds tested, *i.e.* acridine. This is not surprising because this reagent is nearly specific for compounds containing the amine group. This ability

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of the test to be specific for amines makes it useful for detecting this class of compounds, even though many aliphatic amines are not detected at the 50-$\mu$g level.

*2,3-Dichloro-1,4-naphthoquinone test*

The 2,3-dichloro-1,4-naphthoquinone test used in this study was devised for the detection of *n*-alkylvinylamines[22]. The reaction produces purple 2-chloro-3,2′-alkylvinylnaphthoquinones. The reagent has not been generally used for detection of amines.

No *n*-alkylvinylamines were included in the 28 amines tested, but Table IV shows that this reagent will give colors with a wide variety of amines. Colors produced varied from orange to pink to purple. Aliphatic amines generally gave light orange or pink colors, while aromatic amines gave more intense violet or purple colors. Limits of detection were not as low as with *p*-dimethylaminobenzaldehyde for aromatic amines.

The mechanism that has been proposed[22] for the reaction of 2,3-dichloro-1,4-naphthoquinone with *n*-alkylvinylamines—splitting out HCl to form a monochloro-derivative—cannot be the mechanism for reaction with these amines. The wide variety of compounds that will react with the reagent suggests that molecular association complexes may be formed. It is futile to speculate further regarding the mechanism without more information about the reaction, which these studies were not designed to produce. The reaction mechanism should be investigated in detail.

Unfortunately, the usefulness of this chromogenic reagent for detecting amines is limited because, as shown in Table V, it also reacts with other types of compounds, among them disulfides, heterocyclics, phenols, polynuclear aromatics, and thiols. Nevertheless, the compound should receive consideration as a possible chromogenic reagent for amines in systems where other types of compounds are not present.

*Salicylaldehyde test*

The salicylaldehyde test used in this study was devised for primary arylalkylamines[14]. It has also been used for detecting phloroglucinol[31]. Table IV shows that this reagent gives colors with primary alkyl and aryl amines. It does not give colors with secondary or tertiary alkyl or aromatic amines under the conditions studied. The proposed reaction is the formulation of aldimines[14] by reaction with primary amines. Evidently other chromogenic reactions cannot take place with secondary and tertiary amines.

As already stated, it reacted only with acridine, 1-naphthol, and phloroglucinol among the other types of compounds tested. Salicylaldehyde is thus a useful chromogenic reagent only for detection of primary amines.

*Furfural test*

The furfural test selected for this study was devised for detecting aromatic amines[7,8]. A condensation reaction takes place between furfural and the aromatic amine to give a Schiff base. FEIGL[32] reports that furfural will also react with secondary aromatic amines, aliphatic amines, and amino acids. Table IV shows that furfural reacts with all of the primary, none of the secondary, and two of the three tertiary aromatic amines under the reaction conditions employed in these studies. The only other type of amine that gave any color formation with furfural at the levels studied was 2-hydroxyethylhydrazine.

As was previously stated, only acridine and naphthol among the other classes of compounds gave a chromogenic reaction with furfural. Unfortunately, this lack of interference is of little consequence since furfural is not a useful chromogenic reagent for more different types of amines.

*Changes in test colors with concentration*

Perhaps the most surprising result of these studies is the observation that some of the chromogenic reagents produce different colors for different amounts of the same compound. In several of these instances the intensity of the color merely decreases as the relative amount of the compound to be detected decreases. The apparent change in color is probably produced by the presence of an excess amount of the chromogenic reagent in the area on the plate containing the other compound, or by the effect of the white silica gel. Examples of this type of change are the reactions of salicylaldehyde with allylamine and *p*-phenylenediamine with 2,3-dichloro-1,4-naphthoquinone.

However, in other systems, a definite change in color occurs. For example, the color produced by the reaction between *p*-phenylenediamine and *p*-dimethylaminobenzaldehyde changes from medium reddish orange to light orange to deep yellowish pink to pale yellowish pink to pinkish white as the amount of *p*-phenylenediamine decreases, and the color produced by the reaction between 2-aminonaphthalene and *p*-dimethylaminobenzaldehyde changes from strong orange to bright orange yellow to brilliant yellow to pale orange yellow to pale yellow. Two of the most striking color changes occur in the reactions between 2,4-toluenediamine and furfural, in which the color changes from very deep purplish red to deep purplish red to light orange yellow to pale yellow, and in the reaction between 2-aminonaphthalene and furfural, in which the color changes from dark purple to deep purplish red to pinkish white to pale yellow. The most dramatic color changes occur in reactions with furfural. FEIGL[32] reports that transient colors are formed in reactions between furfural and *m*-hydroxybenzenes. However, he also reports that the Schiff bases formed between furfural and aryl amines are purple, whereas our results show that the violet color is produced with large amounts of the amine and a yellow color is produced with smaller amounts.

These remarkable color changes suggest that a different compound is formed as the ratio of the reactants changes. The nature of the compound produced with smaller amounts of chromogenic reagents is not known and could be the subject of a future investigation. The important corollary pertinent to these studies is that colors produced by some chromogenic reagents cannot be simply reported.

*Comparison of tests for usefulness*

The principal criteria for selection of a general purpose chromogenic reagent for detecting amines are (1) sensitivity, (2) general applicability to a variety of amines, and (3) lack of colors produced by other types of compounds. Critical examination of the results in Tables IV and V shows that the most widely useful reagent is 2,3-dichloro-1,4-naphthoquinone, although it also reacts with classes of compounds other than amines. The *p*-dimethylaminobenzaldehyde test will detect most aromatic amines and a few aliphatic amines; it gives the best sensitivity of any of the tests, but only for aromatic amines. Salicylaldehyde is useful only for detecting primary aliphatic and aromatic amines. Hence, if a variety of amines are to be detected, the 2,3-dichloro-1,4-naphthoquinone test should be used. If maximum sensitivity is desired in the

reagent and only aromatic amines are to be detected, then the *p*-dimethylaminobenzaldehyde test is the best.

*Precautions*

It should be pointed out that the results presented herein were obtained for the seven chromogenic reagents under the stated experimental conditions. The changes in colors with amount and the known dependence of the reactions involved on pH and relative amounts of reactant indicate that different colors and lower limits of detection may be obtained under different experimental conditions. To obtain the results presented here, the stated experimental conditions should be followed carefully.

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CHROM. 4344

# SÉPARATION ET IDENTIFICATION PAR CHROMATOGRAPHIE SUR COUCHE MINCE DE CINQ ANTIDÉPRESSEURS THYMOANALEPTIQUES DÉRIVÉS DE LA DIBENZOAZÉPINE

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SUMMARY

*Separation and identification by thin-layer chromatography of five thymoanaleptic drugs derived from dibenzoazepine*

Two thin-layer chromatographic procedures on activated Silica Gel G are presented. They are used for the separation and identification of: imipramine, desipramine, clomipramine, trimeprimine, and opipramol. Developing solvents consist of: (A) dehydrated peroxide-free diethyl ether–acetone–diethylamine (90:10:1); (B) benzene–acetone (100:20), shaken with 10 ml of 5% aqueous ammonia solution. Spots are detected by spraying first with diluted iodoplatinum reagent, then with 50% sulphuric acid. Fluorescence can be performed after 24 h. Positive results are obtained with only 100 ng of product. Migration of the five mixed compounds does not significantly affect the $R_F$ values with respect to those obtained in case that every substance migrated separately.

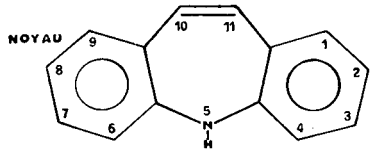
## INTRODUCTION

Les médicaments groupés sous la dénomination clinique de "thymoanaleptiques"[1] sont parmi les plus utilisés des agents chimiques psychotropes destinés au traitement des états dépressifs. La nécessité de les utiliser à doses relativement élevées pendant de longues périodes fait qu'ils sont souvent en quantités très importantes entre les mains des malades. Ceux-ci peuvent songer à attenter à leurs jours et possèdent alors, avec ces drogues, une arme redoutable et efficace, hélas trop souvent utilisée. En effet, le nombre d'intoxications volontaires dues à cette classe de médicaments, en particulier à son chef de file, l'imipramine, est actuellement très important. Nous n'en voulons pour preuve que les chiffres donnés par FREJAVILLE *et al.*[2] sur la part de responsabilité de ces composés dans les intoxications traitées à la Clinique toxicologique de l'Hôpital Fernand Widal en 1966: selon ces auteurs, 5% des malades traités durant cette période auraient été les victimes de tentatives de suicide à l'imipramine; le taux de mortalité chez ces malades serait supérieur à 15% alors qu'il atteint seulement 6% pour l'ensemble des intoxications aiguës reçues.

La fréquence des intoxications de ce type et leur gravité rend donc impérative la possession de techniques toxicologiques simples et rapides pour la recherche de ces poisons dans les milieux biologiques. Plusieurs auteurs se sont déjà attachés à la séparation chromatographique sur papier[3–5] ou sur couche mince[3,6–10] de l'imipramine, de quelques uns de ses dérivés ou de composés à structure chimique voisine. Il nous a semblé intéressant de compléter les résultats obtenus en analysant, par chromatographie sur couche mince, cinq antidépresseurs de la série de la dibenzoazépine, nous réservant d'appliquer par la suite ces procédés aux milieux biologiques.

*Structure chimique des composés étudiés*

Le groupe des antidépresseurs dérivés de la dibenzoazépine, homogène sur le plan clinique, l'est aussi dans sa structure chimique. Tous ces représentants possèdent




*5H* DIBENZOAZEPINE

CHAINES LATERALES

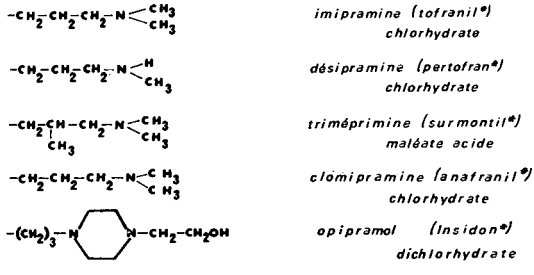



Fig. 1. Noyau de la 5H-dibenzoazépine et chaînes latérales portées par l'azote chez les composés antidépresseurs étudiés. I = imipramine (Tofranil) chlorhydrate; II = désipramine (Pertofran) chlorhydrate; III = triméprimine (Surmontil) maléate acide; IV = clomipramine (Anafranil) chlorhydrate; V = opipramol (Insidon) dichlorhydrate.

un noyau tricyclique dont la partie centrale est constituée par un hétérocycle heptagonal azoté (Fig. 1). On peut, structuralement y distinguer deux séries:

(1) les dibenzodihydroazépines (ou iminodibenzyles) chez lesquels la double liaison en 10–11 est saturée;

(2) les dibenzoazépines proprement dites.

Les seuls représentants de la première série actuellement utilisés sont: l'imipramine ("Tofranil" N.D.); la clomipramine ("Anafranil" N.D.); la desipramine ("Pertofran" N.D.); le triméprimine ("Surmontil" N.D.).

Ils ne diffèrent entre eux que par des modifications légères de la chaîne latérale substituée sur l'azote du noyau, à l'exception de la clomipramine qui possède en outre un chlore en position 3 sur l'un des noyaux benzène (Fig. 1).

La deuxième série comprend deux représentants: la carbamazépine ("Tegretol" N.D.) qui, uniquement utilisée comme antiépileptique, ne nous intéresse pas ici, et l'opipramol ("Insidon" N.D.) dont la chaîne latérale comporte une structure pipéridinique analogue à celle existant chez certaines phénothiazines à propriétés neuroleptiques puissantes (Fig. 1).

## PARAMÈTRES EXPÉRIMENTAUX

*Technique employée*

Chromatographie monodimensionnelle ascendante en atmosphère sursaturée et sans équilibration préalable des plaques (migration effectuée sur 15 cm).

*Phases adsorbantes*

(1) Silica Gel G Merck activé.

(2) Silica Gel G Merck activé, additionné d'un indicateur fluorescent excitable à 254 nm (nous avons employé dans ce cas des plaques de 20 × 20 cm prêtes à l'emploi, commercialisées par les laboratoires Camag sous le sigle DSF-A).

*Phases mobiles*

(A) Éther éthylique déperoxydé et déshydraté–acetone–diéthylamine (90:10:1)*;

(B) Benzène–acétone (100:20), chargé en $NH_3$ par agitation avec 10 ml d'une solution aqueuse ammoniacale à 5% pour une température de 18 à 20°, titrée juste avant l'emploi**.

*Dépôts*

Des solutions mères de chaque composé à 1 mg par ml (exprimé en base) dans le mélange acétone–méthanol (2:1) ont été déposées sous forme de taches à raison de 10 $\mu$l. Le mélange des solutions mères à parties égales fut déposé à raison de 30 $\mu$l. Pour les essais de sensibilité, des dilutions comprises entre 1/10 et 1/200 ont été réalisées dans le même solvant à partir des solutions mères.

Enfin, pour la mesure des $R_X$ des médicaments testés, nous avons déposé une solution de papavérine, préparée dans les mêmes conditions.

*Procédés de révélation*

Plusieurs procédés ont été expérimentés.

*Examen en lumière UV.* Avec les couches ne comportant pas d'indicateur de fluorescence l'irradiation à 350 nm est inexploitable, seuls l'opipramol et la papavérine émettant alors une fluorescence jaune d'intensité convenable. En revanche l'excitation sous 254 nm est plus valable: l'émission jaune de la papavérine et de l'opipramol est intense, tandis que les dibenzodihydroazépines apparaissent en bleuté.

Avec les couches additionnées d'un indicateur de fluorescence, l'irradiation à

* Ce mélange avait déjà été utilisé par l'un de nous à l'occasion de l'étude de médicaments psychotropes variés[11].

** Ce solvant est une modification de la phase préconisée par DANHIER pour l'imipramine et d'autres antidépresseurs[12] et précédemment utilisée par PAULUS *et al.* pour la séparation des dérivés de la phénothiazine[13].

254 nm fait apparaître le fond en jaune tandis que les emplacements de tous les composés essayés se manifestent par des taches sombres.

*Révélation par voie chimique.* Nous avons utilisé deux réactions: (1) Pulvérisation d'acide nitrique concentré: les antidépresseurs dibenzodihydroazépiniques se colorent en bleu intense virant au jaune avec le temps, l'opipramol prend immédiatement une teinte jaune, alors que la papavérine ne réagit pas; (2) double pulvérisation, d'abord de réactif iodoplatinique de MUNIER ET MACHEBEUF[14] dilué au tiers dans HCl *N*, puis immédiatement après d'acide sulfurique au demi p.v.[11,15]: dans ces conditions, clomipramine, triméprimine et papavérine apparaissent en rouge brique, l'opipramol en bleu violacé, tandis que l'imipramine et la désipramine se manifestent par une tache possédant un noyau brique entouré, pour les quantités élevées, d'un halo bleu (particulièrement important pour l'imipramine).

*Révélation chimique suivie d'examen en lumière UV.* Les plaques révélées par le réactif iodoplatinique et l'acide sulfurique sont séchées et examinées sous lumière UV au bout de 24 h; les taches correspondant aux composés essayés possèdent alors, autant à 254 nm qu'à 350 nm, une intense fluorescence jaune à l'exception de la papavérine qui émet en rouge faible tirant sur l'orangé.

## RÉSULTATS

Les résultats obtenus sont illustrés par les Fig. 2 et 3.

Ces photographies de chromatogrammes montrent que, quel que soit le système solvant, les résolutions sont parfaitement satisfaisantes, la diffusion des taches en cours de migration étant cependent beaucoup moins importante avec le solvant B qu'après usage du solvant A. En outre la reproduction photographique nous a permis de déceler sur le chromatogramme obtenu avec le solvant A et quel que soit le procédé de révélation employé, deux particularités que l'oeil n'était pas parvenu à détecter: (1) l'existence d'un front de démixtion au niveau de la tache de triméprimine (celle-ci est même partagée par ce front sur la piste du composé isolé); (2) la présence sur la piste de l'opipramol d'une tache parasite possédant le même $R_F$ que l'imipramine;

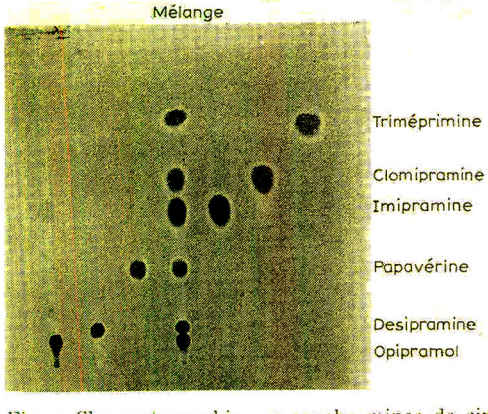



Fig. 2. Chromatographie sur couche mince de cinq antidépresseurs dibenzoazépiniques et d'un étalon de papavérine. Solvant A.

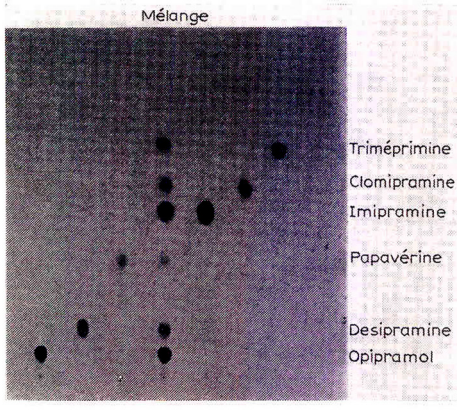



Fig. 3. Chromatographie sur couche mince de cinq antidépresseurs dibenzoazépiniques et d'un étalon de papavérine. Solvant B.

cette tache est vraisemblablement imputable à un produit de décomposition de la dibenzoazépine étudiée, préexistant ou se formant en cours de migration.

## DISCUSSION

*Phases adsorbantes*

L'emploi de Silica Gel G additionné d'un indicateur fluorescent ne présentant aucun avantage particulier sur celui de Silica Gel G simple, nous n'avons donc pas d'intérêt à préconiser l'usage du premier.

*Phases mobiles*

Aucun commentaire spécial n'est à faire sur le mélange A. Pour la mise au point du solvant B, nous nous sommes inspirés de la formule proposée par PAULUS[13] et par DANHIER[12]. Ces auteurs indiquent la composition suivante:

benzène–acétone–ammoniaque à 25% (50:10:5).

Préparé ainsi, ce mélange se sépare en deux phases; de plus le volume total indiqué est tout juste suffisant pour assurer une immersion correcte du bas de la plaque dans les conditions où nous avons opéré. Aussi avons-nous préféré, d'abord doubler le volume total, puis, après agitation en ampoule à décantation du mélange benzène–acétone avec l'ammoniaque, éliminer la phase aqueuse excédentaire. Ainsi obtenu le solvant assure une excellente résolution pour l'opipramol et la désipramine mais ne différencie pas les trois autres thymoanaleptiques.

Pour pallier cet inconvénient nous avons effectué une série d'essais en diminuant progressivement la quantité de $NH_3$ saturant le mélange benzène–acétone. Pour cela nous avons utilisé de "l'ammoniaque RP pour analyses, Prolabo" dont la teneur en $NH_3$ devait se situer entre 26.5 et 28% mais qui n'était en fait que de 18.9% car il s'agissait d'un flacon entamé. A partir de cette solution exactement titrée nous avons préparé une série de dilutions en étendant à 10 ml respectivement 4, 3, 2 et 1 ml du réactif; chaque dilution nous a servi ensuite à la préparation d'un solvant de migration différent par agitation avec la phase organique et décantation. Les chromatogrammes obtenus dans ces différentes conditions sont schématisés sur la Fig. 4.

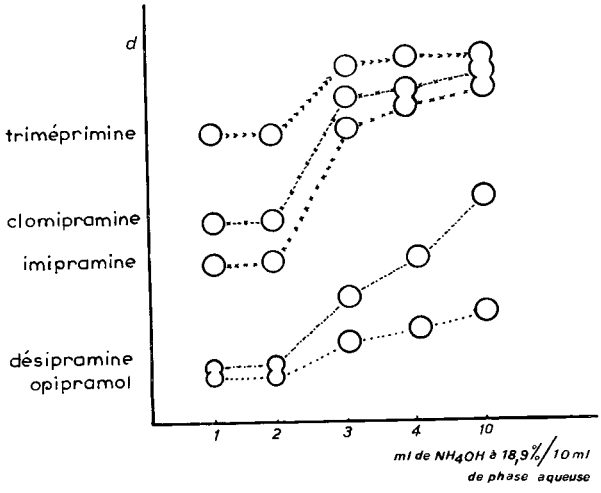



Fig. 4. Schéma des différentes séparations chromatographiques obtenues avec des solvants du type B (benzène–acétone, 100:20) possédant des teneurs en $NH_3$ différentes (charges effectuées par agitation avec des solutions ammoniacales contenant 1, 2, 3, 4 · · · 10 ml d'ammoniaque à 18.9%/10 ml).

On observe sur ce schéma que les valeurs des $R_F$ s'élèvent d'autant plus que la teneur en $NH_3$ du solvant de migration est plus forte. Mais il est encore plus intéressant de constater que les taches de l'imipramine, de la clomipramine et de la triméprimine sont beaucoup mieux séparées que celles de la désipramine et de l'opipramol pour les faibles teneurs en $NH_3$ du solvant, le phénomène étant exactement inversé pour les fortes teneurs. Nous avons émis à ce sujet l'hypothèse que les séparations sont en

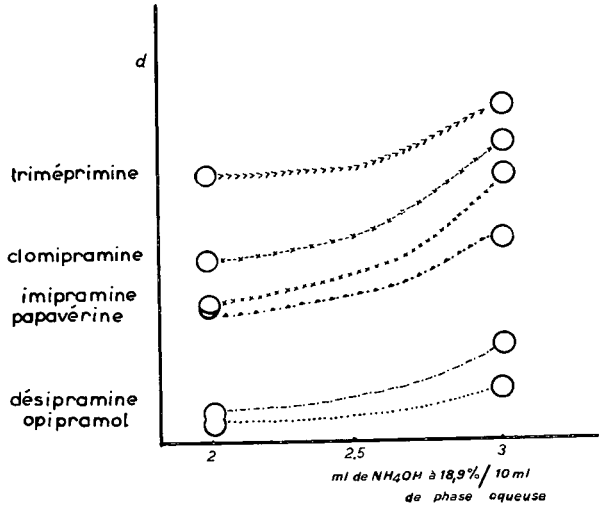



Fig. 5. Schéma des différentes séparations chromatographiques obtenues avec des solvants du type B (benzène–acétone, 100:20) possédant des teneurs en $NH_3$ différentes (charges effectuées par agitation avec des solutions ammoniacales contenant 2.5, 2.6, 2.7, · · · 3 ml d'ammoniaque à 18.9%/100 ml).

relation avec la structure chimique des composés étudiés (influence des groupements $CH_3$ et Cl) et avec l'existence possible de fronts de démixtion. Sa vérification expérimentale est en cours et le tout fera l'objet d'un mémoire ultérieur.

Quoiqu'il en soit, la résolution la plus satisfaisante pour les cinq composés (et pour la papavérine) se réalise avec des solvants traités par des solutions ammoniacales de concentration comprise entre 2.5 et 3 ml d'ammoniaque à 18.9% pour 10 ml. Pour préciser la teneur optimale en ammoniaque nous avons fait une nouvelle série d'essais en faisant varier de 0.1 ml en 0.1 ml entre ces limites la teneur en ammoniaque à 18.9% de la phase aqueuse destinée à charger le solvant en alcali. La Fig. 5 schématise les résultats de cette expérimentation.

Les meilleures résolutions sont obtenues avec des concentrations de 2.5 à 2.9 ml d'ammoniaque à 18.9% pour 10 ml, soit 4.7 à 5.5% de $NH_3$ dans 10 ml de phase aqueuse. Nous avons estimé légitime d'adopter une teneur moyenne de 5% et nous avons fixé le protocole de préparation du solvant de migration comme suit: Benzène–acétone (100:20) chargé en $NH_3$ par agitation avec 10 ml exactement mesurés d'une solution aqueuse ammoniacale à 5%, titrée immédiatement avant l'emploi, et élimination de la phase aqueuse excédentaire par décantation.

*Sensibilité des différents procédés de révélation*

Le Tableau I mentionne les limites de sensibilité des différents procédés de révélation employés.

Ces limites ont été évaluées à l'examen visuel, mais il est possible qu'elles soient encore reculées en ayant recours à la photographie des plaques. Quoiqu'il en soit, le procédé de révélation le plus sensible paraît être, pour cette série médicamenteuse, la double pulvérisation iodoplatinique/sulfurique suivie ou non d'un examen en lumière UV. Il faut également souligner un autre intérêt de ce dernier procédé sur la révélation par la seule voie chimique: alors que les taches deviennent invisibles à la lumière du jour après quelques heures, tout au moins pour les plus faibles quantités, elles demeurent encore apparentes plusieurs jours en examinant les plaques sous lumière UV.

TABLEAU I

LIMITES DE SENSIBILITÉS (ng) POUR LES DIFFÉRENTS PROCÉDÉS DE RÉVÉLATION EMPLOYÉS

| | *Irradiation à 254 nm* | *Procédés chimiques* | | | *Réactif iodoplatinique + $H_2SO_4$ au demi; séchage et repos de 24 h; examen sous irradiation à 254 ou 350 nm* |
|---|---|---|---|---|---|
| | | *$NHO_3$ concentré* | *Réactif iodoplatinique* | *Réactif iodoplatinique + $H_2SO_4$ au demi* | |
| Opipramol | 100 | 200 | 250 | 100 | 100 |
| Désipramine | 1000 | | | | 100 |
| Autres dibenzoazépines | 1000 | | | | 350 |

*Valeurs moyennes des $R_F$ et des $R_X$; calcul des paramètres statistiques et analyse de variance pour les composés chromatographiés isolément ou en mélange*

Nous avons effectué une interprétation statistique sur les différentes valeurs des $R_F$ et des $R_X$ établis par rapport à la papavérine à l'aide des solvants standardisés. Son exploitation nous a permis:

(1) de chiffrer la reproductibilité des paramètres mesurés ($R_F$ et $R_X$) pour chaque composé étudié et dans chaque système chromatographique;

(2) de comparer entre eux les deux groupes de mesures ($R_F$ et $R_X$) et de déduire la confronce qui peut être attachée à chacun de ces deux paramètres;

(3) d'établir enfin s'il est licite de considérer que les valeurs des $R_F$ (et des $R_X$) mesurées sur les composés migrant isolément ou en mélange font partie de la même population statistique et donc si le mélange des composés a ou n'a pas d'influence sur la valeur des $R_F$ (et des $R_X$).

Les valeurs des paramètres statistiques essentiels (moyennes et coefficients de variation), calculées à partir des $R_F$ et des $R_X$ mesurés, sont mentionnés dans le Tableau II.

L'examen comparatif des coefficients de variation montre que la dispersion des résultats augmente en règle générale lorsque les valeurs des $R_F$ (ou des $R_X$) diminuent, quel que soit le solvant employé. Cette constatation est particulièrement évidente

TABLEAU II

PARAMÈTRES STATISTIQUES (MOYENNES ET COEFFICIENTS DE VARIATION )CALCULÉS À PARTIR DES MESURES DE $R_F$ DE CINQ ANTIDÉPRESSEURS DIBENZOAZÉPINIQUES ET DES MESURES DE LEURS $R_X$ PAR RAPPORT À LA PAPAVÉRINE PRISE COMME ÉTALON

Système A: 10 essais; système B: 8 essais.

| | | *Système chromatographique A* | | | | *Système chromatographique B* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $R_F$ | | $R_X$ | | $R_F$ | | $R_X$ | |
| | | *Moyennes* | *Coeff. de variation de la moyenne (%)* | *Moyennes* | *Coeff. de variation de la moyenne (%)* | *Moyennes* | *Coeff. de variation de la moyenne (%)* | *Moyennes* | *Coeff. de variation de la moyenne (%)* |
| Triméprimine | Produit isolé | 0.866 | 3.6 | 2.614 | 5.6 | 0.781 | 2.5 | 1.927 | 4.2 |
| | Mélange | 0.872 | 2.65 | 2.235 | 6.75 | 0.784 | 2.5 | 1.945 | 4.4 |
| Clomipramine | Produit isolé | 0.717 | 6.0 | 2.158 | 8.3 | 0.634 | 3.5 | 1.580 | 5.6 |
| | Mélange | 0.666 | 9.5 | 1.946 | 8.8 | 0.626 | 3.5 | 1.564 | 3.3 |
| Imipramine | Produit isolé | 0.552 | 11.9 | 1.693 | 13.4 | 0.541 | 5.2 | 1.349 | 4.9 |
| | Mélange | 0.554 | 10.9 | 1.631 | 11.2 | 0.531 | 5.8 | 1.332 | 4.1 |
| Papavérine (étalon) | Produit isolé | 0.335 | 8.1 | | | 0.402 | 5.2 | | |
| | Mélange | 0.345 | 8.2 | | | 0.400 | 5.9 | | |
| Désipramine | Produit isolé | 0.170 | 5.8 | 0.555 | 9.4 | 0.154 | 12.6 | 0.406 | 14.8 |
| | Mélange | 0.176 | 5.4 | 0.535 | 5.9 | 0.154 | 14.2 | 0.397 | 12.1 |
| Opipramol | Produit isolé | 0.115 | 11.2 | 0.344 | 8.1 | 0.079 | 16.1 | 0.194 | 12.4 |
| | Mélange | 0.104 | 6.6 | 0.318 | 5.15 | 0.076 | 19.6 | 0.189 | 12.0 |

avec le système B. Avec le solvant A, cependant, l'imipramine se singularise, les valeurs mesurées des $R_F$ ayant une dispersion beaucoup plus importante que celle des composés de plus faible mobilité; ce phénomène peut s'expliquer par le fait que les taches de désipramine et d'opipramol sont bloquées l'une contre l'autre (probablement par l'effet d'un front de démixtion situé immédiatement au dessus de la tache de désipramine): ainsi leurs $R_F$ sont relativement peu sujets à variations alors que dans le système B, elles sont nettement détachées l'une de l'autre et ainsi plus libres.

Quant à la comparaison entre elles des valeurs des $R_F$ et des $R_X$ par l'intermédiaire des coefficients de variation, elle nous permet de conclure, quel que soit le solvant, que l'emploi d'un étalon interne de papavérine et le calcul des $R_X$ n'améliorent en rien la reproductibilité des paramètres mesurés.

Enfin, dans le but de savoir si nous pouvions légitimement considérer que les produits en mélange possèdent le même $R_F$ (ou $R_X$) que lorsqu'ils migrent isolés, nous avons soumis nos résultats à une analyse de variance par la méthode du "test F". Nous avons ainsi pu conclure qu'il n'existait aucune différence significative entre les $R_F$ (et les $R_X$) d'un même composé migrant isolément ou en mélange, sauf dans les cas de la clomipramine et de l'opipramol en système A; il est apparu alors une très légère différence significative. Un complément d'information analytique s'imposerait donc pour ces deux composés afin de confirmer ou infirmer l'analyse de variance actuelle, et dans le cas d'une confirmation, afin de rechercher la nature du facteur de perturbation intervenant lorsque ces composés sont mélangés aux autres.

## CONCLUSION

Le grand intérêt toxicologique actuel des antidépresseurs thymoanaleptiques à noyau de dibenzoazépine nous a incités à présenter deux méthodes de chromatographie sur couche mince assurant la séparation et l'identification de l'imipramine, de la désipramine, de la clomipramine, de la triméprimine et de l'opipramol.

L'une d'elles nécessite que des précautions rigoureuses soient prises en ce qui concerne la teneur en agent alcalin du solvant de migration. Avec les deux techniques la reproductibilité des $R_F$ et la résolution des taches sont pleinement satisfaisantes. La sensibilité est extrême puisque certains procédés de révélation permettent de déceler jusqu'à 100 ng de produit.

Enfin une analyse statistique nous a permis de montrer que, à l'exception de deux composés sur lesquels nous n'avons pu conclure avec l'un des solvants, le fait que les cinq corps étudiés soient en mélange ne perturbe pas significativement la migration de chacun d'entre eux.

## RÉSUMÉ

Les auteurs présentent deux méthodes chromatographiques sur Silica Gel G activé permettant la séparation et l'identification de cinq antidépresseurs à structure dibenzoazépinique: imipramine, désipramine, clomipramine, triméprimine, opipramol. Les mélanges solvants utilisés sont les suivants: (A) éther éthylique déperoxydé et déshydraté–acétone–diéthylamine (90:10:1); (B) benzène–acétone (100:20), chargé en ammoniac par agitation avec 10 ml d'eau ammoniacale à 5%. Le meilleur procédé de révélation consiste à pulvériser d'abord un réactif iodoplatinique dilué puis de

l'acide sulfurique au demi; éventuellement un examen de fluorescence est effectué au bout de 24 h. Dans ces conditions, il est encore possible d'obtenir un résultat positif avec seulement 100 ng de produit. Les deux méthodes sont de valeurs pratiquement égales et, en règle générale, la migration des cinq composés en mélange ne perturbe pas significativement les $R_F$ par rapport à ceux qui sont obtenus lorsque chaque substance migre isolément.

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CHROM. 4313

# CHROMATOGRAPHIC BEHAVIOUR AND CHEMICAL STRUCTURE

## I. THIN-LAYER CHROMATOGRAPHY OF ALIPHATIC ACIDS

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SUMMARY

About sixty aliphatic acids, predominantly fatty acids, hydroxy acids, amino acids and halogen acids, were separated on cellulose layers with the solvent mixture *n*-butanol–diethylamine–water (85:1:14). The $R_M$ values so obtained illustrate in most cases the validity of MARTIN's theoretical postulates for the relationship between chemical structure and $R_F$ value in partition chromatography. Thus when $R_M$ is plotted against the number of substituent groups of any one kind a close approximation to linearity results, except in the case of acids with vicinal hydrophilic groups. The calculation of group constants and binding increments was done by direct comparison of the $R_M$ values of substances differing by only one group or increment. These data facilitate the chromatographic elucidation of structural problems; only very small amounts of the substances which do not even need to be isolated in pure form are required.

## INTRODUCTION

In the course of our investigations of radiation induced carboxylation of organic acids with $^{14}C$ labelled $CO_2$ an analytical procedure was required to separate the organic acids formed by this process. The method was also required to indicate how many COOH-groups were introduced into the model substrate and in what position relative to other functional groups, they were located. A chromatographic procedure appeared to be especially suited, since there were already a number of papers on the relationship between molecular structure and chromatographic behaviour[1–18].

Based on predictions of CONSDEN *et al.*[19] and MARTIN[20], a linear relationship between the $R_M$ value and the number of identical groups has been proved experimentally by many authors[1–18,21–29]:

$$R_M = G_0 + nG_x + mG_y + \cdots\cdots\cdots \quad (1)$$

$G_x, G_y, \cdots\cdots\cdots$ = group constant of X, Y, $\cdots\cdots\cdots$

$G_0$ = basic constant.

Some of these group constants are known in the case of paper chromatography (PC)[1,4–6,23], but for TLC which is better suited for the separation of organic acids[28,30,31]

only a few constants have been published[10,13]. Furthermore, in TLC the contribution of one group to the $R_M$ value of the molecule is dependent on the other groups in the molecule, as was shown by PATAKI[13] who compared the $R_M$ values of amino acids differing by specific groups.

It is the aim of this and a following paper[32] to establish the group constants from the $R_F$ values of more than a hundred organic acids. In addition, the effect of criteria so far hardly considered, for instance binding increments, such as double bonds, chain branching, ring closure and the relative positions of functional groups are taken into account. These data can facilitate the elucidation of structural problems.

## EXPERIMENTAL

The separation of the acids was carried out on cellulose layers for two reasons. First, the best results for the chromatography of acids were obtained on cellulose layers[33–35]. Secondly, cellulose layers correspond well with PC in their chromatographic behaviour, thus facilitating the transferability of PC experience to TLC[36]. Cellulose layers, however, have the advantages of giving better separations, and having shorter development times and greater sensitivity than PC.

### *Preparation of the plates*

Fifteen grams cellulose powder HR 300 (Macherey, Nagel) were stirred vigorously with 75 ml water. Adsorbent layers of 0.25 mm thickness were prepared on $20 \times 20$ cm glass plates with Desaga equipment, (Heidelberg). The plates were air-dried horizontally, at room temperature overnight. Almost all the acids were spotted as their diethylamine salts, dissolved in methanol, along a line 2.5 cm from the lower end of the plate. Approximately 10 $\mu$g of test substance (1 $\mu$l of 1% solution) were used for each spot. The plates were then equilibrated for 4 h at constant temperature ($23° \pm 1°$) and developed by the ascending technique in a tank lined with solvent-soaked filter paper.

### *Solvent system*

System: *n*-butanol–diethylamine–water (85:1:14). The duration of development is 85 min. After that time the front is at a distance of 10 cm from the starting line. After development the solvent is allowed to evaporate at 60°, which is below the decomposition temperature of the diethylamine salts.

The use of diethylamine as the volatile alkali instead of the otherwise more common ammonia was first proposed by JONES *et al.*[37] for the PC of some volatile organic acids. It prevents the partial separation of the eluting system possible under certain conditions, since diethylamine dissolves well in both phases. Furthermore it facilitates the identification of the acids as diethylamine salts after spraying with ninhydrin reagent.

### *Detection reagent*

Various reagents were tried, *e.g.*, pH-indicators such as bromocresol purple or bromocresol green, sodium nitroprusside and ninhydrin reagent. The best results, however, for minute amounts of even very weak acids were obtained with ninhydrin. After spraying with ninhydrin the plates were warmed for 30 min at 60°. Ninhydrin

reveals the acids as blue violet spots on a white or pale rose background. The diethylamine salts appear as more durable and more clearly defined spots than the ammonium salts. The detection limit is about 0.5 $\mu$g acid.

To correlate the chromatographic behaviour of substances with their chemical structure an accurate knowledge of the $R_F$ values is necessary. Recently, new techniques for the exact determination of $R_F$ values have therefore been proposed, such as tankless or flatbed chromatography in the case of PC[11]. We worked with conventional equipment, but strictly observed the following points to achieve reproducible and accurate results: constant temperature, time of saturation and size of spots. A control substance, in most cases glycolic acid, was always run alongside and if its $R_F$ value differed by more than 0.02 from the standard value, the run was discarded.

## RESULTS AND DISCUSSION

### *Monocarboxylic acids*

The separation of organic acids by chromatography has been reported in a number of publications. Some of them make use of PC, *e.g.*, the work by LONG *et al.*[22], HOWE[27] and HARTLEY[38], other authors however prefer TLC. The separation of straight chain carboxylic acids by TLC was carried out by HROMATKA AND AUE[28] as well as by LYNES[39] and BRAUN AND VORENDOHRE[40], who obtained good results. Branched carboxylic acids however have been investigated less.

TABLE I

UNSUBSTITUTED MONOCARBOXYLIC ACIDS

| *No.* | *Acid* | $R_F$ *exp.* | $R_M$ *measured* | $R_M$ *calc.* | $R_F$ *calc.* | $\Delta R_M$ | $\Delta R_F$ |
|---|---|---|---|---|---|---|---|
| 1 | Formic | 0.41 | 0.16 | 0.30 | 0.33 | 0.14 | 0.08 |
| 2 | Acetic | 0.45 | 0.09 | 0.08 | 0.45 | 0.01 | 0.00 |
| 3 | Propionic | 0.58 | −0.14 | −0.14 | 0.58 | 0.00 | 0.00 |
| 4 | Butyric | 0.69 | −0.35 | −0.36 | 0.70 | 0.01 | 0.01 |
| 5 | Valeric | 0.79 | −0.58 | −0.58 | 0.79 | 0.00 | 0.00 |
| 6 | Caproic | 0.86 | −0.79 | −0.80 | 0.86 | 0.01 | 0.00 |
| 7 | Enanthic | 0.92 | −1.06 | −1.02 | 0.91 | 0.04 | 0.01 |
| 8 | Caprylic | 0.95 | −1.28 | −1.24 | 0.95 | 0.04 | 0.00 |
| 9 | 2-Methylpropionic | 0.71 | −0.39 | −0.39 | 0.71 | 0.00 | 0.00 |
| 10 | 2-Methylbutyric | 0.80 | −0.60 | −0.61 | 0.80 | 0.01 | 0.00 |
| 11 | 2,2-Dimethylpropionic | 0.81 | −0.63 | −0.64 | 0.81 | 0.01 | 0.00 |
| 12 | 2,2-Dimethylbutyric | 0.88 | −0.87 | −0.86 | 0.88 | 0.01 | 0.00 |
| 13 | 2-Ethylbutyric | 0.87 | −0.83 | −0.83 | 0.87 | 0.00 | 0.00 |
| 14 | 2-Methyl-2-ethylbutyric | 0.92 | −1.06 | −1.08 | 0.92 | 0.02 | 0.00 |
| 15 | 2,2-Dimethylvaleric | 0.92 | −1.06 | −1.08 | 0.92 | 0.02 | 0.00 |
| 16 | 2-Ethylpropionic | 0.95 | −1.28 | −1.27 | 0.95 | 0.01 | 0.00 |
| 17 | 3,3-Dimethylbutyric | 0.78 | −0.55 | −0.52 | 0.77 | 0.03 | 0.01 |
| 18 | 4-Methylvaleric | 0.82 | −0.66 | −0.66 | 0.82 | 0.00 | 0.00 |
| 19 | 2,2,4,4-Tetramethylcaproic | 0.97 | −1.51 | −1.46 | 0.96 | 0.05 | 0.01 |
| 20 | Acrylic | 0.53 | −0.05 | −0.05 | 0.53 | 0.00 | 0.00 |
| 21 | Crotonic | 0.64 | −0.25 | −0.27 | 0.65 | 0.02 | 0.01 |

The experimental $R_F$ and $R_M$ values of straight chain and branched monocarboxylic acids, as well as the $R_M$ and $R_F$ values calculated by means of the established group constants, are given in Table I (Nos. 1–19). The differences, *i.e.* $\Delta R_M$ and $\Delta R_F$, are presented in the last two columns. It can be seen that only formic acid shows a discrepancy between the experimental and calculated $R_M$ value. This can be explained by the fact, that the calculated $R_M$ value of formic acid is obtained by the sum of the basic constant and the $R_M$ constant of the carboxylic group. Thus the hydrogen atom of formic acid is not accounted for.

The method described does not give a satisfactory separation of formic and acetic acid. This is in agreement with BAYZER[41] who found that for the complete separation of the alkali derivatives of $C_1$–$C_5$ carboxylic acids a combination of TLC and electrophoresis is necessary. The separation of the hydroxamates however can be achieved by TLC alone.

When the $R_M$ values of the fatty acids are plotted against their carbon number a close approximation to linearity is obtained. The difference of the $R_M$ values between these homologues gives the constant for the $CH_2$-group, $G_{(CH_2)} = -0.23$. Based on the data of other homologous compounds, especially hydroxy acids and dicarboxylic acids[32], the $R_M$ constant of the $CH_2$-group as well as of any other aliphatic C-atom was established as −0.22 (Table VI). For an example might be mentioned:

$$G_{(CH_2)} = R_{M\ \text{leucine}} - R_{M\ \text{valine}} = -0.22 \qquad (2)$$

The calculation of other group constants was carried out in the same manner, by using the difference between the $R_M$ values of two compounds, differing only by the group in question.

The $R_M$ constants for chain branching were determined by way of the $R_F$ values of branched fatty acids (Nos. 9–19) and branched amino acids. For a branching in the $\alpha$-position the value is −0.03, for any other branching it is +0.12 (Table VI). The data available were not sufficient to establish these values very accurately or to distinguish between chain branching in the $\beta$- and $\gamma$-position. One can assume that these positions would also show rather different values for chain branching, an assumption which is supported by the special value of the $\alpha$-position. A comparison

TABLE II

HYDROXY ACIDS

| No. | Acid | $R_F$ exp. | $R_M$ measured | $R_M$ calc. | $R_F$ calc. | $\Delta R_M$ | $\Delta R_F$ |
|---|---|---|---|---|---|---|---|
| 22 | Glycolic | 0.25 | 0.48 | 0.57 | 0.21 | 0.09 | 0.04 |
| 23 | Lactic | 0.31 | 0.35 | 0.35 | 0.31 | 0.00 | 0.00 |
| 24 | 3-Hydroxypropionic | 0.27 | 0.43 | 0.42 | 0.27 | 0.01 | 0.00 |
| 25 | 2-Hydroxybutyric | 0.42 | 0.14 | 0.14 | 0.42 | 0.00 | 0.00 |
| 26 | 3-Hydroxybutyric | 0.38 | 0.21 | 0.20 | 0.39 | 0.01 | 0.01 |
| 27 | 4-Hydroxybutyric | 0.28 | 0.41 | 0.40 | 0.28 | 0.01 | 0.00 |
| 28 | 2-Hydroxyvaleric | 0.55 | −0.09 | −0.09 | 0.55 | 0.00 | 0.00 |
| 29 | 2-Hydroxycaproic | 0.66 | −0.29 | −0.31 | 0.67 | 0.02 | 0.01 |
| 30 | 2-Hydroxyhexanoic | 0.77 | −0.52 | −0.53 | 0.77 | 0.01 | 0.00 |
| 31 | Glyceric | 0.12 | 0.87 | 0.91 | 0.11 | 0.04 | 0.01 |

between the $R_M$ values of acrylic and crotonic acid and the corresponding saturated compounds leads to a mean value of +0.09 for the $R_M$ constant of the double bond.

*Hydroxy acids*

The $R_F$ and $R_M$ values of 10 hydroxy acids (Nos. 22–31), predominantly $\alpha$-hydroxy acids are reported in Table II. With the exception of the first member of the series, glycolic acid, and glyceric acid, there is a good agreement between the experimental and calculated $R_F$ values. Exceptions from the rule of additivity have already been noted before in the case of vicinal groups, as in glycolic and glyceric acid.

Although REICHL[4], from the very limited data available to him, differentiated between primary and secondary hydroxy groups, it appears to be much more important to take into account the position of the hydroxy group, if one regards the data given in this paper.

In calculating the $R_M$ value of the $\alpha$-hydroxy group, glycolic acid was not accounted for due to its vicinal groups. The differences between $R_M$ values of the other $\alpha$-hydroxy acids and the corresponding unsubstituted carboxylic acids lead to a $G_{(\alpha\text{-OH})}$ of +0.49. 3-Hydroxy propionic acid and 3-hydroxy butyric acid were used to calculate the constant for the $\beta$-OH group:

$$G_{(\beta\text{-OH})} = R_{M\ \text{3-hydroxy propionic}} - R_{M\ \text{propionic}} = 0.57 \quad (3)$$

$$G_{(\beta\text{-OH})} = R_{M\ \text{3-hydroxy butyric}} - R_{M\ \text{butyric}} = 0.56 \quad (4)$$

According to the above equations it does not appear to matter whether the $\beta$-OH groups are primary or secondary. Owing to the lower $\beta$-OH value of the amino acid pair threonine/2-aminobutyric acid, the $G_{(\beta\text{-OH})}$ was defined as 0.56. Using 4-hydroxy butyric acid according to the method described above, $G_{(\gamma\text{-OH})}$ was found to be +0.76.

*Amino acids*

A thorough study of the relationship between molecular structure and the $R_M$ values of the amino acids has been carried out by SCHAUER AND BULIRSCH[5], PATAKI[13] and TRZASKA AND KOWKABANY[29]. The latter authors also used butanol in

TABLE III

AMINO ACIDS

| No. | Acid | $R_F$ exp. | $R_M$ measured | $R_M$ calc. | $R_F$ calc. | $\Delta R_M$ | $\Delta R_F$ |
|---|---|---|---|---|---|---|---|
| 32 | Glycine | 0.07 | 1.12 | 1.06 | 0.08 | 0.06 | 0.01 |
| 33 | Alanine | 0.13 | 0.83 | 0.84 | 0.13 | 0.01 | 0.00 |
| 34 | 2-Aminobutyric | 0.19 | 0.62 | 0.62 | 0.19 | 0.00 | 0.00 |
| 35 | 3-Aminobutyric | 0.17 | 0.69 | 0.68 | 0.17 | 0.01 | 0.00 |
| 36 | 4-Aminobutyric | 0.14 | 0.79 | 0.78 | 0.14 | 0.01 | 0.00 |
| 37 | 2-Aminoisobutyric | 0.21 | 0.58 | 0.59 | 0.20 | 0.01 | 0.01 |
| 38 | Norvaline | 0.27 | 0.43 | 0.40 | 0.28 | 0.03 | 0.01 |
| 39 | Valine | 0.22 | 0.55 | 0.54 | 0.22 | 0.01 | 0.00 |
| 40 | Leucine | 0.32 | 0.33 | 0.32 | 0.32 | 0.01 | 0.00 |
| 41 | Isoleucine | 0.31 | 0.35 | 0.32 | 0.32 | 0.03 | 0.01 |
| 42 | 6-Aminocaproic | 0.14 | 0.79 | 0.78 | 0.14 | 0.01 | 0.00 |

their solvent system but in an acid solution, *n*-butanol–acetic acid–water (4:1:5), and obtained a similar mean value for $G_{(CH_2)}$ of −0.20. Due to the small number of compounds investigated, they did not calculate any other group constants.

Table III shows the $R_F$ and $R_M$ values of 11 amino acids (Nos. 32–42). The values of phenylalanine and tyrosine are listed in the second part of this work[32] under aromatic acids in order to make it possible to calculate the $R_M$ constant of the phenyl group. In this other paper the data of other amino acids run in methanol solutions can also be found.

Comparison of experimental and calculated $R_M$ values shows that a high discrepancy is only observed with glycine due to its vicinal groups. The calculation of the $NH_2$-group constants was done in the same way as for hydroxy compounds. Here too, emphasis was put on the relative positions of the amino and carboxyl groups (Table VI). The high positive value of $G_{(\varepsilon\text{-}NH_2)}$ which was obtained according to the following equation is notable:

$$G_{(\varepsilon\text{-}NH_2)} = R_{M\ 6\text{-amino caproic}} - R_{M\ \text{caproic}} = +1.58 \qquad (5)$$

A similar relation between the group constants for the $NH_2$-group was observed by SCHAUER AND BULIRSCH[5]. They too, found $G_{(\alpha\text{-}NH_2)}$ and $G_{(\beta\text{-}NH_2)}$ to be close together, whereas the group constants of the remaining positions showed distinct differences.

*Halogen acids*

The halogen acids (Nos. 43–53) display a rather complex behaviour. Chloro-, bromo- and iodoacetic acid for instance have almost identical $R_F$ values. The $R_F$

TABLE IV

HALOGEN ACIDS

| *No.* | *Acid* | $R_F$ *exp.* | $R_M$ *measured* | $R_M$ *calc.* | $R_F$ *calc.* | $\Delta R_M$ | $\Delta R_F$ |
|---|---|---|---|---|---|---|---|
| 43 | Difluoroacetic | 0.67 | −0.31 | −0.28 | 0.66 | 0.03 | 0.01 |
| 44 | Trifluoroacetic | 0.78 | −0.55 | −0.50 | 0.76 | 0.05 | 0.02 |
| 45 | Chloroacetic | 0.52 | −0.04 | −0.06 | 0.53 | 0.02 | 0.01 |
| 46 | 2-Chloropropionic | 0.64 | −0.25 | −0.28 | 0.65 | 0.03 | 0.01 |
| 47 | 2-Chlorobutyric | 0.75 | −0.48 | −0.50 | 0.76 | 0.02 | 0.01 |
| 48 | Dichloroacetic | 0.64 | −0.25 | −0.28 | 0.66 | 0.03 | 0.02 |
| 49 | Trichloroacetic | 0.74 | −0.45 | −0.50 | 0.76 | 0.05 | 0.02 |
| 50 | Trichlorolactic | 0.69 | −0.35 | −0.31 | 0.67 | 0.04 | 0.02 |
| 51 | Bromoacetic | 0.54 | −0.07 | −0.06 | 0.56 | 0.01 | 0.01 |
| 52 | Tribromoacetic | 0.75 | −0.48 | −0.50 | 0.73 | 0.02 | 0.01 |
| 53 | Iodoacetic | 0.54 | −0.07 | −0.06 | 0.53 | 0.01 | 0.01 |

values obtained experimentally lead to the conclusion, that the number of halogen atoms rather than kind and position of the halogens are of more importance for the $R_F$ value. Therefore an attempt was made to obtain good agreement between measured and calculated $R_F$ values with a small number of group constants and to keep the calculation of the theoretical $R_F$ values of halogen acids as simple as possible. As a first approximation it appears to be sufficient to introduce, apart from the group

constant of the first halogen atom in $\alpha$-position ($G = -0.14$), only one more constant for any further halogen atom independent of its position with respect to any other functional group in the compound ($G = -0.22$). This simplified approach only causes greater deviations in the case of the trisubstituted acids, whereas good agreement is obtained with the other compounds.

*Other acids*

$R_F$ and $R_M$ values of six organic acids (Nos. 54–59) with different functional groups were also investigated (Table V). The first three contained a carbonyl group. The successful TLC separation of ketocarboxylic acids on cellulose layers has already been described: RINK AND HERRMANN[42] separated the acids as rhodamine derivates, and CHIARI AND RÖHR[43] separated the 2,4-DPHs of $\alpha$-ketocarboxylic acids.

TABLE V

OTHER ACIDS

| No. | Acid | $R_F$ exp. | $R_M$ measured | $R_M$ calc. | $R_F$ calc. | $\Delta R_M$ | $\Delta R_F$ |
|---|---|---|---|---|---|---|---|
| 54 | Glyoxylic | 0.30 | 0.37 | 0.37 | 0.30 | 0.00 | 0.00 |
| 55 | Pyruvic | 0.49 | 0.02 | 0.01 | 0.49 | 0.01 | 0.00 |
| 56 | Levulinic | 0.57 | −0.12 | −0.13 | 0.57 | 0.01 | 0.00 |
| 57 | Ethoxyacetic | 0.55 | −0.08 | −0.09 | 0.55 | 0.01 | 0.00 |
| 58 | Thioglycolic | 0.52 | −0.04 | −0.05 | 0.53 | 0.01 | 0.01 |
| 59 | Butane-1-sulfonic | 0.64 | −0.25 | −0.25 | 0.64 | 0.00 | 0.00 |

The calculation of the group constant for $\alpha$-CHO could not be done by direct comparison of the $R_M$ values of formic acid and glyoxylic acid, since, as already mentioned, the $R_M$ value measured for formic acid differs greatly from the theoretical. Therefore $G_{(\alpha\text{-CHO})}$ was directly determined from glyoxylic acid:

$$G_{(\alpha\text{-CHO})} = R_{M\ \text{glyoxylic}} - G_{(\text{COOH})} - G_0 = +0.07 \qquad (6)$$

The difference between the $R_F$ values of pyruvic and acetic acid leads to a $G_{(\alpha\text{-CO})}$ of −0.07, that between levulinic and butyric acid to a $G_{(\gamma\text{-CO})}$ of +0.23. Here too, one can observe, that the hydrophilic properties of a group are greatly weakened, when in the $\alpha$-position.

The group constant for the ether group was obtained by subtracting the $R_M$ value of butyric acid from the $R_M$ of ethoxyacetic acid ($G_{\text{ether}} = +0.27$).

Thioglycolic acid, when compared with acetic acid, yields a $G_{(\alpha\text{-SH})}$ of −0.13; and butane-1-sulfonic acid a value for $G_{(SO_3H)} = +1.59$.

*Calculation of the basic constant*

For the calculation of group constants and binding increments by direct comparison of the $R_M$ values of substances differing by only one group or binding increment, the knowledge of the value for the basic constant is not necessary. For its determination, straight chain fatty acids, hydroxy acids and other simple acids were

TABLE VI

BASIC CONSTANT, GROUP CONSTANTS AND BINDING INCREMENTS

Adsorbent: Cellulose HR 300 (Macherey, Nagel). Solvent: *n*-Butanol–diethylamine–water (84:1:14). Temperature: 23° ± 1°.

| | |
|---|---|
| Basic constant | −0.96 |
| Aliphatic C-atom | −0.22 |
| Chain branching, in $\alpha$-position | −0.03 |
| in other positions | +0.14 |
| C = C double bond | +0.09 |
| OH in $\alpha$-position | +0.49 |
| in $\beta$-position | +0.56 |
| in $\gamma$-position | +0.76 |
| $NH_2$ in $\alpha$-position | +0.98 |
| in $\beta$-position | +1.04 |
| in $\gamma$-position | +1.14 |
| in $\varepsilon$-position | +1.58 |
| CHO in $\alpha$-position | +0.07 |
| CO in $\alpha$-position | −0.07 |
| in $\gamma$-position | +0.23 |
| COOH | +1.26 |
| Halogen atom, the first if in $\alpha$-position | −0.14 |
| any other one | −0.22 |
| —O—, ether groups | +0.27 |
| SH in $\alpha$-position | −0.13 |
| $—SO_3H$ | +1.59 |

*E.g.* the $R_M$ value for valine is calculated thus: $R_M = G_0 + G_{(COOH)} + 4G_{(C)} + G_{(\alpha\text{-}NH_2)} + G_{(branching)}$. $R_M = -0.96 + 1.26 - 0.88 + 0.98 + 0.14 = +0.54$ (see No. 39).

used. The calculation was done by inserting the experimental $R_M$ value and the group constants determined (Table VI) in equation (1). For instance one can determine the basic constant $G_0$ from butyric acid and alanine in the following way:

$$R_{M\ \text{butyric}} = G_0 + G_{(COOH)} + 3G_{(C)} \qquad G_0 = -0.96 \qquad (7)$$

$$R_{M\ \text{alanine}} = G_0 + G_{(COOH)} + G_{(\alpha\text{-}NH_2)} + 2G_{(C)} \qquad G_0 = -0.96 \qquad (8)$$

The mean value obtained from 14 different acids by this method was found to be $G_0 = -0.96$.

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CHROM. 4347

# PAPIERCHROMATOGRAPHISCHE TRENNUNG DER STELLUNGSISOMEREN ALKANDISULFONSÄUREN DER KETTENLÄNGE VON $C_1$–$C_5$*

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SUMMARY

*Paper chromatographic separation of the positional isomers of alkanedisulphonic acids with chain lengths of 1–5 carbon atoms*

The positional isomers of alkanemono- and -disulphonic acids theoretically obtainable on sulphoxidation of $C_1$–$C_5$ paraffinic hydrocarbons can be separated by means of paper chromatography. This has been confirmed with synthesized test samples. The mixture *tert.*-butanol–*n*-butanol–ammonia–water (11:11:1:5) is a suitable solvent system for the homologous alkanemonosulphonic acids. The separation of the alkanedisulphonic acids is mostly achieved by repeated elution with *tert.*-butanol–*n*-propanol–ammonia (1:4:1).

## EINLEITUNG

Bei Substitutionsreaktionen an Paraffinkohlenwasserstoffen entstehen neben den Monosubstitutionsprodukten auch disubstituierte Verbindungen. Innerhalb einer Reihe systematischer Arbeiten zur Untersuchung der Isomerenverteilung bei Disubstitutionsreaktionen an Paraffinkohlenwasserstoffen (vgl. Lit. 1) prüften wir auch die Verhältnisse bei der Disulfoxydation, über die wir an anderer Stelle berichten werden.

Eine quantitative Untersuchung und einwandfreie Zuordnung der bei der Disulfoxydation auftretenden stellungsisomeren Verbindungen setzt die Erfüllung folgender Forderungen voraus:

(a) Synthese aller theoretisch möglichen Alkanmono- und -disulfonsäuren des zu untersuchenden Kettenlängenbereiches;

(b) vollständige, reproduzierbare und analytisch quantitativ auswertbare Trennung der unter (a) genannten Verbindungen;

(c) quantitative Bestimmung aller durch Sulfoxydation des betreffenden Paraffinkohlenwasserstoffs bzw. der betreffenden Alkanmonosulfonsäuren erhaltenen stellungsisomeren Alkandisulfonsäuren.

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Im folgenden beschreiben wir die Herstellung und papierchromatographische Trennung der theoretisch möglichen Alkanmono- und -disulfonsäuren von $C_1$–$C_5$.

## DARSTELLUNG DER TESTSUBSTANZEN

Bei der Sulfoxydation bzw. der Disulfoxydation der homologen Alkane von $C_1$–$C_5$ können theoretisch die in der Tabelle I aufgeführten Alkanmono- bzw. -disulfonsäuren entstehen. Alle dort aufgeführten Alkansulfonsäuren wurden nach Literaturvorschriften synthetisiert und durch Elementaranalyse oder durch Schmelzpunktsbestimmung geeigneter, bereits in der Literatur beschriebener Derivate oder durch gaschromatographische Analyse der reinen, stellungsisomeren Sulfonsäuremethylester (vgl. Lit. 2) charakterisiert.

Von den zahlreichen Möglichkeiten (s. vollständige Literaturübersicht in Lit. 3) zur Synthese reiner, stellungsisomerer Alkanmonosulfonsäuren wandten wir die gebräuchlichste Methode an, die darin besteht, dass man die entsprechenden Brom-

TABELLE I

ALKANMONO- UND -DISULFONSÄUREN DER KETTENLÄNGE $C_1$–$C_5$

| *Sulfonsäure* | *Darstellungsverfahren*[a] | *Literatur* |
|---|---|---|
| Methan- | A | 4 |
| Methan-(1,1)-di- | | 15 |
| Äthan- | A | 4 |
| Äthan-(1,1)-di- | E | 16 |
| Äthan-(1,2)-di- | A | — |
| Propan-(1)- | A | 17 |
| Propan-(2)- | A | 18 |
| Propan-(1,1)-di- | E | 16 |
| Propan-(1,2)-di | A | 19 |
| Propan-(1,3)-di- | A | 19 |
| *n*-Butan-(1)- | A | 20 |
| *n*-Butan-(2)- | A | 21 |
| *n*-Butan-(1,1)-di- | E | 16 |
| *n*-Butan-(1,2)-di | A | — |
| *n*-Butan-(1,3)-di- | A, D | 19 |
| *n*-Butan-(1,4)-di- | A | 19 |
| *n*-Butan-(2,3)-di- | A, D | — |
| *n*-Pentan-(1)- | A | 20 |
| *n*-Pentan-(2)- | A | — |
| *n*-Pentan-(3)- | A | — |
| *n*-Pentan-(1,1)-di- | E | 16 |
| *n*-Pentan-(1,2)-di- | A, D | — |
| *n*-Pentan-(1,3)-di- | A, D | — |
| *n*-Pentan-(1,4)-di- | A, B, C, D | 22 |
| *n*-Pentan-(1,5)-di- | A | — |
| *n*-Pentan-(2,3)-di- | A, D | — |
| *n*-Pentan-(2,4)-di- | A, C | — |

[a] Zur Bedeutung der Abkürzungen s. Text.

alkane mit wässriger Natriumsulfit-Lösung in der Siedehitze umsetzt[4] (Verfahren A).

Dieses Verfahren eignet sich auch in vielen Fällen zur Darstellung der stellungsisomeren Alkandisulfonsäuren. Die Disulfonsäuren werden teilweise besser und in glatt verlaufender Reaktion durch Umsetzung der entsprechenden Alkandisulfochloride mit Methanol[5] erhalten.

Die Alkandisulfochloride wurden nach einem der folgenden Verfahren dargestellt:

Nach einer Methode von JOHNSON UND SPRAGUE[6,7] erhält man durch Behandeln einer wässrigen Lösung eines S-Isothiuroniumsalzes mit Chlor ein definiertes Sulfochlorid. Die Isothiuroniumsalze entstehen bei der Umsetzung von Mono- bzw. Dihalogenalkanen mit Thioharnstoff in Gegenwart von *p*-Toluolsulfonsäureestern[8] (Verfahren B). Eine Variante dieses Verfahrens besteht darin, dass man an Stelle des Halogenalkans den entsprechenden Alkohol (Diol) mit *p*-Toluolsulfochlorid in Gegenwart von Pyridin in das Alkan-*p*-toluolsulfonat überführt, das beim Erhitzen mit Thioharnstoff in alkoholischer Lösung S-Alkylthiuronium-*p*-toluolsulfonat bildet. Daraus entsteht durch oxydative Chlorierung im wässrigen Medium das betreffende Alkanmono- bzw. -disulfochlorid[9] (Verfahren C).

Definierte Alkanmono- bzw. -disulfochloride bilden sich auch bei der Umsetzung von Alkanmono- bzw. -dirhodaniden mit Chlor im wässrigen Medium (vgl. Lit. 10) (Verfahren D).

Als Ausgangsbasis für die Synthese der 1,1-Alkandisulfonsäuren diente die Methionsäure; diese wird am besten nach dem Verfahren von BACKER[11] in Form des Natriumsalzes durch Umsetzung von Methylenchlorid mit wässriger Kaliumsulfit-Lösung[12,13] bei 150–160° im Schüttelautoklaven bei einem Druck von 25–30 atm hergestellt. Mittels Ionenaustauscher (H-Form, z.B. Lewatit S 100, Farbenfabriken Bayer) erhält man aus dem Kaliummethionat die freie, hygroskopische Methionsäure. Die Methionsäure wird durch Umsetzung mit Phosphorpentachlorid in das Methionsäuredisulfochlorid[12,14] übergeführt, das mit Äthylanilin zum Methionsäurediäthylanilid reagiert[15]. Diese Verbindung zeichnet sich durch ihre C–H-Acidität aus; es gelingt glatt, in Analogie zur Malonestersynthese den Wasserstoff an der Methylengruppe durch Natrium zu ersetzen und durch Umsetzung der Natriumverbindung mit primären Halogenalkanen die höheren Alkanmethionsäurediäthylanilide darzustellen. Diese liefern bei der sauren Hydrolyse und anschliessenden Neutralisation die Salze der betreffenden 1,1-Alkandisulfonsäuren (Verfahren E). Mittels Ionenaustauscher (H-Form) werden die freien Säuren als dunkelbraune, viskose Öle erhalten.

## PAPIERCHROMATOGRAPHISCHE TRENNUNG DER AUS DEN TESTSUBSTANZEN HERGESTELLTEN GEMISCHE AUS ALKANMONO- UND -DISULFONSÄUREN

Papierchromatographische Trennungen von aliphatischen Sulfonsäuren wurden von COYNE UND MAW[23] durchgeführt. Sie untersuchten die Abhängigkeit des $R_F$-Wertes vom angewandten Laufmittel. Die dort angegebenen und als vorteilhaft erkannten Laufmittelgemische eignen sich zwar, wie wir feststellten, zur Trennung der homologen Alkanmonosulfonsäuren, aber für die Trennung der Alkandisulfonsäuren mussten andere Systeme aufgefunden werden.

Wegen der hohen Dissoziationskonstanten der Alkansulfonsäuren sind saure Laufmittelsysteme für deren Chromatographie wenig geeignet; sie ergeben in den

TABELLE II

$R_F$-WERTE DER ALKANSULFONSÄUREN BEI ANWENDUNG VERSCHIEDENER LAUFMITTELGEMISCHE

Laufmittel:
1 = *tert.*-Butanol–*n*-Butanol–$H_2O$—$NH_4OH$ (28%) (30:30:20:3).
2 = *n*-Propanol–$NH_4OH$ (28%)–$H_2O$ (20:5:5).
3 = *tert.*-Butanol–$NH_4OH$ (28%)–*n*-Propanol (1:1:4).
4 = *tert.*-Butanol–$NH_4OH$ (28%)–*n*-Butanol (2:0.5:0.5).
5 = *tert.*-Butanol–$NH_4OH$ (28%) (2:0.5).
6 = *n*-Propanol–$NH_4OH$ (28%) (3:1).
7 = Methanol–*tert.*-Butanol–$NH_4OH$ (28%) (10:5:2).
8 = *n*-Propanol–$NH_4OH$ (28%) (30:5).
9 = Äthanol–$NH_4OH$ (28%)–$H_2O$ (20:10:5).
10 = *n*-Propanol–$CHCl_3$–$NH_4OH$ (28%) (30:10:5).
11 = *n*-Butanol–Aceton–$NH_4OH$ (28%) (3:1:2).
12 = *n*-Butanol–Aceton–$NH_4OH$ (28%) (3:1:1).
13 = *tert.*-Butanol–HCOOH–$H_2O$ (20:10:5).

| *Sulfonsäure* | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* | *10* | *11* | *12* | *13* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Methan- | 0.16 | 0.576 | — | 0.32 | 0.53 | 0.72 | 0.59 | 0.45 | 0.84 | 0.32 | 0.33 | 0.24 | 0.61 |
| Äthan- | 0.26 | 0.64 | — | 0.42 | 0.61 | 0.79 | 0.70 | 0.55 | 0.86 | 0.31 | 0.38 | 0.32 | 0.73 |
| Propan-(1)- | 0.39 | 0.73 | 0.55 | 0.58 | 0.59 | 0.85 | 0.75 | 0.65 | 0.87 | 0.42 | 0.47 | 0.41 | 0.80 |
| Propan-(2)- | — | 0.70 | — | 0.51 | — | 0.83 | 0.74 | 0.63 | 0.86 | 0.37 | 0.43 | 0.39 | 0.76 |
| Butan-(1)- | 0.57 | 0.79 | 0.40 | 0.68 | 0.75 | 0.86 | 0.78 | 0.74 | 0.87 | 0.51 | 0.55 | 0.52 | 0.86 |
| Butan-(2)- | — | 0.77 | 0.36 | 0.63 | — | 0.84 | 0.75 | 0.72 | 0.87 | 0.50 | 0.51 | 0.48 | 0.83 |
| Butan-(1,4)-di- | — | — | 0.06 | 0.05 | 0.14 | 0.45 | 0.31 | 0.09 | 0.87 | — | 0.15 | 0.57 | 0.20 |
| Pentan-(1,4)-di- | 0.075 | — | — | 0.08 | — | 0.56 | 0.37 | 0.17 | 0.84 | — | 0.14 | 0.08 | 0.30 |
| Pentan-(1,5)-di- | — | — | — | 0.08 | — | 0.56 | 0.37 | 0.16 | 0.83 | — | 0.14 | 0.07 | 0.31 |

meisten Fällen langgezogene Flecken. Unsere Untersuchungen zeigen, dass Gemische aus ein bzw. zwei niedermolekularen Alkoholen und verschiedenen Volumenanteilen an konz. Ammoniak (28%) besonders günstig sind. Eine Auswahl der verschiedensten von uns angewendeten Laufmittel zeigt Tabelle II.

Bei Verwendung verhältnismässig hydrophiler Laufmittel haben Alkanmono- und -disulfonsäuren hohe und dicht beieinanderliegende $R_F$-Werte. Mit zunehmender Hydrophobizität nehmen die $R_F$-Werte ab, aber die Differenz zwischen den $R_F$-Werten der einzelnen Verbindungen vergrössert sich, d.h., der Trenneffekt wird verbessert. Die Alkanmonosulfonsäuren besitzen bei Verwendung basischer Laufmittel höhere $R_F$-Werte als die Alkandisulfonsäuren gleicher Kettenlänge. Bei den homologen Alkanmonosulfonsäuren steigen die $R_F$-Werte wie bei den Monocarbonsäuren (vgl. Lit. 24–27) mit zunehmender C-Zahl.

Bei den stellungsisomeren Alkanmonosulfonsäuren bestimmter Kettenlänge fallen die $R_F$-Werte in dem Masse, in dem die Sulfonsäuregruppe zum Innern des Moleküls hin wandert; sie liegen dann so dicht beieinander, dass zur einwandfreien Trennung mehrmals chromatographiert werden muss. Es empfiehlt sich, nach jedem Lauf vom unteren Teil des Papiers soviel abzuschneiden, dass jeweils der unterste Fleck sich etwa 2 cm oberhalb der Laufmitteloberfläche befindet. Die Trennung verbessert sich dadurch zwar wesentlich, aber eine Zuordnung der Flecken aufgrund von $R_F$-Werten wird gleichzeitig unmöglich gemacht.

Zur Trennung der niedermolekularen Alkandisulfonsäuren haben sich, wie wir fanden, ebenfalls basische Laufmittelsysteme bewährt. Von den untersuchten Disulfonsäuren von $C_1$–$C_5$ besitzt die Methionsäure in allen Fällen den kleinsten $R_F$-Wert.

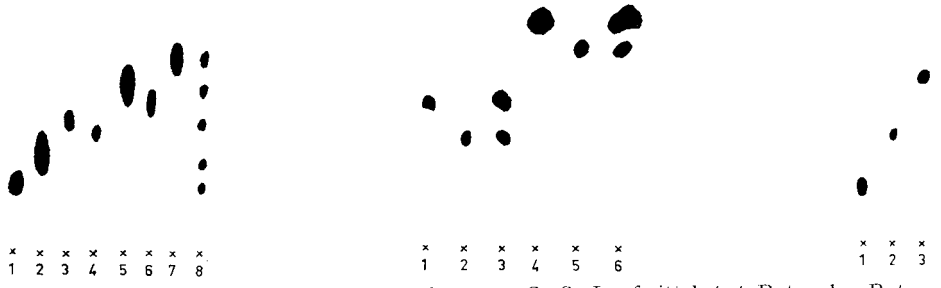


Fig. 1. Chromatogramm der Alkanmonosulfonsäuren $C_1$–$C_6$. Laufmittel: *tert.*-Butanol–*n*-Butanol–Ammoniak (28%)–Wasser (11:11:1:5). Temperatur: 22°. Sulfonsäure: 1 = Methan-, 2 = Äthan-, 3 = Propan-(1), 4 = Propan-(2), 5 = Butan-(1), 6 = Butan-(2), 7 = Pentan-(1), 8 = Gemisch aus 1 bis 7.

Fig. 2. Chromatogramm der isomeren Propan- und Butanmonosulfonsäuren. Laufmittel: *tert.*-Butanol–*n*-Butanol–Ammoniak (28%) (4:1:1). Temperatur: 22°. Sechs mal chromatographiert. Laufzeit 60 Std. Sulfonsäure: 1 = Propan-(1), 2 = Propan-(2), 3 = Gemisch aus 1 und 2, 4 = Butan-(1), 5 = Butan-(2), 6 = Gemisch aus 4 und 5.

Fig. 3. Chromatogramm der $C_1$-Sulfonsäuren. Laufmittel: *tert.*-Butanol–*n*-Propanol–Ammoniak (28%)–Wasser (4:1:1:2). Temperatur 22°. Sulfonsäure: 1 = Methantrisulfonsäure, 2 = Methionsäure, 3 = Methansulfonsäure, 4 = Gemisch aus 1 bis 3.

Mit zunehmender Kettenlänge steigen die $R_F$-Werte an. Bei den stellungsisomeren Alkandisulfonsäuren gleicher C-Zahl sinken die $R_F$-Werte beim Übergang von den endständigen geminalen zu den $\alpha,\omega$-Alkandisulfonsäuren ab. Auch deren Trennung gelingt nicht durch einmaliges Chromatographieren. Die Anwendung hydrophiler Laufmittel bewirkt zwar eine rasche Wanderung der Disulfonsäuren, aber die Trennungen sind schlecht. Am günstigsten sind sehr langsam laufende Lösungsmittelgemische.

In allen Fällen bewährte sich die aufsteigende Chromatographie mit MN 218-Papier*; das absteigende Verfahren lieferte stets schlechtere Ergebnisse. Zum Entwickeln der Substanzflecken verwendet man vorteilhaft eine alkoholische Lösung von

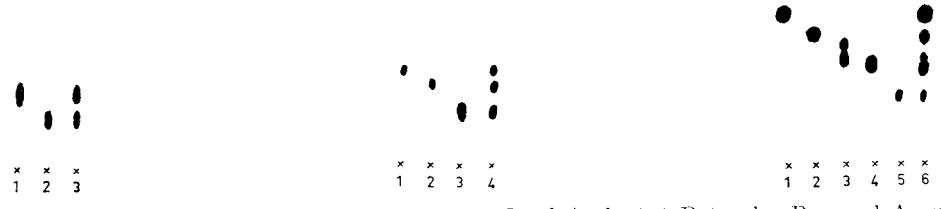


Fig. 4. Chromatogramm der $C_2$-Disulfonsäuren. Laufmittel: *tert.*-Butanol–*n*-Propanol–Ammoniak (28%) (1:4:1). Temperatur 22°. Fünf mal chromatographiert. Laufzeit 60 Std. Disulfonsäure: 1 = Äthan-(1,1), 2 = Äthan-(1,2), 3 = Gemisch aus 1 und 2.

Fig. 5. Chromatogramm der $C_3$-Disulfonsäuren. Laufmittel: *tert.*-Butanol–*n*-Propanol–Ammoniak (28%) (1:4:1). Temperatur 22°. Acht mal chromatographiert. Laufzeit 85 Std. Disulfonsäure: 1 = Propan-(1,1), 2 = Propan-(1,2), 3 = Propan-(1,3), 4 = Gemisch aus 1 bis 3.

Fig. 6. Chromatogramm der $C_4$-Disulfonsäuren. Laufmittel: *tert.*-Butanol–*n*-Propanol–Ammoniak (28%) (1:4:1). Temperatur 22°. Achtmal chromatographiert. Laufzeit 85 Std. Disulfonsäure: 1 = Butan-(1,1), 2 = Butan-(1,2), 3 = Butan-(2,3), 4 = Butan-(1,3), 5 = Butan-(1,4), 6 = Gemisch aus 1 bis 5, 7 = Gemisch aus 1, 2, 4 und 5.

* Fa. Macherey, Nagel & Co., Düren/Rheinland.


Fig. 7. Chromatogramm der $C_5$-Disulfonsäuren. Laufmittel: *tert.*-Butanol–*n*-Propanol–Ammoniak (28%) (1:4:1). Temperatur 22°. Acht mal chromatographiert. Laufzeit 85 Std. Disulfonsäure: 1 = Pentan-(1,1), 2 = Pentan-(1,2), 3 = Pentan-(1,3), 4 = Pentan-(1,4), 5 = Pentan-(1,5), 6 = Gemisch aus 1 bis 5, 7 = Gemisch aus Pentan-(2,4) und -(2,3).

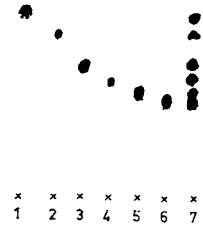


Fig. 8. Chromatogramm der $C_5$-Disulfonsäuren. Laufmittel: *tert.*-Butanol–*n*-Propanol–Ammoniak (28%) (1:4:1). Temperatur 22°. Acht mal chromatographiert. Laufzeit 85 Std. Disulfonsäure: 1 = Pentan-(1,1), 2 = Pentan-(1,2), 3 = Pentan-(1,3), 4 = Pentan-(2,4), 5 = Pentan-(1,4), 6 = Pentan-(1,5), 7 = Gemisch aus 1 bis 6.

Bromkresolgrün; es zeigen sich gelbe, unbeständige Flecken auf blauem Untergrund. Die Nachweisempfindlichkeit war, wie am Beispiel der Butandisulfonsäure-(1,4) gezeigt werden konnte, bei 2.5 $\mu$g Substanz noch gut.

Die Fig. 1–6 zeigen, dass mittels der jeweils angegebenen Laufmittelsysteme eine exakte Trennung der betreffenden Verbindungen erreicht werden konnte. Lediglich die Trennung von Pentandisulfonsäure-(1,4) und -(1,5) (Fig. 7 und 8) gelang nur unvollständig.

TABELLE III

$R_M$-WERTE[a] VON ALKANMONOSULFONSÄUREN $CH_3(CH_2)_xSO_3H$ IN ABHÄNGIGKEIT VON DER ANZAHL $x$ DER METHYLENGRUPPEN

Laufmittelsystem: *tert.*-Butanol–*n*-Butanol–Ammoniak (28%)–Wasser (11:11:1:5).

| | $x$ | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| $R_M$ | 0.25 | 0.36 | 0.18 | 0.03 | −0.13 | −0.21 | −0.29 |

[a] $R_M = \log\left(\frac{1}{R_F} - 1\right)$

Die bereits nach einmaligem Chromatographieren erreichbare Trennung der homologen Alkanmonosulfonsäuren der Kettenlänge $C_1$–$C_6$ (s. Fig. 1) ermöglicht die Prüfung eines Zusammenhanges zwischen den $R_F$-Werten und der Konstitution der Säuren. Ähnliche Versuche an Alkancarbonsäuren zeigten, dass eine lineare Beziehung zwischen den $R_M$-Werten und der Anzahl der Kohlenstoffatome besteht[25,28].

Die aus der Fig. 1 errechneten $R_M$-Werte für die dort angegebenen Alkanmonosulfonsäuren sind in Tabelle III in Abhängigkeit von der Anzahl der Methylengruppen wiedergegeben. Innerhalb kleiner Bereiche besteht ein linearer Zusammenhang zwischen den $R_M$-Werten und der Anzahl der Methylengruppen (Fig. 9).

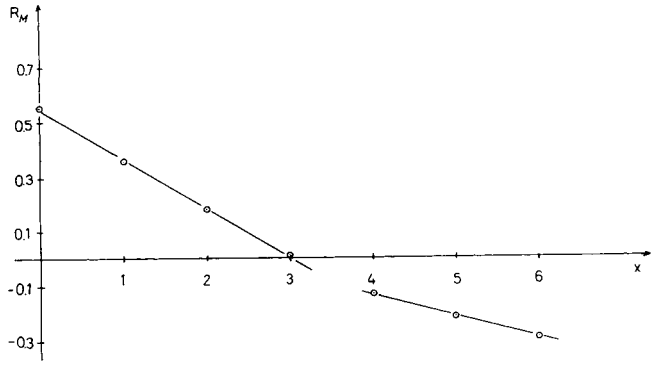


Fig. 9. Abhängigkeit der $R_M$-Werte der homologen Alkansulfonsäuren $CH_3(CH_2)_xSO_3H$ von der Anzahl $x$ der Methylengruppen.

ZUSAMMENFASSUNG

Die bei der Sulfoxydation von Paraffinkohlenwasserstoffen der Kettenlänge von $C_1$–$C_5$ theoretisch möglichen stellungsisomeren Alkanmono- und -disulfonsäuren lassen sich papierchromatographisch mittels basischer Laufmittelsysteme trennen, wie an Hand von Modellverbindungen gezeigt wird. Als Laufmittel für die homologen Alkanmonosulfonsäuren eignet sich das Gemisch *tert.*-Butanol–*n*-Butanol–Ammoniak–Wasser (11:11:1:5). Die Trennung der Alkandisulfonsäuren gelingt zumeist mit dem Gemisch *tert.*-Butanol–*n*-Propanol–Ammoniak (1:4:1) nach mehrmaligem Chromatographieren.

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CHROM. 4330

# INVESTIGATIONS ON THE RELATIONSHIP BETWEEN MOLECULAR STRUCTURE AND CHROMATOGRAPHIC PARAMETERS

## III. PARTITION OF PYRIDYL ALKYL KETONES BETWEEN ORGANIC SOLVENTS AND HYDROCHLORIC ACID

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SUMMARY

$R_M$ *vs.* $H_0$ relationships of two homologous series of pyridyl alkyl ketones were determined for systems of the type organic solvent/aqueous hydrochloric acid. In accordance with theoretical anticipations, linear $R_M$ *vs.* $H_0$ plots were obtained for $H_0$ values in the range $-1$, $+1$. $R_M$ values for methylene groups were found to be constant in the systems studied so that the homologous series, especially 2-pyridyl alkyl ketones, can be used as reference compounds for the chromatography of weak lipophilic organic bases in strongly acidic liquid–liquid partition systems.

## INTRODUCTION

In investigations on the structural effects in liquid–liquid partition chromatography, relatively little notice has been paid to simple derivatives of pyridine. There were some reports on the separation of pyridine bases in the form of N-oxides or N-methyl iodides; on the other hand, few authors separated free pyridine bases or their salts. A review of papers concerning the chromatographic seperation of pyridines has recently been given by NEUHÄUSER AND WOLF[1].

In the first paper in this series[2], we have reported the $R_M$ values of several homologous 4-pyridyl alkyl ketones and 4-pyridyl alkyl alcohols in liquid–liquid partition systems, the polar phase being MacIlvaine's buffer solution, water, formamide, ethylene glycol or dimethylformamide. For stronger solvents such as chloroform, high $R_F$ values of the ketones have been obtained even for the lower extreme of the MacIlvaine's buffer solutions (pH 2); therefore, in the present investigations we have extended the range of acidity by employing moderately concentrated solutions of hydrochloric acid (0.2–10.0%, which corresponds to Hammett's $H_0$ values of $+1$ and $-1$, respectively[3]) as polar phase. The use of such systems proved to give good results in the chromatography of weak hydrophobic bases[4]. As the developing liquid, ten non-polar or weakly polar solvents with various donor–acceptor properties were used.

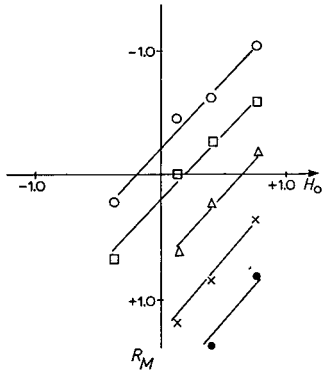


Fig. 1. $R_M$ *vs.* $H_0$ relationships of five 2-pyridyl alkyl ketones. Mobile phase: cyclohexane. The alkyl group is denoted as follows: ● = Me; × = Et; △ = Pr; □ = Bu; ○ = Am.

In view of the low mutual solubility of the phases, the partition systems employed were expected to give better selectivities[5] than the solvent systems employed by NEUHÄUSER AND WOLF[1], who chromatographed some homologues of pyridine in systems of the type lower alcohols–hydrochloric acid–water. Furthermore, the increasing interest in olumn liquid–liquid chromatography[6] is a stimulus to investigate par-

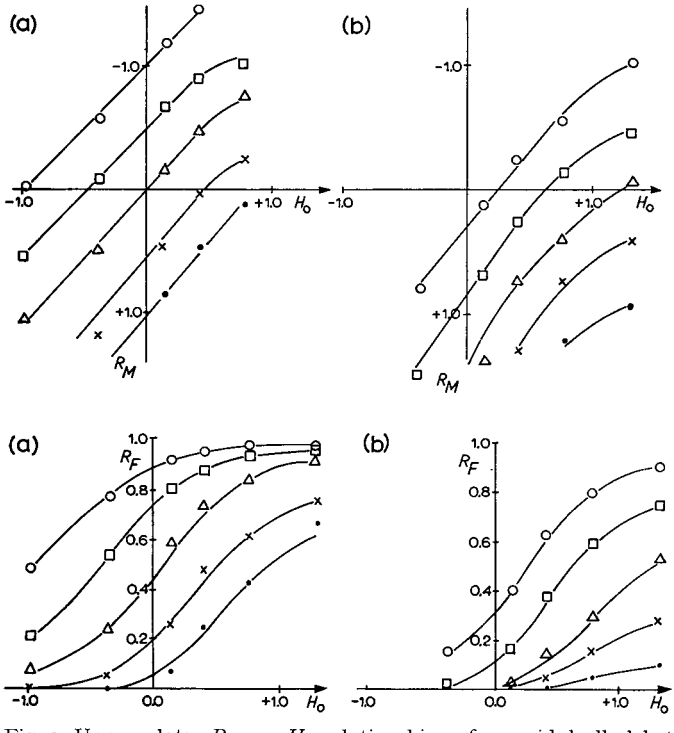


Fig. 2. Upper plots: $R_M$ *vs.* $H_0$ relationships of 2-pyridyl alkyl ketones (a) and 4-pyridyl alkyl ketones (b). Lower plots: corresponding $R_F$ *vs.* $H_0$ relationships. Mobile phase: benzene.

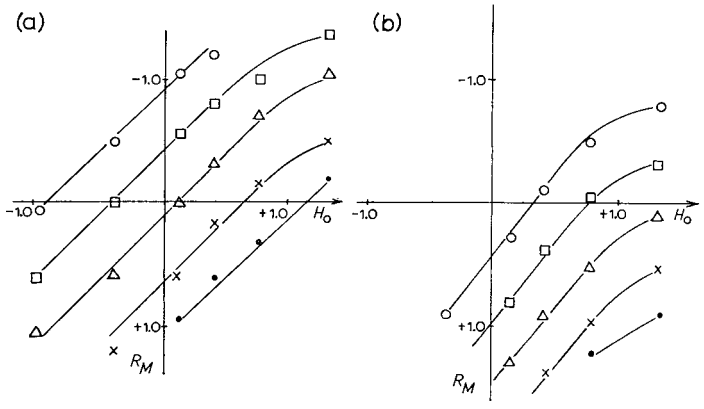


Fig. 3. $R_M$ *vs.* $H_0$ relationships of 2-pyridyl alkyl ketones (a) and 4-pyridyl alkyl ketones (b). Mobile phase: toluene.

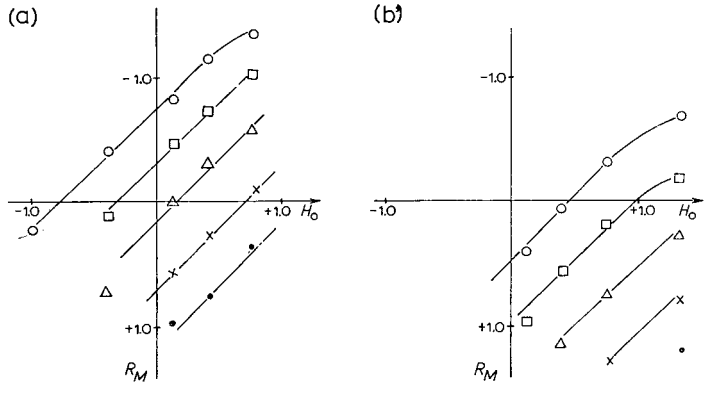


Fig. 4. As in Fig. 3. Mobile phase: carbon tetrachloride.

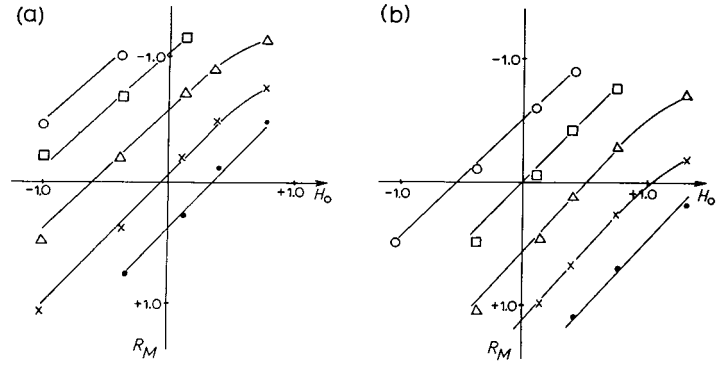


Fig. 5. As in Fig. 3. Mobile phase: chloroform.

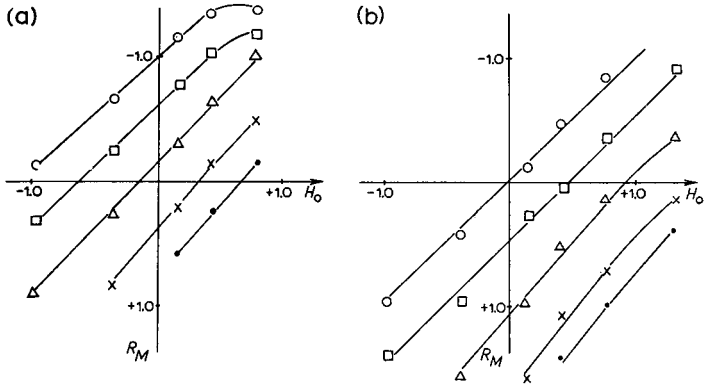


Fig. 6. As in Fig. 3. Mobile phase: 1,2-dichloroethane.

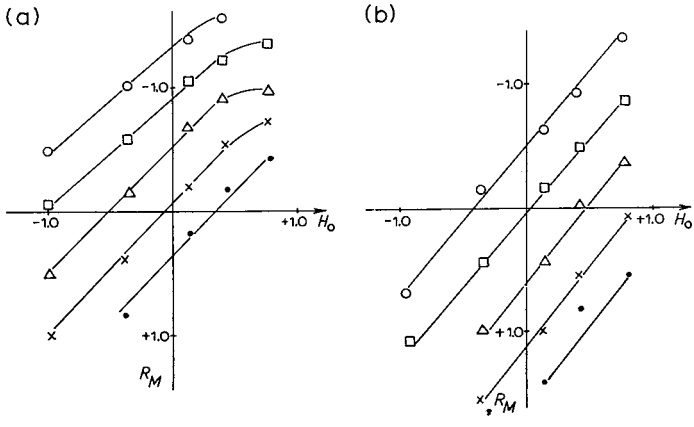


Fig. 7. As in Fig. 3. Mobile phase: tetrachloroethane.

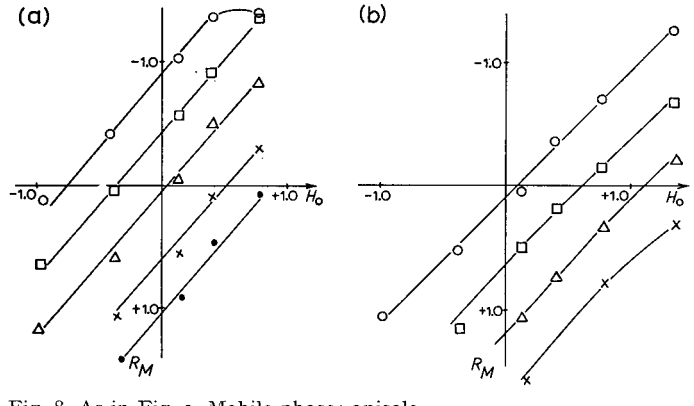


Fig. 8. As in Fig. 3. Mobile phase: anisole.

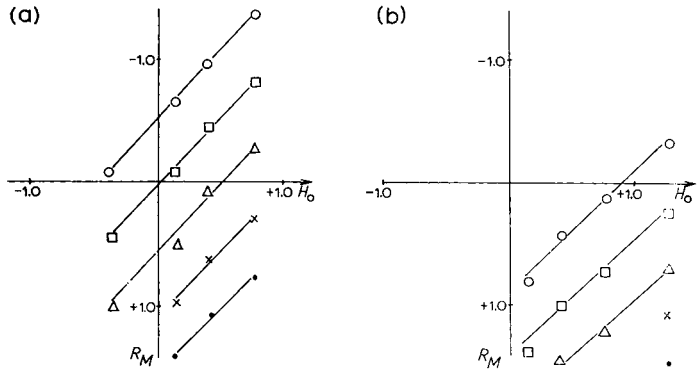


Fig. 9. As in Fig. 3. Mobile phase: di-*n*-butyl ether.

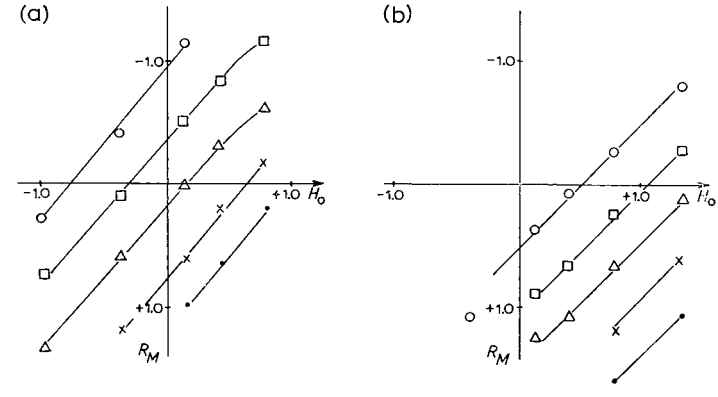


Fig. 10. As in Fig. 3. Mobile phase: diethyl ether.

tition systems of minimal mutual solubility of the two phases, since the commonly employed onephase systems, or systems of marked mutual solubility, introduce serious complications in the technical problems of column chromatography.

The use of acidic aqueous phases permits the application of liquid–liquid partition chromatography to be extended to solutes which are strongly hydrophobic and/or have very weak basic properties and are thus strongly extracted into the organic phase in the usual pH range of buffer solutions.

## EXPERIMENTAL

The pyridyl alkyl ketones were obtained by methods described in the first part of this series[2], the 2-pyridyl alkyl ketones being synthetized from the ethyl ester of $\alpha$-picolinic acid.

Whatman No. 4 paper strips were impregnated with solutions of hydrochloric acid of the following concentrations: 0.02 *M*, 0.05 *M*, 0.1 *M*, 0.2 *M*, 0.5 *M*, 1.0 *M* and 2.0 *M*. 'Moist paper' technique was employed, the strips being dried after impregnation

to a humidity of 0.5 g of aqueous solution per 1.0 g of dry paper, as in an earlier paper on paper chromatography of lipophylic acridine and quinoline bases in analogous solvent systems[4].

To determine the actual concentration of HCl in the stationary phase during the development, a calibration line was obtained by plotting the initial concentration of HCl in the impregnating solution ($C_0$) (multiplied by a factor accounting for partial evaporation of water from the strip) against the final concentration of HCl ($C$) determined by titrating the acid from the partially dried strip with NaOH. For low and moderate concentrations of HCl, the plot was practically linear, the slope being *ca.* 1.0, which indicated that under these conditions only water evaporated from the strip during the partial drying so that the actual concentration of HCl during development, $C$, could be calculated from the equation:

$$C = \frac{W_i - W_d}{0.5\, W_d} \cdot C_0$$

where $W_d$ is the weight of the dry strip and $W_i$ is the weight just after impregnation and blotting. ($W_i$ = 2.0–2.2 $W_d$; 0.5 $W_d$ is the weight of the aqueous phase during development.)

For concentrations of HCl higher than 15% ($H_0 < -1.5$) some loss of HCl during the partial drying was observed, and the calibration line deviated from linearity.

From the actual concentration of HCl, $C$, the values of $H_0$ of the aqueous phase were estimated[7].

To avoid elution of the aqueous phase from the chromatogram, more polar solvents were saturated with solutions of HCl of concentrations expected in the strip after partial drying (*ca.* 2 $C_0$). Final equilibration of the phases was expected to occur in the distance between the solvent level and the start line (*ca.* 6 cm).

All-glass tanks (5 × 9 × 24 cm) were used for descending development. The temperature was 22 ± 1°.

Experimental $R_F$ values, average from three or more runs, were plotted directly on $R_M$ *vs.* $H_0$ diagrams (Figs. 1–10), using an $R_F$ scale subordinate to proportional $R_M$ scale. As in the preceding papers from this series, the $R_M$ axis is directed downward so that the $R_M$ and $R_F$ both increase ($R_M = \log (1 - R_F)/R_F$).

## RESULTS AND DISCUSSION

### *Effect of acidity of the aqueous phase*

In most cases linear $R_M$ *vs.* $H_0$ relationships are observed, in accordance with theoretical anticipations (see KEMULA AND BUCHOWSKI[8]); for higher values of $H_0$, the lines tend to deviate to a horizontal asymptote, like $R_M$ *vs.* pH plots[9]. However, for some cases, this deviation may be due to gradient effects observed at higher values of $R_F$ (*e.g.*, $R_M < 0.5$, *i.e.*, $R_F > 0.7$). The respective $R_F$ *vs.* $H_0$ plots are thus sigmoidal in shape[2], like $R_F$ *vs.* pH plots (see Fig. 2).

### *Structural effects of the solutes*

It can be seen from the figures that, for both homologous series, the $R_M$ *vs.* $H_0$ lines are regularly spaced and parallel, which indicates that for all solvent systems the $R_M$ is linearly dependent on the number of carbon atoms in the alkyl chain. The $R_M$

values of the 4-pyridyl alkyl ketones vary in the range of optimal accuracy for stronger solvents (*e.g.*, chloroform); for benzene and toluene the points of the first two homologues are beyond the range of accurate $R_M$ values ($-1 < R_M < +1$). Nonpolar solvents such as carbon tetrachloride and cyclohexane (class N) are not suitable for 4-pyridyl alkyl ketones; also the solvent power of aliphatic ethers (class B) is too low.

The $\Delta R_M$ ($CH_2$) value for the solvents used, both weakly polar and nonpolar, is approx. $-0.52$ to $-0.56$ $R_M$ units, as in the preceding paper[2]. The selectivity of the partition systems for the solvents investigated is almost constant since it depends mostly on the squeezing effect of the aqueous phase, although their extraction power is differentiated over quite a wide range. In most systems, the value of $\Delta R_M$ (Et→Me) is slightly different from $\Delta R_M$ ($CH_2$) for the higher homologues ($-0.40$ to $-0.46$).

The $R_F$ values obtained for the other homologous series of 2-pyridyl alkyl ketones are considerably higher and for almost all solvents used are in the range of optimal accuracy for $H_0$ values in the range of $-1$, $+1$. The $R_M$ *vs.* $H_0$ plots are also in this case linear, parallel and almost equidistant; the $\Delta R_M$ ($CH_2$) values are approx. $-0.50$ to $-0.52$ $R_M$ units. A slightly higher selectivity is observed for cyclohexane, benzene and di-*n*-butyl ether; however, the solvents are weak extractants and the $\Delta R_M$ ($CH_2$) values, especially for lower homologues, are less accurate.

Comparing the chromatographic results for 2- and 4-pyridyl alkyl ketones, the $\Delta R_M$ values due to 4–2 isomerism could be estimated. The $\Delta R_M$ ($\gamma \rightarrow \alpha$) is usually in the range of 1.1 to 1.3 $R_M$ units. The $\Delta R_M$ values for the corresponding 2- and 4-isomers, determined from the $R_M$ *vs.* $H_0$ plots by linear interpolation (or extrapolation) to $H_0 = 0$, are presented in Table I.

The observed differences of $R_M$ values of the corresponding 4- and 2-isomers are presumably due to an *ortho* effect involving the shielding of the aromatic nitrogen by the vicinal R–CO–group. The inductive effects in the 2- and 4-positions are probably similar[10]; however, some difference in the p$K_A$ values of 2- and 4-isomers can also contribute to the value of $\Delta R_M$ (4→2).

*Extraction power of the solvents*

For both homologous series, the solvents can be arranged in the following sequence of decreasing solvent power:

TABLE I

$\Delta R_M$ VALUES OF ISOMERIC 2- AND 4-PYRIDYL ALKYL KETONES
The numbers in parentheses are less accurate.

| *Fig. No.* | *Solvent (mobile phase)* | *Homologue* | | | | |
|---|---|---|---|---|---|---|
| | | *Me* | *Et* | *Pr* | *Bu* | *Am* |
| 1 | Cyclohexane | | | | | |
| 2 | Benzene | | | 1.30 | 1.30 | 1.30 |
| 3 | Toluene | | (1.34) | 1.38 | 1.38 | 1.34 |
| 4 | Carbon tetrachloride | | (1.30) | 1.30 | 1.30 | 1.30 |
| 5 | Chloroform | 1.20 | 1.20 | 1.12 | 1.06 | (0.94) |
| 6 | 1,2-Dichloroethane | 1.24 | 1.24 | 1.24 | 1.12 | 0.98 |
| 7 | Tetrachloroethane | 1.12 | 1.10 | 1.10 | 0.90 | 0.82 |
| 8 | Anisole | | 1.10 | 1.14 | 1.04 | 1.04 |
| 9 | Di-*n*-butyl ether | | | 1.30 | 1.40 | 1.40 |
| 10 | Diethyl ether | | (1.10) | 1.30 | 1.38 | 1.40 |

chloroform, tetrachloroethane > 1,2-dichloroethane > anisole > benzene, toluene > ethyl ether, carbon tetrachloride > di-*n*-butyl ether > cyclohexane.

The sequence is in agreement with the chromatographic spectra obtained for the partition of quinoline bases[11]. The extraction power of the solvents is largely determined by the electron-donor properties of the solutes which are thus better extracted by solvents of class A and weakly extracted by solvents of classes N and B, even more polar ones.

When *n*-hexanol (class AB) was used as the mobile phase, the $R_F$ coefficients were found to be constant at lower $H_0$ values, which indicated extraction of ion pairs (*cf.* ref. 4).

## CONCLUSIONS

The experiments demonstrate that in using solutions of hydrochloric acid as the stationary phase and organic solvents of various polarities as the developing phase suitable ranges of $R_M$ values of the two homologous series investigated were obtainable.

In the range of moderate acidity of the aqueous phase ($-1 < H_0 < +1$), accurate $R_M$ values of the first five 4-pyridyl alkyl ketones could be determined for stronger solvents (class A, *e.g.*, chloroform) while for 2-pyridyl alkyl ketones the range of solvents could be extended for weaker proton-donor solvents (*e.g.*, ethylene chloride) electron-donor and neutral solvents (classes B and N, respectively).

Constancy of $\Delta R_M$ ($CH_2$) values qualifies the compounds and especially 2-pyridyl alkyl ketones, as reference series for the determination of parameters analogous to KOVATS' indices[2] in chromatography of weak lipophilic bases in liquid–liquid partition systems with moderately acidic aqueous phase ($-1 < H_0 < +1$).

The selectivity to molecular size of the system investigated was found to be much higher than in the case of usual systems of lower alcohol–hydrochloric acid–water; thus, $\Delta R_M$ ($CH_2$) was *ca.* 0.5 units while NEUHÄUSER AND WOLF[1] report $\Delta R_M$ ($CH_2$) values in the range of $-0.1$ to $-0.3$.

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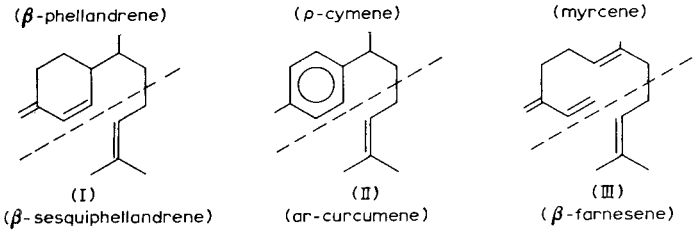
## Notes

CHROM. 4358

### A guide for the prediction of the gas chromatographic retention times of selected sesquiterpenes

During recent studies on essential oils[1] it has been found that the most readily available characteristic of a sesquiterpene is its gas chromatographic retention time since this can usually be measured from a crude mixture. In some cases a considerable amount of useful information can be derived from this property alone by correlation with the retention times of known monoterpenes.

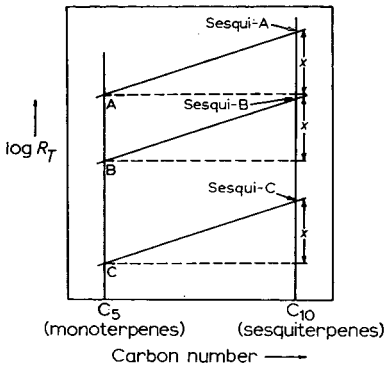
Examination of the structures of sesquiterpenes based on the bisabolane and farnesane skeletons reveals that each can be regarded as a monoterpene extended by an isoprene unit attached to the isopropyl or isopropylidene grouping, *e.g.* I, II and III.




This addition of a uniform increment to each of the monoterpenes would be expected to increase the gas chromatographic retention times in a consistent way. Thus ideally the retention times of the sesquiterpenes can be regarded as being made up of two contributions: (1) a variable contribution arising from the monoterpene unit and (2) a consistent contribution from the additional isoprene unit. In consequence it may be expected that the customary semi-log plot of retention times *versus* carbon number[2] if applied to terpene–sesquiterpene pairs will yield approximately parallel lines (see Fig. 1).

The data for a number of known mono- and sesqui-terpenes was plotted in this fashion giving lines which were found to be approximately parallel. Therefore if a plot is made for one corresponding pair the retention times for other sesquiterpenes can be derived. Lines constructed parallel to the known reference line and passing through the retention times of the known monoterpenes will intersect the sesquiterpene line at approximately the retention times of the corresponding sesquiterpenes (see Fig. 2). Most satisfactory results are obtained by using a reference pair as closely related as possible to the sesquiterpene, the retention time of which is to be predicted.

A comparison between predicted and observed retention times is shown in Table I. The close correlation obtained indicates that for these selected sesquiterpenes this relationship can be used to obtain approximate retention times from those of corresponding monoterpenes. In addition it suggests that a similar relationship would probably exist between analogous structurally related monoterpene–sesquiterpene



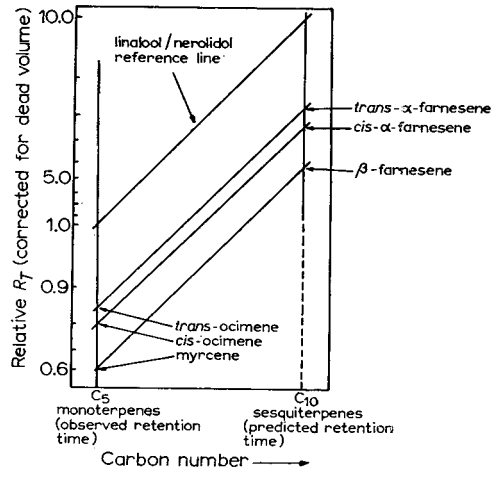




Fig. 1. Hypothetical plots of the retention times of mono- and sesquiterpenes. The retention times of A, B and C represent the variable contributions while $x$ represents the consistent contribution from the additional isoprene unit.

Fig. 2. Plot used to predict the retention times of acyclic sesquiterpenes.

groups, *e.g.* camphene–$\beta$-santalene and $\alpha$-pinene–$\alpha$-bergamotene. However, it should be noted that information obtained in this way cannot be regarded as conclusive but as a useful guide for isolation procedures or for further work aimed at a positive identification or structural characterisation.

## *Experimental*

*Gas chromatography.* An Aerograph Model A-600-B instrument incorporating a

TABLE I

OBSERVED AND PREDICTED SESQUITERPENE RETENTION TIMES

Corrected for dead volume.

| *Monoterpene used for primary retention time* | *Sesquiterpene* | *Retention times* | |
|---|---|---|---|
| | | *Predicted* | *Observed* |
| *Monocyclic group*[a] | | | |
| $\beta$-Phellandrene | $\beta$-sesquiphellandrene | 10.4 | 10.4 |
| Limonene | $\beta$-bisabolene | 9.2 | 9.5 |
| Terpinolene | $\gamma$-bisabolene | 12.4 | — |
| Terpineol-4 | anymol | 23.4 | 22.0 |
| *Acyclic group*[b] | | | |
| Myrcene | $\beta$-farnesene | 6.0 | 6.0 |
| *cis*-Ocimene | *cis*-$\alpha$-farnesene | 7.8 | 7.8 |
| *trans*-Ocimene | *trans*-$\alpha$-farnesene | 8.6 | 8.4 |

[a] Calculated from a zingiberene–$\alpha$-phellandrene reference line; retention times relative to $\alpha$-phellandrene = 1.

[b] Calculated from a linalool–nerolidol reference line, see Fig. 1; retention times relative to linalool = 1.

flame ionisation detector and fitted with stainless steel columns (6 ft. $\times \frac{1}{8}$ in.) was used. The column packing was Gas-Chrom 80–100 mesh size containing 15% by weight of Apiezon M. A column temperature of 170° was maintained while nitrogen was used as carrier gas.

*Terpenoid samples.* $\beta$-Sesquiphellandrene and $\beta$-bisabolene were contained in fractions from the chromatography on silver nitrate treated alumina (234 g containing 34% silver nitrate) of distillation fractions (b.p. 89–90.5° at 1.0 mm) from the essential oil of ginger. Benzene in hexane (1:5) was used as eluant. Purity was demonstrated by the usual spectroscopic and chromatographic procedures and by satisfactory combustion analyses.

Nerolidol (75 ml) in pyridine (375 ml) was added to phosphorus oxychloride (150 ml) in pyridine (375 ml) and allowed to stand for 48 h then heated at 100° for 1 h. Subsequent working up yielded a yellow oil containing two major products. These were separated using an Auto-Prep Model 705 preparative gas chromatograph fitted with a 20 ft. $\times \frac{3}{8}$ in. column packed with 20% Apiezon M on Gas-Chrom. Satisfactory combustion analyses combined with close ultraviolet, infrared and proton magnetic resonance spectral correlations with results reported in the literature[3–5] revealed these compounds to be *trans*-$\alpha$-farnesene and $\beta$-farnesene. Another compound present was tentatively identified as *cis*-$\alpha$-farnesene.

The author wishes to thank Dr. M. D. SUTHERLAND of the University of Queensland for many helpful suggestions and kindly supplying samples of the various monoterpenes and anymol.

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CHROM. 4352

## The preparation of 2-methyl-4-chlorophenoxyacetic acid and 2,3,6-trichlorobenzoic acid methyl esters by pyrolysis of their tetramethylammonium salts

The preparation of methyl esters by heating the tetramethyl ammonium salts of carboxylic acids was reported by PRELOG AND PICENTANIDA in 1936[1]. In this reaction heat causes trimethylamine to be driven off leaving the methyl ester. The reaction was applied to the direct production of a number of aryl and alkyl acid esters in the injection port of a gas chromatograph by ROBB AND WESTBROOK[2]. It was found that while a quantitative yield of ester could be determined, the temperature of the injection port was critical. Even so the response of the acids to treatment was somewhat variable and quantitative conversion was not attained in many cases. DOWNING[3] modified the ROBB AND WESTBROOK procedure so that it could be applied to most simple organic acids. The essential feature of his modification was the removal of water before pyrolysis, which had the advantage that the efficiency of the conversion to methyl esters was unaffected by sample size or concentration. The yields were also largely insensitive to reagent concentration or to variation, within wide limits, of injection port temperature.

BAILEY[4], again working with non-aqueous conditions, showed that the method could be applied to the determination of mono- and dibasic aromatic acids when present as major components in mixtures containing non-acidic components, and to similar mixtures containing mono- and dibasic aliphatic acids and esters.

This paper describes an attempt to apply the findings of these workers to the determination of the phenoxy aliphatic and substituted benzoic acids normally used in herbicidal formulations.

*Experimental*

The apparatus used throughout this work was a Perkin Elmer Model F.11 gas chromatograph fitted with the analytical module appropriate to the column selected. In all cases the flame ionisation detector was used to monitor column effluents. The columns used were either a 2-m glass ($\frac{1}{4}$ in. O.D.) or a 2-m stainless steel ($\frac{1}{8}$ in. O.D.). Both columns were packed with 10% Silicone Oil SE-30 on 80–100 mesh Chromosorb W. The temperature of the oven was maintained at 150° and the nitrogen carrier gas inlet pressure was kept constant at 1.0 kg/cm$^2$ throughout these experiments. The temperature of the injection block and oven were measured using the Perkin Elmer temperature readout attachment.

For the purpose of these experiments two acids were used, *viz.* pure 2-methyl-4-chlorophenoxyacetic acid (MCPA) and pure 2,3,6-trichlorobenzoic acid (TBA).

*Use of the glass column.* A glass column was prepared as outlined above and the inlet arm was packed with quartz wool to increase the area available for heat transfer. The injection block was maintained at a temperature of 380°. The sample was prepared by dissolving a known weight of MCPA in methanol and adding a calculated 50% excess of tetramethylammonium hydroxide in methanol solution. Aliquots of this solution were injected onto the quartz wool at a point just above the hottest zone of the injection block. A typical chromatogram is reproduced in Fig. 1.

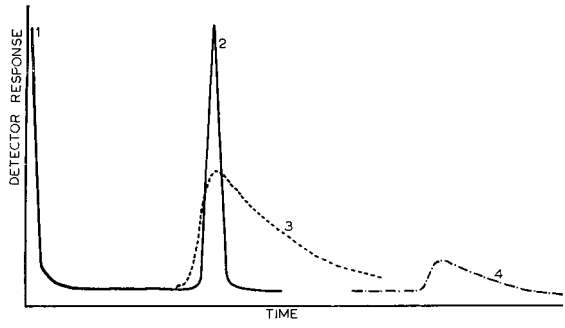


Fig. 1. Typical chromatograms obtained from pure MCPA (4), MCPA methyl ester (2) and MCPA tetramethylammonium salt (3). 1 = solvent.

Also shown in Fig. 1 are typical peaks obtained from prepared methyl ester of MCPA and MCPA free acid. This work was not continued because it was felt that the tailing of the peak was due to the small length of the heating zone being insufficient for the pyrolysis reaction to go to completion.

A similar experiment was carried out using a solution of TBA in methanol containing a 50% excess of tetramethylammonium hydroxide, but no conversion to the methyl ester was achieved.

*Use of the stainless steel column.* Methanolic solutions of MCPA in excess tetramethylammonium hydroxide were gas chromatographed at a number of different injection block temperatures. The analytical module for the stainless steel columns has a much longer injection block heating zone and consequently the sample has more time to undergo pyrolysis before being swept into the column. It was found that the temperature of the pyrolysis was critical. Too high a temperature resulted in a considerable quantity of 4-chlorocresol being produced by pyrolytic decomposition of the MCPA, whereas too low a temperature resulted in a poor yield of MCPA methyl ester. The rate of production of MCPA methyl ester and 4-chlorocresol at different injection block temperatures is shown in Table I. This table shows that a maximum production of methyl ester occurs at about 240°.

TABLE I

MCPA METHYL ESTER PRODUCED BY PYROLYSIS OF THE TETRAMETHYL AMMONIUM SALT AT VARIOUS INJECTION BLOCK TEMPERATURES

| *Injection block temperature (°C)* | *Methyl ester produced (%)* | *4-Chlorocresol (%)* |
|---|---|---|
| 150 | 72 | — |
| 210 | 86 | — |
| 240 | 96 | — |
| 270 | 94 | Trace |
| 305 | 85 | 1 |
| 335 | 81 | 2 |

The percentage ester produced was determined by comparing the peak area obtained from a known weight of MCPA in tetramethylammonium hydroxide with that obtained from the same weight of pure MCPA converted to the methyl ester by treatment with diazomethane and chromatographed at an injection block temperature of 175°. The amount of 4-chlorocresol was estimated as a percentage of the methyl ester peak assuming the same response factor. The production of 4-chlorocresol at relatively low injection block temperatures was not anticipated and in order to see if this was due to decomposition of the free acid or the methyl ester, a separate experiment was carried out.

A known weight of MCPA was converted to the methyl ester by treatment with diazomethane and chromatographed at a series of different injection block temperatures. The results again showed formation of 4-chlorocresol at temperatures above 270°. It is evident from this that careful control of injection block temperature is essential even during routine gas chromatographic analyses of MCPA methyl esters if decomposition is to be avoided.

Similar experiments were carried out with TBA but again the conversion to the methyl ester was minimal. The reason for this is almost certainly steric hindrance since it is well known that di-*ortho* substituted benzoic acids are resistant to all but the strongest methylating agents.

*Conclusion*

In the experiments described above it is evident that the temperature of pyrolysis is highly critical. With the simple control of injection block temperature available on the gas chromatograph used, the fine temperature control necessary for reproducible conversions is not likely to be achieved.

The method appears to work qualitatively in the case of MCPA but not for TBA, probably on the grounds of steric hindrance. This method of preparing methyl esters does not, however, lend itself to the quantitative determination of phenoxyacetic acids on a routine basis.

In addition, it has been demonstrated that the methyl ester of MCPA is decomposed at injection block temperatures above 270°. The effects of high injection block temperatures in the decomposition of the methyl esters of other herbicidally active acids is at present under investigation.

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## Pressure-elution pump for spacecraft chromatograph

The *in situ* analysis of extraterrestrial soil samples by means of soft-landed spacecrafts requires automatic chemical equipment which consumes minimum weight, volume, and electrical power.

During a feasibility study of a miniaturized amino acid analyzer[1] employing ion-exchange chromatography, the high-pressure metering pumps normally used in commercial instruments seemed prohibitive because of their weight and electrical power requirements.

An alternate way of forcing the eluent solution through the column is by means of pressure elution. The term 'pressure elution' implies that a gas under constant pressure is used to push the eluent liquid through the column.

The simple application of gas pressure to the eluent leads to several problems. For example, if one subjects the eluent solution to direct gas pressure any gas dissolved at the high-pressure column input will bubble out at the low pressure in the column exit causing the column to develop air pockets. Thus HARE[2], using nitrogen gas in direct contact with the eluent buffers, had to load the buffers into long coils to prevent bubble formation at the low-pressure column exit. In this way, presumably, the time required for the gas to diffuse through the buffer reservoir was too long to interfere. Another problem is that bubble formation would be troublesome with any flow-through detector cell.

The gas diffusion can be prevented by using a movable barrier of some sort to separate the driving gas and eluent liquid. JENTOFT AND GOUW[3] used a mercury barrier. A commercial pressure-elution pump (Waters Associates, Framingham, Mass.) using a polyethylene barrier is also available.

The approach in this paper is to use a movable metal barrier in the form of a metal bellows to separate the gas from the eluent solution.

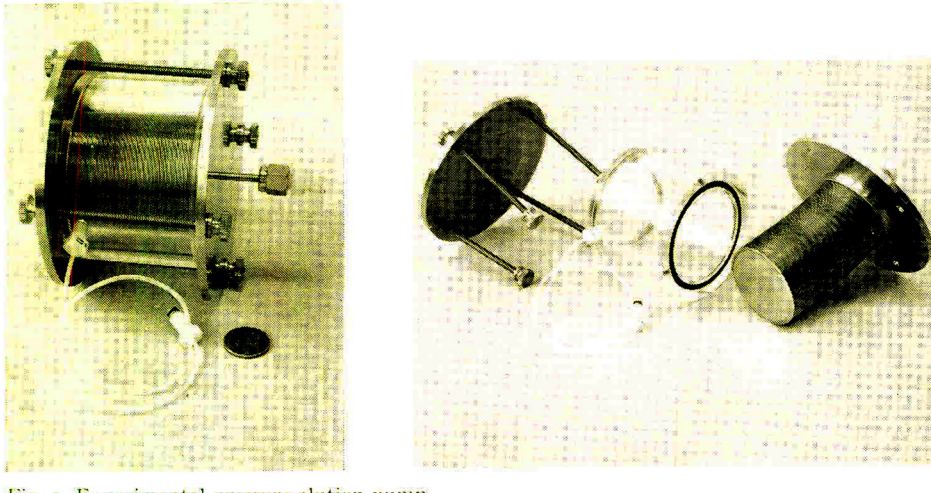

Fig. 1. Experimental pressure-elution pump.

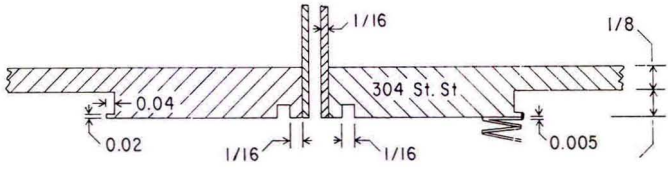


Fig. 2. Cross-section of top plate and gas inlet showing design necessary for arc welding. Dimensions are in inches.

*Apparatus*

*Pump description.* Photographs of the assembled and disassembled pump are shown in Fig. 1. It consists of a metal bellows operating inside a Plexiglass (Rohm and Haas acrylic plastic) cylinder. The cylinder is closed off at the ends by stainless steel end plates. The flange of the open end of the bellows is arc welded onto one of the end plates, the top plate, as shown in Fig. 2. A Teflon O-ring at each end provides a seal between the Plexiglass cylinder and the end plate. The O-rings are located in grooves in the cylinder as recommended by the manufacturer (Parker Seal Company, Culver City, Calif.).

In this experimental pump, the cylinder (length $2\frac{1}{2}$ in., I.D. 2 in., wall 0.5 in.) was made of transparent Plexiglass to allow inspection for gas bubbles and bellows position during loading and elution.

The best commercial bellows for this pump was a so-called welded bellows (Model No. 61050, Metal Bellows Corporation, Chatsworth, Calif.). The bellows was so elastic that the pressure absorbed during one full stroke (1.25 in.) within the cylinder was only 1.8 p.s.i. Hence, at say 180 p.s.i., the flow rates at initial contraction and final complete expansion differ by only 1%.

*Pump loading.* The pump is loaded with eluent by applying partial vacuum to the gas port in the top plate. This causes the bellows to contract and produce suction in the liquid port in the Plexiglass cylinder.

The initial air in the liquid chamber is easily removed by a few filling cycles before connecting the pump to the column. After loading with eluent, the liquid port is connected to the center port of a 3-way valve, the loading valve, from which one exit leads to the column, and the other exit to the bottom of an eluent reservoir. In this manner, air is prevented from entering the liquid chamber during successive loadings. A nitrogen gas tank is connected to the gas port through a pressure-reducing valve and gas pressure increased until the desired liquid flow rate is obtained at the column exit.

*Test solutions.* The pump was tested by chromatographing a mixture of the principal bases from DNA and RNA.

Two test buffers were used: 1.6 *M* citrate adjusted to pH 6.5, and 0.05 *M* citrate adjusted to pH 5.58 with HCl.

*Column.* The microcolumn consisted of a $0.191 \times 112$ cm resin bed of Aminex A-4 spherical cation-exchange resin (Bio-Rad Laboratories, Richmond, Calif.). The resin bed was contained in nylon pressure tubing (1000 p.s.i. rating; 1/8 in. O.D.; 0.075 in. I.D.). The nylon column was formed into a loop (4 in. in diameter) and kept in a water bath at 55° by means of a Haake circulating pump.

*Sampling valve.* A sample injection valve was connected between the 3-way

loading valve previously mentioned and the column. This valve consisted of two 4-way valves connected in the standard manner recommended by the manufacturer for manual operation (Model CAV-4031 4-way Cheminert Valve, Chromatronix Inc., Berkeley, Calif.). The sample loop had a capacity of 150 $\mu$l.

*Detection system.* A UV monitor (Model UA-2, ISCO Inc., Lincoln, Nebr.), normally used for fraction collection, served as the detector. In this instrument a 100-$\mu$l flow-through cell with a 2-mm light path is illuminated with light at 254 m$\mu$ from a mercury lamp.

*Results and discussion*

The chromatograms (Fig. 3) show the results obtained when the pump is used

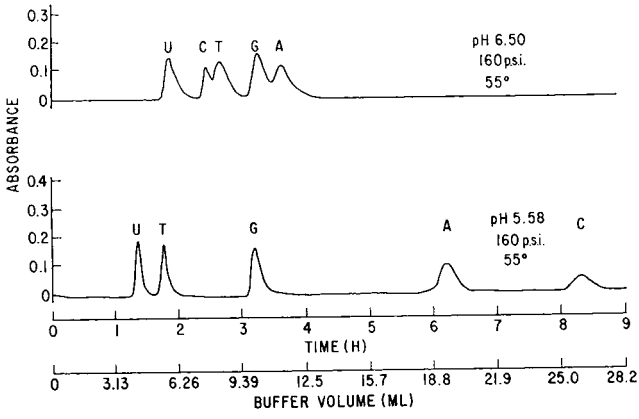


Fig. 3. Pressure-elution chromatograms. U, 43 nanomoles of uracil; T, 58 nanomoles of thymine; G, 54 nanomoles of guanine; A, 32 nanomoles of adenine; C, 41 nanomoles of cytosine; $\lambda$ = 254 m$\mu$.

on the microcolumn. The 'column gain', due to the small diameter of the column, is apparent since the compounds are detected at the nanomole level despite the rather simple detection system.

The pump was developed primarily for a spacecraft chromatograph employing single-buffer elution. For this purpose, the pump has the following advantages:

(1) It requires no electrical power. The driving energy is stored in the form of compressed gas.

(2) It is efficient. The slow isothermal expansion of the gas—an almost reversible process in the thermodynamic sense—results in nearly the maximum available PV-work.

(3) It is an 'integrated' device in the sense that the liquid reservoir is indistinguishable from the pump. Hence a separate liquid reservoir has been eliminated.

(4) The pump is compatible with microcolumns (I.D. $<$ 1 mm) which entail small volumes of eluent per run and low flow rates ($<$ 1 ml/h). The flow rate can be made as slow as one wishes. The upper pressure is limited only by the strength of the materials since no leakage is possible through the metal barrier. This particular pump was tested up to 200 p.s.i.

(5) The pump is non-pulsating.

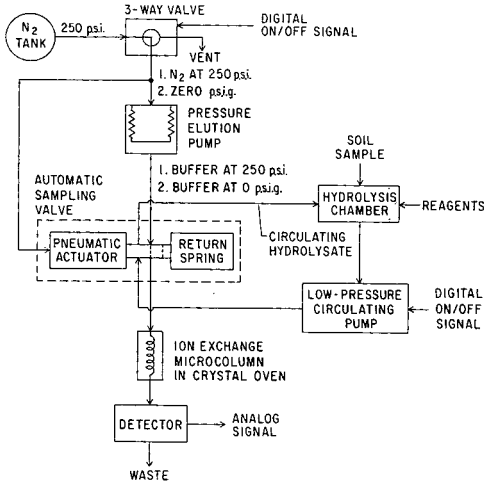


Fig. 4. Gross description of spacecraft chromatograph.

If pH-programming of the eluent is desired, several pumps could conceivably be operated in parallel. If several such pumps are operated in parallel at the same pressure, the pump has the disadvantage that the flow rates will differ if the column resistance is pH-dependent.

A possible system description for the analysis of soil hydrolysates is shown in Fig. 4.

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## Improvement of the chromatographic properties of Sephadex G-15

Tightly cross-linked dextran gels are useful for the separation of substances of low molecular weight. However, the general utility of this chromatographic medium is somewhat limited by the poor resolution which it affords. A substantial improvement in chromatographic resolution may be achieved by boiling the gel in hydrochloric acid.

*Experimental*

One hundred grams of Sephadex G-15 xerogel (Pharmacia Fine Chemicals, Piscataway, N.J.) were expanded to 300 $cm^3$ in distilled water by allowing it to stand at room temperature for 24 h. One hundred and fifty cubic centimeters of the gel were packed by sedimentation in a $1.5 \times 85$ cm column at room temperature. The eluent, 0.15 *M* saline–0.01 *M* acetic acid, was passed through the column at 6 ml/h for 48 h before use. One hundred and fifty milliliters of 1 *N* HCl were added to an equal volume of the remaining expanded xerogel and placed in a boiling water bath for 2 h with intermittent stirring. After acid boiling, the gel was allowed to settle and the supernatent decanted. Repeated washes with distilled water were performed until the supernatent acidity was reduced to less than 0.01 *M*. This material was packed in an identical column in the same manner as the former procedure and washed with eluent until the refractive index of the effluent returned to that of the eluent. A test sample containing the materials listed in Table I was layered on the gel surface of each column and chromatographed at 6 ml/h.

TABLE I

COMPOSITION OF THE TEST SAMPLE CHROMATOGRAPHED IN FIG. 1

| *Compound* | *Quantity* | $M_w$ | *Source* |
|---|---|---|---|
| Blue Dextran 2000 | 1 mg | $10^6$ | Pharmacia Fine Chemicals |
| Stachyose tetrahydrate | 50 mg | 666 | Sigma Chemical Co. |
| Maltose monohydrate | 50 mg | 342 | Eastman Kodak Co. |
| Glucose | 50 mg | 180 | Baker Chemical Co. |
| Ethylene glycol | 100 µl | 62 | Fisher Chemical Co. |
| Deuterium oxide | 900 µl | 20 | Volk Radiochemical Co. |
| Sodium chloride | 9 mg | — | Baker Chemical Co. |

The elution position of the test molecules was indicated by the maximum deflection of a refractive index monitor (Ec211, E-C Apparatus Co., Philadelphia, Pa.) operating at a sensitivity of 0.00068 refractive index units per cm of pen deflection. The void volume ($V_0$) was measured as the peak elution of blue dextran and the internal volume ($V_i$) was estimated by the elution of deuterium oxide ($V_i = V_e(D_2O) - V_0$). The diffusion coefficients ($K_d$) were computed by the relationship[1]:

$$K_d = \frac{V_e - V_0}{V_i} = \frac{V_e - V_e(\text{blue dextran})}{V_e(D_2O) - V_e(\text{blue dextran})} \quad (1)$$

The number of theoretical plates ($N$) was computed from the standard deviation ($\sigma$) of the stachyose elution peak by the relationship[2]:

$$N = \left(\frac{V_e}{\sigma}\right)^2 \qquad (2)$$

The height equivalent to a theoretical plate (HETP) is obtained by dividing $N$ by the column height.

*Results and discussion*

Tightly cross-linked Sephadex gels exhibit a remarkable resistance to acid hydrolysis. Unlike the more loosely cross-linked gels, Sephadex G-15 and G-10 withstand boiling in 1 $N$ HCl for up to 10 h without noticeable deterioration; complete hydrolysis could be achieved by boiling in 6 $N$ HCl for a comparable length of time. The resistance of these gels to acid hydrolysis suggested that gentle hydrolysis might improve the chromatographic characteristics by the removal of hydrolyzable contaminants. The existence of non-dextran contaminants in Sephadex may be inferred from its anomolous adsorption of aromatic[3] and alphatic substances[4], the presence of fixed negative charges[3] and the release of UV-absorbing material[5].

Acid hydrolysis resulted in three major effects: an increased number of theoretical plates, an increased internal volume and an increased effective pore size. From a practical standpoint, the most important modification is a 127% increase in the total number of theoretical plates ($N$, Table II). By doubling the number of theoretical plates, the resolution becomes equivalent to that of a column twice its length without the dilution and prolonged elution time which occur as the column length is increased. A factor contributing to improved resolution is a 17% increase in internal volume ($V_i$, Table II). However, this factor alone does not explain the major improvement seen above. The increase in effective pore size is seen in Fig. 1 as a displacement of the relative elution positions of the test molecules. Fig. 2 indicates that this increase in

TABLE II

THE ELUTION VOLUMES ($V_e$) AND DIFFUSION COEFFICIENTS ($K_d$) OF TEST MOLECULES CHROMATOGRAPHED UNDER TWO DIFFERENT CONDITIONS

Columns: Sephadex G-15, 1.5 × 85 cm. (A) $V_o$ = 49.6 ml; $V_i$ = 61.7 ml; $N$ (stachyose)[a] = 1100 plates; HETP = 0.77 mm/plate. Expanded in distilled water for 24 h. (B) $V_o$ = 51.8 ml; $V_i$ = 72.2 ml; $N$ (stachyose) = 2500 plates; HETP = 0.34 mm/plate. Expanded as column A and then boiled in hydrochloric acid for 2 h.

| | *Column A* | | *Column B* | |
|---|---|---|---|---|
| | $V_e$ | $K_d$ | $V_e$ | $K_d$ |
| Blue dextran | 49.6 | 0.0 | 51.8 | 0.0 |
| Stachyose | 68.5 | 0.31 | 81.2 | 0.41 |
| Maltose | 81.5 | 0.52 | 95.5 | 0.60 |
| Glucose | 88.0 | 0.62 | 102.3 | 0.70 |
| Ethylene glycol | 96.0 | 0.75 | 109.8 | 0.80 |
| Deuterium oxide | 101.3 | 1.0 | 124.0 | 1.0 |

[a] Number of theoretical plates as determined for stachyose.

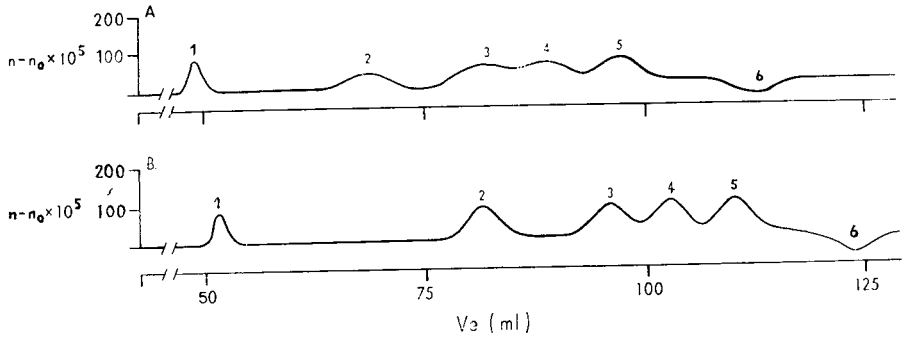


Fig. 1. The elution volume ($V_e$) of blue dextran (1), stachyose (2), maltose (3), glucose (4), ethylene glycol (5) and deuterium oxide (6) from 1.5 × 85 cm columns of Sephadex G-15. (A) Expanded in distilled water at room temperature for 24 h. The better resolution seen in (B) is obtained by boiling in hydrochloric acid for 2 h before packing. The change in refractive index of the column effluent ($n - n_0$) is used to indicate the elution position of the test molecules.

effective pore size is not associated with a change in the basic geometry but represents an overall enlargement. Thus, the effect of partial acid hydrolysis of Sephadex G-15 appears to be a significant increase in gel swelling as indicated by the larger effective pores and increased internal volume along with an unexplained increase in resolution which may result from decreased gel interaction or improved flow characteristics.

Two methods for gel swelling are suggested by the manufacturer[6]. For tightly cross-linked gels either 3 h at room temperature or 1 h in a boiling water bath is recommended. In our experience, either method produces results comparable to those

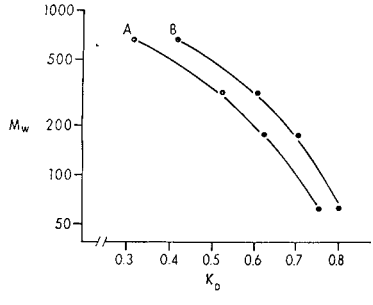


Fig. 2. The change in diffusion coefficients ($K_d$) for molecules of a given molecular weight ($M_w$) resulting from partial acid hydrolysis of Sephadex G-15 (B) compared with that obtained by conventional swelling procedures (A) indicating an enlargement of the effective pore size.

seen in Fig. 1A. The increased internal volume seen after boiling in hydrochloric acid suggests that gel swelling is significantly greater by this procedure than that seen with the recommended procedures. It has been noted by several authors[3,4,7,8] that the theoretical relationship $V_i = aW_r$, which is used to estimate the internal volume from the weight of xerogel ($a$) and the solvent regain ($W_r$) is in error by approximately 20%.

By this computation, a column containing 50 g G-15 xerogel should have an internal volume of 75 ml. This is very close to the internal volume measured by deu-

terium oxide (72.2 ml) on the acid-treated column and 22% greater than that of the untreated column (61.7 ml) suggesting that the theoretically available diffusion space is not achieved by normal swelling techniques due to internal hindrance which is largely eliminated by acid hydrolysis.

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## Neue Erkenntnisse bei der Verwendung von Ninhydrin–Hydrindantin als Farbreagenz zur Aminosäure-Autoanalyse

Die Nachweisempfindlichkeit einer Aminosäure im Eluat automatisch betriebener Ionenaustauschergeräten (Analyzer) konnte laufend erhöht werden und liegt heute bei $10^{-11}$ Mol. Derartige Empfindlichkeitssteigerungen waren möglich:

(1) Durch Variation des Säulendurchmessers.

(2) Durch Verwendung besonders einheitlicher Ionenaustauscherharze.

(3) Durch Bereitstellung möglichst einer einzigen, sehr kleinen, kugelförmigen Austauscherfraktion, wobei die Teilchendurchmesser um nicht mehr als 1 $\mu$ voneinander abweichen sollten.

(4) Durch Verstärkung der elektrischen Signale, am Colorimeterausgang.

(5) Durch empfindlichere und weniger oxydationsanfällige Anfärbereagentien.

Die Parameter 1–4 sind heute soweit optimiert, dass hier die Grenzen des Möglichen so gut wie erreicht worden sind. Anders beim Nachweisreagenz. Diesem Problem werden wohl in jedem analytischen Laboratorium, das mit Aminosäureautoanalyzern arbeitet, die meisten Untersuchungen gewidmet.

Im allgemeinen werden eluierte Aminosäuren mit einem Ninhydrinreagenz angefärbt. So auch bei allen bekannten, automatischen Aminosäureanalyzern. Wir selbst

arbeiten mit einem "Technicon-Mehrkanal-Aminosäure-Autoanalyzer". Das für dieses System verwendete Ninhydrinreagenz ist etwa wie folgt zusammengesetzt: 20.0 g Ninhydrin, 1.6 g Hydrindantin, 650 ml Äthylenglycolmonomethyläther (peroxydfrei) und 350 ml Natriumacetatpuffer pH 5.5 (ammoniakfrei), verdünnt mit einer Lösung aus 1500 ml Äthylenglycolmonomethyläther (peroxydfrei) und 1500 ml Wasser (ammoniakfrei).

Der grösste Nachteil beim quantitativen Arbeiten mit diesem Reagenz liegt in der enormen Instabilität des Ninhydrins und Hydrindantins gegenüber Sauerstoff und Licht, ein weiterer Nachteil am verwendeten "Methylcellosolve". Dieses Lösungsmittel ist verhältnismässig teuer (in peroxydfreier Form 42,— DM pro Liter). Es muss deshalb laufend im Laboratorium aus p.A. Ware in hochgereinigter Form hergestellt werden. Dazu ist es erforderlich, das Lösungsmittel mit Eisensulfat und Phosphorsäure peroxydfrei zu machen. Nach dem Abfiltrieren von dem ausgefallenen Eisensulfatschlamm muss es dann im Vakuum unter extrem gereinigtem Stickstoff destilliert werden. Das so gereinigte Methylcellosolve ist auch im Kühlschrank aufbewahrt nur begrenzte Zeit stabil, vor allem nur begrenzte Zeit peroxydfrei zu halten. Nicht jedes Laboratorium, in dem Methylcellosolve verwendet wird, kann sich diese sehr zeit-, arbeits- und geräteaufwendige Reinigungsprozedur leisten. Und doch hat sich bis heute keine wesentlich andere Zusammensetzung des Reagenzes für die Autoanalyse gefunden.

Ein weiterer Nachteil der Verwendung von Methylcellosolve, insbesondere beim Arbeiten mit Technicon-Geräten, ist das durch das Lösungsmittel hervorgerufene Pulsieren des segmentierten Gas-Flüssigkeitsstromes hinter dem Heizbad. Dieses Phänomen ist der Herstellerfirma bekannt und es wurde verschiedentlich versucht, die Fliessunregelmässigkeit im Flüssigkeitsstrom zu eliminieren, u.a. durch Nachschaltung von Kapillarschlauch. Wie allgemein bekannt ohne allzu grossen Erfolg. Das Pulsieren lässt sich einigermassen verhindern durch probeweise Variation des Stickstoffzuführungsschlauches zum Mischkaktus nach der Proportionierpumpe.

Wir selbst haben lange mit den verschiedensten Lösungsmitteln und Puffern gearbeitet und versucht, beide Nachteile einigermassen zu eliminieren. Insbesondere aber wollten wir die Stabilität des verwendeten Reagenzes erhöhen und—wenn möglich—das Reinigen des Lösungsmittels ganz umgehen. Dass wir dabei gleichzeitig eine Reagenz-Lösungsmittelkombination fanden, die auch das Pulsieren des segmentierten Flüssigkeits-Gasstromes verhinderte, hatten wir nicht ohne weiteres erwartet.

*Zusammensetzung des Ninhydrinreagenzes*

Das Reagenz ist wie folgt zusammengesetzt: 16.0 g Ninhydrin, 1.2 g Hydrindantin, 1650 ml Äthylenglycol p.A. (Merck), 1000 ml 0.5 *M* Citratpuffer pH 5.5 und 650 ml dest. Wasser (ammoniakfrei).

*Vorbereitung des Reagenzes*

Das Ninhydrin und das Hydrindantin werden unter Rühren mit einem Magneten in dem auf *ca.* 100° erwärmten Äthylenglycol gelöst. Danach wird die Lösung mit 0.5 *M* Citratpuffer und Wasser vermischt. Durch die Verwendung von Citratpuffer erreichen wir gleichzeitig die Ausschaltung von Fremdsalzen im System. Die Zusammensetzung des Reagenzes geht konform mit der Zusammensetzung aller Elutionspufferlösungen.

Das so hergestellte Ninhydrinreagenz ist frei von Peroxyden und bleibt auch bei längerer Lagerung frei davon. Eine Aufbewahrung bei Kühlschranktemperatur ist nicht erforderlich. Der angegebene Ansatz von 3300 ml Reagenz ist, bei 16 Stundenchromatogrammen und der Verwendung von einem 2-Säulensystem ausreichend für eine Arbeitswoche. Die Mischung 50% v/v Äthylenglycol mit dem Puffer-Wasser-Gemisch ist absolut klar und zeigt keinerlei Opaleszenz, wie das mitunter bei der Verwendung von Methylcellosolve als Lösungsmittel auftritt. Eine Entfernung dieser äusserst unerwünschten Opaleszenz bei einem Methylcellosolve/Wassergemisch ist nicht möglich. Diese Lösung ist daher nicht zur Autoanalyse geeignet. Ein weiterer entscheidender Vorteil unseres Reagenzsystems ist in der Verwendung des Citratpuffers zu sehen. Die Herstellung des im alten Reagenz benötigten Acetatpuffers ist umständlich und verhältnismässig zeitaufwendig. Ausserdem ist Natriumacetat ein Fremdsalz im System, während Citrat eine Komponente des Gradienten und damit des gesamten analytischen Systems ist.

Der hochkonzentrierte Natriumacetatpuffer muss nach dem Kochen filtriert werden, nicht so der Citratpuffer. Die zum Filtrieren notwendigen grossen Faltenfilter enthalten zudem selbst Ammoniakverunreinigungen, in noch stärkerem Masse aber, z.B. die grossen Rundfilter oder evtl. verwendete Filterbögen (zu mindestens enthalten sie ninhydrinpositive Substanzen, die im Ammoniakbereich eluiert werden). Ausserdem kommt der Puffer beim Filtrieren zwangsläufig wieder mit atmosphärischer Luft und damit mit Sauerstoff und evtl. Ammoniak in Berührung. Bei der Herstellung des Acetatpuffers darf bei der Zugabe des wasserfreien Natriumacetates zu dem heissen Aqua dest. keine Kristallisation des Acetats auftreten, weil sonst der fertige Puffer selbst stark zur Kristallisation neigt, und diese Kristalle nur sehr schwierig wieder zu entfernen sind. Derartige Probleme treten bei der Verwendung von Citratpuffer nicht auf.

TABELLE I

ERGEBNISSE VON 20 AMINOSÄUREANALYSEN

| *Aminosäure* | *Flächenwert 0.05 µM* | *Standard abweichung* | *Variationskoeffizient* | *2 s-Wert* |
|---|---|---|---|---|
| Asparaginsäure | 11.94 | 0.53 | 4.3 | 1.06 |
| Threonin | 11.64 | 0.37 | 3.1 | 0.74 |
| Serin | 12.71 | 0.59 | 4.6 | 1.18 |
| Glutaminsäure | 11.81 | 0.50 | 4.1 | 1.00 |
| Prolin | 3.78 | 0.40 | 10.4 | 0.80 |
| Glycin | 12.66 | 0.53 | 4.1 | 1.06 |
| Alanin | 12.10 | 0.58 | 4.7 | 1.16 |
| Valin | 11.46 | 0.53 | 4.6 | 1.06 |
| Cystin | 12.36 | 0.56 | 6.1 | 1.12 |
| Methionin | 12.50 | 0.30 | 2.4 | 0.60 |
| Isoleucin | 11.87 | 0.40 | 3.3 | 0.80 |
| *n*-Leucin | 12.64 | 0.36 | 2.8 | 0.72 |
| Tyrosin | 12.38 | 0.44 | 3.5 | 0.88 |
| Phenylalanin | 12.46 | 0.44 | 3.5 | 0.88 |
| $NH_4^+$ | 7.43 | 1.02 | 13.7 | 2.04 |
| Lysin | 13.39 | 2.46 | 18.0 | 4.52 |
| Histidin | 13.07 | 0.85 | 6.4 | 1.70 |
| Arginin | 13.99 | 0.74 | 5.6 | 1.48 |

Die Segmentierung der zugeführten flüssigen Phase mit Stickstoff behalten wir bei, da schon geringe Sauerstoffanteile zur Zerstörung des Hydrindantins führen. Beim Arbeiten mit nachgereinigtem Stickstoff ist es aber nicht nötig, diesen weiter über Pyrogallol zu reinigen, wie vielfach empfohlen wurde.

Beim Arbeiten mit Methylcellosolve als Ninhydrinlösungsmittel nimmt die absolute Peakhöhe jeder einzelnen Aminosäure von Chromatogramm zu Chromatogramm ab. Nicht so beim Äthylenglycol. Die Peakhöhe bleibt über den gesamten Chargenzeitraum nahezu konstant (zu jeder vorhergehenden Analyse und Säule).

Bisher haben wir aus bekannten Gründen das Ninhydrinreagenz immer Freitags für die kommende Woche neu angesetzt. Dass sich, durch den Alterungsprozess über das Wochenende einstellende Gleichgewicht im Reagenz und die dadurch erreichte grössere Stabilität, sind noch ungeklärte Faktoren. Es liegen bei uns zu diesem Zeitpunkt noch keine eingehenderen Untersuchungen darüber vor, ob sich das Reagenz bei Verwendung von Äthylenglycol in diesem Punkt anders verhält als bei Verwendung von Methylcellosolve. So viel lässt sich aber sagen, dass die Verwendung, auch von frisch hergestelltem Äthylenglycol-Ninhydrin-Reagenz keine steigende oder fallende Basislinie ergibt.

Bei Äthylenglycol als Lösungsmittel ist die Eigenfärbung des Ninhydrins im 440 nm-Bereich etwas stärker als bei Methylcellosolve. Arbeitet man im gleichen Colorimeterempfindlichkeitsbereich wie bei Verwendung von Methylcellosolve, so tritt eine Verschiebung der Basislinie um 0.05 bis 0.07 Extinktionseinheiten auf. Das macht sich aber nicht störend auf die Analyse bemerkbar.

Die Löslichkeit des Hydrindantins in den gängigen Lösungsmitteln, insbesondere wenn sie Wasser enthalten, ist ausserordentlich gering. Das kann sehr leicht zum Auskristallisieren dieses Salzes, insbesondere in den Schlauchleitungen führen, und diese nach einer gewissen Zeit blockieren. Um festzustellen, wie lange das Hydrindantin bei unserer Reagenzzusammensetzung in Lösung bleibt, haben wir das Reagenz unter ungünstigen Bedingungen eine Woche bei $-4°$ und Atmosphärenluft aufbewahrt. Während dieser Zeit traten keinerlei Kristallisationskeime auf. Danach wurde dieses Reagenz zur Analyse verwendet. Die Nachweisempfindlichkeit hatte sich gegenüber dem normalerweise hergestellten Reagenz nicht verändert.

Mit diesem Äthylenglycol–Ninhydrinreagenz wurde, unter Verwendung von Technicon-Aminosäure-Standard-Lösungen die "Präzision" oder "Reproduzierbar-

TABELLE II

NOR-LEUCIN FARBAEQUIVALENTE

In Klammern die Werte für Methylcellosolve.

| *Aminosäure* | *Farbaequivalent* | *Aminosäure* | *Farbaequivalent* |
|---|---|---|---|
| Asparaginsäure | 0.965 (1.03) | Methionin | 1.011 (1.02) |
| Threonin | 0.941 (1.04) | Isoleucin | 0.960 (0.98) |
| Serin | 1.027 (0.99) | *n*-Leucin | 1.022 (1.01) |
| Glutaminsäure | 0.955 (1.01) | Tyrosin | 1.000 (1.04) |
| Prolin | 3.056 (4.66) (440 nm) | Phenylalanin | 1.007 (1.05) |
| Glycin | 1.023 (0.97) | $NH_4^+$ | (1.09) |
| Alanin | 0.978 (0.97) | Lysin | 1.082 (0.89) |
| Valin | 0.926 (1.00) | Histidin | 1.056 (1.03) |
| Cystin | 0.999 (1.98) | Arginin | 1.066 (1.07) |

keit'' unseres Verfahrens über mehrere Wochen überprüft. In der Tabelle I und II sind die Ergebnisse von 20 Aminosäurenalysen aufgeführt. Je fünf Analysen wurden unter Verwendung eines neu angesetzten Ninhydrinreagenzes durchgeführt, so dass in diesen Werten vier Chargen Ninhydrinreagenz enthalten sind.

Bei den von uns durchzuführenden Analysen an biologischen Materialien finden sich auch seltenere Aminosäuren, z.B. bei den Basen: 1-Methyl-histidin und 3-Methyl-histidin, bei den Säuren nach Oxydation: Cysteinsäure, und weiter z.B.: $\beta$-Alanin und $\gamma$-Amino-buttersäure. Bei Vorhandensein dieser Aminosäuren treten bei der Trennung mit dem üblichen Gradientensystem Schwierigkeiten auf. Wir haben deshalb den Gradienten wie folgt geändert und erreichen damit eine verhältnismässig gute Trennung, besonders auch im Basenbereich (Tabelle III). Als sehr vorteilhaft erweist es

TABELLE III

VERWENDETES KAMMERSYSTEM

| *Kammer*[a] | *Puffer (ml)* | | | |
|---|---|---|---|---|
| | *pH 2.875* | *pH 3.880* | *pH 5.000* | *pH 6.300* |
| 1 | 70 | | | |
| 2 | 72 | | | |
| 3 | 75 | | | |
| 4 | 75 | | | |
| 5 | | 70 | 5 | |
| 6 | 6 | 9 | 60 | |
| 7 | | | 75 | |
| 8 | | | 75 | |
| 9 | | | 40 | 35 |

[a] Kammer 1 enthält 5 ml Methanol; Kammer 2 enthält 3 ml Methanol.

sich, dass die Cysteinsäure *ca.* 10 Min. vor dem Zuckerpeak erscheint und auch Citrullin einwandfrei vom Prolin getrennt wird, wenn der Puffer in der Kammer 1 auf pH 2.70 zurückgepuffert wird.

Tabelle III zeigt das von uns verwendete Kammersystem.

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CHROM. 4340

## A simplified method for the preparation of immunoglobulin G by gel filtration–centrifugation

There is an extensive bibliography on the methods used in the preparation of $\gamma$-globulin. SCHULTZE AND HEREMANS[1] presented an up-to-date review. KISTLER AND NITSCHMANN[2] emphasized the preparation of $\gamma$-globulin for therapeutic purposes. Due to the high degree of purification of the finished product, each of these methods requires the application of accurate techniques or results in poor efficiency (see also BAUMSTARK *et al.*[3]).

Here a simplified technique for the rapid preparation in the laboratory of an immunoelectrophoretically almost pure $\gamma$-globulin G is described.

Euglobulin was precipitated from human serum by saturating one-third of the serum with ammonium sulphate. The precipitate was washed with a 30% solution of ammonium sulphate and then dissolved in aqua dest. The euglobulin solution was centrifuged with Sephadex G-200 (40–120 $\mu$) which had been equilibrated in distilled water with a specific conduction of $\sigma = 2.693 \cdot 10^{-7}$ ($1/\Omega$ cm) (For conductivity measurements see Table I.)

TABLE I

RESULTS OF CONDUCTIVITY MEASUREMENTS

For the measurements a Wheatstone bridge of up to 1000 c/sec was employed.

| *Material* | *Conductivity* ($1/\Omega cm$) |
|---|---|
| Human serum pool | $6.640 \cdot 10^{-3}$ |
| $F_1$ | $0.168 \cdot 10^{-3}$ |
| $F_2$ | $2.048 \cdot 10^{-3}$ |
| $F_3$ | $5.760 \cdot 10^{-3}$ |

Ten millilitres of human serum pooled from clinically healthy donors were mixed with 5 ml of a saturated solution of ammonium sulphate, and the precipitate was washed twice in a 30% solution of ammonium sulphate. (Sufficient centrifugation is necessary for adequate separation of the proteins in solution.) After washing, the precipitate was collected in 3 ml of aqua dest. or in a 1% solution of NaCl.

The serum of patients with $\gamma$ G-myeloma was used in other tests. For the results of these tests see Fig. 2.

For centrifugation, the upper part of the centrifugation tube (see Fig. 1) was filled to the brim with a dense Sephadex gel and centrifuged for 5–6 min at a velocity of 500–600 r.p.m. (swing-out centrifuge; radius, 20 cm). Too many revolutions or the use of other types of gel may produce separation of gel surface.

After the first centrifugation of the Sephadex gel, the euglobulin solution was placed on the gel surface and then centrifuged again at a velocity of 500 r.p.m. for 5 min. The filtrate obtained was designated $F_1$. In order to obtain a complete elution of proteins, 3 ml of distilled water were added to the gel surface, and centrifuged for

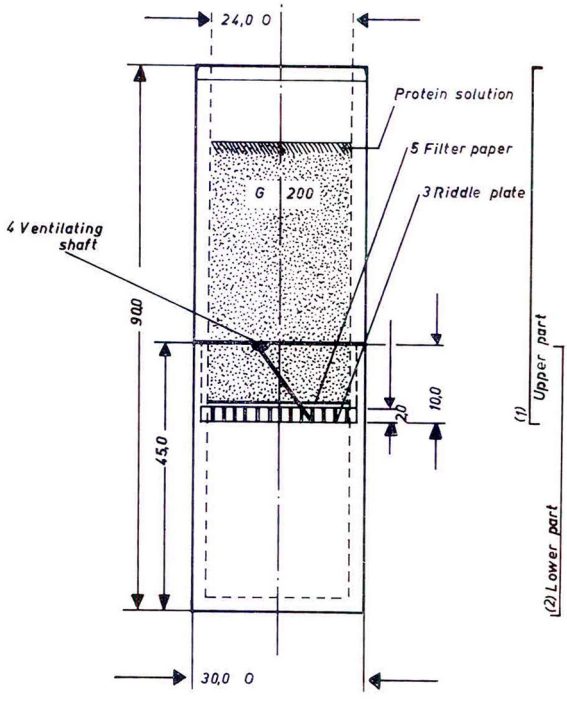


Fig. 1. Centrifugation tube with riddle plate. (1) Upper part with a fixed riddle plate (3); (2) lower part with an air-balanced channel (4); (5) paper filter on the riddle plate. The diameter of the riddle holes is 1 mm. The data are expressed in mm. Material: plexiglass.

5 min (500 r.p.m.). This filtrate was designated $F_2$. Filtrates $F_3$ and $F_4$ were obtained in the same way.

Immunoelectrophoresis was performed in agar Difco-Noble and Veronal buffers (pH 8.2). The filtrates were placed directly in the start position (in the other tests after concentration). Horse antihuman serum (see BUNDSCHUH *et al.*[4] with respect to the immunization plan) as well as rabbit antihuman serum were used as immune sera.

As shown in Fig. 2, the first filtrate contains immunoelectrophoretically pure

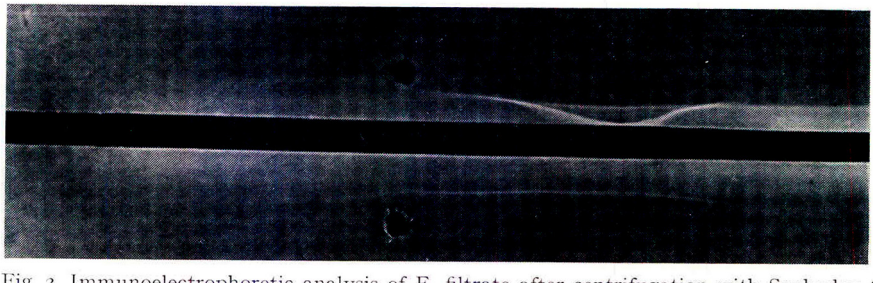

Fig. 2. Immunoelectrophoretic analysis of $F_1$ filtrate after centrifugation with Sephadex G-200. Above: $F_1$ from $\gamma$G-myeloma serum. Below: $F_1$ from normal serum. Horse antihuman serum, see text. The $F_1$ filtrate of the myeloma serum shows the paraprotein (precipitation arc near the antibody channel) as well as the normal $\gamma$-globulin G.

$\gamma$-globulin G. In consecutive filtrates $\gamma$-globulin G is concentrated; however, other euglobulin traces as well as albumin traces can be found.

The results of the tests permit us to conclude that a part of the $\gamma$-globulin G is still soluble in a dilute salt medium. The conductivity of filtrates $F_1$ and $F_2$ is usually less than that of the initial material. The other euglobulin components are considered similar to a protein insoluble in the small amount of salt present in the Sephadex gel. According to HEIMBURGER AND SCHMIDTBERGER[5], these components can be obtained by means of an increase in the salt gradients during the elution phase.

Contrary to the usual filtration procedure of Sephadex gel, the basis of separation of the protein components by the method described here does not depend on the size of the molecule of the different proteins but on the protein being soluble in a medium poor in salt.

The paraprotein may be obtained from the $\gamma$ G-myeloma serum in relation to normal $\gamma$-globulin G (see Fig. 2) by the method described.

In a further communication we shall report on the purity of $\gamma$-globulin, obtained by this method, which is determined by means of the hyperimmunization of rabbits as well as by the quantitative efficiency of the material obtained.

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CHROM. 4354

## Visualization reagents for quantitation of carbohydrates on thin-layer chromatograms by transmission densitometry

The quantitation of carbohydrates by transmission densitometry of thin-layer chromatograms has been reported recently. MIZELLE *et al.*[1] described an aniline–oxalic acid reagent for identifying and quantitating the sugars of flavonoid glycosides, and LAMKIN *et al.*[2] an aniline phthalate reagent for quantitation of the neutral monosaccharides in glycoproteins and glycopeptides. Unfortunately, the use of these aniline reagents was limited to reducing sugars. HUBER *et al.*[3] reported the quantitation of D-glucose and its oligomers that were visualized by spraying with 50% sulfuric acid and heating at 140° for 30 min. LEHRFELD[4] quantitated trimethylsilylated carbohydrates that were visualized by spraying with 5% sulfuric acid in ethanol and heating. Although sulfuric acid reagents are applicable to both reducing and nonreducing carbohydrates, no study to determine the optimum application of sulfuric acid has been reported. Other charring techniques for visualization of chromatograms are known[5–8]. This investigation was undertaken to evaluate four sulfuric acid charring techniques for their suitability in the quantitation of carbohydrates by transmission densitometry.

*Experimental*

*Cleaning of glass plates.* Grease on our glass TLC plates caused spots on our chromatoplates and also a flaking of the adsorbent layer from apparently good chromatoplates. A careful scouring of each plate eliminated this problem but was tedious, time-consuming, and often scratched the plates. Boiling the glass plates in a detergent solution followed by rinsing with distilled water effectively eliminated all grease spots.

The cleansing procedure is simple, fast, and efficient when the plate holder and tank shown in Fig. 1 are used. The holder separates the plates and ensures that all surfaces are exposed to the detergent solution. The holder containing the plates is placed in a boiling detergent solution for one-half hour. After the solution cools, the plate holder is removed; the plates are rinsed with distilled water and left to dry. The entire cleaning is carried out with the plates in the holder.

The holder and tank were fabricated from stainless steel. The tank is 9 in. wide × 6 in. deep × 11 in. high. The basic dimensions of the holder are 8 $\frac{3}{4}$ in. wide × 5 $\frac{3}{4}$ in. deep × 7 in. high. A handle projects 7 in. above the top plate of the holder.

The holder consists of two slotted plates and one perforated plate (8 $\frac{3}{4}$ × 5 $\frac{3}{4}$ in.) separated by 6 in. and $\frac{1}{4}$ in., respectively, and held by four $\frac{3}{8}$ × 7 $\frac{3}{8}$-in. rods threaded and bolted at the ends. The handle is a bent $\frac{3}{8}$ × 32 $\frac{1}{2}$-in. rod threaded at the ends and bolted to hold and separate the perforated plate from the lower slotted plate. The perforated plate supports the TLC plates and facilitates drainage.

This holder accommodates ten 8 × 8-in. plates or forty 2 × 8-in. plates. Although the slots in this apparatus are $\frac{1}{4}$ × 8 $\frac{1}{8}$ in., their size or the space between them may be reduced to accommodate more plates and still use the same basic design and dimensions.

*Preparation of chromatoplates.* Glass plates (20 × 20 cm) were coated with a

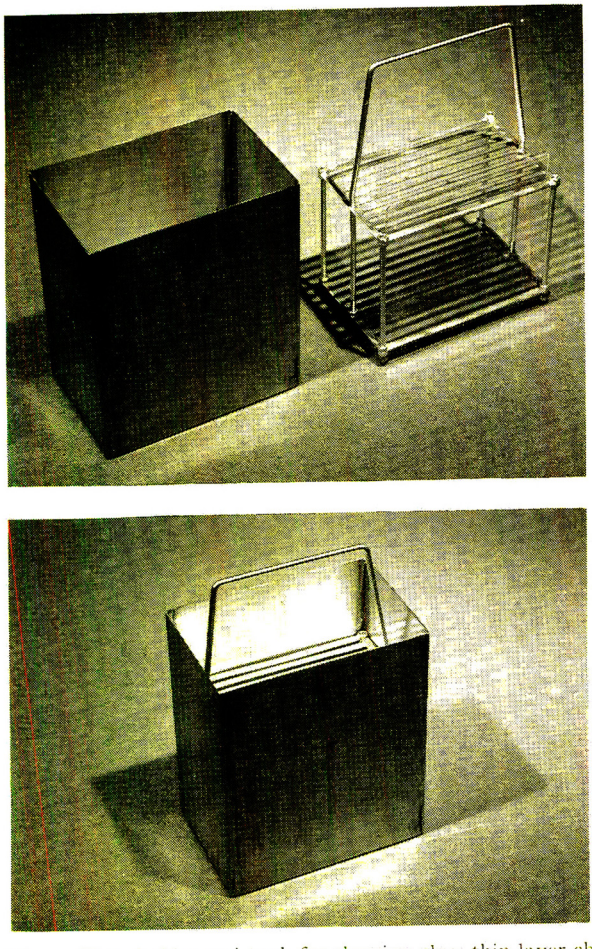

Fig. 1. Plate holder and tank for cleaning glass thin-layer chromatography plates.

0.25 mm layer of Silica Gel G*. The slurry used to coat the plates was prepared from two parts water to one part Silica Gel G[9]. The plates were dried overnight at room temperature and used without activation.

*Procedure.* Standard solutions of maltose, D-glucose, and methyl α-D-glucopyranoside, containing 1, 3, and 5 μg/μl, respectively, were prepared in (60%) ethanol. Aliquots of 3 μl were applied to the plate in duplicate with a 10-μl Hamilton microsyringe.

Chromatograms were developed by the ascending method in a tightly closed rectangular tank lined with filter-paper wicks[10]. The chromatogram was doubly developed to a distance of 15 cm with butanone–water azeotrope/ethanol (23:2, v/v). The plates were allowed to dry 30 min in a forced-air hood.

* Mention of firm names or trade products does not constitute endorsement over similar firms or products not mentioned by the U.S. Department of Agriculture.

*J. Chromatog.*, 45 (1969) 150–154

TABLE I

DENSITOMETRY OF SOME CARBOHYDRATES BY FOUR INDICATOR REAGENTS

| *Reference compounds (μg)* | *3.3% sulfuric acid* | | | | *Ammonium sulfate* | | | | *50% sulfuric acid* | | | | *Sulfuryl chloride* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Peak area (cm²)* | | *Slope* | *Standard deviation* | *Peak area (cm²)* | | *Slope* | *Standard deviation* | *Peak area (cm²)* | | *Slope* | *Standard deviation* | *Peak area (cm²)* | | *Slope* | *Standard deviation* |
| | *Found*[a] | *Calc.*[b] | | | *Found*[a] | *Calc.*[b] | | | *Found*[a] | *Calc.*[b] | | | *Found*[a] | *Calc.*[b] | | |
| Maltose | | | | | | | | | | | | | | | | |
| 3 | 4.45 | 4.52 | 1.04 | 0.184 | 5.65 | 5.80 | 1.03 | 0.367 | 1.90 | 1.95 | 0.61 | 0.122 | 2.75 | 2.65 | 0.70 | 0.245 |
| 9 | 10.95 | 10.8 | | | 12.30 | 12.0 | | | 5.70 | 5.6 | | | 6.65 | 6.85 | | |
| 15 | 17.0 | 17.08 | | | 18.05 | 18.2 | | | 9.20 | 9.25 | | | 11.15 | 11.05 | | |
| D-Glucose | | | | | | | | | | | | | | | | |
| 3 | 5.3 | 5.32 | 0.87 | 0.061 | 5.35 | 5.52 | 1.25 | 0.429 | 1.80 | 1.73 | 0.69 | 0.163 | 2.25 | 2.21 | 0.75 | 0.102 |
| 9 | 10.6 | 10.55 | | | 13.35 | 13.0 | | | 5.75 | 5.88 | | | 6.60 | 6.68 | | |
| 15 | 15.75 | 15.77 | | | 20.30 | 20.47 | | | 10.1 | 10.03 | | | 11.20 | 11.15 | | |
| Methyl α-D-gluco-pyranoside | | | | | | | | | | | | | | | | |
| 3 | 4.50 | 4.62 | 0.98 | 0.306 | 5.92 | 6.02 | 1.07 | 0.163 | 2.0 | 1.83 | 0.56 | 0.408 | 2.25 | 2.32 | 0.65 | 0.184 |
| 9 | 10.75 | 10.5 | | | 12.55 | 12.42 | | | 4.85 | 5.18 | | | 6.40 | 6.25 | | |
| 15 | 16.25 | 16.37 | | | 18.75 | 18.82 | | | 8.70 | 8.53 | | | 10.10 | 10.17 | | |

[a] Average of two determinations.
[b] Calculated from least squares fit curve.

The plates were sprayed evenly by a crisscross method to near translucence with the following visualization indicators described under (1), (2), and (3); or they were saturated sequentially with vapors of sulfuryl chloride and water (4) below:

(1) 3.3% sulfuric acid in 33% ethanol; heating time, 30 min at 150°.

(2) 20% aqueous ammonium sulfate, 4% sulfuric acid[5]; heating time, 1 h at 210°.

(3) 50% sulfuric acid (aqueous, v/v)[6]; heating time, 30 min at 260°.

(4) Sulfuryl chloride and water vapor chambers[8]. The plates were placed in a tank saturated with sulfuryl chloride vapor for 15 min. Then the plates were rapidly removed (the operator using rubber gloves in forced-air hood) and placed in a second tank saturated with water vapor for 15 min. In the first tank sulfuryl chloride saturates the adsorbent layer and, in the second, is hydrolyzed to sulfuric and hydrochloric acids; the plates are then heated at 150° for 30 min. The density of the spots was determined 1 h after the plates had cooled.

$R_F$ values were as follows: maltose, 0.16; D-glucose, 0.32; and methyl α-D-glucopyranoside, 0.43.

*Densitometer.* A Photovolt TLC densitometer, Model 530, equipped with a collimating slit aperture of 0.1 × 15 mm was used. The TLC stage was driven 1 in./min. The signal from the photometer was recorded on a 10-in. Beckman linear–log recorder on the log scale with a chart speed of 2 in./min. Peak areas (spot densities) were measured with a Keuffel and Esser compensating polar planimeter.

*Results and discussion*

For each of the visualization reagents a linear relationship was established between the weight of the standard reference compounds (3–15 mg) and the area of the peaks (Table I). The lines do not coincide, are not parallel, and do not go through the origin. Even though the basic visualization reagent is sulfuric acid, its form and the temperature of charring markedly affect the degree of color development. The color intensity for a given amount of reference compound decreased in the following order: ammonium sulfate, 3.3% sulfuric acid, sulfuryl chloride, 50% sulfuric acid.

Spot intensity decreased with time (Table II). The rate of fading is related to the spray reagent. The per cent decrease after 22 h is greatest with sulfuryl chloride and least with 50% sulfuric acid. The spots from the three sugars visualized by 3.3% sulfuric acid faded at about the same rate. Consequently, an analysis that extends over any period of time would best be carried out with 3.3% sulfuric acid.

The reference compounds are also a significant source of variation. The lines

TABLE II

PER CENT DECREASE IN SPOT INTENSITY AFTER 22 HOURS

| *Reference compounds* | *Visualization indicators* | | | |
|---|---|---|---|---|
| | *Sulfuryl chloride* | *50% sulfuric acid* | *3.3% sulfuric acid* | *20% ammonium sulfate* |
| D-Glucose | 33 | 7.1 | 13.2 | 16.8 |
| Maltose | 23 | 2.5 | 14.7 | 11.8 |
| Methyl α-D-glucopyranoside | 25 | 1.5 | 14.1 | 13.5 |

derived from the three reference compounds with any one reagent spray do not coincide (see Table I). The least squares difference between the slopes is 0.0668 (95% level).

The average standard deviation is largest for the ammonium sulfate spray. In this series the largest deviations occurred with the two reducing sugars. Therefore, ammonium sulfate is the least satisfactory reagent for quantitation.

Larger deviations occur when data from several plates are compared. The effect is random and unpredictable. Consequently, it is advisable to run an internal standard. When a series was run on one plate, duplicate spots varied about $\pm$ 3% in recorded peak area. Deviations from a curve constructed by a least squares fit varied about $\pm$ 2.8%. The standard error was 0.26.

Spraying technique markedly affects color development. Either overspraying or underspraying a section of the plate causes color intensity to vary. Because of this variation, the sulfuryl chloride procedure should give the most consistent results. JONES *et al.*[8] suggested holding the sulfuryl chloride saturated plate over a steam bath to hydrolyze the sulfuryl chloride to sulfuric and hydrochloric acids. We found that a chamber saturated with water gave more consistent results without the danger of splattering from a steam bath.

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## The separation of neomycin sulphate, polymyxin B sulphate and zinc bacitracin

There are several pharmaceutical formulations which contain neomycin, polymyxin B and zinc bacitracin in various admixtures and dosage forms[1] and the separation of these into their components could facilitate their routine estimation.

The separation of antibiotics by paper chromatography has been reviewed in detail by BETINA[2]. Other chromatographic techniques have been widely used for the separation of antibiotics in general. FOPPIANI AND BROWN[3] used a thin-layer technique for the separation and potency determination of neomycin sulphate. GUVEN AND OZSARI[4] separated neomycin, polymyxin B and bacitracin using thin-layer chromatography on silica gel using a mixture of benzene, water and acetic acid. BRAMMER AND HEMSON[5] were unable to separate polymyxin B and neomycin, using electrophoresis on cellulose acetate with 0.07 *M* barbital buffer (pH 8.6). They used Ponceau S for the detection of the antibiotics. However, LIGHTBOWN AND DE RISSI[6] were able to separate bacitracin, polymyxin B and neomycin using agar gel electrophoresis with Tris–maleate buffer at pH 5.6. In this case the antibiotics were located using the production of inhibition zones after inoculation with the appropriate organism and subsequent incubation. The production of colour with ninhydrin has been used extensively for the detection of peptide antibiotics and for the quantitative estimation of neomycin by MAEHR AND SCHAFFNER[7] and THORBURN BURNS, LLOYD AND WATSON-WALKER[8].

*Experimental*

*Antibiotics and reagents.* Polymyxin B sulphate (690 μg/mg), neomycin sulphate (810.6 units/mg), and zinc bacitracin (60 units/mg) were dissolved in 1% sodium ethylenediamine tetracetate to give a final concentration per millilitre of: neomycin sulphate B.P., 483 units; zinc bacitracin B.P., 360 units and Polymyxin B sulphate, 146 units, approximating the commercial aerosol spray under investigation.

All reagents were of analytical reagent grade. Ninhydrin, 0.2 g dissolved in 100 ml *n*-butanol, was used as spray reagent. Ninhydrin stabilising reagent was according to Merck[9] and the ninhydrin quantitative reagent was prepared according to the method of JACOBS[10]. Of Ponceau S 200 mg were dissolved in 100 ml of 3% trichloroacetic acid and excess dye was removed with 5% acetic acid.

*Paper chromatography.* All separations were carried out on Whatman No. 3 paper, using the following solvents: $S_1$ = *n*-butanol–water–glacial acetic acid (30:13:8); $S_2$ = *n*-butanol–glacial acetic acid–pyridine–water–ethanol (60:15:6:5:5); $S_3$ = *n*-butanol–water–glacial acetic acid–pyridine–NaCl (30:12:7:2:0.1).

*Thin-layer chromatography.* For thin-layer chromatography 5 cm × 20 cm Kieselgel G (Merck) plates were used. The following solvents were used: $S_4$ = *n*-butanol–glacial acetic acid–water–pyridine (30:22:38:6) and $S_5$ = *n*-butanol–water–pyridine–glacial acetic acid–ethanol (60:10:6:15:5).

*Electrophoresis.* For electrophoresis a Shandon (Kohn Mark II) apparatus was used. Cellulose acetate (Oxoid) strips of 2.5 × 20 cm or Whatman No. 1 paper, strips of 5 × 20 cm were used. For paper the solvent $S_6$ = glacial acetic acid–formic acid–

water (60:30:910) and for cellulose acetate the solvent $S_7$ = pyridine–glacial acetic acid–water (75:2.5:922.5), pH 6.6, was taken.

*Procedure*

Paper chromatography was carried out by the ascending technique in the normal way. The antibiotic solutions were applied in 5-$\mu$l aliquots, using a micro syringe. The resulting chromatograms were developed using the ninhydrin spray reagent followed by heating at 105° for 5 min. The developed chromatograms were stabilised using the stabilising spray reagent.

Thin layers of Kieselgel G, 250 $\mu$ thick, were prepared in the normal way, activated by heating at 110° for 45 min, and stored over silica gel. Antibiotic samples were applied in 1-$\mu$l aliquots. The chromatograms were developed and visualised as above.

Electrophoretic separations were carried out on either paper strips, using solvent $S_6$, at a constant voltage of 700 V for 40 min, or on cellulose acetate strips using 400 V, constant voltage, for 60 min. In each case the temperature of the electrophoretic solutions were 12°. The samples were applied in 3.5-$\mu$l aliquots as a band across the strip. The antibiotic bands were visualised using either ninhydrin, which stained all three antibiotics, or by staining with Ponceau S reagent for 10 min and removing excess stain by soaking for 15 min in 5% acetic acid. In the latter case only polymyxin B and neomycin sulphate retained the dye, giving red and orange bands, respectively.

Quantitative estimations of the antibiotics present in the electrophoresis strips were made by eluting the Ponceau S stained material with 2 ml of 0.1 *N* NaOH and estimating the colour produced at 510 nm. The results were in agreement with those obtained by BRAMMER AND HEMSON[5]. The system was found to be suitable for quantitative estimation of polymyxin B.

Alternatively two strips were run in parallel, the presence of the antibiotics located on one and the corresponding section removed from the other and the antibiotic eluted from this with 2 ml of pH 5.0 acetate buffer. To this solution 1 ml of quantitative ninhydrin reagent was added and the colour was developed by heating at 98° for 10 min. The colour produced was estimated at 570 nm and by comparison with calibration curves of the pure antibiotics the amount in each sample could be determined for all three antibiotics in the same manner as has been described for neomycin[7,8].

The calibration curves obtained for the ninhydrin method were linear over the range of 10 to 800 $\mu$g. Beyond these limits there was considerable departure from linearity.

*Results*

The results obtained are represented by Tables I–III.

*Discussion*

The presence of the antibiotics polymyxin B, neomycin and zinc bacitracin may be detected using either paper or thin-layer chromatography. In the case of paper chromatography the addition of small amounts of sodium chloride to the solvent system was found to reduce the tailing and to give discrete spots.

The electrophoretic separation was rapid and allowed for quantitative elution

TABLE I

$R_F$ VALUES FOR THE PAPER CHROMATOGRAPHIC SEPARATION OF THE ANTIBIOTICS

| *Solvent system* | *Neomycin* | *Polymyxin B* | *Bacitracin* |
|---|---|---|---|
| $S_1$ | 0.02 | 0.16 | 0.30 |
| $S_2$ | 0.04 | 0.70 | 0.97 |
| $S_3$ | 0.05 | 0.56 | 0.75 |

TABLE II

$R_F$ VALUES FOR THE THIN-LAYER CHROMATOGRAPHIC SEPARATION OF THE ANTIBIOTICS

| *Solvent system* | *Neomycin* | *Polymyxin B* | *Bacitracin* |
|---|---|---|---|
| $S_4$ | 0.14 | 0.50 | 0.61 |
| $S_5$ | 0.05 | 0.34 | 0.66 |

TABLE III

DISTANCE TRAVELLED (CM) FROM POINT OF APPLICATION TOWARDS THE CATHODE BY THE ANTIBIOTICS ON ELECTROPHORESIS

| *Medium* | *Neomycin* | *Polymyxin B* | *Bacitracin* |
|---|---|---|---|
| Cellulose acetate | 11.2 | 8.5 | 5.5 |
| Paper | 17.7 | 14.0 | 11.0 |

TABLE IV

CORRELATION BETWEEN THE AMOUNT OF ANTIBIOTIC PRESENT ON AN ELECTROPHORETIC STRIP AND THE AMOUNT ELUTED ESTIMATED BY THE NINHYDRIN METHOD

| *Amount present (μg)* | *Amount detected after elution (μg)* | | |
|---|---|---|---|
| | *Neomycin* | *Bacitracin* | *Polymyxin B* |
| 10 | 9 | 8 | 10 |
| 20 | 18 | 17 | 19 |
| 30 | 29 | 29 | 30 |
| 40 | 35 | 35 | 38 |
| 50 | 48 | 50 | 51 |
| 100 | 98 | 101 | 100 |
| 200 | 205 | 200 | 202 |
| 400 | 400 | 404 | 395 |
| 500 | 505 | 501 | 498 |

of the antibiotics for estimation (Table IV). The use of Ponceau S for quantitative work is limited by the narrow range of compounds giving a stained product. The ninhydrin reaction is more versatile. Whilst it has been widely studied for the estimation of neomycin[7,8], it can be used to estimate other peptide antibiotics. The elution of the antibiotics was carried out in pH 5 acetate buffer in preference to the normal 0.1 *N* NaOH[5] because it was found that zinc bacitracin decomposed rapidly in alkaline solution.

The electrophoretic separation allows a rapid and quantitative estimation for routine quality control of the antibiotics present in the aerosol preparation investigated in comparison to the normal microbiological methods of assay.

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## Dünnschichtchromatographie von 4-Acetyl-2-nitrophenyl-Derivaten phenolischer Verbindungen

In Analogie zum 2,4-Dinitrofluorbenzol ist 4-Fluor-3-nitroacetophenon zur Abscheidung phenolischer Verbindungen geeignet[1,2], wobei die Reaktionsfähigkeit durch deren Nucleophilie und sterischen Bau bestimmt wird. Enthält die phenolische Komponente Aminogruppen, so treten auch diese in Reaktion. Im Falle des *o*-Aminophenols erfolgt lediglich Substitution am Stickstoff. Die entstehenden 4-Acetyl-2-nitrophenyl-Derivate (ANP-Derivate) liefern bei der UV-spektroskopischen Charakterisierung im Gegensatz zu den entsprechenden 2,4-Dinitrophenyläthern wertvolle Informationen zur Struktur der ursprünglichen Phenole[3]. Aus diesem Grund ist ihr dünnschichtchromatographisches Verhalten von Interesse. Von einer Vielzahl an Sorbentien erwies sich Kieselgel am geeignetsten, das als manuell gefertigte Dünnschicht sowie

TABELLE I

($R_F \times 100$)-WERTE DER ANP-DERIVATE PHENOLISCHER VERBINDUNGEN

| | *S1* | | *S2* | |
|---|---|---|---|---|
| | *Kieselgel G* | *Polygram SIL S-HR/ $UV_{254}$* | *Kieselgel G* | *Polygram SIL S-HR/ $UV_{254}$* |
| *ANP-Äther von* | | | | |
| Phenol | 62 | 60 | 82 | 65 |
| *o*-Kresol | 65 | 65 | 81 | 86 |
| *m*-Kresol | 60 | 52 | 71 | 70 |
| *p*-Kresol | 65 | 65 | 82 | 85 |
| 2,3-Dimethylphenol | 65 | 46 | 69 | 70 |
| 2,4-Dimethylphenol | 69 | 62 | 82 | 80 |
| 2,5-Dimethylphenol | 70 | 65 | 83 | 80 |
| 3,4-Dimethylphenol | 62 | 64 | 83 | 81 |
| Thymol | 72 | 74 | 76 | 69 |
| Guajacol | 44 | 34 | 61 | 60 |
| 3-Methoxyphenol | 50 | 60 | 73 | 70 |
| 2,3-Dimethoxyphenol | 50 | 28 | 54 | 20 |
| 2,6-Dimethoxyphenol | 46 | 29 | 53 | 25 |
| *o*-Chlorphenol | 53 | 52 | 65 | 49 |
| 2,4,6-Trichlorphenol | 69 | 75 | 70 | 56 |
| *o*-Nitrophenol | 34 | 19 | 45 | 69 |
| *m*-Nitrophenol | 49 | 38 | 50 | 63 |
| *p*-Nitrophenol | 55 | 40 | 53 | 64 |
| *p*-Hydroxybenzaldehyd | 32 | 22 | 54 | 51 |
| Vanillin | 29 | 23 | 65 | 36 |
| 4-Hydroxy-3-methoxyacetophenon | 34 | 13 | 41 | 15 |
| 4-Hydroxy-2-methoxyacetophenon | 42 | 21 | 36 | 14 |
| 2-Hydroxy-4-methoxyacetophenon | 40 | 19 | 38 | 24 |
| $\alpha$-Naphthol | 58 | 53 | 73 | 81 |
| $\beta$-Naphthol | 69 | 62 | 65 | 51 |
| *p*-Hydroxybenzoesäure | 0 | 7 | 0 | 0 |
| Vanillinsäure | 0 | 4 | 0 | 0 |
| Syringasäure | 0 | 3 | 0 | 8 |
| *p*-Hydroxyzimtsäure | 0 | 4 | 0 | 13 |
| Ferulasäure | 0 | 4 | 5 | 0 |
| N-ANP-*m*-Aminopheno' | 28 | 27 | 36 | 10 |
| N-ANP-*p*-Aminophenol | 45 | 25 | 44 | 44 |
| *Bis-ANP-Äther von* | | | | |
| Brenzcatechin | 20 | 10 | 39 | 11 |
| Resorcin | 16 | 14 | 54 | 29 |
| Hydrochinon | 34 | 20 | 45 | 45 |
| Orcin | 34 | 17 | 48 | 29 |
| 2-Methylresorcin | 22 | 23 | 49 | 19 |
| Methylhydrochinon | 26 | 28 | 45 | 16 |
| *Tris-ANP-Äther von* | | | | |
| Phloroglucin | 12 | 7 | 22 | 43 |
| 2,4,6-Trihydroxyacetophenon | 5 | 3 | 13 | 29 |
| *N-ANP-Derivat von* | | | | |
| *o*-Aminophenol | 56 | 38 | 11 | 8 |

als Fertigfolie verwendet wurde. Tabelle I gibt einen Überblick über die Trennung verschiedener ANP-Derivate in den Laufmittelsystemen Cyclohexan–Cyclohexanon (3:1) (S 1) und Methylenchlorid–Cyclohexan (5:1) (S 2).

*Diskussion der $R_F$-Werte*

ANP-*m*-Kresol weist gegenüber ANP-*o*- und ANP-*p*-Kresol eine geringere Wanderungsgeschwindigkeit auf. 2.3-Dimethylphenol lässt sich nach Verätherung mit S 2 und S 1 (nur auf Polygram SIL S-HR/$UV_{254}$) von den isomeren Dimethylphenolen abtrennen. Methoxylgruppen bewirken in *o*- und *o,o'*-Stellung zur O-ANP-Funktion eine wesentliche Erhöhung der Adsorptionsaffinität. Die Trennung der ANP-Nitrophenole wird auf Kieselgel G mit S 1 ermöglicht. Eine Acylgruppe im Substrat setzt den $R_F$-Wert beträchtlich herab. ANP-$\alpha$- und ANP-$\beta$-Naphthol können gut differenziert werden. Carbonsäuren laufen nicht oder nur geringfügig. Sie sind somit leicht von den übrigen phenolischen Verbindungen abzutrennen. In polaren Laufmittelsystemen besitzen Carbonsäuren höhere aber wenig unterschiedliche $R_F$-Werte. *o*-, *m*- und *p*-Aminophenol lassen sich als ANP-Derivate auf Kieselgel G separieren. Mit zunehmender Zahl an 4-Acetyl-2-nitrophenoxygruppen wird im allgemeinen die Adsorption erhöht. Auf MN-Kieselgel S-HR/$UV_{254}$ als Polygram-Fertigfolie erscheinen die Substanzen als punktförmige scharfe Flecken, so dass auch bei geringen $R_F$-Wert-Unterschieden gute Trennungen möglich sind.

*Methodik*

20 × 20 cm-Platten werden in der üblichen Weise mit je 8 g Kieselgel G (Merck) in 16 ml Wasser beschichtet und an der Luft getrocknet. Als Fertigfolie verwendet man MN-Polygram SIL S-HR/$UV_{254}$ (5 × 20 cm). Die ANP-Derivate werden in 0.5%iger acetonischer oder dimethylformamidhaltiger Lösung aufgetragen. Bei Kammersättigung chromatographiert man bis zu einer Laufhöhe von 13 cm. Zum Nachweis der ANP-Derivate auf Kieselgel G dient Rhodamin B im UV-Licht.

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## Polyamide–silica gel thin-layer chromatography of water-soluble vitamins

Thin-layer chromatography has proved to be a valuable analytical technique in the separation of vitamins. However, relatively few publications appeared concerning the separation of water-soluble vitamins by this technique; the use of silica gel (*e.g.* refs. 1–4), aluminum oxide[4], starch[5] and polyamide[6] has been reported.

Recently, polyamide–silica gel mixed layers have been successfully used for the separation of red food dyes[7]. Therefore the mixed-layer method was further applied to separate eleven water-soluble vitamins. For comparison thin-layer chromatography using only polyamide and silica gel was performed under the same conditions. Separation on polyamide–silica gel mixed layers was found to be preferable.

*Experimental*

*Preparation of polyamide–silica gel (2:1) mixed layer.* Twenty grams of polyamide chip (Nylon 6, type 1022B of UBE Industrial Ltd., Osaka, Japan) were dissolved in 80 ml of 90% formic acid; then 20 ml of distilled water were added. After warming (below 40°) and stirring, a homogeneous solution was obtained; after cooling it to room temperature, 10 g of Silica Gel G (E. Merck) were added. Two hundred milliliters of the above-mentioned solution were poured into a dish (14.5 × 19.5 × 2.5 cm), and a glass plate (12 × 14 × 0.1 cm) was dipped into it. Both sides of the glass were covered homogeneously. The glass was hung for 2 min over the dish to let the excess solution drain back before suspending it in a water-saturated cabinet (50 × 50 × 50 cm) for half a day. It was then taken out of the cabinet and heated at 100° for 30 min.

*Preparation of polyamide layer.* The above-mentioned method was employed but without the addition of Silica Gel G.

*Preparation of silica gel layer.* Plates of Silica Gel G were prepared by using Desaga S 11 spreader, pre-set to give an applied layer 250 $\mu$ thick, and then were heated at 100° for 30 min.

*Chromatographic procedure*

Two percent orotic acid dimethylamine (*ca.* 40%) solution, 2% ascorbic acid solution and 0.5% of other vitamin solutions were applied to the start line 1.5 cm from the bottom of the layer. The plates were developed in the dark by ascending techniques. The chamber had been equilibrated with the respective solvent for 30 min before use.

*Visualization.* Rutin, riboflavin, riboflavin 5'-phosphate sodium and cyanocobalamine can be recognized under long-wave length UV light at 365 m$\mu$. The layers were sprayed with a 0.07% Rhodamine B alcoholic solution and all the spots could be observed under UV light at 254 m$\mu$.

*Results and discussion*

$R_F$ values of polyamide–silica gel mixed layers, silica gel layers and polyamide layers with two solvent systems are given in Table I. It has been found that the results obtaining using the mixed layers show better separation and sharper spots. The spots on the silica gel layers are rather diffused and larger. In the preparation of polyamide

TABLE I

$R_F$ VALUES OF VITAMINS ON DIFFERENT LAYERS

P–S = polyamide–silica gel layer; S = silica gel layer; P = polyamide layer.

| *No.* | *Samples* | *10% NaCl solution* | | | *10% sodium acetate solution* | | |
|---|---|---|---|---|---|---|---|
| | | *P–S* | *S* | *P* | *P–S* | *S* | *P* |
| 1 | Orotic acid | 0.00 | 0.86 | 0.00 | 0.00 | 0.90 | 0.00 |
| 2 | Rutin | 0.01 | 0.43[a] | 0.30[a] | 0.10 | 0.54[a] | 0.05 |
| 3 | *p*-Aminobenzoic acid | 0.06 | 0.64 | 0.11 | 0.65 | 0.80 | 0.78 |
| 4 | Riboflavin | 0.18 | 0.22 | 0.29 | 0.20 | 0.29 | 0.24 |
| 5 | Cyanocobalamine | 0.27 | 0.10 | 0.04 | 0.40 | 0.30 | 0.06 |
| 6 | Thiamine HCl | 0.40 | 0.15 | 0.86 | 0.32 | 0.10 | 0.93 |
| 7 | Riboflavin 5′-phosphate sodium | 0.43 | 0.22 | 0.79 | 0.47 | 0.29 | 0.67 |
| 8 | Nicotinamide | 0.53 | 0.43 | 0.60 | 0.52 | 0.55 | 0.70 |
| 9 | Pyridoxine HCl | 0.60 | 0.45 | 0.74 | 0.58 | 0.59 | 0.77 |
| 10 | Nicotinic acid | 0.74 | 0.47 | 0.77 | 0.73 | 0.62 | 0.88 |
| 11 | Ascorbic acid | 0.98 | 0.88 | 0.90 | 0.90 | 0.85 | 0.98 |
| Time required (min)[b] | | 110 | 10 | 40 | 110 | 15 | 55 |

[a] Tailing.
[b] Time required to ascend 10 cm from origin.

layers, slow drying of layers in the water-saturated cabinet is essential to reduce the developing time of these layers.

The content of polyamide (*ca.* 66%) in these mixed layers is greatly increased compared to that of the previous report (12%)[7] for getting suitable results. The layer did not crack or peel and can be stored easily. The method is suitable for the identification of various water-soluble vitamins.

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## Book Review

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*Thin-Layer Chromatography*, edited by E. STAHL, translated by M. R. F. ASHWORTH, 2nd Ed., Springer-Verlag, Berlin, 1969, 1041 pages, price $ 32.00 (in English).

The second German edition of this compendium was published in 1967 and has been reviewed earlier (*cf. J. Chromatog.*, 35 (1968) 448). Many non German-speaking users of thin-layer chromatography will welcome the appearance of this translation of an invaluable reference book. M. R. F. ASHWORTH is to be congratulated on the fluency of his translation and the book is well organised and indexed and attractively presented.

For the benefit of newcomers to the technique who may not be familiar with earlier versions of this book it consists of two sections; just under 200 pages are devoted to the techniques and materials used and the remaining 800 pages to a systematic coverage of applications of TLC. Many authorities have made contributions to the book which the Editor, STAHL, has combined well. Inevitably, after the process of translation, the book does not include references to recent work and other deficiencies which strike the reviewer are the sketchy treatments of sample transfer from thin layers and of problems associated with impurities in or on adsorbents, eluents and apparatus. In any further edition of this book one would hope that attention might be given to the relation between thin-layer and column chromatography, particularly noting recent developments in the latter field. Notwithstanding these criticisms the book represents a tremendous effort by many people and is, and will remain, an essential source book of information about an invaluable technique.

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## News

## Meetings

ON-STREAM ANALYSIS—A FEATURE TOPIC AT IMEKO V

The next Congress of the International Measurement Confederation has set as its goal a scientific programme around the highly topical fields of measurement and instrumentation. One of these topics is a section concerning *On-stream composition and analysis* which includes recent advances in instruments in this general field, but particularly its suitability for computer control. This section will be arranged jointly with IFAC.

The next Congress, IMEKO V, will be held at Versailles (France) between 25th and 30th of May, 1970. The organization of IMEKO V will be in the hands of the association AFCET. Papers presented within the section on *On-stream composition and analysis* will be followed by a round-table discussion with M. L. CHOVIN of France as chairman. This discussion will deal with *Metrological basis of gas chromatography and evaluation of accuracy of measuring data from composition of mixtures.* A survey paper will be presented by an outstanding 'connoisseur' of this general field, and will serve as a keynote to the technical section at the round-table discussion.

Specialists concerned directly or indirectly with the timely problems of on-stream composition analysis will have ample opportunity to meet and discuss their immediate problems with their colleagues from several of the 23 countries usually present at IMEKO Congresses.

Among other current topics to be found on the programme of IMEKO V, those of interest to process analysts will be

(1) The industrial measurement technique for on-line computation;

(2) Advances in flow measurement for optimisation of material balances.

An unusually rich crop of papers has been collected by the 17 member organizations of IMEKO, and these will go through a final selection by the International Papers Committee in Paris, early September.

Instrumentation experts of the chemical and allied process industries are invited to meet in Versailles in May 1970, where at the same time the international MSSUCORA exhibition will also take place.

Intending participants should write to the IMEKO secretariat: IMEKO secretariat, P.O. Box 457, Budapest, 5 (Hungary).

## New Apparatus

A new exponential *automatic dilutor* from Carlo Erba S.p.A. has been announced in order to make calibration curve preparation for gaseous mixtures easier and more accurate. Using the dilutor it is possible to supply the gas chromatograph sampling device with gaseous mixtures of known concentrations from p.p.b. to some per cent. A complete calibration graph may be plotted in only 30 min.

For further information apply to the publisher under reference No. Chrom. N-235.

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CHROM. 4381

# OPTICAL NOISE IN PHOTOMETRIC SCANNING OF THIN MEDIA CHROMATOGRAMS

## I. GENERAL THEORY AND ITS APPLICATION TO SINGLE-BEAM TRANSMISSION MEASUREMENTS

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SUMMARY

An attempt has been made to apply the concepts of "signal" and "noise" so important in communication theory to the analysis of the performance of photodensitometers used for the quantitative assessment of thin media chromatograms. Very low concentrations of separated substances are considered thus permitting the linearization of the relationships involved. It has been shown that for the best performance under these conditions a highly stabilized light source is essential and that the spectral width of the scanning beam should be the same, or somewhat less than that of the absorbing zone of interest. At low concentrations flying-spot scanning as opposed to fixed-slit scanning is of no real advantage. The double-beam system described in the following paper is vastly superior to any single-beam arrangement.

## INTRODUCTION

There exist numerous devices for the scanning of thin media chromatograms (see recent review[1]); nearly all of them are single-beam devices. Though they are rather inferior in their performance to the double-beam arrangements as described by SALGANICOFF *et al.*[2], based on the time-sharing dual wavelength spectrophotometer of CHANCE[3,4] they still represent the vast majority of all instruments in practical use.

In this paper an effort has been made to analyse the performance of photoelectric densitometers from a theoretical point of view and to determine those factors which affect their sensitivity, stability and reproducibility. The concepts and analytical procedures used are borrowed to some extent from the field of communication theory. In this paper the general theory will be discussed and applied to the analysis

of single-beam devices operating in the transmission mode. Double-beam devices are considered separately in the following paper.

The basic purpose of any physical measuring procedure is to supply information about the quantity to be measured. Regardless of the physical nature of the output signal obtained from the measuring assembly, it is frequently convenient to express it in terms of electrical parameters. The information content of any datum obtained from the measurement is thus determined by the ratio of the amplitude of the useful signal obtained to that of the sum of all undesirable and disturbing signals of any kind, which tend to obliterate the desired signal. These unwanted signals can be divided into two groups: the first, and the one which will be our main concern here, is of a random nature and may comprehensively be called "noise". The other, which will be dealt with in a subsequent paper, is deterministic in nature and essentially the result of non-linearities in the transfer function of the system involved. Neglecting the latter (as is done in this analysis) is tantamount to assuming a strictly linear transfer function of the system. In practical terms this means that we shall here restrict ourselves to the analysis of very small signals (this being the case for very low concentrations of absorbing substances), because in these cases virtually any transfer characteristic can be approximated by a straight line.

Regardless of this restriction it may be shown that the lower the ratio of useful signal amplitude to noise amplitude, the less is the amount of information that may be extracted from the individual measured result. The noise amplitudes determine the lowest signal level that may be detected and the resolving power of the method, in other words the smallest absolute difference in useful signal value that may be reliably distinguished. The signal to noise ratio, therefore, decides the obtainable accuracy.

The general rules mentioned above apply, of course, also to photometric methods of quantitative evaluation of chromatograms. In order to determine the inherent limitations upon the sensitivity, accuracy and resolving power of the evaluation a detailed study of the noise encountered is required. The output signal is the amplified output of the photoelectric detector unit. We have to consider, therefore, noise contributions from both the optical and the electrical parts of the instrument. The study, the results of which are presented here, was undertaken with the aim of analysing the sources of noise encountered, to assess it in a quantitative way and to determine possible approaches to reduce its detrimental influence. In these, the first two papers of a series, we are concerned only with the noise arising in the optical part of the system. An analysis of the electrical noise will be given in a later publication.

Our principal aim in this study was to investigate the feasibility of considerably reducing the levels of detection and quantitative evaluation of absorbing zones separated on various chromatographic media. It is, of course, in these regions of very low concentrations where noise considerations are of primary importance, whilst non-linearity effects may in first approximation be disregarded.

## THEORETICAL

Let the absorbance of the chromatogram at a certain wavelength be $\alpha_c$. Assuming that Beer's law is valid the resulting transmission is:

$$A_c = A_0 e^{-\alpha_c} = A_0 \left(1 - \frac{\alpha_c}{1!} + \frac{\alpha_c^2}{2!} - + \cdots\right) \qquad (1)$$

The series expansion for $e^{-\alpha}$ shown in eqn. 1 possesses alternating signs and converges uniformly. It can be shown that the error involved by terminating the series after the $n$th term is less than the absolute value of the $(n + 1)$th term.

As we are concerned with low concentrations and, therefore, low values of incremental absorption $\alpha_c$ in an absorbing zone, the case where $\alpha_c$ is small is of special interest. In this case we may terminate the series in eqn. 1 after the second term.

$$\Delta A = A_0 - A_c = A_0 \left(1 - \frac{A_c}{A_0}\right) \approx A_0 \alpha_c \qquad (\alpha_c \ll 1) \tag{2}$$

The error committed will be less than $(A_0 \alpha_c^2)/2$. It can be shown that this approximation is also valid for all other laws of transmission, deviating from Beer's law, provided $\alpha_c \ll 1$. As a matter of fact it can be shown that (see later papers in this series), other conditions being equal, the error committed in these cases is always less than that committed with a purely exponential dependence.

The adoption of eqn. 2 results in a linearization of the relationship between the decrement in transmission and concentration and consequently it becomes permissible to use average (mean) values of concentration (absorbance) to obtain the average value of transmission or other characteristic optical parameters. Inversely, measured average optical values may be used to determine the average values of absorbance and concentration. The implications and limitations of this assumption will be explained in more detail in a subsequent paper of this series.

Furthermore, let us assume that the spectral density $\varepsilon$ of the illuminating beam is constant over a certain spectral width $\Delta\lambda$; this results in an illuminating flux of $\varepsilon\Delta\lambda$. If the spectral density in this region is not a constant $\varepsilon$ has to be taken equal to its average value over this region. $\varepsilon\Delta\lambda$ is also assumed to be constant over the spatial cross section of the beam. In practical use this condition may be difficult to achieve specially with long slits.

The background transmission $A_0$ of the material to be scanned may vary widely from a value of the order of $10^{-3}$ for paper chromatograms up to close to 1.0 for some films. In a given medium the value of transmittance may vary spatially from one part of the medium to another (see Fig. 1). $A_0$ is, however, also a function of the spectral wavelength and for paper chromatographic strips a steady increase in transmission occurs with increasing $\lambda$ over which there may be superimposed random fluctuations. A similar situation is obtained with other types of support material.

When a coloured zone is encountered the transmission of the chromatogram decreases selectively over a certain region of the spectrum the width of which is determined by the effective width of the principal absorption band of the absorbing substance. In most cases this band is fairly wide, of the order of 50 m$\mu$ or more.

The absorbance of the investigated substance, however, is not constant throughout this region, but a function of the spectral position inside the absorbing band. The result of this is that the loss in transmission encountered is also a function of wavelength. To avoid the difficulties arising from this condition it is customary to employ

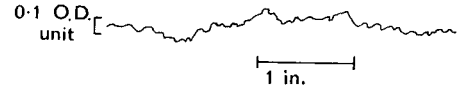


Fig. 1. Transmittance of Whatman No. 2 paper (scanning spot size 1 mm × 1 mm).

a very narrow spectral width of the illuminating beam. With low concentrations, however, where the linear approximation shown in eqn. 2 is reasonably valid, it becomes possible to use a fairly broad spectral bandwidth $\Delta\lambda_c$ and to operate with the average value of transmission over this band. Using a broader spectral width of the scanning beam offers a higher light output to the photoelectric receiver and therefore, a better electrical signal to noise ratio; an additional advantage is a slight decrease in optical background noise; this will be discussed later.

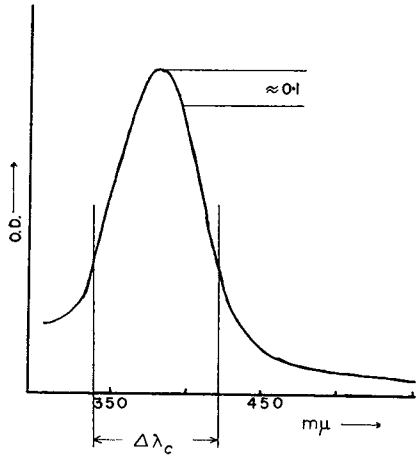


Fig. 2. Typical shape of the absorption band of a chromogen. (4-Hydroxy-4′-nitroazobenzene separated on Whatman No. 2 during 4 h in the solvent system light petroleum–toluene–acetic acid–water (133:66:170:30).)

The concentration of the absorbing substance varies over the coloured zone. In order to obtain meaningful data from current scanning devices, therefore, efforts are made in the practical methodology of chromatography (deposition of the solutes in bands, equilibration, constant temperature, etc.; see ref. 5 for further details) to ensure that the distribution of concentration within the illuminated scanning slit remains as constant as possible. With low concentrations where again eqn. 2 may be assumed to hold with reasonable accuracy, it is possible to relate the average concentration over the illuminated slit area linearly to the average decrease in transmission. This means that to a large extent the geometry of the zone and the distribution of concentration within the zone can be disregarded.

The transmission $A_0$ of the medium, as already mentioned, is spatially not constant but may vary from one point of the illuminated area to another in a random fashion. These fluctuations are caused partly by variations in the thickness of the medium, partly by local inhomogeneities. Variations in thickness produce transmission fluctuations, which are virtually independent of the wavelength of the scanning beam, whilst irregularities in composition may have effects, which are strongly dependent upon wavelength.

Let the mean value of $A_0$ over a very large (infinite) area of a particular type of chromatogram (paper, film, etc.) and at a given wavelength $\lambda_0$ of the scanning

beam be $\bar{A}$ and the standard (r.m.s.) deviation from this value be $\bar{\bar{A}}$. Since $\bar{\bar{A}}$ is usually quite small, we can again apply the linear approximation as in eqn. 2.

$$A \approx \bar{A}e^{\alpha} \simeq \bar{A}(1 + \alpha) \qquad \alpha \ll 1$$

$$\bar{\bar{A}} \approx \bar{A}(1 + \bar{\bar{\alpha}}) - \bar{A} = \bar{A}\bar{\bar{\alpha}} \tag{2a}$$

Let the mean value of transmission over a given slit of area $F$ be $A_s$. $F = b\Delta W$ as illustrated in Fig. 3.

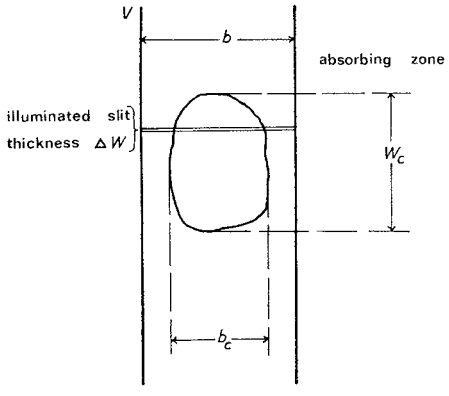


Fig. 3. Usual arrangement for fixed-slit scanning of chromatogram strips.

Since the area $F$ is finite, $A_s$ will vary. The mean value of $A_s$ over many areas of the same size will, however, be $\bar{A}$. In accordance with the central limiting theorem of probability theory, $A_s$ will approach a normal (Gaussian) distribution around $\bar{A}$ with a standard deviation $\bar{\bar{A}}_s$.

It then follows that $\bar{\bar{A}}_s$ will be approximately inversely proportional to the square root of the area of the illuminated slit.

$$\bar{\bar{A}}_s = \bar{\bar{A}}\sqrt{\frac{b^*\Delta W^*}{b\Delta W}} = \bar{\bar{A}}\sqrt{F} \tag{3}$$

$b^*\Delta W^*$ is the area of the smallest slit which may be used to define $\bar{A}$. A decrease below this value does not increase the measured value of $\bar{\bar{A}}_s$. The values $b^*$ and $\Delta W^*$ correspond approximately to the average dimensions of the irregularities in the density of the medium. These may of course vary considerably from one type of medium to another. They may also be interpreted as the fundamental spatial frequency of the noise caused by background irregularities. It is convenient if all the areas and cross sections are measured in multiples of $b^*\Delta W^*$; this means that on all subsequent occasions $b^*\Delta W^*$ is considered equal to unity. Since there is no reason to prefer one of the directions in the medium (provided it is reasonably homogeneous and isotropic), we may postulate that $b^* = \Delta W^* = 1$. Eqn. 3 is based on the assumption that

the variations in transmission $A$ from one part of the chromatogram strip to another are random and uncorrelated provided their mutual distance is larger than one unit. Since $A_s$ varies, the intensity $I_s$ of the light transmitted by the strip will also vary from one strip to another. Let the mean value of $I_s$ be $\bar{I}_s$ and its standard deviation $\bar{\bar{I}}_s$; let the light intensity entering the medium be $I_0$; due to a certain amount of surface reflection $I_0$ is smaller than the illuminating light intensity; the amount of light lost in this way is expressed in the following equation by the coefficient $\psi$. We thus obtain:

$$\varepsilon_0 = \varepsilon \cdot b^* \Delta W^* \cdot \psi$$

$$I_0 = \varepsilon_0 \Delta\lambda \cdot b \Delta W = \varepsilon_0 \Delta\lambda \cdot F$$

$$\bar{I}_s = I_0 \bar{A}_s(\Delta\lambda)$$

$$\bar{\bar{I}}_s = I_0 \bar{\bar{A}}_s(\Delta\lambda) = I_0 \bar{\bar{A}}(\Delta\lambda) \cdot 1/\sqrt{F} \tag{4}$$

$F$ denotes the illuminated slit area, measured in multiples of the area unit $b^* \Delta W^*$.

$\bar{\bar{I}}_s$ is the r.m.s. value of the light fluctuations at the input of the photoelectric receiver; it represents, therefore, the effective (r.m.s.) amplitude of the optical noise signal.

As already mentioned none of the media used in chromatography is ideally "grey"; this means, therefore, that the transmission $A$ will, to a certain extent, be dependent upon the wavelength of the scanning beam. This dependence may be expressed as follows:

$$A(\lambda) = A(\lambda_0)\,[1 + g(\lambda - \lambda_0)] \tag{5}$$

Over a reasonably small spectral distance we may approximate $g(\lambda - \lambda_0)$ by a linear trend component $(g_0)$ superimposed by a random term $(\gamma)$. The latter term as previously mentioned is a consequence of the slight inhomogeneities in the medium thus resulting in random changes in the transmission for different wavelengths in different parts of the medium.

$$g(\lambda - \lambda_0) \simeq g_0(\lambda - \lambda_0) + \gamma(\lambda) \tag{6}$$

From the way $\gamma(\lambda)$ is defined it becomes evident that the spatial average of $\gamma(\lambda)$ obtained over a slit of infinite area is equal to zero. By a reasoning, which is completely analogous to that used in eqn. 3, it may be shown that the standard deviation $\bar{\bar{\gamma}}_s(\lambda)$ of $\gamma_s(\lambda)$, as measured over slits of finite area $F$, is proportional to $1/\sqrt{F}$. By analogy it may also be shown that spectral averaging of $\gamma_s(\lambda)$ over a spectral window of finite width $\Delta\lambda$ decreases the r.m.s. value $\bar{\bar{\gamma}}_s(\lambda,\Delta\lambda)$ approximately in proportion to $\sqrt{(1/\Delta\lambda)}$. Since $\bar{\bar{A}}_s$ contains a component due to $\gamma_s(\lambda)$, it follows that an increase in spectral width of the scanning beam should decrease $\bar{\bar{A}}_s$ and therefore improve the optical noise conditions. The improvement to be expected will depend upon the degree of inhomogeneity of the medium.

In a recent scanning device of more sophisticated design a double-beam arrangement is used[2]. In this device the two beams are arranged to have different spectral positions. It is the fluctuation of the difference in transmission between both

beams which is here of key importance. From the arguments developed above it is evident that this difference is made up from two terms: the first being variations in transmission, affecting both beams proportionally, *e.g.* random variations in thickness of the medium, and the second being local inhomogeneities affecting both beams in a randomly different way. Both terms may be considered as statistically independent. The standard deviation of the difference can, therefore, be calculated as the sum of the squared terms:

$$\overline{\overline{A_s(\lambda_0) - A_s(\lambda)}} = \frac{1}{\sqrt{F}} [g_0^2(\lambda - \lambda_0)\bar{\bar{A}}^2(\Delta\lambda) + \bar{\bar{\gamma}}^2(\lambda_0,\Delta\lambda)\bar{A}^2(\lambda_0) + \bar{\bar{\gamma}}^2(\lambda,\Delta\lambda)\bar{A}^2(\lambda_0)]^{1/2}$$

$$= \frac{1}{\sqrt{F}} [g_0^2(\lambda - \lambda_0)\bar{\bar{A}}^2(\Delta\lambda) + 2\bar{\bar{\gamma}}^2(\lambda,\Delta\lambda)\bar{A}^2(\lambda_0)]^{1/2} \quad (7)$$

In most cases one of the two terms in this expression will prevail; because of the quadratic law of addition, the other term may then be neglected especially if the spectral width $\Delta\lambda$ of the scanning beams is not too small. This will usually apply to the second term.

From the reasoning given above it appears that for the investigation of absorbents in low concentrations as separated on chromatograms a relatively large spectral width of the scanning beam is desirable. This reduces the amount of optical noise produced by possible inhomogeneities of the chromatograms with a non-grey absorbance characteristic. That the increased light intensity at the photodetector, which is obtained in this way, will decrease the relative weight of the electrical noise, originating in the photodetector and the associated amplifying equipment, has already been mentioned.

For high concentrations of absorbent, where consequently strong optical signals are encountered, both optical and electrical noise become less important. Here the linear approximation of eqn. 2 may entail large errors. To avoid these the spectral width of the scanning beam should then be confined to a region where the absorbance of the substances investigated is reasonably constant. In general a compromise between these conflicting requirements has to be made. More details on this will be presented in a subsequent paper.

In the next section and the following paper the performance of the two basic transmission design alternatives for chromatogram scanning devices, the single-beam and the double-beam arrangement, will be discussed with regard to the obtainable optical noise performance.

## SINGLE-BEAM ARRANGEMENT

First let us consider a single-beam arrangement in which the light source is illuminating a slit of length $\Delta W$ and extending across the full width $b$ of the chromatogram strip as shown in Fig. 3.

The basic arrangement of a scanning device of this type is illustrated in Fig. 4.

In the receiving photoelectric device (Ph) the transmitted light intensity is converted to a proportional electrical signal. The random component of $I_s$ produces fluctuations in the electrical output which are for all practical purposes equivalent

to the noise in a communication system; they superimpose upon the electrical noise generated here.

There is, however, one important difference to be noted. In an electrical communication system most of the noise originates independently of the useful signal and therefore merely adds to it. The optical noise considered here, however, is multiplicative in nature (see eqn. 8).

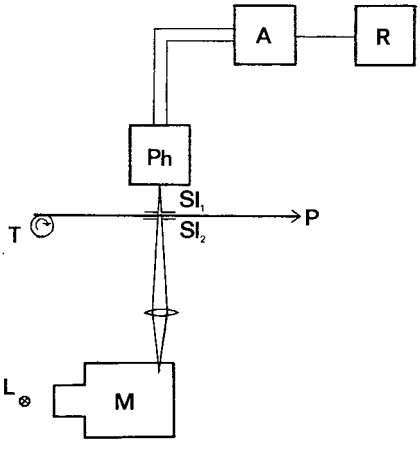


Fig. 4. Schematic representation of a single-beam chromatogram scanning device. L = light source; M = monochromator or filter; P = thin medium chromatogram; T = chromatogram transport mechanism; $Sl_1$ and $Sl_2$ = entry and exit slits respectively; Ph = photoelectric device; A = amplifier; R = analogue recorder.

What is important is not so much the amplitude of the noise signal itself but, as already pointed out in the introduction, the ratio of the amplitude of the useful signal to that of the noise. We, therefore, have first to determine the useful signal amplitude. For this purpose let us consider an illuminated slit of length $\Delta W$ and width $b$, covering part of an absorbing zone with the dimensions $b_c$ and $\Delta W_c$ (see Fig. 3). Let the concentration in this area be $c$ (not necessarily homogeneously constant) and the corresponding increase in absorbance $\alpha_c$. $\alpha_c$ is the mean value of absorbance, measured over the full width $\Delta\lambda_c$ of the spectral absorption characteristic, covered by the illuminating beam. Let the spectral width of the illuminating beam be $\Delta\lambda$ and it is assumed that it comprises the region of absorption of the investigated substance.

For small concentrations the transmission of any point within the absorbing zone may be written in the form shown in eqn. 2. The decrease in transmission produced by the absorbing material is therefore:

$$\Delta A = A_0 \left(1 - \frac{A_c}{A_0}\right) = A_0 \alpha_c \tag{8}$$

The decrease in light intensity at the photodetector is obtained by integrating eqn. 8 over the whole illuminated part of the absorbing zone. It represents the useful optical signal amplitude $I_c$.

$$b_c \Delta W_c = F_c$$

$$I_c = \varepsilon_0 \Delta\lambda_c \cdot F_c \cdot \bar{A}\bar{\alpha}_c = I_0 \cdot \frac{F_c}{\bar{F}} \cdot \frac{\Delta\lambda_c}{\Delta\lambda} \cdot \bar{A}\bar{\alpha}_c \tag{9}$$

$\bar{A}\bar{\alpha}_c$ is the mean value of the decrease in transmission over the illuminated part of the zone. The use of $\bar{A}$ instead of $A_s$ implies that $I_c$ represents the mean value of the useful signal, disregarding local changes in transmission $A_s$ from strip to strip.

From Fig. 3 it is apparent that the illuminated slit may also include parts of the medium outside of the absorbing zone. For the mean total light input $It$ to the photodetector we obtain therefore:

$$It = I_s - I_c = I_0 \bar{A} \left(1 - \frac{F_c}{\bar{F}} \cdot \frac{\Delta\lambda_c}{\Delta\lambda} \cdot \bar{\alpha}_c\right) \tag{10}$$

$It$ will of course fluctuate from one strip to the other, since the transmission $A_s$ of the strips varies around the mean value $\bar{A}$. The optical noise signal produced in this way is designated $Iv$. Usually the second term in eqn. 10 is small as compared with unity; thus we obtain:

$$Iv \simeq I_0 \bar{\bar{A}} \cdot \frac{1}{\sqrt{F}} \tag{11}$$

We can now obtain the optical signal to noise ratio $\sigma$ by forming the ratio of $I_c/Iv$.

$$\sigma = \frac{I_c}{Iv} = \alpha_c \cdot \frac{\bar{A}}{\bar{\bar{A}}} \cdot \frac{\Delta\lambda_c}{\Delta\lambda} \cdot \frac{F_c}{F} \cdot \sqrt{F}$$

$$= \frac{\alpha_c}{\bar{\bar{\alpha}}} \cdot \frac{\Delta\lambda_c}{\Delta\lambda} \cdot \frac{F_c}{F} \cdot \sqrt{F} \tag{12}$$

$\bar{\alpha}_c/\bar{\bar{\alpha}}$ is the basic densitometric signal to noise ratio of the chromatogram.

For low concentration, where eqn. 2 holds, we can assume that the quantity of investigated substance $Q_s$ in the illuminated part of the zone is proportional to $\bar{\alpha}_c$ (eqn. 2).

$$Q_s = k\bar{\alpha}_c F_c \tag{13}$$

$k$ is a proportionality constant, taking into consideration the absorbance of the investigated substance per unit of concentration. In actual measurements $k$ has to be found by calibration against a known concentration. If the linear approximation in eqn. 2 holds, the value of $Q_s$ obtained will be independent of the distribution of the investigated substance over the slit. For higher concentrations an error will be committed; but this will be discussed in more detail in a later paper in this series.

Introducing eqn. 13 into eqns. 9 and 12 we obtain:

$$I_c = I_0 \cdot \frac{\bar{A} Q_s}{kF} \cdot \frac{\Delta\lambda_c}{\Delta\lambda}$$

$$\sigma = \frac{Q_s}{k\bar{\bar{\alpha}}} \cdot \frac{\Delta\lambda_c}{\Delta\lambda} \cdot \frac{1}{\sqrt{F}} \tag{14}$$

To determine the total amount of absorbing substance present in the zone, we must, of course, integrate over all the slits in contact with this zone.

DISCUSSION

The most important relationship so far derived is that exhibited in eqn. 12, which determines the optical signal to noise ratio obtained. This ratio is the principal factor which limits the sensitivity, accuracy and resolving power of a photodensitometer of the type described. It is apparently dependent upon several factors.

The first and most important one is the relative non-uniformity of the transmission of the chromatogram itself, as expressed by the relation $\bar{A}/\bar{\bar{A}}$; this factor is clearly a characteristic parameter of the medium used, depending in general only to a minor degree upon the spectral width of the scanning beam.

The second factor is the ratio $\Delta\lambda_c/\Delta\lambda$; the largest value obtainable is evidently 1. To obtain it, the spectral width of the illuminating beam $\Delta\lambda$ must be equal to or smaller than the width $\Delta\lambda_c$ of the absorption characteristic of the substance investigated. With the nearly monochromatic light, which is conventionally used for scanning, this condition is always fulfilled. For the best results the spectral position of the illuminating beam should coincide with the reasonably flat part of the absorption characteristic. For low concentrations $\Delta\lambda$ should be made almost equal to $\Delta\lambda_c$, in order to obtain the highest input signal at the photodetector. It can be shown that the best signal to noise ratio is achieved, if the scanning beam is spectrally shaped in such a way that it models the absorption characteristic of the substance involved. This is of course feasible only if linearity errors are of no concern.

For higher concentrations, where the linear approximation of eqn. 2 cannot be applied, $\Delta\lambda$ should be smaller than $\Delta\lambda_c$ and cover that part of the absorption characteristic where $\alpha_c(\lambda)$ does not change too much. The error committed by using a finite value of $\Delta\lambda$ is analysed in detail in another paper to appear later. At higher concentrations and consequently higher values of the useful output signal $I_c$ the electrical noise becomes less important and the decrease in $I_v$, caused by a smaller value of $\Delta\lambda$ is of less significance.

It appears to be immaterial whether the spectral shaping of the scanning beam is done on the primary side, that is between the light source and the chromatogram, or on the secondary side, that is between the chromatogram and the photoelectric converter. In the case of fluorescence measurements filtering on the secondary side is, of course, mandatory.

The last important factor to be examined is the ratio of the total area illuminated by the scanning beam to that part of it which contains the absorbing zone of interest. For this purpose we may write:

$$F = \mu F_c \qquad (\mu \geqslant 1)$$

$$\frac{F_c}{\sqrt{F}} = \sqrt{\frac{F_c}{\mu}} \tag{15}$$

The highest value is evidently obtained if $\mu = 1$, that is if all the illuminated area belongs to a stained zone. At the same time $F_c$ should be as large as possible. In

practice this condition is met by a slit aperture, extending over the full width of the chromatogram and applying the solution to be chromatographed in bands over the whole width of the medium. At higher concentrations, however, errors are incurred if the distribution of concentration is not exactly uniform over the band. This error can be reduced or avoided if the area of the scanning beam is reduced to virtually a point. This arrangement is usually termed "flying-spot scanning". Subsequent integration of the signals $I_c$ obtained for each spot has to be performed over the whole zone. If Beer's law can be assumed to be valid, the usable range can be extended to higher concentrations by using a logarithmic converter before summing of the individual spot signals. If considerable deviations from Beer's law are to be expected, more complicated procedures than simply forming the logarithms of the spot signals should be used. More details about this will be found in a subsequent paper to be published soon.

The difficulty with spot scanning and subsequent integration is to determine the area over which integration has to be performed. The criterion, therefore, has to be derived from the spot-output signal $I_c$ exceeding a certain preset threshold value. Since the area of the scanning spot is small, the noise content in $I_c$ is high and the decision whether a particular $I_c$ is above or below threshold is affected with a high degree of uncertainty. Some smoothing and curve fitting operations on the set of values $I_c$ obtained may alleviate this problem; they will, however, need processing by a computer.

There are still other sources of error to be mentioned, namely variations in the coefficient of surface reflection $\psi$ (see eqn. 4) and instability of the light source $I_0$. They are discussed in the following paper. These sources of error are of equal influence in single- and double-beam difference forming instruments; the latter device, however, produces a much better optical signal to noise ratio. Double-beam instruments appear, therefore, to be preferable for all but the most unsophisticated measurements and our further attention will be centred on them.

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# OPTICAL NOISE IN PHOTOMETRIC SCANNING OF THIN MEDIA CHROMATOGRAMS

## II. DOUBLE-BEAM DIFFERENCE SYSTEMS

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SUMMARY

In this the second part of a theoretical treatment of the quantitative analysis of thin media chromatograms the double-beam difference system of scanning has been investigated. This system is much more sensitive than any single-beam arrangement. The incorporation of a "flying-spot" system as opposed to fixed slits permits the quantitative analysis regardless of zone geometry. Perfect balance between both scanning beams and a high degree of stabilization of the light source are essential for good performance. The limits in sensitivity are obtained when the optical noise approaches the electrical noise.

## INTRODUCTION

In the preceding paper[1] the concepts of "signal" and "noise" as they are used in communication theory have been applied to single-beam transmission photodensitometers. It was shown that for the quantitative analysis of very small amounts of absorbing substances a linear relationship may be assumed between the resultant electrical signal and concentration and that in this case the parameter of greatest importance is $\bar{A}/\bar{\bar{A}}$ which refers to the relative non-uniformity of the medium itself.

In this paper the double-beam difference system[2–8] first utilized by SALGANICOFF *et al.*[2] is investigated. In this device (see Fig. 1) in addition to the principal measuring beam arranged to have a wavelength corresponding to that of the peak absorption of the substance of interest there is also a reference beam. The wavelength of this reference beam is selected so as to be virtually unabsorbed by the zones of interest. In practice there may be some difficulty in achieving this at high concentration levels. In order to cancel out the optical noise arising from the irregularities of the paper background the difference between the electrical output signals of both beams is formed and recorded.

The double-beam method appears to possess important advantages in comparison with any presently conceivable single-beam arrangement. This paper is intended, therefore, to extend the results obtained for the single-beam device to the double-beam difference system. The meaning of the symbols is the same as in the preceding paper and in addition the general theoretical relationships given there will be used here again. Those equations developed in the preceding paper and used here are denoted by adding [1] to their number.

## BALANCE OF BEAMS

For efficient noise rejection it is evidently necessary that both beams should ideally possess equal spectral energy density $\varepsilon_0$ and $\varepsilon_R$, and also an equal spectral bandwidth and equal spatial cross section $F = F_R$ (the index $R$ will here always refer to the reference beam).

In practice, of course, all those conditions cannot ideally be met and a finite difference in radiant flux $I_0$ remains.

$$I_{0R} = I_0(1 + \beta) \qquad (\beta \ll 1) \tag{1}$$

In addition to the differences in the radiant flux between the two beams there is also a certain difference in transmission for these two wavelengths.

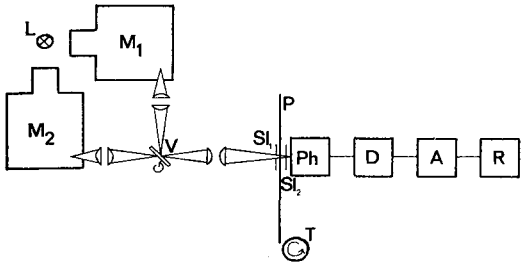


Fig. 1. Schematic representation of a dual beam chromatogram scanner with difference system. (according to SALGANICOFF *et al.*[2]). L = light source; $M_1$,$M_2$ = monochromators; V = vibrating mirror; $Sl_1$ = slit 1; P = chromatogram; $Sl_2$ = slit 2; Ph = photodevice; T = chromatogram transport mechanism; A = amplifier; R = recorder; D = device for obtaining difference signal.

In order to keep this difference small, the two beams should be spectrally as close together as the width of the absorption band permits. In agreement with eqn. 5 [1] we may now write

$$\begin{aligned} g(\lambda_0 - \lambda_R) &= g_0 + \gamma \\ A_R &= A(1 + g_0 + \gamma) \\ \bar{A}_R &= \bar{A}(1 + g_0) \qquad (g_0 \ll 1) \end{aligned} \tag{2}$$

$$\begin{aligned} I_{0R}A_{Rs} &= I_0A_s(1 + \beta)(1 + g_0) \\ &\approx I_0A_s(1 + \beta + g_0) \end{aligned} \tag{3}$$

In order to cancel out as much of the background optical noise as possible, it is desirable that in the average both sides of eqn. 3 should be equal. For this purpose a

mechanically adjustable diaphragm or two polaroids may be used. This permits the adjustment of the relative intensity of both beams so that

$$I_{0R}\bar{A}_R - I_0\bar{A} = I_0(\beta + g_0 + C) = I_0\Delta\beta \to 0 \tag{4}$$

The coefficient $C$ takes care of the adjustment of the diaphragm.

In order to avoid the stability problems which might occur when d.c. amplification of the output signal is used, the two illuminating beams are generally chopped. SALGANICOFF *et al.*[2] use a vibrating mirror and a common photodetector for this purpose. The adjustable diaphragm in this case, however, as well as other minor differences in the optical pathways of the two beams tend to introduce a phase difference into the two signals reaching the photodetector. The result of this phase shift is that even with ideal balancing the output signal does not become zero (see Fig. 2).

$$|I_0| = |I_{0R}| \text{ but } \hat{I}_0 - \hat{I}_{0R} \neq 0$$

This effect may be abolished by introducing a phase-sensitive (synchronous) detector circuit which only responds to the in-phase component $I_0'$ of both signals. The diaphragm is then adjusted to make $I_0' = I_{0R}$.

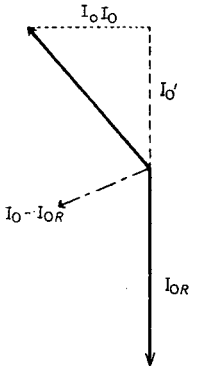


Fig. 2. Effect of a phase shift on the difference of two signals.

However, even with synchronous detection a complete equalization of both signals over any extended period of time is not possible. One of the reasons is inconstancy in time of the parameters involved in the term $\beta$. Another reason is the difficulty in adjusting in practice to the true value of $g_0$, which may vary to a certain degree from one chromatogram to another, even if samples from the same batch are used. As a consequence $\Delta\beta$ behaves to a certain degree as a chance variable and the value used in the equations should be understood as the most unfavourable value, both with regard to amplitude and sign, which may be expected with some reasonable probability.

To minimize $\Delta\beta$, the two beams should be spectrally close together and their optical pathways nearly identical. A common photodetector, serving both beams on a time-sharing basis, as schematically shown in Fig. 1 causes both beams to pass slightly different areas of the chromatogram. The difference is dependent upon the alternating frequency $f$ and the speed $u$ with which the illuminated area is moving.

$$\xi = \frac{u}{f}/\Delta W_c \tag{5}$$

The result is equivalent, at least as far as noise cancellation is concerned, to a minimum value of $\Delta\beta \simeq \xi$. To avoid this two independent photodetectors with simultaneous chopping of both beams can be used. Another possibility is of course to make $f$ sufficiently large. A minimum condition is evidently:

$$f \geqslant u/\Delta W_c \tag{5a}$$

If eqn. 5a is violated, both beams pass different areas of the medium, jeopardizing altogether the advantage of the double-beam method. For all these reasons a complete equalization of the two output signals is not feasible; and it, therefore, becomes necessary to consider a finite difference in the final output signal.

$$I_0\bar{A}\Delta\beta = |(I_{0R}\bar{A}_R - I_0\bar{A})|_{\min} \tag{6}$$

The minimum value of this expression has to be found by suitably adjusting the intensity of one of the beams [*e.g.* by changing the diaphragm (co-efficient $C$ in eqn. 4)].

## THE OPTICAL SIGNAL TO NOISE RATIO

The overall noise is also here essentially determined by expression 7[1] except that $g_0\cdot(\lambda-\lambda_0)$ is replaced by $\Delta\beta$. Further we have to consider the additional local unbalance caused by the useful signal $I_c$. In this way we obtain:

$$I_0 = I_0\frac{1}{\sqrt{F}}\left[\bar{\bar{A}}^2(\Delta\lambda)\left\{\Delta\beta + \bar{\alpha}_c\cdot\frac{F_c}{F}\cdot\frac{\Delta\lambda_c}{\Delta\lambda}\right\}^2 + 2\bar{\bar{\gamma}}^2(\Delta\lambda)\bar{A}^2(\lambda_0)\right]^{1/2} \tag{7}$$

As a consequence of the definition of $\Delta\beta$ given earlier, here and in all the following formulae that sign of $\Delta\beta$ has to be considered which gives the most unfavourable result. As already mentioned in connection with eqn. 7[1] usually one of the terms in 7 will prevail and then only this term need be considered. This permits a considerable simplification of the expressions involved.

In most practical cases it will be the first term which dominates. The straight addition of the two terms in the brace is justified if $F_c/F \simeq 1$, which is the most important case. If $F_c/F \ll 1$ the square root of the sum of the squared terms would be more appropriate. It should be kept in mind that $\bar{\bar{A}}(\Delta\lambda)$ decreases to a certain degree with increasing spectral bandwidth $\Delta\lambda$. If the illuminated region does not contain any absorbent, the second term in the brace becomes zero. In order to obtain minimum noise under this condition, $\Delta\beta$ should be as small as possible, as is of course expected. The total differential output signal of the optical system is:

$$It = I_s - I_c = I_0\bar{A}\left[\Delta\beta - \bar{\alpha}_c\cdot\frac{F_c}{F}\cdot\frac{\Delta\lambda_c}{\Delta\lambda}\right] \tag{8}$$

and the signal to noise ratio $\sigma_1$, with the term containing $\bar{\bar{\gamma}}$ being neglected, becomes:

$$\sigma_1 \simeq \frac{I_c}{Iv} = \frac{\bar{A}}{\bar{\bar{A}}(\Delta\lambda)}\cdot\frac{a_c\cdot\dfrac{F_c}{F}\cdot\dfrac{\Delta\lambda_c}{\Delta\lambda}}{\Delta\beta + a_c\cdot\dfrac{F_c}{F}\cdot\dfrac{\Delta\lambda_c}{\Delta\lambda}}\cdot\sqrt{F} \tag{9}$$

To maximize this expression we put as before $F_c/F = \Delta\lambda_c/\Delta\lambda = 1$. All further expressions will refer to this condition. Using expression 2a[1] we obtain

$$\sigma_1 = \frac{\bar{a}_c}{\bar{\bar{a}}} \cdot \frac{\sqrt{F}}{\Delta\beta + \bar{a}_c} \tag{9a}$$

The way in which this can be achieved was explained in the discussion section of the preceding paper. Either the solute has to be applied in bands which extend across the full width of the chromatogram after development or the fixed illuminating slit has to be replaced (by a flying-spot arrangement with subsequent integration) over the whole zone area.

The two procedures are, however, equivalent only if a sufficiently large amount of investigated substance is available. If very small samples are to be analyzed, a small spot-shaped zone may provide a higher value of average concentration and consequently a better signal to noise ratio if flying-spot scanning is employed. The reason is of course that the useful signal is—with a limited amount of analyzed substance $Q_s$ available—proportional to $Q_s$, regardless of the area over which $Q_s$ is spread, whilst the optical noise increases proportionally with the square root of the area $\sqrt{F}$. A closer inspection shows that there is no contradiction to expression 9, since the latter is based upon the assumption that there is sufficient solute available, so that spreading does not change the concentration.

In a double-beam arrangement, as opposed to the single-beam system it is relatively easy to discriminate between zone and non-zone parts of the chromatogram. From eqn. 8 it is apparent that $It$ is mainly dependent upon $\alpha_c$ so long as $\Delta\beta$ is small enough. To obtain the signal to noise ratio with external integration, $\sqrt{F}$ in formula 9a has to be replaced by $\sqrt{S}$, where $S$ is the area of integration. There is no necessity to emphasize that when logarithmic forming of the output signal is employed this has to be done before averaging is carried out.

With very low concentrations, that is for small values of $\alpha_c$, the second term in the denominator may be neglected; this gives:

$$\sigma_1 \text{ (low)} \simeq \frac{\bar{a}_c}{\bar{\bar{a}}} \cdot \frac{\sqrt{S}}{\Delta\beta} \tag{10}$$

For larger concentrations $\Delta\beta$ may be neglected, resulting in

$$\sigma_1 \text{ (high)} \simeq \frac{\sqrt{S}}{\bar{\bar{a}}} \tag{11}$$

Inspection of the original expression 9 shows that in this case the fraction $F_c/F$ cancels out; this means that bandzones or flying-spot scanning do not produce here any significant improvement in $\sigma$. Further it is interesting to note that the signal to noise ratio at high concentrations appears to be independent of the amplitude of the useful signal. The explanation of this fact is, of course, that the chromatogram background noise affects the useful signal in a multiplicative rather than an additive way. As a consequence of this the noise signal is proportional to the useful signal. At the same time the noise contribution from the rest of the chromatogram virtually cancels out provided $\Delta\beta$ is sufficiently small. The noise produced by the irregular transmission of $I_c$ within the absorbing area, however, is not affected at all by the difference forming

procedure. A qualitative illustration of the dependence of the signal to noise ratio upon the ratio $\bar{\alpha}_c/\Delta\beta$ is shown in Fig. 3.

In some cases the second term in eqn. 7 will dominate; we then obtain a signal to noise ratio:

$$\sigma_2 \simeq \frac{a_c}{2\bar{\bar{\gamma}}(\Delta\lambda)} \sqrt{S} \qquad (12)$$

It should be noted that $\bar{\bar{\gamma}}$ can be reduced by the same means used to make $\Delta\beta$ small and by using the maximum spectral width $\Delta\lambda$ compatible with the linearity requirements.

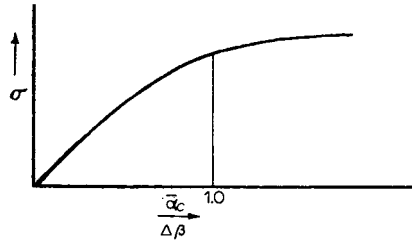


Fig. 3. Signal to noise ratio ($\sigma$) in dependence upon the ratio of concentration to beam equalization.

## OTHER SOURCES OF ERRORS

Another possible source of error is the varying average transmittance of the medium, which may show considerable fluctuation from one chromatogram to another (see eqn. 8). These deviations will produce proportional errors in the optical output signal similar to those produced by variations in the illuminating light density. With present chromatographic media such as the current qualities of papers, loaded papers, coated sheets etc., it appears that the calibration should be repeated whenever a new paper is inserted into the scanning device. As the changing value of transmittance appears as a multiplicative factor in the useful output signal $I_c$ the percentage of error introduced in the measurement of $A_c$ is independent of concentration. With higher concentrations where the linear approximation in eqn. 2[1] is no longer valid the error in the result from this source tends to become smaller.

A further factor to be considered is the surface reflection factor $\psi$ (see eqn. 4[1]), which may change from one chromatogram to another. It results in a change in the proportion of light entering into and transmitted by the medium; it is therefore equivalent to a variation in the intensity of the light source. Suitable calibration procedures at the beginning of each measurement are the best remedy.

## STABILITY REQUIREMENTS FOR THE LIGHT SOURCE

From the arguments developed above it appears that the optical signal to noise ratio deteriorates rather rapidly if $\alpha_c$ becomes small against $\Delta\beta$. $\Delta\beta$ may therefore in a certain sense be considered as a threshold value, which imposes a limit upon the sensitivity that may be obtained in quantitative scanning, using the double-beam difference method.

To ensure good stability of $\Delta\beta$, a few precautions have to be taken: the voltage supplying the light source should be highly stabilized. Care should be taken to derive both beams from the same area of the lamp, because the temperature distribution and emission density within the lamp are not constant and may vary with time, supply voltage, etc. The lamp should be replaced in advance of obvious aging effects. An important source of error is the instability of the illuminating light source $I_0$. A change $\Delta I_0$ in $I_0$ produces a change in detector output $\Delta It$ (eqn. 8) proportional to the change $\Delta I_0$. The observer, however, attributes this change to an apparent change in useful signal output $\Delta I_c$. For $\Delta I_c$ we obtain the relation

$$\Delta It = \Delta I_0 \bar{A}(\Delta\beta - a_c) = \Delta I_c$$

$$\frac{\Delta I_c}{I_c} = \frac{\Delta I_0}{I_0}\left(\frac{\Delta\beta - a_c}{a_c}\right)$$

$$= \frac{\Delta I_0}{I_0}\left(\frac{\Delta\beta}{a_c} - 1\right) \tag{13}$$

From this expression it follows that the percentage error in the measured output will in general be larger than the percentage change in $I_0$, depending upon the ratio $\Delta\beta/a_c$. Again the consequences will be more serious with weaker concentrations.

For a very crude estimate of the value of $\Delta I_0$ let us assume an incandescent lamp where most of the energy supplied is emitted as radiant energy. If the supply voltage changes by $e$ % the emitted radiant flux $I_0$ changes approximately by $2e$ %. This underlines the importance of good stabilization of the supply voltages.

## NUMERICAL EXAMPLE

The best way to illustrate the results obtained above is probably a numerical example. The first value we have to consider in this context is the optical noise value of the medium itself. According to our own measurements as well as data obtained from the literature[2,9] the value of $\bar{\bar{\alpha}}$ with Whatman No. 3 paper is of the order of 0.05 optical density units. In natural units this is about 0.15. The spatial fundamental $F^*$ of the noise appears to be approximately 2 × 2 mm. The optical density of this type of paper is according to SALGANICOFF *et al.*[2] of the order of 3.4 units; this corresponds to a transmittance $\bar{A} \simeq 3 \cdot 10^{-4}$.

Let us now assume a single-beam instrument with an illuminated slit area 2 × 50 mm. The solute is assumed to be applied in bands, so that $F_c/F = 1$ and the spectral width of the beam shall be sufficiently narrow, so that $\Delta\lambda_c/\Delta\lambda = 1$. Using eqn. 12[1] we obtain a signal to noise ratio.

$$\frac{\sigma}{\sqrt{25}} \geqslant 2 \simeq \frac{\bar{a}_c}{0.15} \qquad (\sigma \text{ min} \simeq 10) \tag{14}$$

Assuming that for reasonable accuracy a minimum signal to noise ratio of 10 is required, we obtain the value of the smallest signal which can still be measured to about 0.30, that is $\approx 0.13$ optical density units.

Passing now to a double-beam difference forming device with the same area of illumination we obtain from eqn. 9a.

$$\sigma_1 = \frac{\bar{\alpha}_c}{0.15} \cdot \frac{\sqrt{2\bar{5}}}{\Delta\beta + \alpha_c}$$

$$\simeq 33 \cdot \frac{1}{1 + \frac{\Delta\beta}{\bar{\alpha}_c}} \qquad (15)$$

According to SALGANICOFF *et al.*[2] it seems that values of $\Delta\beta \approx 0.02$ may be obtained in practical operation. For a minimum value of $\sigma_1 \gtrsim 10$ we obtain a minimum value of $\bar{\alpha}_c \approx 1 \cdot 10^{-2}$, that is about 0.004 optical density units, as the weakest signal to be processed. Realization of this value, however, requires very careful optical design and an electrical arrangement with a low enough noise figure.

Subsequent integration over the area of the zone could improve those values by a factor of 2 to 3, depending upon the area of the zone; this applies of course to single-beam devices as well. Against the single-beam instrument the double-beam method offers an improvement of about 32 times. The error in determining a signal of this intensity will be about $\pm 1/\sigma \approx 10$ % due to optical noise plus a certain amount due to surface reflection, instability of the light source, etc., disregarding both $\bar{\bar{\gamma}}$ and the electrical noise. To obtain a value of $\Delta\beta$ of the order mentioned $\xi$ (see eqn. 5) has to be well below this value. Again assuming $\Delta W_c = 2$ mm and a paper velocity of 1 mm/sec, we obtain for a single photodetector arrangement a chopping frequency

$$\xi \leq 0.01$$

$$f = 200$$

To obtain improved accuracies at the same sensitivity or a higher sensitivity at the same accuracy, $\Delta\beta$ has to be decreased. Further improvement can be obtained by making use of the fact that the optical noise is affecting the useful signal in a multiplicative way. The residual noise in eqn. 15 can then be decreased by replacing the difference signal at the output by a ratio signal and using different chopping techniques. A planned device incorporating these features will be described shortly. If the optical and electrical noises are of comparable amplitude, their powers have to be added; this amounts to reducing the signal to noise ratio in eqn. 15 by a factor of $\sqrt{2}$. By the same factor, of course, accuracy is decreased and the minimum amount of investigated substance is increased. In general a design with both noise components equal will give the best trade off between performance and cost.

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# STANDARD COLUMNS AND OPERATING CONDITIONS FOR DIVERSE ANALYSES BY GAS CHROMATOGRAPHY

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SUMMARY

The construction and characteristics of standard gas chromatographic columns suitable for use in the separation of diverse mixtures of volatile compounds are given. A method for determining the best isothermal period and temperature programme rate for the standard columns is described and the operating conditions that are suitable for use with a wide range of stationary phases separating all types of solutes are tabulated. Chromatograms indicating the relative performance of such columns are given and an example demonstrating the reproducibility of the standardised columns and operating conditions is included.

## INTRODUCTION

Where a gas chromatograph is used for diverse applications ranging from the analysis of multi-component mixtures of wide boiling range to that of simple four or five component samples, much effort can be wasted in determining the optimum temperature programme to effect the required separation. Considerable time can also be spent in exploring the possibilities of different stationary phases when the required separation could have been obtained more quickly by using a standard stationary phase and choosing the correct column length. In the majority of gas chromatographic separations, special stationary phases are not usually necessary and if a number of columns of different lengths are available then two stationary phases are generally all that are needed. The two typical stationary phases that are normally used are a non-polar phase, *e.g.* Apiezon Grease, and a polar phase, *e.g.* PEG 20M. In order to standardize programming conditions, however, columns of known and reproducible resolving power must be available.

This paper describes a procedure for producing columns of different lengths having known and reproducible resolutions. An experimental method for determining the optimum initial isothermal period and programme rate is also described and the optimum programming conditions for each column length are given. Details of the construction and characteristics of seventeen columns are included, together with the respective optimum operating conditions that will give the best separation of the components of any type of sample without prior knowledge of its composition. The

only undefined variables left to the choice of the operator are the column length, the amplifier sensitivity and the chart speed.

## COLUMN CONDITIONS

In order to operate columns under standard temperature programming conditions it is necessary to have a number of standard columns available that can be packed reproducibly to give a specified performance. If columns differ in performance, then they will require a different set of programming conditions to obtain optimum resolution and this would be very time consuming to determine.

The choice of column material depends on the solutes to be chromatographed. Metal columns are rugged, easily packed and may be used provided thermally labile or easily adsorbed solutes are not present. Glass columns are more fragile and in some instruments can be difficult to change. If thick-walled glass tube is used (1 mm wall thickness) such columns can be operated at gas pressures of up to 250 p.s.i. If mixtures are to be separated that may contain thermally labile substances, glass columns should be employed.

Theoretically the column diameter should be as small as possible to provide the maximum efficiency but in order to allow the column to be effectively packed and cope with adequate loads the minimum diameter is limited. McKENNA AND IDLEMAN[1], and SCOTT[2] have suggested optimum diameters of 4 and 2 mm, respectively. As the 4 mm diameter column was easier to pack reproducibly and can carry charges up to 50 $\mu$l this was chosen as the diameter of the standard columns.

The support must be as inert as possible and so acid-washed, silanised Celite was used. The effect of particle diameter on column efficiency has been studied by MELLOR[3], DESTY *et al.*[4], and CHESHIRE AND SCOTT[5], who show generally that the smaller the particle size, the higher the efficiency obtained from the column. However, supports of small diameter produce a high resistance to carrier gas flow and a compromise has to be reached with respect to the inlet pressures available to the column. The support sizes that were used for the standard columns are shown in Table I.

TABLE I

SUPPORT SIZES FOR THE STANDARD COLUMNS

| *Column length (ft.)* | *Support size* |
|---|---|
| 5 | 100–120 BS mesh, 152–124 $\mu$ |
| 18 | 100–120 BS mesh, 152–124 $\mu$ |
| 50 | 80–100 BS mesh, 185–152 $\mu$ |

The support was coated in the normal manner, the stationary phase being dissolved in a suitable solvent. Care was taken to treat the support as gently as possible during the evaporation of the solvent and in subsequent handling, to prevent the production of "fines" by attrition. An even coating of stationary phase on the support is not essential as might be supposed. In Fig. 1 the HETP curve is shown for methyl and propyl acetates on a column packed with 12.5% w/w stationary phase on the support. One set of points is for the support coated directly with 12.5% w/w stationary phase, the other for a packing consisting of a mixture of equal quantities

of supports carrying 7.5%, 10.0%, 12.5%, 15.0%, and 17.5% w/w of stationary phase, respectively. It can be seen that both sets of points lie on the same curve, although one set is that from a column having a very wide range of film thickness and represents what might be considered a very poorly coated support.

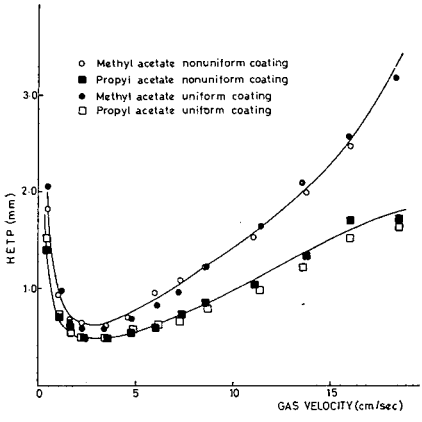


Fig. 1. HETP curves showing effect of homogeneity of support coating. Column temperature, 55°; support loading, 12.5% stationary phase.

The optimum quantity of stationary phase for maximum resolution and minimum analysis time varies with the type and molecular weight of the solute being separated, and the operating temperature. The optimum loading of stationary phase ranges from about 5 to 15% w/w, and to cope with mixtures of solutes having a wide range of molecular weights and polarities under temperature programming conditions, 10% w/w of stationary phase was taken as the standard support loading.

Each column was packed by attaching it to a pressurised funnel loaded with packing and applying a vacuum to the column exit. The packing was then transferred from the funnel into the column. A small wad of quartz wool at the end of the column prevented the packing from being lost down the vacuum line. After filling the column a pressure of 100 p.s.i. was applied to the inlet and the maximum packing density obtained by gentle tapping. The maximum column efficiency was then determined for a series of fatty acid methyl esters from $C_{10}$ to $C_{18}$ at 200° using the appropriate gas velocities for each column length as described later. A minimum efficiency of 600 plates/ft. was accepted; if this was not achieved the column was removed from the oven, and reattached to the vacuum and pressure lines. The column was again vibrated, more packing added and the efficiency measured again. This process was repeated until the required efficiency of 600 plates/ft. was achieved. It was found that all columns could eventually be made to give the required efficiency, but in some instances, particularly for the longer columns, it was found that the packing procedure had to be repeated as many as six times. The packing efficiencies obtained from a series of 5-ft., 18-ft., and 50-ft. columns are shown in Table II.

The relative lengths of the standard columns had to be determined by the increase in resolution required on changing from one column to another. Defining resolution as the ratio of the distance between two adjacent peaks to the average

TABLE II

EFFICIENCY OBTAINED FROM PEG COLUMNS OF DIFFERENT LENGTHS RECORDED IN PLATES/FOOT

| | *5-ft. column* | *18-ft. column* | *50-ft. column* |
|---|---|---|---|
| 1 | 625 | 660 | 659 |
| 2 | 640 | 600 | 600 |
| 3 | 640 | 600 | 570 |
| 4 | 690 | 711 | |
| 5 | 685 | 645 | |
| 6 | 680 | 744 | |
| 7 | 630 | 680 | |

peak width at the base, then the resolution is given by the following equation[6]:

$$R = \frac{(K_A - K_B)v_l n}{4(n)^{1/2}(v_g + Kv_l)}$$

where $R$ = resolution,
$K_A$ and $K_B$ distribution coefficients of solutes A and B, respectively,
$v_g$ = volume of gas per plate,
$v_l$ = volume of stationary phase per plate,
$n$ = efficiency in theoretical plates,

$$K = \frac{K_A + K_B}{2}$$

Thus

$$R = \frac{(K_A - K_B)v_l(n)^{1/2}}{4(v_g + Kv_l)}$$

If the same packing is employed and all columns are packed to give the same number of theoretical plates/ft. then $K_A$, $K_B$, $v_l$ and $v_g$ are all constant and $n$ will be proportional to $l$, the column length. Thus, as the resolution $R$ is proportional to $\sqrt{n}$, the resolution will also be proportional to $\sqrt{l}$.

Fig. 2 shows how the resolution of two peaks increases as the length of the column is increased. The improvement required in resolution by increasing the column length is very much a matter of arbitrary choice; for a significant increase in resolution it can be seen that the column length must be increased by a factor of 3 or 4 (*cf.* Fig. 2). As the standard columns were to be used with the Pye 104 gas chromatographs, the standard lengths were taken to suit the sizes available for these instruments, *viz.* 5 ft., 18 ft., and 50 ft. These columns give ratios of increase in length of 3.6 and 2.8, respectively, and thus the increase in resolution from column to column is 1.9 and 1.72, respectively.

Each column had to be used at a specific gas velocity and this was determined from the HETP curves of the respective columns. In Fig. 3, the HETP curves are shown for three standard columns packed with the standard packing carrying PEG 20M as the stationary phase. The curves were obtained by chromatographing a series of fatty acid methyl esters ($C_{10}$-$C_{18}$) at 200° at different gas velocities. The maximum

efficiency was obtained at each gas velocity by plotting efficiency against the retention time of each ester.

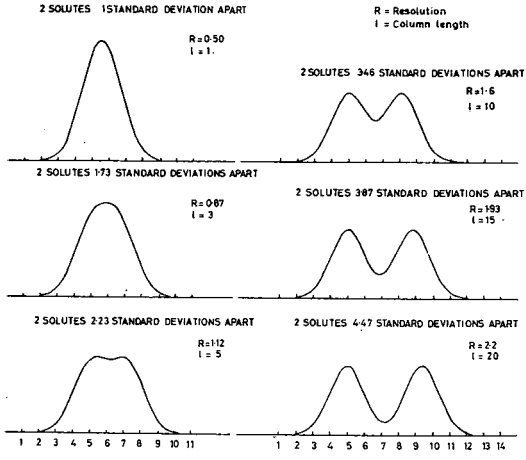


Fig. 2. Elution curves showing change in resolution with column length.

The efficiency increases at first and then levels to a constant value at about $C_{14}$ to $C_{18}$. The constant value was taken as the maximum efficiency at that particular flow rate and from the series of such values the HETP was calculated at each gas velocity in the usual way[7]. It is seen from Fig. 3 that the gas velocities to be used for the 5-ft., 18-ft. and 50-ft. columns are 4.5, 3.5 and 3.0 cm/sec, respectively. These velocities correspond to values about half way between the optimum gas velocity and the optimum practical gas velocity for each column[8]. The decrease in standard gas velocity with column length reflects the increase in resistance to mass transfer affecting the HETP of the larger columns due to the higher column pressures reducing the diffusivity of the solute in the gas phase.

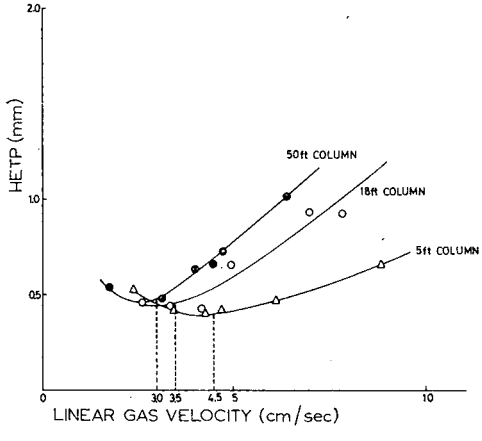


Fig. 3. HETP curves for standard columns, 5, 18 and 50 ft. long.

STANDARD CONDITIONS FOR TEMPERATURE PROGRAMMING

Any standard programming condition that would be applicable to the separation of diverse mixtures must be suitable for use with solutes and stationary phases of all polarities. It was therefore necessary to establish that the conditions would apply to solute types ranging from paraffins to alcohols, and stationary phases ranging from the non-polar, *e.g.* Apiezon Grease, to the polar, *e.g.* PEG 20M. For this reason, work on the 5-ft. column was carried out using both Apiezon Grease and PEG 20M as stationary phases and homologous series of hydrocarbons, esters and alcohols as solutes. Having established that the same optimum conditions applied for both Apiezon Grease and PEG 20M on the 5-ft. column, to conserve effort the optimum conditions for the 18-ft. column were determined using only PEG 20M as the stationary phase. However, the homologous series of paraffins, esters and alcohols were still used as solutes. The experiments carried out on the 5- and 18-ft. columns established that the same optimum programming conditions applied to all solutes and therefore the standard programming conditions were determined for the 50-ft. column using only PEG 20M as the stationary phase and the homologous series of esters as the solutes.

*Initial isothermal period*

The initial isothermal period had to be determined such that the maximum column resolution was obtained before the temperature programme was commenced. The homologous series of solutes were as follows: *n*-paraffins $C_6$–$C_{20}$; *n*-fatty acid methyl esters $C_2$–$C_{18}$; *n*-alcohols $C_1$–$C_{16}$. Samples of each of the homologous series were chromatographed on the 5-ft. column at approximately 50, 100, 150 and 200°, respectively, using both Apiezon Grease and PEG 20M as stationary phase. The normal alcohols were not chromatographed on the Apiezon Grease due to peak asymmetry. From the chromatograms obtained, the resolution of the column was calculated, for each carbon number of each homologous series and for each stationary phase, using the following equation:

$$R_n = \frac{y_{(n+1)} - y_n}{x_{(n+1)} + x_n}$$

where $y_{n+1}$ is the retention distance of homologue $(n+1)$ from injection;

$y_n$ is the retention distance of homologue $(n)$ from injection;

$x_{n+1}$ is the peak width of homologue $(n+1)$ taken at the points of inflexion;

$x$ is the peak width of homologue $(n)$ taken at the points of inflexion.

The graphs of resolution against retention time for each homologue of each series on each stationary phase for the 5-ft. column are shown in Fig. 4. It is seen that over the range of temperatures used the resolution between carbon numbers for the three series of solutes on both stationary phases at first increases with retention time and then tends to level to a constant value. Although the absolute values for the maximum resolution obtained for each series on each phase differ as they are different solute types, the maxima are achieved at about the same retention time.

The isothermal period for the 5-ft. column was taken as the mean value of the retention times where the resolution of the column reached 95% of its maximum

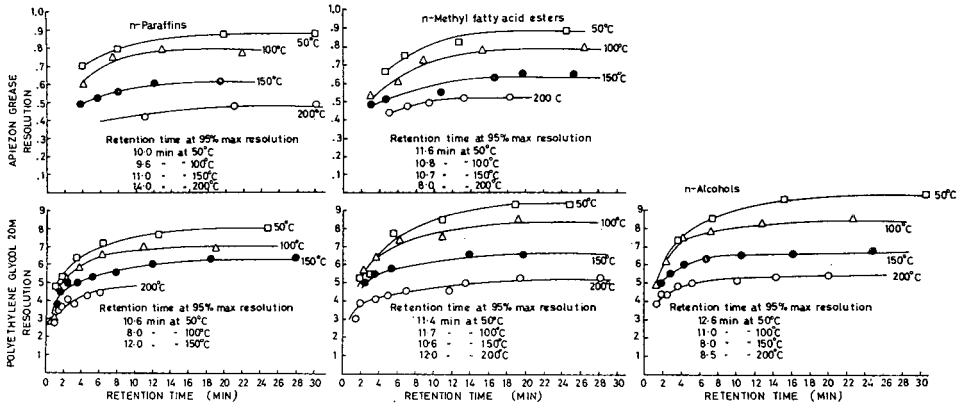


Fig. 4. Graphs of resolution against retention time for different solutes and stationary phases on a 5-ft. column.

value. The value of 95% maximum resolution is an arbitrary choice that attempts to compromise between adequate resolution and reasonable analysis time. (It is seen that to realise the extra 5% resolution would extend the isothermal period by about 50%.) On this basis the isothermal period for the 5-ft. column was taken as 11.0 min, the range between the different series and different stationary phases being min. 8 min–max. 14.0 min.

The same experiments were carried out on the 18-ft. column with PEG 20M as the stationary phase and the three homologous series of solutes. The 50-ft. PEG column was also examined using PEG 20M as the stationary phase at 200° but only the ester series was used as solutes. The initial isothermal periods were determined in the same way and found to be 39 min (min. 25 min–max. 57 min) and 60 min for the 18-ft. and 50-ft. columns, respectively. The graphs relating resolution and retention time for the 18-ft. and the 50-ft. columns are shown in Fig. 5.

It is seen that the intervals taken for the isothermal period are the averages of series of values that have a significant standard deviation. However, as the curves are fairly flat over this range, the corresponding standard deviation of the values for resolution about the mean interval that is taken for the isothermal period is much smaller, and constitutes only 2–3% of the maximum resolution. This justifies taking the intervals given above as standard isothermal periods for the column concerned.

*The optimum programme rate*

To determine the optimum programme rate, the homologous series of solutes were chromatographed on the different length columns at a series of different programme rates and the resolution between carbon numbers of each series was calculated. The details of the set of experiments which was carried out are shown in Table III. The results for the 18-ft. column packed with PEG as a stationary phase will be discussed first. The results obtained for this column, separating the three homologous series of alcohols, esters and alkanes, are shown in Fig. 6. The results for the normal alcohols show that the slower the programme rate the greater the resolution between each solute. At the higher programme rates there is a minimum in the resolution

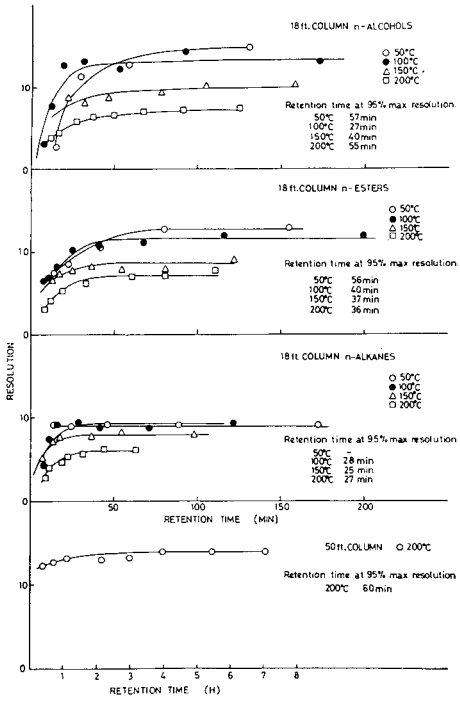


Fig. 5. Graph of resolution against retention time for different solutes on the 18- and 50-ft. columns.

TABLE III

EXPERIMENTAL CONDITIONS TO EXAMINE EFFECT OF PROGRAMME RATE ON THE RESOLUTION OBTAINED FROM EACH COLUMN

| | *5-ft. column* | *18-ft. column* | *50-ft. column* |
|---|---|---|---|
| Stationary phase used | Apiezon L grease<br>PEG 20M | PEG 20M | PEG 20M |
| Homologous series of solutes used | *n*-Alkanes $C_6$–$C_{20}$<br>*n*-Methyl esters $C_2$–$C_{18}$<br>*n*-Alcohols $C_1$–$C_{16}$ | *n*-Alkanes $C_6$–$C_{20}$<br>*n*-Methyl esters $C_2$–$C_{18}$<br>*n*-Alcohols $C_1$–$C_{16}$ | *n*-Methyl esters $C_2$–$C_{18}$ |
| Initial isothermal period (min) | 11 | 39 | 60 |
| Temperature programme rate (°C/min) | 0.5, 1, 2, 3, 4 | 0.25, 0.5, 1, 2, 3 | 0.15, 0.25, 0.5, 1, 2 |

that occurs between carbon numbers 10 and 12. This is due to the fact that the rapid rise in temperature has reduced the separation ratios to a greater extent than it has reduced the peak width. When the maximum temperature has been reached, however, the column is operating under isothermal conditions and the resolution increases again up to carbon number 15. The same situation occurs with the curves for the

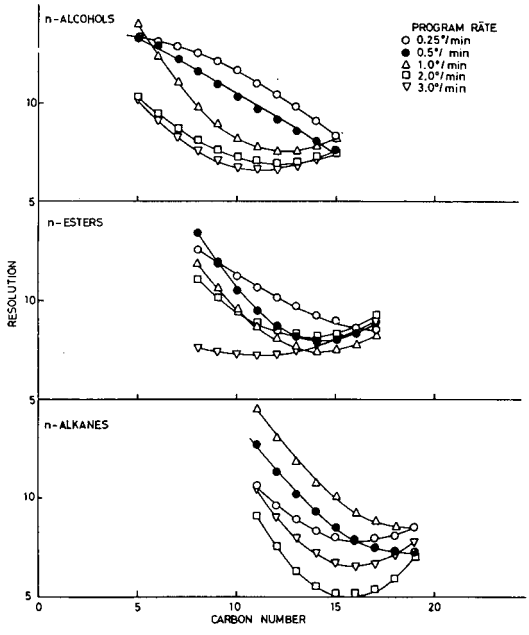


Fig. 6. Graph of resolution against carbon number for the 18-ft. standard column operated at different programme rates.

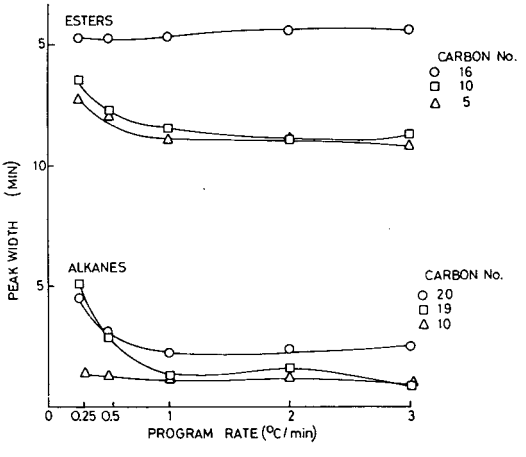


Fig. 7. Graph of peak width against programme rate for the 18-ft. standard column separating an homologous series of esters and *n*-paraffins.

esters. Again the higher the programme rate the higher the resolution and again minima occur in the resolution curves at the higher programme rates. For the normal alkanes, however, the situation is quite different. The maximum resolution is obtained at a programme rate of 1°/min and poorer resolution obtained at lower or higher programme rates than this. The reason for this can be seen from the results given in

Fig. 7, which are plots of peak width against programme rate for three members of the ester and alkane series. For the ester it is seen that as the programme rate increases the band width either remains constant or falls. In the case of alkanes, however, although the same is true for carbon numbers 10 and 20, the intermediate homologue 14 shows a maximum in the peak width at a programme rate of 2°/min. Now it was shown by SCOTT AND HAZELDEAN[8] that the resistance to mass transfer factor affecting the band width had a maximum value at a particular distribution coefficient or carbon number for a given homologous series. Furthermore this maximum in the resistance to mass transfer varied with the absolute temperature[9]. Thus for the alkanes we see this curious effect of a primary fall in band width as the programme rate increases and subsequent increase in band width. Because of this we have the optimum programme rate shown in Fig. 6 for the alkanes.

The best programme rate must always be a compromise between resolution and analysis time, so to determine this compromise the average resolution for the entire homologous series at each programme rate was plotted against programme rate for each column in Fig. 8.

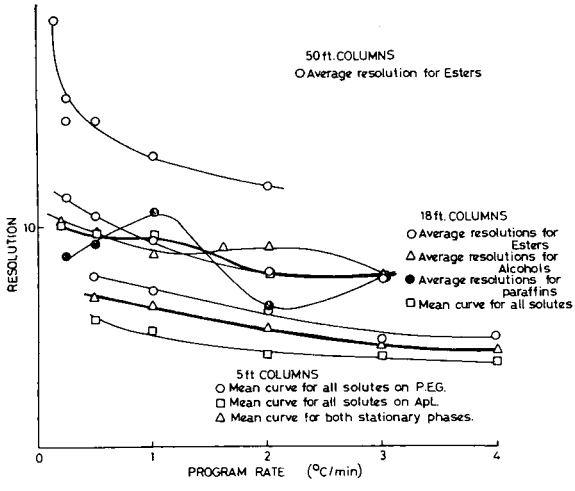


Fig. 8. Graph of resolution against programme rates for different length columns, different homologous series of solutes and different stationary phases.

Still concerning ourselves with the 18-ft. column we see that the average value for the resolution of the homologous series of esters falls continuously as the programme rate increases whereas the average value for the alkanes shows the S shape curve that follows logically from the results in Fig. 6. The heavy line for the 18-ft. column in Fig. 8 shows the mean curve obtained by averaging the three individual curves for each homologous series. As the columns have to cope with a complete range of solutes over a range of polarities, this mean curve will give the best programme rate for the 18-ft. column. It can be seen that this must be chosen at 1°/min as this gives 95% of the maximum resolution obtainable and that a further 5% increase in resolution can be obtained only at a sacrifice of four times the analysis time.

Considering now the 5-ft. column, the curves for each homologous series on

each stationary phase were averaged giving the light curves shown in Fig. 8. It is seen that the shape of these curves for both the polar stationary phase PEG and the non-polar stationary phase Apiezon L is the same and thus the optimum programme rate can be deduced from a mean of these two curves (shown as the heavy line for the 5-ft. column in Fig. 8). Arguing on the basis of the best resolution commensurate with a reasonable analysis time, it follows that the optimum programme rate will be 2°/min. Although operating at a programme rate of ½°/min would increase the resolution by about 8%, this would result in the analysis time being four times as long.

Finally, considering the 50-ft. column, which to economise on time was only examined using the methyl esters as solutes, the optimum programme rate is more of an arbitrary choice. The highest resolution was obtained for a programme rate of 0.15°/min but this resulted in an analysis time of nearly 26 h. However, to obtain as high a resolution as possible commensurate with reasonable analysis time a ¼°/min programme rate was chosen as the standard.

## CONCLUSION

The results indicate that for the analysis of diverse mixtures the standard columns and operating conditions shown in Table IV should be used. These conditions have been used with six chromatographs over a period of a year and found to significantly reduce the time spent on chromatographic analysis and to provide more reliable

TABLE IV

CHARACTERISTICS OF STANDARD COLUMNS AND OPERATING CONDITIONS

| | *5-ft. column* | *18-ft. column* | *50-ft. column* |
|---|---|---|---|
| Column diameter (mm) | 4 | 4 | 4 |
| Support particle size (BS mesh) | 100–120 | 100–120 | 80–100 |
| Stationary phase loading (%) | 10 | 10 | 10 |
| Minimum packing efficiency (plates/ft.) | 600 | 600 | 600 |
| Gas velocity (cm/sec) | 4.5 | 3.5 | 3.0 |
| Initial isothermal period (min) | 11 | 39 | 60 |
| Temperature programme rate (°C/min) | 2 | 1 | 0.25 |

results from the point of view of reproducibility. During this period a wide variety of samples have been analysed and for no sample was it found necessary to deviate in any way from the standard conditions to obtain satisfactory results. An example of the results obtained using the standard columns for the separation of a complex essential oil, inchigrass oil, is shown in Fig. 9a, b and d. One sees the advantage of increasing lengths from 5 ft. to 18 ft. and then to 50 ft., the analysis time being 1 h 30 min, 4 h, and 13 h for each column, respectively. These columns can operate with loads ranging from 50 μg to 50 mg, maintaining approximately the same resolution. The reproducibility of a standard column is shown by comparing the chromatograms shown in Fig. 9b and c. These two chromatograms were obtained from two different standard 18-ft. columns in two completely different Pye 104 gas chromatographs using the standard operating conditions. The reproducibility can be seen to

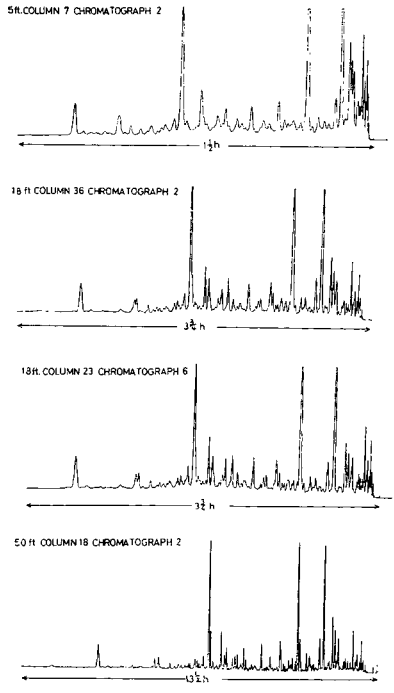


Fig. 9. Chromatograms of inchigrass oil on different standard columns.

be extremely good. This ease of reproducing identical chromatograms from the same sample with different instruments has been a great help when subsequent to preliminary analysis, the mass spectra or infrared spectra are required from specific solute peaks. With the use of standard columns and operating conditions the peaks of interest can be easily and reliably picked out even though run on another instrument in another laboratory.

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CHROM. 4369

# THE CHROMATOGRAPHIC PROPERTIES OF POLYETHYLENE AND POLYPROPYLENE

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SUMMARY

The chromatographic properties of polyolefin molding powders have been determined. Polyethylene molding powders, both high- and low-density, appear to be useless. Uncoated polypropylene on the other hand appears to separate organic compounds from one another by a gas–liquid partition mechanism. The uncoated polypropylene columns are very good for trace water analysis in organic compounds. Coated polypropylene is useful for some separations. Theoretical considerations of the separations are discussed.

## INTRODUCTION

In the past few years there has been a great deal of research on nondiatomite solid supports for gas chromatography. The reason for this research is the fact that there has been increasing interest in the analysis of polar compounds as well as compounds which attack siliceous surfaces. OTTENSTEIN has reviewed much of this research up to 1965 (ref. 1).

Of the nondiatomite supports, the most widely used have been the fluorine-containing polymers. These materials have very non-adsorptive surfaces; hence, compounds as polar as water do not interact or tail seriously. These materials do have their disadvantages however. They are costly, some have very bad handling properties and some lack efficiency. Considering the disadvantages of the fluorine-containing supports, the authors became interested in the possible use of the various polyolefin polymers.

A review of the Gas Chromatography Abstracts yielded only two papers on the subject. In 1963 BAUM reported the separation of lacquer thinner on columns containing low-density polyethylene[2]. The next year LECHNER-DECHATEL reported the separation of perchlorocyclopentane from mixtures of chlorinated cyclopentadiene on 0.2–0.3 mm (55–75 mesh) polyethylene powder coated with 1 % Apiezon N[3].

In an effort to understand better the mechanism of separation and extend the

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work on polyolefins the authors felt that a critical evaluation of the materials was in order.

## EXPERIMENTAL

The polyolefins used in this study were solicited from the primary manufacturers of the polymers in the United States. The polymers received were full-range powders and required screening. The 30–60 mesh cuts were used for the study because most of the powders had a good percentage of powder in this range. Surface areas were determined after drying at 60° under reduced pressure (about 20 mm Hg) (see Table I). The surface areas of the various polymers can be easily explained in terms of their history of manufacture. Of the many samples of polymer received, three samples of low-density polyethylene, three samples of high-density polyethylene and two samples of polypropylene were chosen for chromatographic study.

TABLE I

SAMPLES OF POLYOLEFINS EVALUATED

| *Manufacturer* | *Polyolefin* | *Type* | *Surface area* ($m^2/g$)[a] |
|---|---|---|---|
| | *Polyethylene* | | |
| DuPont | Alathon 1730 | Low-density | 0.05 |
| DuPont | Alathon 7050 | High-density | 0.05 |
| U.S.I. | Microthene 710-20 | Low-density | 0.03 |
| U.S.I. | Microthene 722 | Low-density | 0.04 |
| Phillips | Marlex 525 | High-density | 0.27 |
| Celanese | Fortiflex A60-500 | High-density | 0.61 |
| | *Polypropylene* | | |
| Phillips | PD 500 | | 0.33 |
| Chevron | 9094F | | 1.82 |

[a] Determined by a variation of the dynamic BET method. The numbers are based on one BET plot.

The columns evaluated were prepared from the various polymers and stationary phases and were packed in 6-ft. lengths of 1/4-in. copper tubing. The squalene columns were prepared from Eastman practical grade squalane and were deposited from hexane. The Carbowax 600 columns were prepared from Union Carbide Carbowax 600 and were deposited from acetone. The diglycerol columns were prepared from diglycerol obtained from the Applied Science Laboratory and were deposited from methanol. All the coating was done in polypropylene beakers on a steam bath. The polypropylene beakers are necessary because glass competes for the stationary phase at the expense of the polymer packing. The columns were packed vertically with mechanical agitation. The handling properties of the polymers are very good. They are hard particles which handle much like the common supports. They develop static charge only slightly.

A MicroTek Model 2500R with a Sargent Model SR (1 mV) recorder was used in the study. Flow rates were measured with a soap film flow meter. The instrumental conditions were as follows: injection port temp., 130°; detector block temp., 150°;

detector current, 400 mA; the column temperature was variable. Dry helium was used as the carrier gas. Samples were introduced with a 10 $\mu$l Hamilton syringe.

Several test mixtures were used in the study. Unless otherwise stated, 1-$\mu$l samples of the test mixtures were used. The test mixtures are listed in Table II.

TABLE II

COMPOSITION OF TEST MIXTURES

| *No.* | *Component* | *Amount (g)* |
|---|---|---|
| 1 | *n*-Butanol | 16.9 |
| | Water | 12.0 |
| | Acetone | 6.6 |
| | Ethyl acetate | 15.4 |
| 2 | Ethanol | 12.7 |
| | Methanol | 11.2 |
| | *n*-Propanol | 12.7 |
| 3 | Water | 5.4 |
| | Acetone | 13.4 |
| | *n*-Propanol | 15.2 |
| | Ethyl acetate | 19.6 |
| 4 | Water | 5.9 |
| | Acetone | 17.5 |
| | Ethyl acetate | 19.9 |
| 5 | Water | 20.2 |
| | Methanol | 23.8 |

## RESULTS AND DISCUSSION

In this paper, the authors will use three common approaches to the systematic description of a gas chromatographic support. The first approach used will be a description of what the columns will separate. While this approach is probably of the most utility to the practical gas chromatographer, there are also two other quite useful theoretical approaches. The first theoretical approach used will be a study of the variation in efficiency with the variation in other experimental parameters. The second theoretical approach used will be a study of the variation of specific retention volume with increasing loading.

Before a discussion of specific data is presented it should be pointed out that a support to be used in classic gas–liquid chromatography must be very inert with respect to the sample. It is in this respect that the diatomaceous earth support fall short in the analysis of polar compounds. Unfortunately the authors have found that the polyolefins also suffer to varying degrees from a lack of inertness. In direct contrast to BAUM's work, we have found that low-density polyethylene (Mn 710-20, Mn 722 and 1730) is useless as a support. Of the compounds in the test mixtures only water is rapidly eluted, with alcohols eluted as broad bands. Hydrocarbons are not eluted at all or are eluted at concentrations below the limits of detections. The differences between our work and BAUM's may be due to the differences in polymers or additives in his polymer. His polymer was Mipor No. 14PN-G (ESB-Reeves Corporation, Glenside, Pa., U.S.A.) and is said to have a pore size of approximately 10 $\mu$, while our polymers are of very low surface area.

High-density polyethylene (M525, A60-500 and 7050), while more inert than

low-density polyethylene, is still useless as a support. This is surprising in view of higher surface areas of the two samples. This also makes the significances of the surface area explanation in the preceding paragraph questionable.

On the other hand, the lack of inertness has not been as serious for polypropylene. While few compounds are not retained, those which are retained give good peaks with respect to tailing. Since some important separations can be made on the coated as well as the uncoated polymer, studies on both have been carried out.

*Uncoated polypropylene*

While it was found that uncoated polypropylene was not very inert toward most organic compounds, it was found that it does have the ability to separate many compounds. Consider the typical chromatograms in Figs. 1–3.

In an effort to understand better the separations taking place, efficiency studies were carried out. The variations in efficiency with temperature, flow rate, sample

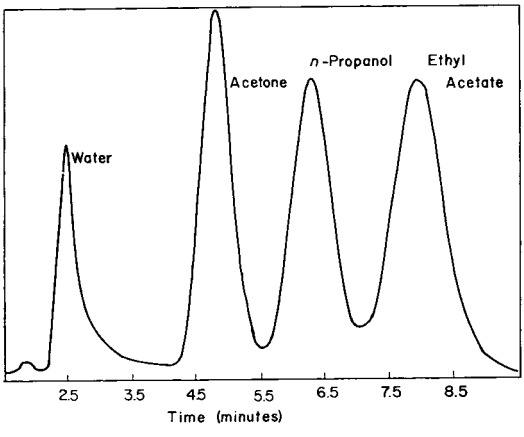


Fig. 1. Separation of Text Mixture 3 on 30–60 mesh Polypropylene PD 500. Column temp., 130°; flow rate, 10 ml/min; sample size, 1 $\mu$l.

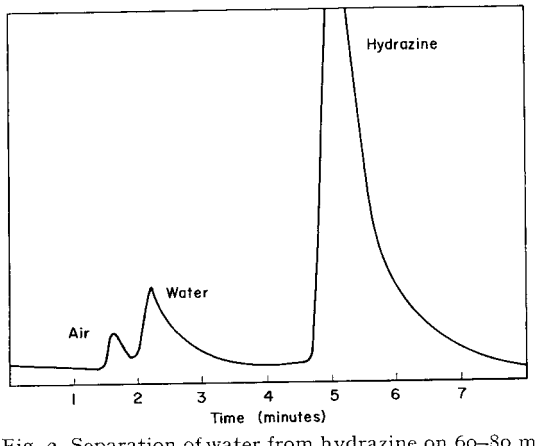


Fig. 2. Separation of water from hydrazine on 60–80 mesh Polypropylene PD 500. Column temp., 105°; flow rate, 25 ml/min; sample size, 1 $\mu$l.

size and mesh size of the support have all been determined. It must be pointed out, however, that the interpretation of the data may be somewhat artificial. The theory of gas–liquid chromatography is based on columns containing a stationary phase; we speak of diffusion in the liquid phase and liquid film thickness in these theories– hence the discussion of data on polypropylene columns is not realistic in the classical sense.

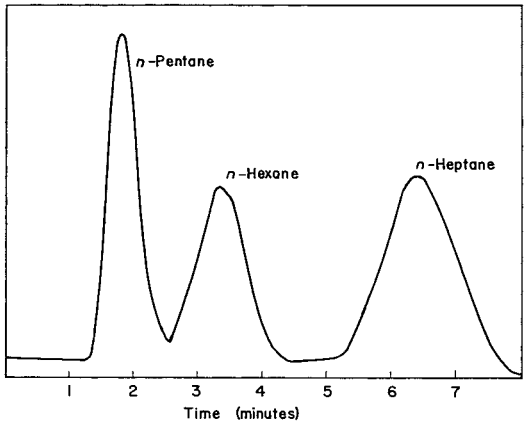


Fig. 3. Separation of *n*-pentane, *n*-hexane and *n*-heptane on 30–60 mesh Polypropylene PD 500. Column temp., 100°; flow rate, 50 ml/min; sample size, 1 $\mu$l.

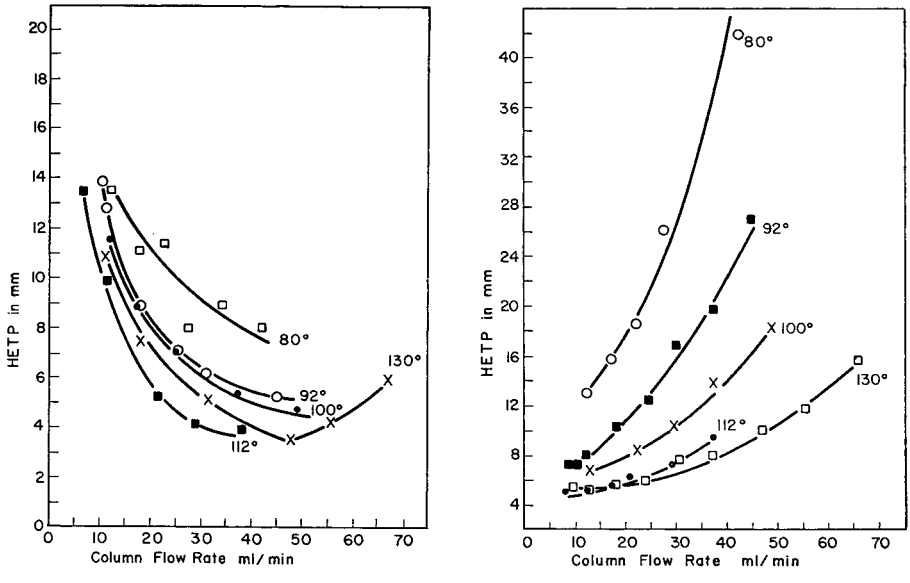


Fig. 4. Van Deemter plot showing the variation of efficiency (HETP) with changes in column flow rate (ml/min) at five temperatures. Column: 30–60 mesh Polypropylene PD 500; sample: water from 1 $\mu$l of Test Mixture 3.

Fig. 5. Van Deemter plot showing the variation of efficiency (HETP) with changes in column flow rate at five temperatures. Column: 30–60 mesh Polypropylene PD 500; sample: ethyl acetate from 1 $\mu$l of Test Mixture 3.

On the other hand, the column appears to be separating the organic compounds on the basis of a gas–liquid partition mechanism rather than adsorption. The peaks are very symmetrical and in addition many of the trends found in gas–liquid chromatography data also appear in this evaluation. Hence, it appears reasonable to view the separations as gas–liquid in nature.

In the study of the polypropylene columns, Test Mixture 3 containing water, acetone, *n*-propanol and ethyl acetate was injected and the efficiency was calculated from the chromatograms. All of the organic compounds gave much the same shaped curves; hence only one plot for organic compounds is shown. In Figs. 4 and 5 Van Deemter plots for water and ethyl acetate of Test Mixture 3 are shown at five different temperatures. The plots for the organic compounds deviate from the hyperbolic shape predicted by theory. The fact that the polypropylene column is limited as to transfer probably accounts for the atypical shape.

The improvement of efficiency with increasing column temperature is often found for gas–liquid columns and is explained by the decrease in the ratio of $d_1^2/D_1$, where $d_1$ is the liquid film thickness and $D_1$ is the solute diffusivity in the liquid phase. This same explanation is not too unrealistic for plastic materials if the gas–liquid mode of separation is allowed for such a column.

With respect to the Van Deemter plots for water, the data are much the same as the data found on all columns for a nonpartitioned material. The anomalous curve at 130° can only be explained by increased interaction of water vapor with the polymer at higher temperatures where the polymer is softer.

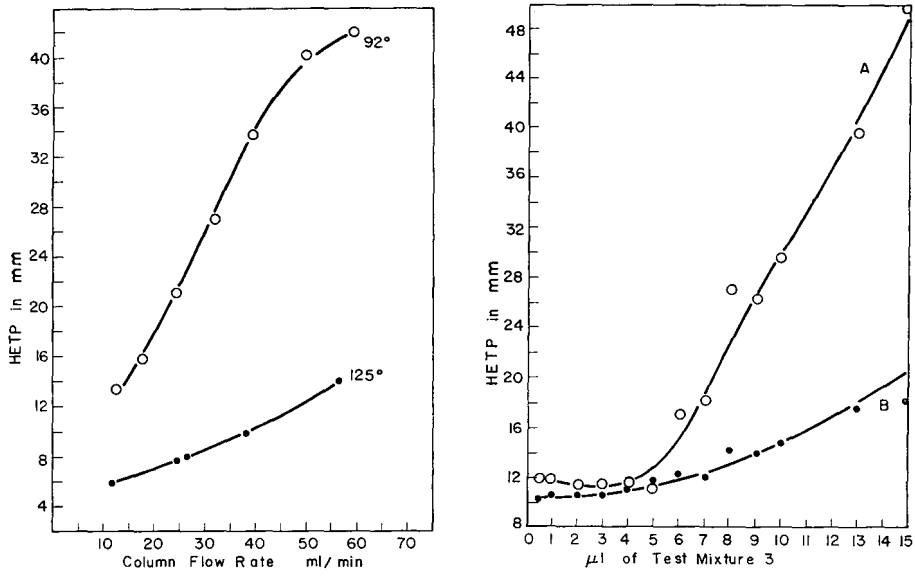


Fig. 6. Van Deemter plot showing the variation of efficiency (HETP) with changes in column flow rate (ml/min) at two temperatures. Column: 30–60 mesh Polypropylene PD 500; sample: 1 μl hexane.

Fig. 7. The variation of efficiency with increasing sample size. Column, 30–60 mesh Polypropylene PD 500; column temp., 100°; column flow rate, 13 ml/min; sample, (A) water from Test Mixture 3; (B) *n*-propanol from Test Mixture 3.

To confirm these findings, the author studied the variation in efficiency for a normal hydrocarbon. The data are shown in Fig. 6; they are in complete agreement with the previous data.

In Fig. 7 the variations of efficiency with sample size are reported for water and *n*-propanol of Test Mixture 3. The organic compounds all show much the same dependence. This finding agrees with the gas–liquid partition mechanism proposed. On the other hand, the effect of the sample size on the shape of the water peak is quite different. The shape of the curve could be explained by the limited solubility of water in the polymer or by a nonlinear partition isotherm. Peak shape variations of the type found are often found in gas–solid chromatography.

To prove that the separations were being caused by polypropylene and not by some unknown phenomenon, a second sample of different manufacture was considered. The material was Chevron Polypropylene 9094F with a surface area of 1.8 $m^2/g$. (The previous work was on Phillips Polypropylene PD 500 with a surface area of 0.33 $m^2/g$.) The results were similar to those found on PD 500. The peaks from organic compounds were still very symmetrical, and the water peak was still a little tailed.

In Fig. 8 the efficiency for 60–80 mesh PD 500 *vs.* flow rate is shown. This is atypical with respect to common coated columns because the efficiency for 60–80 mesh material is no better than for 30–60 mesh material. This indicates that the small particles that make up the larger particles are limiting the efficiency.

Summarizing the data, it has been found that polypropylene can separate mixtures of various compounds. Water for all practical purposes is not retained by the columns but does tail slightly. The origin of the tailing is thought to be either adsorption, or the limited solubility of water in the polymer.

On the other hand, the separation of organic compounds appears to be caused

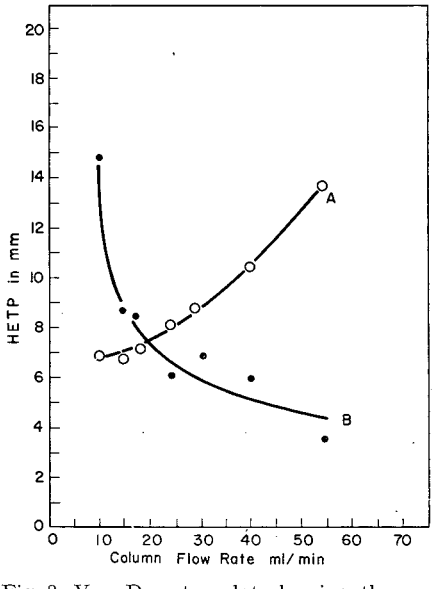


Fig. 8. Van Deemter plot showing the variation of efficiency (HETP) with column flow rate (ml/min). Column, 60–80 mesh Polypropylene PD 500; column temp., 100°; sample, acetone and water from 1 $\mu$l of Test Mixture 3. A=acetone; B=water.

by a gas–liquid partition mechanism. Their behavior can be successfully explained on the basis of gas–liquid chromatography theory.

While the partition of a gas on a plastic seems a little strange, it has been reported earlier. LYSYJ AND NEWTON[4] prepared a column packing from 50 % Kel-F 300 solid support and 50 % halocarbon oil. The packing was plasticized by refluxing with trichloroethylene. The finished packing was a 50:50 mixture and was a free flowing powder. This monophase gel, as they called it, had good separating ability for halogenated samples. The author believes that separations on polypropylene are similar in nature to those described by LYSYJ AND NEWTON.

Efficient separations are favored by high column temperature and slow flow rates. The practical temperature limit is above 130°. The softening point of polypropylene is 150°. Although the author never worked above 130°, operation up to 150° appears practical. This might improve efficiency even more if the general trend holds up to 150°.

From the practical point of view, the most significant finding of this study is that polypropylene, in the dry state, can separate most organic compounds from water. Hence, these columns are quite useful for water analysis. Using the 60–80 mesh PD 500 polymer, the author set up a standard curve for water in absolute ethanol.

From the theoretic point of view, the most significant finding of this study is that polypropylene is behaving much like other polymer supports. This means that polypropylene that has been specially prepared for chromatography could be very useful.

*Coated polypropylene*

While the use of polypropylene as a generally useful support has been precluded by its lack of inertness, the material is useful as a coated support in several specific cases. As was stated earlier, water and most one-carbon organic compounds are not retained significantly by the polymer. In addition, some inorganics are not retained. A good example of a difficult separation which is possible on a Carbowax polypropylene

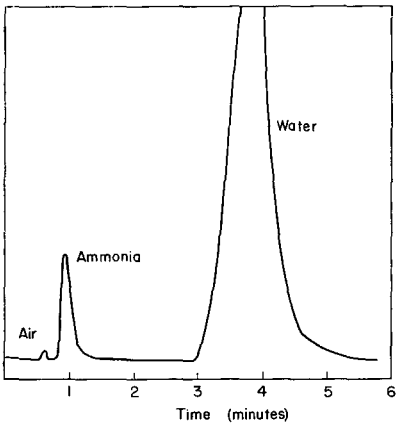


Fig. 9. Representative chromatogram showing the separation of ammonia from dilute ammonium hydroxide. Column, 2.5% Carbowax 600 on 30–60 mesh Polypropylene PD 500; column temperature, 85°; flow rate, 30 ml/min; sample, 1 $\mu$l dilute ammonium hydroxide.

column is the separation of ammonia from dilute ammonium hydroxide solution, shown in Fig. 9.

The feasibility of useful separations on coated polypropylene columns warranted a study of the columns. The author wished to determine the optimum operating conditions and the effect of the stationary phase surface energy.

In order to determine the optimum flow rate for columns prepared from polypropylene, three columns were prepared which contained 5 % loading of squalene, Carbowax, and diglycerol, respectively. The variation in efficiency with changing flow rate was determined using one-microliter samples of Test Mixture 5. The chromatograms and Van Deemter plots show that the columns are all badly limited by mass transfer. Only the plot for the squalene column shows any indication of passing through a minimum. Although an absolute comparison of the HETP data for the three columns is not possible owing to differences in stationary phase viscosity and column temperature, the general trends can be explained in terms of the wetting ability of the stationary phase on the low surface energy support. Diglycerol with its very high surface energy probably cannot wet the polypropylene surface. The fact that the surface is not wetted means that the "film" thickness will be very large, hence the column is badly mass transfer limited. Carbowax and squalene appear to wet the polypropylene surface somewhat better. The Van Deemter plot for the squalene column is typical. Squalane probably wets the support much better than Carbowax.

A second series of polypropylene columns was prepared from squalane and Carbowax 600. Columns containing 2.5 %, 5 %, 10 %, 15 % and 20 % loadings of the stationary phases were studied to determine the variation of efficiency with loading. Performance of columns with more than 5 % diglycerol was too poor to warrant further study. The data indicate that the efficiency decreases with increasing loadings. This fact can be accounted for by the increase of the film thickness of the stationary phase.

To clarify further the behavior of coated polypropylene columns, specific retention volumes for columns of varying loading were obtained. The data indicate that the sample is being adsorbed on or dissolved in the polymer when the columns are lightly loaded.

The work on coated polypropylene columns has shown that polypropylene is useful as a gas chromatography support in some applications. The low surface energy of the polymer causes nonlinear relationships between loading and specific retention volumes. In addition, the resistance to mass transfer severely limits the efficiency. The high mass transfer resistance is ascribed to the low surface area and low surface energy of the polymer. Optimum conditions for separations are generally favored by low flow rates, low loading, and the use of a stationary phase with a low surface energy.

## GENERAL CONCLUSIONS

In view of the extensive research that has been done on polyolefins, some interesting conclusions can be made. It appears that polyolefins are separating compounds in much the same way as the porous polymer bead supports. The very polar compounds are eluted first with some evidence of adsorption while organic compounds are partitioned by solution in the polymer. This is borne out by the monophase gel

column of LYSYJ AND NEWTON, the work on various divinylbenzenestyrene polymer beads, our research and the work of BAUM. Judging from BAUM's typical chromatograms the HETP values for his separations are in the same order of magnitude as in our polypropylene work. From the practical standpoint, this makes polypropylene the polyolefin of choice because of its greater temperature limit.

## DEFINITION OF TERMS

*Column flow rate.* The volume flow rate that has been corrected to the temperature in the column.

*Flow rate.* The volume flow rate measured by a soap bubble flow rate meter at the column exit.

*Efficiency (HETP).* The HETP used in this paper is the normal HETP as opposed to the relative HETP which is measured from the air peak.

*Specific retention volume.* The volume of gas at 0° required to elute one-half of a solute from a column which contains 1 g of liquid phase and has no pressure drop or free gas space.

*Conversion from column flow rate to linear flow rate for 30–60 mesh PD 500 columns.* 1 cm/sec = 7.5 ml/min.

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CHROM. 4379

# THE GAS CHROMATOGRAPHY OF AROMATIC ACIDS AS THEIR TRIMETHYLSILYL DERIVATIVES, INCLUDING APPLICATIONS TO URINE ANALYSIS

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SUMMARY

The preparation of trimethylsilyl derivatives from aromatic acids and a few related lactones was examined under various conditions. Indoles and amides frequently gave more than one derivative, particularly with bis-(trimethylsilyl)-acetamide; the formation and stability of derivatives were also influenced by time, solvent and the presence of water or of substances possessing catalytic activity. The most suitable silylating reagent examined appeared to be hexamethyldisilazane in the presence of trifluoroacetate.

Methylene unit values on OV-17 and OV-1 were recorded for derivatives of 79 compounds, mostly acidic, containing a phenyl or indolyl nucleus together with various combinations of unsaturated, amide, methoxyl and phenolic, alcoholic or enolic hydroxyl groups. Values were higher on OV-17 to an extent determined by the number of hydroxyl, methoxyl and especially NH groups in a molecule. Small variations on a given liquid phase with experimental conditions appeared to be related largely to the number of hydroxyl groups in a molecule.

With urine extracts, quantitative results satisfactory for many purposes were obtained for 4-hydroxy-3-methoxymandelic acid on OV-17 and for *p*-hydroxyphenyllactic acid on OV-1 or, less satisfactorily, OV-17.

## INTRODUCTION

Since chromatographic techniques first began to be applied to urine analysis no group of urinary constituents has attracted more interest than the aromatic acids. Phenols and indoles, both easily detected by paper chromatography, have received most attention but gas chromatographic methods are now beginning to facilitate studies in a wider field.

The extraction of aromatic acids from urine and their gas chromatography as trimethylsilyl derivatives have been the partial subjects of previous publications[1–4]. However the chromatographic behaviour of only a limited number of authentic substances has been recorded as yet. Moreover, although the simpler compounds appear to undergo trimethylsilylation at carboxyl and hydroxyl groups with reagents such as bis-(trimethylsilyl)-acetamide or hexamethyl-disilazane, discrepancies in the re-

corded[2,3] behaviour of compounds containing the NH group appear most readily to be explained in terms of the silylation process.

More detailed investigations into the behaviour of aromatic acids form the subject of this paper.

## EXPERIMENTAL

### *Definitions and abbreviations*

The term silylation is used rather than the more accurate trimethylsilylation; derivatives are referred to as TMS derivatives. The following abbreviations are also used: BSA, bis-(trimethylsilyl)-acetamide; HMDS, hexamethyldisilazane; TMCS, trimethylchlorosilane; TFA, trifluoroacetic acid; pyr, pyridine. Benzoylglycine and its derivatives are referred to as hippuric acids and phenylacetylglycine and its derivatives as phenaceturic acids. Two physiologically important phenolic acids, 4-hydroxy-3-methoxymandelic acid and *p*-hydroxyphenyllactic acid, are referred to as VMA and PHPL respectively.

The chromatographic properties of each substance, described in terms of methylene unit (MU) values, was determined by the procedure of DALGLIESH *et al.*[2] using as standards even-numbered straight-chain hydrocarbons from $C_{12}$ to $C_{28}$. $\Delta$ MU (OV-17 — OV-1) values were obtained by subtracting values found using long OV-1 columns from those using long OV-17 columns and $\delta$MU (OV-17 or OV-1) values by subtracting values obtained using the short from those using the long columns.

The chromatographic behaviour of some amides and indoles was complicated by their tendency to form two or more peaks. In practice the peak (or major peak) formed using HMDS (Table II) was considered to be normal and additional peaks, all obviously formed more readily with BSA (Tables I and II) were considered to be "difficult".

### *Materials*

All reagents were of the best available quality. Pyridine was stored over solid KOH. BSA and reagents derived from it were prepared from freshly opened 1 ml ampoules (Sigma London Ltd.) and used immediately. The following silylating reagents were employed: BSA; BSA–pyr (1:1); BSA–TMCS (4:1); HMDS–pyr (no fixed composition; sometimes TMCS was added as catalyst); HMDS–TFA prepared by adding TFA (0.5 ml) and HMDS (4.5 ml) to pyridine (5 ml); the mixture was allowed to stand overnight and appeared to be stable indefinitely if protected from moisture.

Most aromatic acids were obtained commercially but many were synthesised or received as gifts for which we are particularly indebted to Dr. J. W. T. SEAKINS. Most substances appeared to be chromatographically pure although small secondary peaks were occasionally observed. A few of the cheaper commercial products such as hippuric, cinnamic and 2,6-dihydroxybenzoic acids gave appreciable secondary peaks. Several aroylamides gave peaks identified as the parent aromatic acids, of whose possible presence we were warned. Minor peaks from ketoacids were probably decomposition products of these unstable compounds. A sample of *m*-hydroxyhippuric acid isolated from the urine of a subject fed *m*-hydroxybenzoic acid contained hippuric acid as impurity. Since no authentic sample was available, *m*-hydroxyphenylhydracrylic acid, a major constituent, was isolated from human urine by paper chromatography.

TABLE I

METHYLENE UNIT VALUES OF AROMATIC ACIDS SILYLATED WITH BSA

Correlations of peaks on OV-1 with those on OV-17 were not attempted where ambiguity was evident.

| *Acid* | *Column* | | | | |
|---|---|---|---|---|---|
| | *OV-17* (*1.5m*) | *OV-1* (*1.5m*) | *OV-17* | *OV-1* | *Δ MU* (*OV-17* – *OV-1*) |
| Benzoic | 13.65 | 12.26 | 13.96 | 12.31 | 1.65 |
| Hippuric[a] | 21.05 | 18.02 | 21.13 | 18.04 | 3.09 |
| Phenylacetic | 14.40 | 12.64 | 14.40 | 12.72 | 1.68 |
| Phenaceturic | 21.67 | 18.47 | 21.77 | 18.43 | |
| | | 18.62 | | 18.57 | |
| Phenylacetylglutamic | 24.68 | 20.67 | 24.91 | 20.68 | |
| | 26.06 | 22.39 | 26.00 | 22.35 | |
| | | 22.96 | | 22.95 | |
| Phenylacetylglutamine[a] | 24.67 | 20.68 | 24.93 | 20.68 | |
| | 24.97 | 20.80 | 25.21 | 20.82 | |
| | 27.96 | 21.95 | *ca.* 28.4 | 22.00 | |
| | | | *ca.* 28.5 | | |
| β-Phenylpropionic | 15.52 | 14.05 | 15.65 | 14.06 | 1.59 |
| Cinnamic | 17.04 | 14.97 | 17.17 | 15.21 | 1.96 |
| Mandelic | 15.91 | 14.69 | 15.90 | 14.57 | 1.33 |
| β-Phenyllactic[a] | 16.88 | 15.87 | 16.83 | 15.82 | 1.01 |
| Phenylpyruvic[a] | 18.28 | 17.02 | 18.32 | 16.99 | 1.33 |
| N-Acetylphenylalanine[a] | 19.39 | 17.88 | 19.31 | 17.90 | |
| | 20.54 | | 20.65 | | |
| *o*-Hydroxybenzoic[a] | 16.26 | 15.01 | 16.30 | 15.00 | 1.30 |
| *o*-Hydroxyhippuric[a] | 22.92 | 20.41 | 23.02 | 20.51 | 2.51 |
| *o*-Hydroxyphenylacetic[a] | 16.92 | 15.56 | 16.85 | 15.53 | 1.32 |
| *o*-Hydroxyphenylpyruvic, lactone | 18.95 | 16.50 | 19.32 | 16.74 | 2.58 |
| *o*-Hydroxycinnamic | 19.57 | 18.00 | 19.62 | 18.03 | 1.59 |
| *m*-Hydroxybenzoic[a] | 16.66 | 15.57 | 16.76 | 15.51 | 1.25 |
| *m*-Hydroxyhippuric[a] | 23.80 | 21.20 | 23.78 | 21.20 | 2.58 |
| *m*-Hydroxyphenylacetic[a] | 17.36 | 15.97 | 17.25 | 15.97 | 1.28 |
| β-(*m*-Hydroxyphenyl)propionic[a] | 18.52 | 17.17 | 18.48 | 17.13 | 1.35 |
| *m*-Hydroxycinnamic | 20.05 | 18.63 | 20.00 | 18.57 | 1.43 |
| β-(*m*-Hydroxyphenyl)hydracrylic[a] | 19.41 | 18.63 | 19.22 | 18.50 | 0.72 |
| *p*-Hydroxybenzoic[a] | 17.32 | 16.20 | 17.25 | 16.20 | 1.05 |
| *p*-Hydroxyphenylacetic[a] | 17.65 | 16.29 | 17.59 | 16.25 | 1.34 |
| β-(*p*-Hydroxyphenyl)propionic[a] | 18.88 | 17.53 | 18.83 | 17.48 | 1.35 |
| *p*-Hydroxycinnamic[a] | 20.84 | 19.28 | 20.84 | 19.30 | 1.54 |
| *p*-Hydroxymandelic[a] | 18.88 | 17.94 | 18.65 | 17.74 | 0.91 |
| β-(*p*-Hydroxyphenyl)lactic[a] | 20.00 | 19.13 | 19.86 | 19.05 | 0.81 |
| *p*-Hydroxyphenylpyruvic[a] | 21.59 | 20.61 | 21.49 | 20.54 | 0.95 |
| N-Acetyltyrosine[a] | 24.86 | 21.29 | 24.90 | 21.24 | 3.66 |
| *p*-Methoxybenzoic | 16.96 | 14.95 | 17.01 | 15.04 | 1.97 |
| *p*-Methoxyhippuric | 24.47 | 20.79 | 24.58 | 20.83 | 3.75 |
| *p*-Methoxyphenylacetic | 17.28 | 15.13 | 17.33 | 15.10 | 2.23 |
| *p*-Methoxyphenaceturic | 24.75 | 20.82 | 24.82 | 20.85 | 3.97 |
| *p*-Methoxyphenylacetylglutamic | 27.80 | 23.19 | *ca.* 28.1 | 23.16 | |
| | *ca.* 28.8 | 24.44 | *ca.* 28.8 | 24.42 | |
| β-(*p*-Methoxyphenyl)propionic | 18.46 | 16.30 | 18.54 | 16.36 | 2.18 |
| *p*-Methoxycinnamic | 20.58 | 17.96 | 20.69 | 18.08 | 2.61 |
| *p*-Methoxymandelic | 18.75 | 16.91 | 18.64 | 16.81 | 1.83 |
| β-(*p*-Methoxyphenyl)lactic | 19.62 | 17.95 | 19.60 | 18.01 | 1.59 |
| *p*-Methoxyphenylpyruvic | 21.42 | 19.54 | 21.46 | 19.54 | 1.92 |
| N-Acetyl-*p*-Methoxyphenylalanine | 23.51 | 20.09 | 23.56 | 20.13 | 3.43 |

TABLE I (*continued*)

| *Acid* | | *Column* | | | | |
|---|---|---|---|---|---|---|
| | | *OV-17* (*1.5m*) | *OV-1* (*1.5m*) | *OV-17* | *OV-1* | *Δ MU*– (*OV-17*– *OV-1*) |
| 2,3-Dihydroxybenzoic | | 18.48 | 17.49 | 18.45 | 17.51 | 0.94 |
| 2,4-Dihydroxybenzoic | | 19.20 | 18.23 | 19.07 | 18.18 | 0.89 |
| 2,5-Dihydroxybenzoic[a] | | 18.78 | 17.85 | 18.60 | 17.74 | 0.86 |
| 2,5-Dihydroxyphenylacetic[a] | | 19.40 | 18.45 | 19.27 | 18.32 | 0.95 |
| 2,5-Dihydroxyphenylacetic, lactone[a] | | 18.70 | 17.30 | 18.71 | 17.30 | 1.41 |
| 2,5-Dihydroxyphenylpyruvic, lactone[a] | | 22.03 | 20.21 | 22.27 | 20.35 | 1.92 |
| 2,6-Dihydroxybenzoic | | 18.86 | 17.68 | 18.81 | 17.64 | 1.17 |
| 3,4-Dihydroxybenzoic[a] | | 19.17 | 18.25 | 19.05 | 18.21 | 0.84 |
| 3,4-Dihydroxyphenylacetic[a] | | 19.39 | 18.35 | 19.31 | 18.30 | 1.01 |
| 3,4-Dihydroxyphenylcinnamic[a] | | 22.48 | 21.49 | 22.43 | 21.40 | 1.03 |
| 3,4-Dihydroxymandelic[a] | | 20.19 | 19.48 | 20.02 | 19.33 | 0.69 |
| 3,5-Dihydroxybenzoic[a] | | 19.28 | 18.28 | 18.99 | 18.11 | 0.88 |
| 3-Hydroxy-4-methoxybenzoic[a] | | 19.33 | 17.60 | 19.23 | 17.52 | 1.71 |
| 3-Hydroxy-4-methoxyhippuric[a] | | 26.34 | 23.26 | 26.31 | 23.18 | 3.13 |
| 3-Hydroxy-4-methoxyphenylacetic | | 19.39 | 17.58 | 19.30 | 17.48 | 1.82 |
| β-(3-Hydroxy-4-methoxyphenyl)propionic[a] | | 20.45 | 18.71 | 20.42 | 18.68 | 1.74 |
| 3-Hydroxy-4-methoxycinnamic[a] | | 22.57 | 20.54 | 22.55 | 20.59 | 1.96 |
| β-(3-Hydroxy-4-methoxyphenyl) hydracrylic[a] | | 21.18 | 20.04 | 21.06 | 19.88 | 1.18 |
| 4-Hydroxy-3-methoxybenzoic[a] | | 19.11 | 17.53 | 19.05 | 17.49 | 1.56 |
| 4-Hydroxy-3-methoxyhippuric[a] | | 24.88 | 23.27 | 24.71 | 23.32 | |
| 4-Hydroxy-3-methoxyphenylacetic[a] | | 19.38 | 17.61 | 19.33 | 17.54 | 1.79 |
| β-(4-Hydroxy-3-methoxyphenyl) propionic[a] | | 20.53 | 18.84 | 20.57 | 18.83 | 1.74 |
| 4-Hydroxy-3-methoxycinnamic[a] | | 22.76 | 20.78 | 22.75 | 20.78 | 1.97 |
| 4-Hydroxy-3-methoxymandelic[a] | | 20.20 | 18.92 | 20.06 | 18.74 | 1.32 |
| β-(4-Hydroxy-3-methoxyphenyl)lactic[a] | | 21.45 | 20.27 | 21.36 | 20.23 | 1.13 |
| 4-Hydroxy-3-methoxyphenylpyruvic[a] | | 23.34 | 21.67 | 23.29 | 21.76 | 1.53 |
| 4-Hydroxy-3-ethoxybenzoic[a] | | 19.55 | 18.04 | 19.46 | 18.02 | 1.44 |
| 3,4-Dimethoxybenzoic | | 19.47 | 16.84 | 19.44 | 16.76 | 2.68 |
| 3,4-Dimethoxyphenylacetic[a] | | 19.61 | 16.87 | 19.56 | 16.78 | 2.78 |
| 4-Hydroxy-3,5-dimethoxybenzoic[a] | | 20.90 | 18.89 | 20.83 | 18.80 | 2.03 |
| 3-Indolylcarboxylic[a] | | 22.60 | 19.95 | 22.78 | 20.01 | 2.77 |
| 3-Indolylacetic[a] | (i) | 21.82 | 18.65 | 22.44 | 18.74 | 3.70 |
| | (ii) | 21.71 | 19.34 | 22.00 | 19.41 | 2.59 |
| β-(3-Indolyl)propionic[a] | (i) | 23.15 | 19.71 | 23.55 | 19.86 | 3.69 |
| | (ii) | 22.76 | 20.53 | 23.07 | 20.81 | 2.26 |
| β-(3-Indolyl)lactic[a] | (i) | 23.92 | 21.07 | 24.17 | 21.18 | 2.99 |
| | (ii) | 23.34 | 21.68 | 23.55 | 21.79 | 1.76 |
| 3-Indolylpyruvic | | 26.33 | 24.30 | 26.41 | 24.34 | 2.07 |
| N-Acetyltryptophan[a] | | 25.65 | 24.00 | 25.82 | 24.11 | |
| | | 27.60 | | 27.62 | | |
| 3-(5-Hydroxyindolyl)acetic[a] | | 24.31 | 22.06 | 24.29 | 22.03 | 2.26 |

[a]The presence, or reported presence, of this compound in human urine is known to the authors. Other compounds such as phenylacetic, phenylacetylglutamic and *m*-hydroxycinnamic acids may be present in urine after hydrolysis.

*Chromatographic conditions*

Chromatograms were run on two Pye 104 series, Model 64 machines with flame-ionisation detectors maintained at 310° and a hydrogen flow rate of 50 ml/min. Argon

TABLE II

METHYLENE UNIT VALUES OF AMIDES AND INDOLES SILYLATED WITH HMDS–TFA

| *Acid* | | *Column* | | |
|---|---|---|---|---|
| | | *OV-17* | *OV-1* | *ΔMU (OV-17–OV-1)* |
| Hippuric | | 21.20 | 18.08 | 3.12 |
| *o*-Hydroxyhippuric | | 23.11 | 20.52 | 2.59 |
| *m*-Hydroxyhippuric | | 23.82 | 21.22 | 2.60 |
| *p*-Methoxyhippuric | | 24.67 | 20.85 | 3.82 |
| 3-Hydroxy-4-methoxyhippuric | | 26.33 | 23.17 | 3.16 |
| 4-Hydroxy-3-methoxyhippuric | | 26.39 | 23.29 | 3.10 |
| Phenaceturic | | 21.84 | 18.45 | 3.39 |
| *p*-Methoxyphenaceturic | | 24.88 | 20.85 | 4.03 |
| Phenylacetylglutamine | | *ca.* 28.5 | 21.86 | *ca.* 6.6 |
| Phenylacetylglutamic | | 26.11 | 23.04 | 3.07 |
| *p*-Methoxyphenylacetylglutamic | | *ca.* 28.8 | 25.23 | *ca.* 3.6 |
| N-Acetylphenylalanine | | 20.72 | 17.84 | 2.88 |
| N-Acetyltyrosine | | 24.97 | 21.24 | 3.73 |
| N-Acetyl-*p*-methoxyphenylalanine | | 23.56 | 20.13 | 3.43 |
| N-Acetyltryptophan | | *ca.* 29.0 | 23.76 | *ca.* 5.8 |
| | | 27.68[a] | | |
| 3-Indolylcarboxylic | (i) | 23.63 | 19.44 | 4.19 |
| | (ii) | 22.76[a] | 20.05[a] | 2.71 |
| 3-Indolylacetic | | 22.51 | 18.68 | 3.83 |
| *β*-(3-Indolyl)propionic | | 23.64 | 19.83 | 3.81 |
| *β*-(3-Indolyl)lactic | | 24.20 | 21.12 | 3.08 |
| 3-Indolylpyruvic | (i) | 27.01 | 23.42 | 3.59 |
| | (ii) | 26.42[a] | 24.30[a] | 2.12 |
| 3-(5-Hydroxyindolyl)acetic | | 25.21 | 21.70 | 3.51 |

[a] This "difficult" peak was formed only in small amount.

at 50 ml/min was used as carrier gas. Injection heaters were used to give an initial temperature at the point of injection about 80° above that at which programming started. The nominal rates of temperature programming on the two machines differed by about 10%.

Coiled columns of 4 mm I.D. were packed with Diatoport S (80–100 mesh) supporting 10% liquid phase. The four variations of liquid phase, column length, temperature of commencement and rate of programming employed were as follows: OV-17, 5.5 m, 180°, 1°/min; OV-17, 1.5 m, 100°, 2°/min; OV-1, 4 m, 170°, 1°/min; OV-1, 1.5 m, 100°, 2°/min.

The simple terms OV-17 and OV-1 employed throughout the text refer only to the longer columns. References to the shorter columns are always qualified since they were used only for subsidiary experiments of a comparative nature.

*Silylations with BSA*

In preliminary experiments employing pure BSA silylation appeared sometimes to be very complex. Thus although *o*-hydroxyhippuric acid dissolved readily in the reagent no peak was observed when the mixture was chromatographed immediately on OV-17. In the course of time, however, no fewer than three peaks made their appearance, in two cases only temporarily. Such complications were largely eliminated

when silylation was carried out in BSA-pyr and this reagent was used for most subsequent experiments.

Compounds (usually 1–4 mg, the larger quantities being used when MUs were high) together with 0.5 mg of each hydrocarbon standard were dissolved in 0.5 ml BSA–pyr and the mixture allowed to stand at least overnight before chromatography. It was frequently possible to silylate and chromatograph several compounds simultaneously without ambiguity. Results are recorded in Table I.

Most members of two groups of compounds, indoles and amides, usually gave multiple peaks although secondary peaks were in many cases neither sufficiently large nor sufficiently consistent in formation to merit inclusion in Table I, particularly in the amide series. Thus, owing to limited supplies of material, an investigation into the unexpectedly large difference in MU values (OV-17) between the isomeric hydroxymethoxy hippuric acids (Table I) necessitated several repeated chromatograms of the original extracts, with and without addition of extra BSA. It was noticed that in some experiments each isomer gave a small secondary peak of similar MU value to that of the major peak from the other isomer. Similarly several examples were noted where amides and indoles present in mixtures gave results differing from those recorded in Table I. For example in various experiments 5-hydroxyindolylacetic acid gave rise to two peaks, N-acetyltryptophan to only one peak, and a third peak MU value 21.12 on OV-17 was observed from N-acetylphenylalanine.

Further observations indicated additional discrepancies between the behaviour of some amides and indoles when alone and when added to urine extracts. Thus whilst 5-hydroxyindolylacetic acid seemed reliably to form only one peak in urine, indolylacetic acid gave sometimes two peaks and at other times only the peak of MU value 19.41 (OV-1) or 22.00 (OV-17). Hippuric acid, invariably present in urine extracts, gave in addition to the normal peak MU 21.13 (OV-17) variable amounts of a "difficult" peak MU 19.88 (17.91 on OV-1) not observed with the pure compound.

This latter peak appeared to be formed more easily if extracts were dried with particular care before silylation. In one such extract the proportion of this peak was increased by the addition of extra BSA, and still further increased when TMCS was then added. When similar extracts were silylated using BSA alone or BSA–TMCS formation of the "difficult" peak appeared to be complete, or nearly so, since only a small peak (which may have been due to some other compound) remained at MU 21.13 (OV-17).

The effect of moisture was investigated in an extract silylated with BSA–pyr which showed a particularly high proportion of the "difficult" peak from hippuric acid and both peaks from added indolylacetic acid. After the initial chromatogram on OV-17 water was gradually added to the extract by breathing gently into the container which was then shaken and allowed to stand 5 min before re-chromatography. Repetition of this process caused the gradual diminution of the "difficult" peak from hippuric acid and its eventual complete replacement by the normal peak. By contrast, the procedure resulted initially in a large increase of the peak MU 22.00 from indolylacetic acid at the expense of the peak MU 22.44, but little further change occurred subsequently. Since that of lower MU must be considered the "difficult" peak in the case of indolylacetic acid, water may well have a catalytic effect on its formation (*e.g.* ref. 5).

*Silylations with HMDS*

Silylation of simpler aromatic acids may be effectively achieved with HMDS–

pyr. The reaction is slow but may be speeded up by addition of TMCS (*e.g.* ref. 2). Neither variation proved satisfactory when applied to indoles and amides, whether pure or added to urine extracts. Results obtained appeared to be a function of time. Indoles gave double peaks, the relative proportions of which varied with time. Peaks were formed slowly from amides, sometimes obviously in very poor yield. Products were unstable in the presence of TMCS (stability was not tested with the uncatalysed reaction). Experiments with the above reagents were abandoned mainly because the insolubility of many substances, and of urine extracts in HMDS and/or pyridine made it impossible to devise any sort of consistent technique when dealing with them. The formation of a precipitate of ammonium chloride when TMCS was used also proved inconvenient.

It seemed possible that a catalyst alternative to TMCS could be found which would be free from the latter objection and would promote the rapid formation of homogeneous solutions. In point of fact the first, and only, two substances tried proved highly efficacious: addition of trifluoro- or trichloroacetic acid to suspensions of even highly insoluble substances such as acetyltryptophan in HMDS–pyr (1:1) resulted in rapid solution within a few minutes. When MUs of indoles and amides were redetermined (Table II) after silylation with HMDS–TFA solution of all substances, with one exception, occurred almost instantaneously and samples were injected after 15 min reaction time. The exception was phenylacetylglutamine which dissolved slowly, probably because the sample assumed the consistency of a gum when in contact with the reagent: reaction was allowed to continue overnight and the mixture was centrifuged to remove undissolved material which was probably inorganic in nature (personal communication from Dr. J. W. T. SEAKINS).

Under the above conditions substances gave single peaks with the exception of three indoles (Table II) which yielded apprecia bleamounts of secondary "difficult" peaks. However slow formation of 'difficult' peaks occurred with other indoles when the solutions were allowed to stand.

*Further experiments on silylation*

When indolyllactic, indolylacetic or 5-hydroxyindolylacetic acids were silylated (HMDS–TFA; 15 min) in the presence of indolylcarboxylic acid the latter two compounds gave traces of "difficult" peaks after 15 min, presumably as the result of a catalytic reaction involving the "difficult" peak formed relatively easily from indolylcarboxylic acid. Ether extracts were prepared in duplicate from 5 ml samples of six normal urines. To one of each pair of extracts was added indolylacetic and 5-hydroxyindolylacetic acids (1 mg each). After silylation (HMDS–TFA; 15 min) chromatography (OV-17) demonstrated the formation of "difficult" peaks from both indoles but in no case was the area estimated to be more than about 2% of that of the corresponding normal peak. Areas of "difficult" peaks were approximately doubled when repeat chromatograms were carried out after 2.5 h reaction time.

A synthetic mixture containing hydrocarbon standards together with *p*-hydroxyphenyl-acetic and -lactic acids, indolylacetic and 5-hydroxyindolylacetic acids, N-acetylphenylalanine and *o*-hydroxy-, *m*-hydroxy- and *p*-methoxyhippuric acids was silylated under various conditions and chromatographed on OV-17 after suitable periods of time. One aliquot was treated with HMDS–TFA for comparison with results obtained when a second sample was silylated with BSA–pyr (24 h). Another aliquot

TABLE III

SILYLATION OF A SYNTHETIC MIXTURE OF ACIDS UNDER VARIOUS CONDITIONS

Equal aliquots of a mixture containing arbitrary quantities of acids were treated with various reagents (0.5 ml) and chromatographed on OV-17 after the time intervals indicated below. The figure recorded for each peak is the ratio of its height to that of a tetracosane standard.

| *Acid* | | *MU* | *HMDS–TFA* | | | | | | *BSA–pyr* | *HMDS–TFA (15 min) then BSA* | | *BSA* | | *BSA–TMCS* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *15 min* | *2.5 h* | *5 h* | *24 h* | *48 h* | *120 h* | *24 h* | *24 h* | *72 h* | *2 h* | *24 h* | *2 h* |
| *p*-Hydroxyphenylacetic | | 17.59 | 1.96 | 1.94 | 1.96 | 1.94 | 1.94 | 1.96 | 2.00 | 1.92 | 1.91 | 2.08 | 2.12 | 2.14 |
| PHPL | | 19.86 | 0.82 | 0.78 | 0.85 | 0.83 | 0.81 | 0.82 | 0.84 | 0.81 | 0.78 | 0.88 | 0.92 | 0.91 |
| Indolylacetic | (i) | 22.48 | 0.83 | 0.83 | 0.82 | 0.73 | 0.68 | 0.50 | 0.05 | 0.00 | 0.00 | 0.77 | 0.67 | 0.78 |
| | (ii) | 22.00 | 0.01 | 0.02 | 0.03 | 0.10 | 0.18 | 0.35 | 0.72 | 0.99 | 0.97 | 0.19 | 0.29 | 0.09 |
| 5-Hydroxyindolylacetic | (i) | 25.21 | 1.00 | 0.96 | 0.95 | 0.85 | 0.84 | 0.57 | 0.08 | 0.00 | 0.00 | 0.76 | 0.64 | 0.78 |
| | (ii) | 24.29 | 0.01 | 0.02 | 0.03 | 0.12 | 0.20 | 0.41 | 0.84 | 1.35 | 1.28 | 0.27 | 0.33 | 0.15 |
| *o*-Hydroxyhippuric | | 21.03 | 0.77 | 0.79 | 0.80 | 0.77 | 0.81 | 0.74 | 0.27 | 0.75 | 0.69 | 0.08 | 0.03 | 0.03 |
| *m*-Hydroxyhippuric | | 23.80 | 0.42 | 0.42 | 0.42 | 0.39 | 0.44 | 0.39 | 0.41 | 0.33 | 0.22 | 0.22 | 0.07 | 0.04 |
| *p*-Methoxyhippuric | | 24.63 | 0.46 | 0.48 | 0.49 | 0.45 | 0.51 | 0.44 | 0.60 | 0.36 | 0.15 | 0.45 | 0.40 | 0.47 |
| N-Acetylphenylalanine | (i) | 20.66 | 0.59 | 0.56 | 0.59 | 0.59 | 0.59 | 0.59 | 0.04 | 0.35 | 0.32 | 0.02 | 0.02 | 0.00 |
| | (ii) | 19.31 | –[a] | –[a] | –[a] | –[a] | –[a] | –[a] | 0.27 | –[a] | –[a] | 0.38[b] | 0.47[b] | 0.58[b] |

[a] Traces of this peak could have been obscured by a small overlapping artifact peak.
[b] An additional peak MU 21.13 was probably derived from N-acetylphenylalanine.

was also treated with HMDS–TFA but after a portion had been withdrawn for chromatography (15 min) an equal volume of BSA was immediately added. Other aliquots were silylated with pure BSA and BSA–TMCS. Results are recorded in Table III; as an indication of quantitative relationships the ratios of peak heights to those of the tetracosane standard were employed.

This experiment demonstrated the satisfactory formation and stability under all conditions of the peaks from the two simple phenolic acids. Indoles formed "difficult" peaks slowly when silylated with HMDS–TFA or pure BSA; their formation with the latter reagent was promoted by the presence of pyridine but not by that of TMCS. The formation of the "difficult" peak (and of a third peak) from N-acetylphenylalanine occurred with BSA but not in a mixture previously silylated with HMDS–TFA although this procedure secured the exclusive formation of "difficult" peaks from indoles. Peaks from the remaining amides tended to be smaller and less stable in the presence of BSA than those formed in the presence of HMDS alone.

It should not be assumed that the various types of behaviour described above necessarily apply to urine extracts. For instance the high degree of stability of all derivatives formed by HMDS–TFA was not confirmed in the case of that from *p*-methoxyhippuric acid which, when formed in a urine extract, disappeared slowly over the course of a few days.

*Applications to urine extracts*

Acidified urines were extracted with ether as described previously[4]. Normally that volume of urine containing 10 mg creatinine was taken and the dried evaporated extract treated with HMDS-TFA (0.5 ml). When aliquots (10 $\mu$l) were chromatographed on OV-1 or OV-17 as described above the ionisation amplifier was set at $2 \times 10^3$.

For quantitative purposes internal standards were employed. Because of overlapping due to peaks naturally present, the choice of standard may vary from urine to urine but docosane appeared to be generally suitable for OV-17 though rather less suitable for OV-1 in some cases.

Using docosane (200 $\mu$g added to each extract) as standard the excretions of VMA (OV-17) and PHPL (OV-1 and OV-17) were determined in a series of urines collected at noon from 10 normal subjects. Quantities of each substance were determined from standard graphs relating quantity to the ratio of the peak heights of the substance to that of docosane. These graphs were linear over the range tested (0–100 $\mu$g substance/ml HMDS–TFA) though slight curves were obtained when columns were presumably not in perfect condition. Since peaks were obviously heterogeneous on OV-1, excretions of VMA were not determined using this column; for comparison with OV-17 the substance was estimated by periodate oxidation[6]. Results are included in Table V.

## DISCUSSION

*Silylation procedures*

The extraction of aromatic acids from aqueous solution, conversion into suitable derivatives and subsequent gas chromatography presents few technical problems and a wide choice of potential methods is available. The principal difficulties lie in the quantitative, or at least reproducible, formation of derivatives and in the selection of

columns which will reliably isolate compounds of interest as single peaks. The ready conversion of phenolic, alcoholic and carboxylic acid groups to TMS derivatives with a variety of silylating reagents and the excellent chromatographic properties of the majority of such derivatives has ensured a permanent place for silylation in this field.

At an early stage in our own work we were very impressed by the simplicity attached to procedures involving the silylation of all appropriate groupings, but did indeed note minor differences in chromatograms from urine extracts silylated under differing conditions[4]. Furthermore we found it profitable to compare results recorded by two other groups working with similar compounds. Both DALGLIESH *et al.*[2] and KAROUM *et al.*[3] chromatographed TMS derivatives of aromatic acids on the liquid phase F60; derivatives were prepared with HMDS catalysed by TMCS, the former group using pyridine and the latter group dioxan, as solvent. MU values were in reasonable agreement, allowing for wide differences in chromatographic conditions, except in the cases of hippuric acid and indoles. It is now clear that such compounds have a tendency to form more than one derivative; the formation of some peaks may be justifiably described as "difficult" since it occurs more readily with the particularly powerful silylating reagent BSA (*cf.* also ref. 7).

There can be little doubt that silylation of NH groups is involved in the chemistry of both amides and indoles and provides a reasonable explanation of the formation of "difficult" peaks from the latter type of compound. However, having regard to the wide differences in behaviour between individual members which makes generalisation dangerous, we would hesitate to draw firm conclusions concerning the chemistry of silylation in the amide series. Thus we have no ready explanation for the formation of three peaks by N-acetylphenylalanine and phenylacetylglutamic acid and are doubtful if peaks of similar MU values formed from one compound by different procedures necessarily always represent the same derivative. For instance we noted that the hippuric acid peak MU 21.13 (OV-17) when formed in urine using BSA appeared to be of a distinctly less symmetrical shape than when formed under any other conditions. Further speculation would appear to be unprofitable in the absence of concrete evidence concerning chemical structure.

Our experiments with indoles and amides illustrate variously that both the formation and stability of TMS derivatives may be influenced by the silylating reagent, solvent and presence of other substances. It seems probable that water has catalytic as well as hydrolytic properties and may be of paramount importance in determining the course of silylations using BSA: our results using this reagent should be regarded as typical rather than invariable. Undoubtedly the behaviour of an indole or amide of interest in urine samples should be checked in detail as the occasion arises: optimum conditions of silylation may vary from compound to compound and from urine to urine. Present evidence suggests HMDS to be a reagent superior to BSA for routine purposes because of its lesser tendency to give "difficult" peaks. In our experience replacement of TMCS by TFA as catalyst greatly enhances the utility and convenience of the reagent. In view of the many unexplored possibilities, it may be that future work will reveal some combination of reagent, solvent and catalyst which will silylate amides and indoles without ambiguity, but chaims in this respect should not be based solely on the behaviour of pure compounds.

As an alternative approach to the gas chromatography of aromatic acids both DALGLIESH *et al.*[2] and KAROUM *et al.*[3] have studied silylation following conversion of

carboxyl groups to their methyl esters. This procedure has the disadvantage that it is not possible to methylate completely all carboxyl groups without simultaneously partially methylating exceptionally reactive phenolic groups. Double bonds are also susceptible to attack by diazomethane. Some compounds may therefore yield multiple peaks. We have not investigated the silylation of methyl esters from indoles and amides since it seems quite clear that problems due to the formation of "difficult" peaks are again present. Not only are discrepancies in recorded MU values for these compounds apparent but the former group of workers observed the formation of two peaks from indolylacrylic and, on storage, indolylpyruvic acids.

*MU values of aromatic acids*

The behaviour of a compound on chromatography under given conditions is most accurately described in terms of its MU value which may readily be determined experimentally when flame-ionisation detection is employed. MU values are most closely related to molecular weights when non-selective liquid phases are employed and for this reason OV-1 may be regarded as a standard with which to compare the behaviour of liquid phases possessing selective properties. A number of such phases are now available in the OV-series: OV-17, a phenylmethylsilicone, was selected for detailed study. Most aromatic acids of biological interest are derived from the amino acids

TABLE IV

CHANGES IN MU VALUES OF CARBOXYLIC ACIDS WITH EXPERIMENTAL CONDITIONS

$\Delta$MU values were usually obtained from Table I but values for amides and indoles were from Table II. $\delta$MU values were obtained from data in Table I.

| *Nucleus* | *OH groups*[a] | *OMe groups* | *Number of examples* | *ΔMU (mean) (OV-17 – OV-1)* | *δMU (mean)* | |
|---|---|---|---|---|---|---|
| | | | | | *OV-17* | *OV-1* |
| *Compounds lacking an NH group* | | | | | | |
| Phenyl | 0 | 0 | 4 | 1.72 | 0.15 | 0.10 |
| Phenyl | 1 | 0 | 14 | 1.31 | −0.03 | −0.04 |
| Phenyl | 2 | 0 | 13 | 0.89 | −0.14 | −0.10 |
| Phenyl | 3 | 0 | 1 | 0.69 | −0.17 | −0.15 |
| Phenyl | 0 | 1 | 4 | 2.25 | 0.08 | 0.06 |
| Phenyl | 1 | 1 | 11 | 1.79 | −0.04 | −0.03 |
| Phenyl | 2 | 1 | 4 | 1.29 | −0.10 | −0.05 |
| Phenyl | 0 | 2 | 2 | 2.73 | −0.04 | −0.09 |
| Phenyl | 1 | 2 | 1 | 2.03 | −0.07 | −0.09 |
| *Compounds containing one NH group* | | | | | | |
| Phenyl | All amides | | 13 | *ca.* 3.3 | –[b] | –[b] |
| Indolyl | All indoles | | 6 | 3.67[c] | 0.26[d] | 0.10[d] |
| *Compounds containing two NH groups* | | | | | | |
| Phenylacetyl-glutamine | | | | *ca.* 6.6 | – | – |
| N-acetyltryptophan | | | | *ca.* 5.8 | – | – |

[a] Including enolisable keto groups.

[b] Data obtained from Table I in a few unambiguous cases do not reveal any marked influence of the amide group on $\delta$MU values.

[c] Excluding data from "difficult" peaks listed in Table II.

[d] Including data from both normal and "difficult" peaks.

phenylalanine, tyrosine and tryptophan and thus contain an aromatic nucleus linked to a side-chain containing no more than three carbon atoms. The list of compounds studied in this work is therefore rather comprehensive: it includes almost all aromatic acids at present known to be constituents of human urine and contains data sufficient to enable the prediction of MU values for many compounds not actually studied.

When a substance of unknown identity is encountered the most practicable approach to a consideration of its possible structural features may lie in a study of the variation of MU values with differing experimental conditions. Differential MU values may be derived in a number of ways; for instance values obtained using a non-selective column may be subtracted from those using a selective column. The $\Delta$MU values (OV-17 – OV-1) listed in Tables I and II are summarised in Table IV and are always positive. However it is apparent that they are increased in magnitude by the presence of methoxyl and, particularly, the NH group present in amides and indoles, and progressively decreased in magnitude by the successive introduction of hydroxyl groups. Interpretation of $\Delta$MU values obtained using OV-17 and OV-1 may be aided by the approximately equal magnitudes but opposite signs of the effects due to hydroxyl and methoxyl groups; differentiation between the presence in a molecule of mutually cancelling hydroxyl and methoxyl groups and the absence of such groups may be aided by a consideration of the approximate molecular weight as revealed by the MU value on OV-1.

Although MU values provide a fair description of the behaviour of a compound on a particular liquid phase they are not absolutely constant even with a given concentration of liquid phase, but depend on such factors as length of column and rate of heating if temperature programming be employed. For the purposes of this paper MU values were determined on short (1.5 m) columns at a fairly high rate of programming, conditions very suitable for routine work, as well as on the longer columns selected as being of more potential use for quantitative work. Differential MUs ($\delta$MU values) were obtained by subtracting values using the shorter from those using the longer columns, and are recorded in Table IV. Such values appear to be dependent almost entirely upon the number of hydroxyl groups present in a molecule, each of which exerts a negative effect. Unfortunately $\delta$MU values as recorded here are of little practical use since in many cases they are only of the same order of magnitude as experimental errors. However it might be possible to obtain values of greater magnitude by further attention to experimental detail.

MU values are influenced not only by the number of substituent groups but also by their nature (*e.g.* phenolic or alcoholic hydroxyl) and position (*e.g.* *o*-, *m*- or *p*-hydroxyl) and by the presence of double bonds as in cinnamic and enolised pyruvic acids. Such effects seem largely to be eliminated when differential MUs are considered though minor influences may be discerned. For instance, cinnamic acids appear to have particularly high $\Delta$MU (OV-17 – OV-1) values and a hydroxyl group in the $\alpha$-side chain position, as in mandelic and hydracrylic acids, seems to exert a particularly potent negative effect on $\delta$MU values. Interestingly, compounds of the above types appear to be revealed when differential MUs obtained by changing from a methylester-TMS derivative to a fully silylated derivative are considered. Examination of the data recorded by DALGLIESH *et al.*[2] and by KAROUM *et al.*[3] indicates that such changes produce increases in MU values which are particularly small in the case of mandelic acids and particular large in the case of cinnamic (and also hippuric) acids.

*Applications to urine analysis*

Previous experience suggested that most aromatic acids may be adequately extracted from urine using ether[4]. A few further experiments did not indicate any substantial advantage to be gained normally through further purification of acids by bicarbonate extraction since non-acidic urinary constituents seem to be excreted generally in much smaller quantities, often in conjugated forms poorly extracted into ether. Silylated ether extracts chromatographed very well (Fig. 1) and the principal difficulties appeared to be those associated with the complex behaviour of some substances as described above. In appropriate pathological urines large peaks were often observed and experience indicated unambiguous identification of such peaks to be greatly facilitated by the use of at least two columns. In some cases such as those of PHPL in tyrosinosis phenyllactic acid in phenylketonuria and homogentisic acid in alkaptonuria compounds were excreted in such large quantity that quantitative determinations could be undertaken without regard to the presence of overlapping normal peaks.

Quantitative determination in less spectacular cases presents considerable difficulty due to the possible heterogeneity of peaks. Given sufficiently extensive data on known compounds many problems may be solved in advance: for instance, reference to Table I indicates the futility of attempting VMA determinations on OV-1. However urines clearly contain many constituents as yet unidentified and under any given set of conditions few peaks on a chromatogram can be expected to be homogeneous. For the most accurate results possible quantitative determinations clearly need supporting evidence from ancilliary techniques such as mass spectrometry. However many unsupported estimations may be acceptable in certain circumstances. This is particularly true in clinical biochemistry where the normal may need to be defined only with sufficient precision to enable its distinction from the abnormal. It is also true when results can be shown to be reasonably close to those obtained by an alternative analytical method.

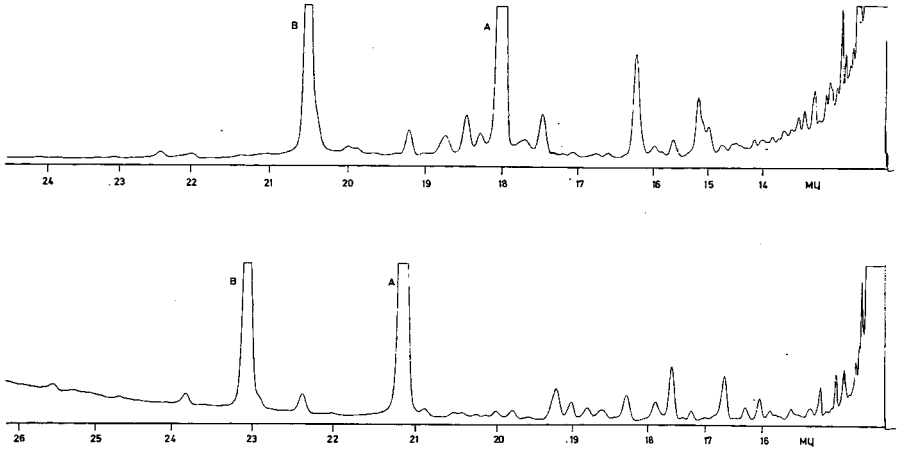


Fig. 1. Ether extract from a mixed normal urine, silylated with HMDS–TFA and chromatographed on OV-1 (above) or OV-17 (below). Base lines are marked in MU values to facilitate correlation of peaks with data in Tables I and II. Urines from normal adults invariably show the large peak A due to hippuric acid. The second large peak B, due to *o*-hydroxyhippuric acid derived from aspirin, is commonplace.

Quantitative aspects of the chromatography of silylated aromatic acids on OV-17 and OV-1 were examined in the cases of two of the most important urinary constituents of physiological interest. Both VMA and PHPL are commonly excreted in abnormal amounts in conditions of stress and in various diseases. Although VMA gave heterogeneous peaks on OV-1 comparison of results using OV-17 with those using a standard periodate-oxidation procedure indicated satisfactory agreement (Table V).

TABLE V

QUANTITATIVE DETERMINATION OF URINARY PHPL AND VMA

All values are expressed as $\mu$g/mg urinary creatinine.

| *Subject* | *PHPL* | | *VMA* | |
|---|---|---|---|---|
| | *OV-17* | *OV-1* | *OV-17* | *Periodate oxidation* |
| 1 | 0.85 | 0.30 | 1.34 | 1.54 |
| 2 | 2.40 | 0.38 | 1.85 | 1.87 |
| 3 | 6.20 | 0.70 | 1.77 | 2.86 |
| 4 | 0.90 | 0.73 | 2.30 | 2.34 |
| 5 | 1.78 | 0.67 | 5.00 | 3.36 |
| 6 | 2.78 | 0.83 | 3.13 | 3.65 |
| 7 | 0.40 | 0.57 | 1.72 | 2.54 |
| 8 | 3.03 | 0.68 | 2.75 | 2.47 |
| 9 | 2.77 | 0.30 | 2.63 | 3.22 |
| 10 | 0.48 | 0.88 | 2.28 | 2.97 |

Determinations of PHPL on OV-1 proved preferable to those on OV-17 (Table V); however since the acid may increase enormously in pathological conditions either column should prove satisfactory for the detection of such conditions. The range of normal values for PHPL on OV-1 (0.30 – 0.88 $\mu$g/mg urinary creatinine) was considerably lower than that of 0.5 – 3.5 $\mu$g/mg creatinine reported recently when the same liquid phase was employed under isothermal conditions[8], despite the fact that in several urines the PHPL peak was obviously partially overlapped by another peak. This discrepancy may well reflect the limited accuracy possible in the determination of very small amounts by gas chromatography due to the difficulty in deciding the position of the base line from which measurements are to be made.

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CHROM. 4378

# STUDIES ON THE ACID STABILITY OF NEUTRAL MONOSACCHARIDES BY GAS CHROMATOGRAPHY, WITH REFERENCE TO THE ANALYSIS OF SUGAR COMPONENTS IN THE POLYSACCHARIDES

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SUMMARY

Acid stability of the monosaccharides used as standards (glucose, mannose and galactose) and also recovery of the sugar components released by hydrolysis with methanolic hydrochloric acid of Copra meal mannan (coconut), Yuri glucomannan (lily bulb) and an immunologically active polysaccharide–peptide complex isolated by us from *Trichophyton mentagrophytes* were examined by gas chromatography.

The results obtained indicate that degradation of free sugars occurs to various extents using 1, 2.5 and 15% concentrations of hydrochloric acid in anhydrous methanol.

## INTRODUCTION

Since the first report by BISHOP and coworkers[1], a gas chromatographic technique has been developed for sugar analysis and has been extensively applied to the identification and quantitation of sugar components in biological materials[2–9].

In order to obtain the most suitable conditions for hydrolysis of the antigenic polysaccharide–peptide complex[10], we have performed some fundamental experiments for elucidating the acid stability of the monosaccharides by carrying out hydrolysis at various concentrations of acid in anhydrous methanol.

The present paper describes the results obtained by GLC for degradation during hydrolysis of the individual monosaccharides released from the polysaccharides.

## EXPERIMENTAL

### *Reagents and materials*

Solvents (reagent grade) were used as supplied unless specially noted. Monosaccharides used as standards in this work were obtained from commercial sources. Silylating reagent (TMS-HT) was purchased from Tokyo Kasei Co. Ltd., Tokyo.

Extraction and separation of the polysaccharide–peptide complex (PPC) with

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with immunological activity were carried out as described previously[10]. The complex was extracted from mycelia of *Trichophyton mentagrophytes* by the slightly modified phenol–water method of WESTPHAL *et al.*[11], and was purified by gel filtration on a Sephadex column. Copra meal mannan and Yuri glucomannan, which were extracted from coconuts and lily bulb, respectively, were kindly supplied by Dr. Y. TAKEUCHI (Department of Agricultural Chemistry, Gifu University).

*Gas chromatography*

A Hitachi Model K-53 gas chromatograph equipped with a temperature programmer and flame ionization detector was employed throughout this investigation. The U-shaped stainless-steel column, 2.0 m long with an I.D. of 3 mm, was packed with 3% Silicone SE-52 (Gaschro Tech. Co. Ltd., Tokyo) on acid-washed, silanized 80–100 mesh Chromosorb W (Gaschro Tech.).

Chromatography was carried out isothermally at 185° with the injection port at 230°. Flow rates, adjusted for optimal efficiency, were 47 ml/min, 43 ml/min and 270 ml/min for nitrogen as carrier gas and for hydrogen and air as detector gas, respectively.

*Preparation of anhydrous methanolic HCl*

Calcium oxide was added to methanol (reagent bottle); the mixture was allowed to stand overnight and was subsequently filtered and distilled. For preparing anhydrous methanolic HCl, hydrogen chloride gas was dissolved into the anhydrous methanol. The hydrogen chloride gas was produced by stepwise dropping concentrated sulphuric acid on NaCl and dried by passing it through U-calcium chloride and phosphorous pentoxide tubes.

*Methanolysis*

Ten milligrams of each of the monosaccharide standards (glucose, galactose and mannose and of the polysaccharides (an immunologically active polysaccharide–peptide complex (PPC), Copra meal mannan and Yuri glucomannan) were refluxed with 2 ml of anhydrous methanolic HCl in a sealed ampoule at 100° for 5 h. The hydrolysates were passed through the column (3.0 × 0.7 cm) containing Amberlite CG 120 Type I ($CH_3COO^-$) 100–200 mesh resin for neutralization.

5 and 2 mg of mannitol as an internal standard were dissolved in the standard sugar and hydrolysate solutions, respectively.

*Preparation of TMS derivatives*

To each methanolyzed sample containing mannitol was added 0.3 ml of silylating reagent (TMS-HT), a mixture of pyridine–hexamethyldisilazane–trimethylchlorosilane (10:2:1). The reaction mixture was shaken vigorously for about 30 sec and then warmed for 5 min at 60°. 1- to 2-$\mu$l aliquots of each mixture after centrifugation were used for injection into the gas chromatograph.

## RESULTS AND DISCUSSION

Although gas chromatography is a useful and suitable technique for analysis of sugar components in biological materials, it is difficult to obtain a satisfactory re-

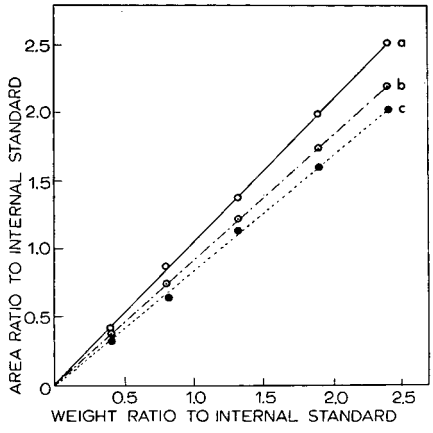


Fig. 1. Calibration curve for free monosaccharides. O—O, glucose; ⊙ – – – ⊙, mannose; ●----●, galactose. Internal standard, mannitol.

covery of sugars liberated by hydrolysis. This disadvantage arises from the facts that acid stability of the glycosidic bonds is dependent on the nature of the sugars involved in the linkages and also that acid degradation of the released sugars occurs. Therefore, attempting to obtain the optimum conditions of hydrolysis, we performed some experiments on the stability of free sugars.

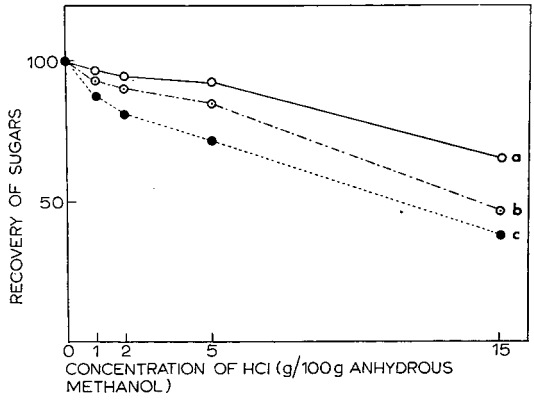


Fig. 2. Recovery of sugars after treatment by methanolic HCl at various concentrations. O—O, glucose; ⊙---⊙, mannose; ●----●, galactose.

The data given in the calibration curve (Fig. 1) show some differences among the standard sugars tested, such as glucose, mannose and galactose. For estimating the loss of the free sugars during hydrolysis of the polysaccharides, the three authentic sugars were treated with various concentrations of hydrogen chloride in anhydrous methanol. Analyses of these treated samples were then compared with those not subjected to the hydrolytic procedure.

As can be seen from the results (Fig. 2, Table I) obtained by calculating the recovery of sugars on the basis of the calibration curve in Fig. 1, it is of importance

TABLE I

RECOVERY OF SUGARS AFTER HYDROLYSIS IN VARYING CONCENTRATIONS OF METHANOLIC HYDROCHLORIC ACID

The weight of each sugar ($S_w$) was calculated from the following equation:

$$S_w \text{ (mg)} = \frac{S_a \cdot I_w}{I_a \cdot R}$$

where $S_a$ = observed area of each sugar, $I_a$ = observed area of internal standard (mannitol), $I_w$ = weight of internal standard added, $R$ = relative response of each sugar. The $R$ values for each sugar were obtained on the basis of the linear calibration curve shown in Fig. 1. $R$ values: mannose, 0.91; galactose, 0.84; glucose, 1.03.

| *Sugar* | *Concentration of HCl (g/100 g anhydrous methanol)* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *0* | | *1* | | *2* | | *5* | | *15* | |
| | % | $S_w$ (mg) | % | $S_w$ (mg) | % | $S_w$ (mg) | % | $S_w$ (mg) | % | $S_w$ (mg) |
| Mannose | 100 | 9.5 | 93.7 | 8.9 | 90.5 | 8.6 | 85.3 | 8.2 | 47.4 | 4.5 |
| Galactose | 100 | 9.7 | 88.7 | 8.6 | 81.4 | 7.9 | 72.2 | 7.0 | 38.1 | 3.7 |
| Glucose | 100 | 8.8 | 96.6 | 8.5 | 95.4 | 8.4 | 93.2 | 8.2 | 65.9 | 5.8 |

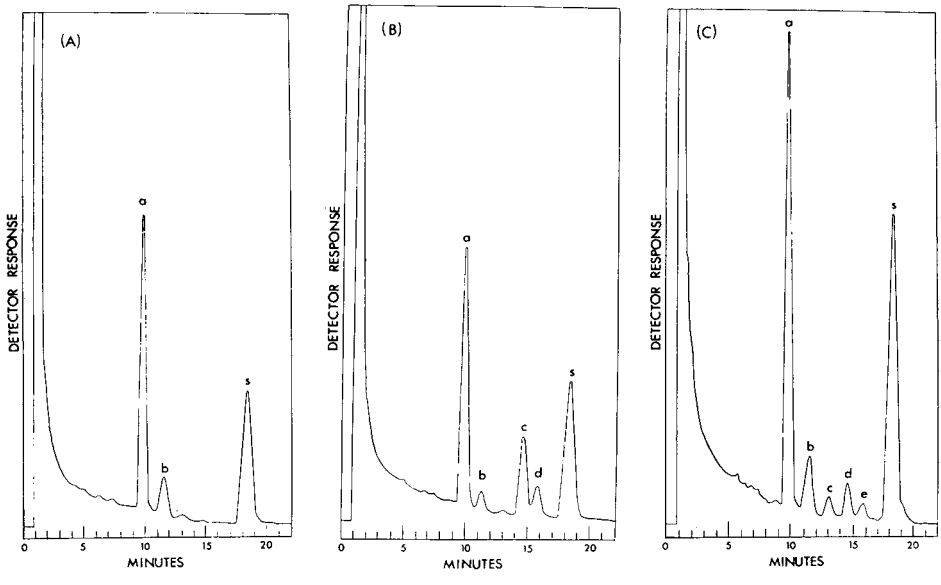


Fig. 3. Typical gas chromatograms of the free sugars released after hydrolysis by 5% methanolic HCl of various polysaccharides. Internal standard(s): mannitol. (A) Copra meal mannan: a = α-mannose, b = β-mannose; (B) Yuri glucomannan: a = α-mannose, b = β-mannose, c = α-glucose, d = β-glucose; (C) An immunologically active polysaccharide–peptide complex (PPC) from *T. mentagrophytes*: a = α-mannose, b = β-mannose + α-galactose, c = β-galactose, d = α-glucose, e = β-glucose. Column: 3% Silicone SE-52 on Chromosorb W (80–100 mesh); column temperature, 185°; injection port temperature, 230°; flow rates of carrier gas ($N_2$), hydrogen and air for detector, 47 ml/min, 43 ml/min and 270 ml/min, respectively.

TABLE II

CHANGES IN THE SUGAR COMPOSITION (IN μMOLES) OF SEVERAL POLYSACCHARIDES AFTER HYDROLYSIS IN METHANOLIC HYDROCHLORIC ACID AT VARIOUS CONCENTRATIONS

| *Polysaccharides* | *Sugar components* | *Concentration of HCl (g/100 g anhydrous methanol)* | | | |
|---|---|---|---|---|---|
| | | *1* | *2* | *5* | *15* |
| Copra mannan (coconut) | total | 18.2 | 18.8 | 26.6 | 10.9 |
| | mannose | 18.2 | 18.8 | 26.6 | 10.9 |
| Yuri glucomannan (lily bulb) | total | 20.9 | 21.0 | 22.3 | 18.6 |
| | mannose | 13.6 | 13.2 | 12.6 | 11.0 |
| | glucose | 7.3 | 7.8 | 9.7 | 7.6 |
| PPC | total | 9.7 | 14.3 | 9.4 | 7.2 |
| (*Trichophyton mentagrophytes*) | mannose | 4.8 | 6.1 | 4.9 | 3.8 |
| | galactose | 2.2 | 3.4 | 2.1 | 1.3 |
| | glucose | 2.7 | 4.8 | 2.4 | 2.1 |

to note that, as the concentration of hydrogen chloride increases, the sugars are destroyed to an extent which depends on the nature of the monosaccharide used. Glucose is in general more acid stable than mannose and galactose.

Galactose, least acid stable, is however found to be destroyed even to the extent of 62% when heated in 15% methanolic HCl at 100° for 5 h, although two other sugars also undergo remarkable decomposition.

Typical gas chromatograms of the sugar components released by hydrolysis of the polysaccharides in 5% methanolic HCl are shown in Fig. 3. Since, as described above, the free sugars liberated are degraded by acid during hydrolysis, the yield of total sugars as well as the ratio of each sugar in them is found to be changed at the concentrations of hydrogen chloride tested. The results of analyses of Copra meal mannan from coconut, Yuri glucomannan from lily bulb and an immunoactive PPC from *T. mentagrophytes* are illustrated in Table II. The best yields of total sugars released from Copra meal mannan and Yuri glucomannan could be obtained by hydrolysis with 5% methanolic HCl.

However, as for the immunoactive PPC containing acid-labile galactose, it was found that hydrolysis was successfully carried out in 2% methanolic HCl where recovery of galactose and yield of total sugar could be obtained at the maximal level. In this case, the presence of peptide moiety in the polysaccharides has been known to complicate the problem because of an undesirable interaction between the free sugars and amino acids such as tryptophan, cysteine and methionine[12,13].

These experimental results indicate that it is difficult to obtain perfect conditions for acid hydrolysis under which all glycosidic linkages involved in the polysaccharides are cleaved and, moreover, all free monosaccharides still remain intact after acid treatment. Therefore, for satisfactory analysis of the sugar composition of polysaccharides, optimum conditions in hydrolysis should be ascertained in advance by preliminary experiments.

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CHROM. 4359

# GAS CHROMATOGRAPHIC SEPARATION OF TRICYCLIC COMPOUNDS WITH BRIDGEHEAD NITROGEN ATOMS

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SUMMARY

The diastereoisomeric mixtures of a series of unsubstituted and substituted perhydrodipyrido[1,2-*c*;2',1'-*e*]imidazoles (I), perhydropyrido[1,2-*c*]pyrrolo[2,1-*e*]imidazoles (II) and perhydrodipyrido[1,2-*c*;2'1'-*f*]pyrimidines (III), have been separated by preparative gas–liquid chromatography. Correlations have been made between the retention times and stereochemistry of the isomers in each mixture.

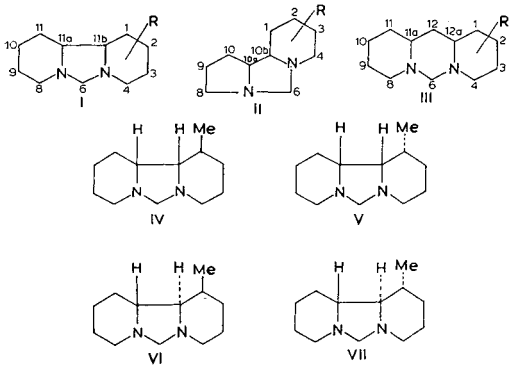
## INTRODUCTION

As part of a detailed investigation into the use of NMR and IR spectroscopy in assigning the configurations and preferred conformations of bicyclic and tricyclic compounds with a nitrogen atom at a ring fusion a series of substituted perhydrodipyrido-[1,2-*c*; 2'1'-*e*]imidazoles (I)[1], perhydropyrido[1,2-*c*]pyrrolo[2,1-*e*]imidazoles (II)[2] and perhydrodipyrido[1,2-*c*; 2'1'-*f*]pyrimidines (III)[3] have been prepared. Since each compound possesses two and usually three asymmetric centres there can exist a number of possible stereoisomers. For example structures IV–VII represent the four diastereoisomeric 1-methylperhydrodipyrido[1,2-*c*; 2',1'-*e*]imidazoles.* In addition, because of the conformational mobility of the bridgehead nitrogen atoms, each isomer may exist in a number of possible conformations with the AB and BC ring fusions *cis cis*, *cis trans*, *trans cis* or *trans trans*.**

The diastereoisomeric relationship between each compound permits separation of a mixture by physical means and this report describes the preparative gas chromatographic separation of mixtures of I, II and of III into their components. Configurations and preferred conformations have been assigned to the pure isomers by NMR and IR spectroscopy and as a result it has been possible, in most instances, to establish a correlation between structures and column retention times. This correlation may then be used as a guide in establishing the stereochemistry of similar types of heterocyclic compounds.

*All the compounds discussed in this paper exist as racemates or in certain case optically inactive forms.

**See Fig. 2 for the various configurations and preferred conformations of these compounds.




## EXPERIMENTAL

*Column packing materials*

(a) 20% w/w Carbowax 1540 on 60–80 Celite (B.D.H. Ltd.) was prepared by adding a solution of Carbowax 1540 (25 g) in chloroform (500 ml) to the Celite (100 g), thoroughly stirring the slurry and then removing the solvent by rotary evaporation, followed by drying the material in an oven at 110° for 6 h.

(b) 12% w/w Carbowax 20M terminated with T.P.A. on 60–72 DMCS (supplied by J.J.'s Ltd., Kings Lynn, Norfolk, Great Britain) was prepared as above except that methylene chloride was used as solvent.

*Construction of columns*

Packing (a) was used to prepare a 22 ft. × 3/8 in. O.D. coiled aluminium column for use in a Wilkens A700 Autoprep. The column was packed under vacuum (25 mm Hg) and the packing added continuously with constant vibration. The column was conditioned at 155° for 48 h in a stream of nitrogen.

Packing (b) was used to prepare a 15 ft. × 3/8 in. O.D. coiled glass column for use in a Pye 105 chromatograph. The packing was introduced under a pressure of 20 p.s.i. of nitrogen and vacuum applied to the outlet. The column was conditioned at 185° for 48 h in a stream of nitrogen.

Analytical data were obtained from a Perkin-Elmer F11 instrument with flame ionisation detector using a 2 m × 1/8 in. O.D. stainless steel column packed with 20% w/w Carbowax 1540 on 60–80 Chromosorb supplied by Perkin-Elmer. Conditions: column temp., 155°; injection temp., 200°; nitrogen carrier with inlet pressure of 30 p.s.i. and a 30 ml/min flow rate. Preparative gas chromatography of the perhydrodipyrido[1,2-*c*;2′,1′-*e*]imidazoles was carried out on the Wilkens Autoprep A700 using the 22-ft. column at 200 ml/min and temperatures between 150 and 165°. In all cases hydrogen was used as carrier gas at an inlet pressure of 40 p.s.i. and an inlet port temperature of 220°. The perhydropyrido[1,2-*c*]pyrrolo[2,1-*e*]imidazoles and perhydrodipyrido[1,2-*c*; 2′,1′-*f*]pyrimidines were separated on a Pye 105 preparative gas chromatograph fitted with the 15-ft. glass column packed with 12% Carbowax 20M terminated with TPA with a 200 ml/min of nitrogen flow at an inlet pressure of 80 p.s.i. and a column temperature between 150–200° depending on the mixture being separated. Sample sizes on both instruments varied from between 50 μl to 300 μl and depended on

the complexity and resolution of the individual mixtures. The Pye 105 instrument was programmed to separate the individual mixtures automatically and gave a total recovery of 75–80%. The Autoprep A700 was used manually and gave poorer recovery of separated components.

## RESULTS AND DISCUSSION

As previously stated the configurations and preferred conformations of the isomers separated from each mixture were assigned using IR and NMR spectroscopy. Although the conformations are those existing in chloroform solution at room temperature the reasonable assumption has been made that the conformational preferences will be the same during the chromatographic separation.

Under the above experimental conditions the analytical column gave similar resolutions to the 15-ft. and 20-ft. preparative columns and the former was used to assign the approximate (±5%) composition of each mixture and the retention times for each isomer. Fig 1. shows chromatograms for some typical mixtures. The details of separation of the mixtures of the perhydrodipyrido[1,2-*c*; 2′1′-*e*]imidazoles (I) is reported in Table I. Not all possible isomers were present in some of the mixtures but in every separation the isomers with an *anti* IIaIIb configuration had shorter retention times than the corresponding *syn* IIaIIb isomers. It is also of note that *syn* and *anti* isomers substituted with an axial methyl group had shorter retention times than the corresponding isomers with an equatorial methyl group.

The mixture of 3-methylperhydrodipyrido [1,2-*c*; 2′,1′-*e*]imidazoles contained all four possible diastereoisomers. However, preparative GLC showed only two peaks and separation gave two fractions each shown by NMR to contain two isomers. These pairs were then separated into the individual components by column chromatography using alumina as adsorbent. In each separation, elution with light petroleum (60–80°)/ether removed the axial methyl-substituted isomer initially, a result consistent with the above observations.

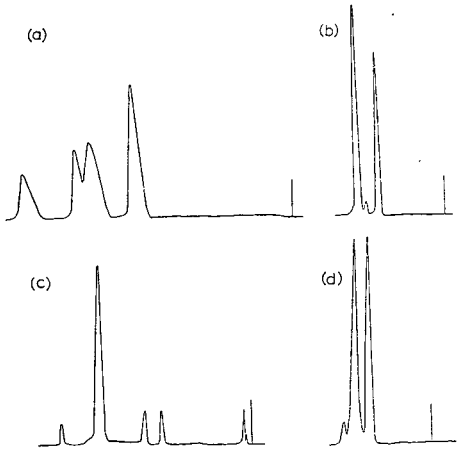



Fig. 1. Chromatograms of some of the mixtures. (a) 3-Methylperhydrodipyrido [1,2-*c*; 2′,1′-*f*]-pyrimidines, (b) 2-methylperhydropyrido[1,2-*c*]pyrrolo[2,1-*e*]imidazoles, (c) 1-methylperhydrodipyrido[1,2-*c*; 2′,1′-*e*]imidazoles, (d) 2,10-dimethylperhydrodipyrido[1,2-*c*; 2′,1′-*e*]imidazoles.

TABLE I

ISOMER DISTRIBUTION AND RETENTION TIMES OF PERHYDRODIPYRIDO[1,2-*c*; 2',1'-*e*]IMIDAZOLES (I)

| *Mixture* | *Retention time (min)* | *Isomer distribution* | *Configuration and preferred conformation* | *Configuration of substituent* |
|---|---|---|---|---|
| Perhydrodipyrido[1,2-*c*; 2'1'-*e*]-imidazoles | 9.2 | 50 | *trans anti trans* | |
| | 13.5 | 50 | *trans syn trans* | |
| 6-methyl | 10.0 | 50 | *trans anti trans* | eq |
| | 12.6 | 45 | *trans syn trans* | eq |
| | 14.7 | 5 | *trans syn trans* | ax |
| 6-isopropyl | 13.2 | 50 | *trans anti trans* | eq |
| | 16.7 | 50 | *trans syn trans* | eq |
| 1-methyl | 10.6 | 10 | *trans anti trans* | ax |
| | 13.4 | 10 | *trans anti trans* | eq |
| | 19.2 | 75 | *trans syn trans* | ax |
| | 26.0 | 5 | *trans syn cis* | ax |
| 3-methyl | 11.4 | 20 | *trans anti trans* | ax |
| | 11.4 | 30 | *trans anti trans* | eq |
| | 15.7 | 5 | *trans syn trans* | ax |
| | 15.7 | 45 | *trans syn trans* | eq |
| 4-methyl | 10.4 | 50 | *trans anti trans* | eq |
| | 14.5 | 50 | *trans syn trans* | eq |
| 2,10-dimethyl | 11.5 | 50 | *trans anti trans* | di-eq |
| | 15.8 | 45 | *trans syn trans* | di-eq |
| | 18.2 | 5 | *trans syn trans* | eq-ax |

TABLE II

ISOMER DISTRIBUTION AND RETENTION TIMES OF PERHYDROPYRIDO[1,2-*c*]PYRROLO[2,1-*e*]IMIDAZOLES (II)

| *Mixture* | *Retention time (min)* | *Isomer distribution* | *Configuration and preferred conformation* | *Configuration of methyl group* |
|---|---|---|---|---|
| II (R = H) | 10.2 | 50 | *trans syn cis* | |
| | 13.4 | 50 | *trans anti cis* | |
| II (R = 1-Me) | 13.2 | 6 | *trans syn cis* | eq |
| | 18.9 | 65 | *trans syn cis* | ax |
| | 20.2 | 29 | *cis anti cis* | eq |
| II (R = 2-Me) | 11.5 | 50 | *trans syn cis* | eq |
| | 15.2 | 46 | *trans anti cis* | eq |
| | 13.7 | 4 | *cis anti cis* | eq |
| II (R = 3-Me) | 11.3 | 25 | *trans syn cis* | ax |
| | 13.1 | 25 | *trans syn cis* | eq |
| | 17.3 | 40 | *trans anti cis* | eq |
| | 17.3 | 10 | *cis anti cis* | eq |
| II (R = 4-Me) | 10.8 | 50 | *trans syn cis* | eq |
| | 15.2 | 47 | *trans anti cis* | eq |
| | 16.6 | 3 | *cis anti cis* | eq |

Table II shows the details of separation and retention times of the mixtures of the perhydropyrido [1,2-*c*]pyrrolo[2,1-*e*]imidazoles (II). As with compounds of type I, there is again a close correlation between the relative retention times of the isomers in a particular mixture and their structure and preferred conformations. Isomers with a

*trans syn cis* stereochemistry have the shortest retention time followed by *trans anti cis* and then *cis anti cis*.

The retention times of the perhydrodipyrido[1,2-*c*; 2′,1′-*f*]pyrimidines (III) are shown in Table III. Mixtures of the 1-methyl- and 4-methyl-substituted isomers were separated by preparative gas chromatography but, for convenience, the parent compounds III (R = H), and the 2- and 3-methyl-substituted isomers, which were solids of long retention times, were separated by column chromatography on alumina using light petroleum (60–80°)/ether as eluant. It is significant that in each separation on alumina the elution pattern was identical to that observed using analytical GLC. Again it is clearly apparent that a correlation exist between the retention times and stereochemistry of the isomers in a particular mixture.

Fig. 2 illustrates the different configurations and preferred conformation observed in the series of perhydrodipyrido[1,2-*c*; 2′,1′-*e*]imidazoles (I), perhydrodipyrido-[1,2-*c*]pyrrolo[2,1-*e*]imidazoles (II) and the perhydrodipyrido[1,2-*c*; 2′,1′-*f*]pyrimidi-

TABLE III

ISOMER DISTRIBUTION AND RETENTION TIMES OF THE PERHYDRODIPYRIDO[1,2-*c*; 2′,1′-*f*]PYRIMIDINES (III)

| *Mixture* | *Retention time (min)* | *Isomer distribution* | *Configuration and preferred conformation* | *Configuration of methyl group* |
|---|---|---|---|---|
| III (R = H) | 26.4 | 50 | *trans syn trans* | |
| | 36.0 | 50 | *trans anti cis* | |
| III (R = 1-Me) | 33.4 | 55 | *trans syn trans* | ax |
| | 40.1 | 30 | *trans anti cis* | ax |
| | 45.0 | 15 | *trans anti cis* | eq |
| III (R = 2-Me) | 31.2 | 50 | *trans syn trans* | eq |
| | 40.4 | 50 | *trans anti cis* | eq |
| III (R = 3-Me) | 27.4 | 25 | *trans syn trans* | ax |
| | 35.4 | 25 | *trans syn trans* | eq |
| | 37.0 | 30 | *trans anti cis* | ax |
| | 46.0 | 20 | *trans anti cis* | eq |
| III (R = 4-Me) | 32.0 | 50 | *trans syn trans* | eq |
| | 40.8 | 50 | *trans anti cis* | eq |

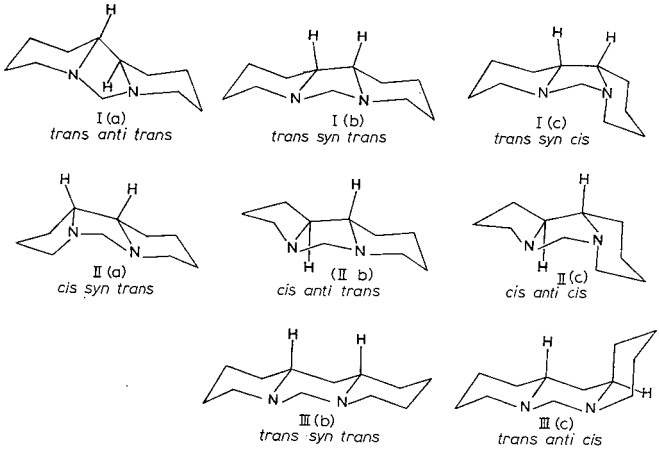



Fig. 2. Configuration and preferred conformations of compounds I, II and III.

nes (III). Each series is in order of increasing retention time (left to right). The close similarity between stereochemistry and relative retention times of compounds of types I and II is easily discernable. Isomers of shortest retention time are those with a 'planar' structure and with the nitrogen lone electron pairs on the opposite side of the molecule (Ia and IIa). A change in conformation to a more 'angled' molecule *e.g.* Ib, IIb and IIIb to Ic, IIc and IIIc, lengthens the retention times still further and isomers with equatorially substituted methyl groups tend to have longer retention times than the corresponding isomers with axial methyl groups. Presumably the 'flat' structures with nitrogen lone pairs on the same side are more strongly attracted by hydrogen bonding to the polar Carbowax stationary phase and puckering of the molecule facilitates this even more. Support for this observation is provided by attempts to separate the mixtures on non-polar columns such as Apiezon L and Silicone SE-30. Much poorer resolutions were obtained and the order of retention times of the various isomers were often changed. The boiling points of the individual isomers separated from a particular mixture also bore no special relationship to their elution pattern.

ACKNOWLEDGEMENT

The authors wish to thank the Science Research Council for a grant for the purchase of the Pye 105 gas chromatograph.

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CHROM. 4377

# TOBACCO CHEMISTRY*

## II. ANALYSIS OF THE GAS PHASE OF TOBACCO SMOKE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

The gas phase of tobacco smoke was injected, without prior condensation, through a glass injection system onto an efficient glass capillary column maintained at low temperature. Components eluted on temperature programming were studied by means of a mass spectrometer coupled to the column via a low-dead-volume molecule separator. By making use of the separation principle of the phase, thirty-four compounds were identified from their mass spectra.

## INTRODUCTION

The gas phase of tobacco smoke is so complex[2–4] that currently the only effective method for the analysis of this material is gas chromatography using highly efficient columns[5–8]. GROB has developed a refined technique for the use of glass capillary columns in the separation of the components of the condensed gas phase[9–15] and in their identification by mass spectrometry[16,17], but a number of problems remain. For example, injection of fresh smoke is preferred[12], since changes may occur during the trapping process, requiring the introduction of large volumes of a diluted gas sample onto a capillary column without danger of serious loss of separating power. Moreover, it has been pointed out[12,13] that decomposition of some smoke constituents is likely if contact with a metal surface is allowed.

Interfacing of capillary columns with a mass spectrometer also presents some problems, particularly if a sample-enrichment device is used to meet sensitivity requirements. Difficulties caused by dead volume in the connection of capillary columns to a mass spectrometer via a molecule separator have also been experienced in the analysis of tobacco smoke[16,17].

The purpose of this work was to overcome the above-mentioned difficulties in analysing the gas phase of tobacco smoke. The problem of on-column concentration

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of a large smoke sample was solved by use of a low-temperature injection method[7,18]. In order to achieve a fairly inert analytical system, a glass injection device and efficient glass capillary columns were used. A mass spectrometer was coupled to the capillary column[19] through a low-dead-volume molecule separator[20], and the spectra of some of the separated components were recorded by means of a novel data-acquisition system[21].

## EXPERIMENTAL

### *Capillary column gas chromatography*

Glass capillaries were prepared on a commercial glass drawing machine (Dr. Hupe Apparatebau, Karlsruhe, G.F.R.). The original pyrex glass tubes were carefully washed with acetone and then dried. The inner surfaces of the glass capillaries were silanised with mixed vapours of hexamethyldisilazane and trimethylchlorosilane[22]. Their coating was carried out with a 10 % solution of Silicone Oil SF-96 in toluene by the dynamic method under controlled conditions[23]. Capillary columns, 48 and 120 m long with an I.D. of 0.2 mm, were used for the analysis of tobacco smoke; they had efficiencies, measured for toluene at room temperature, of 160,000 and 530,000 theoretical plates, respectively.

The all-glass injection splitter illustrated in Fig. 1 was used for mass spectrometry work. The glass-to-metal connections were made with the aid of Kovar alloy.

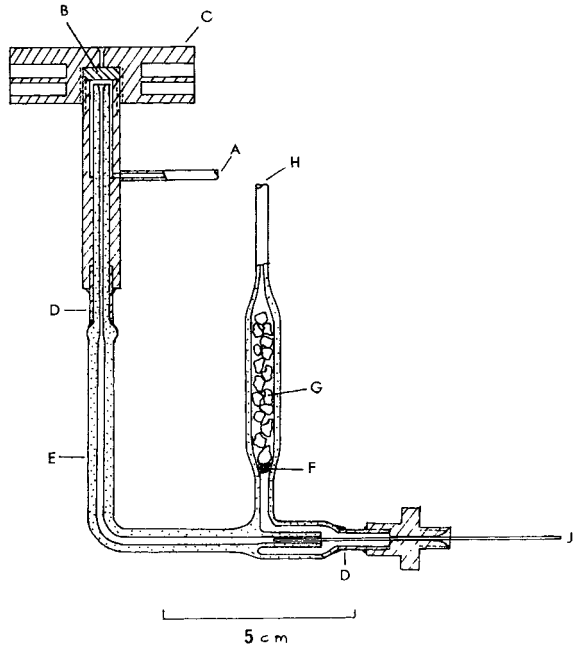



Fig. 1. Glass injection splitter. A = inlet for preheated carrier gas; B = septum; C = flanged nut for septum; D = Kovar alloy; E = glass tube; F = glasswool plug; G = charcoal; H = outlet to control valve; J = capillary column.

The inner glass tube was extended to the septum insert, and the end of the capillary was inserted directly into the mixing chamber so that contact of sample with metal was avoided. The buffer volume of the outlet tubing was filled with charcoal to prevent back diffusion and to protect the regulating valve.

A system which facilitates rapid heating was designed for efficient temperature programming of glass capillary columns. The small oven, shown in Fig. 2, consists of two complementary Marinite parts lined inside with a thin aluminium sheet. The glass capillary column is hooked onto the supports and connected to the splitter, situated in the same block, and the adapter of the flame ionisation detector. This adapter is interchangeable with another one for connection to the mass spectrometer. The column is heated with the aid of a concentrically arranged resistance spiral and an F and M Model 240 temperature programmer. Thermal gradients are minimised by the use of a small fan. The system is cooled by admitting an adjustable stream of liquid carbon dioxide through a jet[24]. The course of heating and cooling is followed with an extra thermocouple.

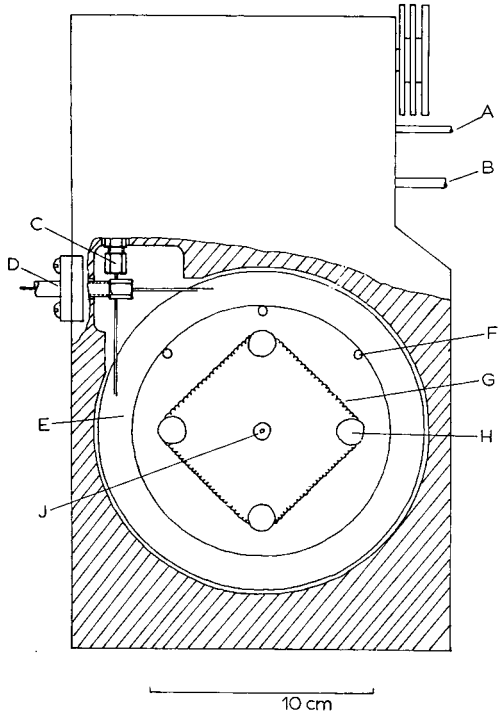



Fig. 2. Oven for capillary column chromatography. A = inlet for carrier gas; B = outlet to control valve; C = connection to injection splitter; D = adapter for FID-detector or connection to the mass spectrometer; E = glass capillary column bundle; F = support for capillary column; G = resistance spiral; H = support for resistance spiral; J = jet nozzle for liquid carbon dioxide.

*Low-temperature injection*

Commercial American blend cigarettes without filter tips were smoked in 30-ml puffs through a Cambridge filter by means of a glass syringe operated manually. Between 1 and 10-ml (usually 5-ml) samples of the gas phase were withdrawn from

this large syringe via a second glass syringe for injection into the column which had been cooled to −70°. The oven temperature was then programmed up to 130°. This enrichment method is essentially that proposed by RUSHNECK[7], but we found that a small splitting ratio was required to attain good resolution.

Problems concerning enrichment were experienced when the 120-m column was used, owing to the high inlet pressure necessary and the low ability of conventional silicone rubber seals to withhold gases at low temperature.

*Coupling of the capillary column to the mass spectrometer*

Capillary columns were connected to the LKB 9000 mass spectrometer through the Becker–Ryhage molecule separator. The performance of this separator in connection with capillary columns has already been studied by one of us[19], and it has been shown that only the second stage should be used, but with the jet nozzle having an I.D. of 0.24 mm replaced by the one from the first stage which has an I.D. of 0.10 mm.

*Mass spectrometry*

The mass spectrometric parameters were adjusted in order to fit the gas chromatographic conditions so that the slit widths were 0.20 and 0.30 mm, respectively, and the scan speed had a value corresponding to *m/e* 12–200 in 2 sec. Sensitivity and scan speed were consequently increased at the expense of resolution, which was 250 (10 % valley definition).

The ion source temperature was 230°, the trap current 60 μA, and the electron energy 70 eV during all runs. The recording of mass spectra was made by means of the data acquisition system described recently by two of us[21]. After some reduction of data, the background was subtracted, and normalized mass spectra were plotted and printed out separately.

## RESULTS AND DISCUSSION

It is generally recognised that the high efficiency of capillary columns, conveniently combined with temperature programming, is the first presupposition in the gas chromatographic separation of a complex mixture containing components with a wide range of boiling points. We have selected Silicone Oil SF-96 as a stationary phase owing to its ability to work over a wide temperature range[24] and to form very effective films on the inner wall of glass capillaries.

First attempts to obtain a good separation of the gas phase of tobacco smoke failed when injecting the sample onto an efficient capillary column at room temperature using a low splitting ratio (Fig. 3), and the need for a technique facilitating sample enrichment was realized. A simple and efficient method is apparently the low-temperature process proposed by RUSHNECK[7]. When the smoke sample was injected onto the same capillary column cooled to −70° and when the column was programmed from −70° to 130°, a considerable improvement was achieved, as can be seen from Fig. 4. The use of this technique is advantageous not only in increasing the possibility of separating the early peaks at low temperature but mainly in efficiently concentrating the sample components at the beginning of the column; no marked change was noted even when two smoke injections separated by a 15-sec interval were made.

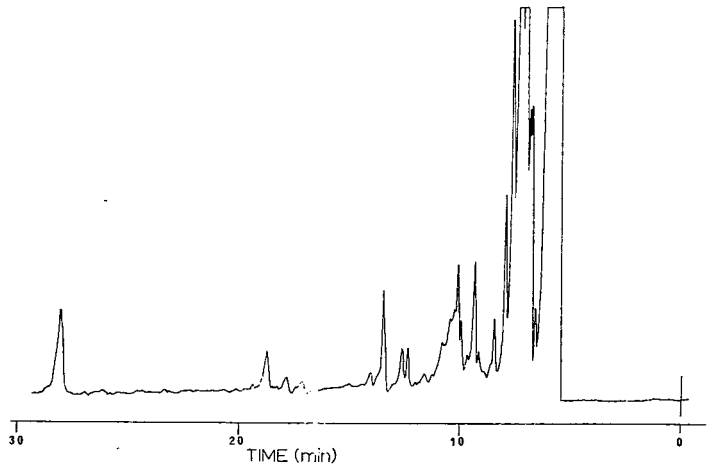



Fig. 3. FID chromatogram of 2 ml of tobacco smoke, split 200:1. Column: 48 m × 0.2 mm I.D. glass capillary, coated with SF-96, operated isothermally at 25°. $N_2$ flow rate, 0.3 ml/min.

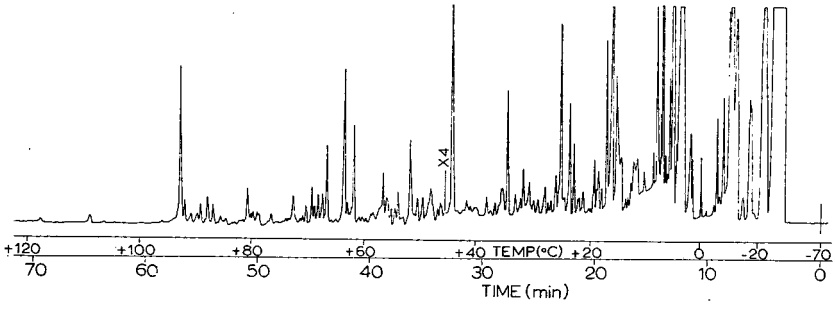



Fig. 4. FID chromatogram of 6 ml of tobacco smoke, split 10:1. Column as in Fig. 3. Initial column temperature, −70°. $N_2$ flow rate at 25°, 0.3 ml/min.

The broadness of the first peaks in the chromatograms with low-temperature injection may be attributable partly to the behaviour of the stationary phase at low temperature, as has been studied in detail by ALTENAU *et al.*[25] and CLAEYS AND FREUND[26], as well as to the overlap of components. The marked increase in the response of the flame ionisation detector for a column at low temperature, also observed in this work, has been discussed by SINGELTON *et al.*[27] and WALSH *et al.*[28].

For the separation and identification of some of the components of tobacco smoke, the 120-m capillary column was used. The chromatogram obtained with the aid of the total ion current (TIC) monitor of the mass spectrometer can be seen in Fig. 5, and some of the identified substances are listed in Table I. The mass spectra were of adequate analytical quality, but more enrichment is necessary for the trace components. The convenience of the method as a whole is exemplified by the identification of the four minor components 16–19 which are all eluted within a period of 40 sec. Fig. 6 shows the spectrum of a small peak (number 7), identified as isobutane,

TABLE I

COMPONENTS IDENTIFIED BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY IN CIGARETTE SMOKE

| *Peak No.*[a] | *Substance* | *Peak No.* | *Substance* |
|---|---|---|---|
| 1 | $CH_4$, $H_2S$, NO | 18 | 1,2-Dimethylcyclopropane (*trans*) |
| 2 | $CO_2$, CO, $NO_2$ | 19 | *n*-Pentane |
| 3 | Ethane | 20 | 2-Methyl-1,3-butadiene |
| 4 | Propene | 21 | $CS_2$ + 1,2-dimethylcyclopropane (*cis*) |
| 5 | Propane | 22 | 2-Methyl-2-butene |
| 6 | Methyl chloride | 23 | Cyclopentadiene |
| 7 | Isobutane | 24 | 2-Propanone |
| 8 | Ethanal | 25 | Acetonitrile + components not yet identified |
| 9 | 1-Butene | 26 | Benzene |
| 10 | 1,3-Butadiene | 27 | 2,5-Dimethylfuran |
| 11 | *n*-Butane | 28 | Toluene |
| 12 | 2-Butene (*trans*) | 29 | Ethylbenzene |
| 13 | 2-Butene (*cis*) | 30 | Methyloctene |
| 14 | 1,1-Dimethylcyclopropane | 31 | *m*-Xylene + *p*-xylene |
| 15 | 2-Methylbutane | 32 | *o*-Xylene |
| 16 | 1-Pentene | 33 | Heptyl chloride |
| 17 | Furan | 34 | 1-Methyl-4-isopropenyl-1-cyclohexene |

[a] See Fig. 5.

while Fig. 7 (peak 18) apparently corresponds to *trans*-1,2-dimethylcyclopropane, which to our knowledge has not been identified in tobacco smoke previously.

It has been pointed out[16,29] that distortion of mass spectra may occur in GC–MS work because of the change in concentration during scanning. In order to study any possible bias in our mass spectra, toluene was injected and its mass spectrum recorded at different points on the elution curve (see Fig. 8). The ratio of the ion intensities

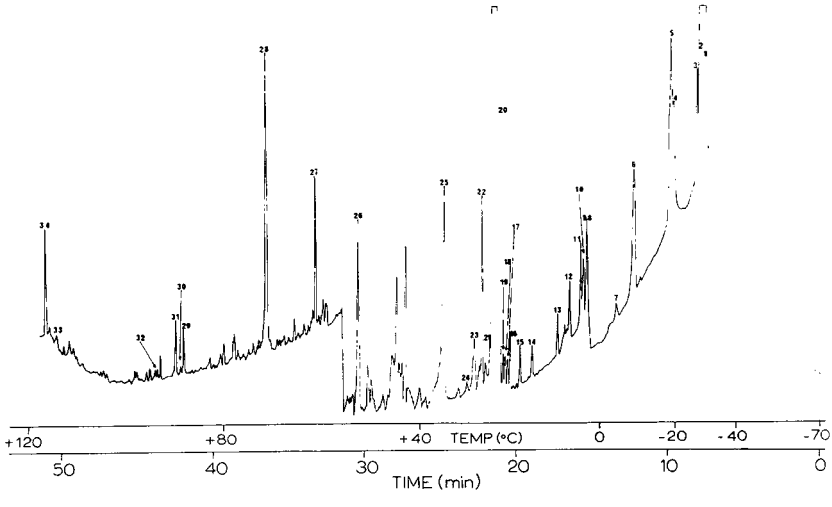
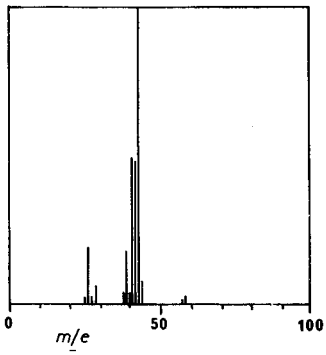



Fig. 5. TIC chromatogram of 5 ml of tobacco smoke split approximately 1:1. Column 120 m × 0.2 mm I.D. glass capillary coated with SF-96. Initial column temperature, −70°. He flow rate at 25°, 0.4 ml/min. Peak numbers refer to Table I.



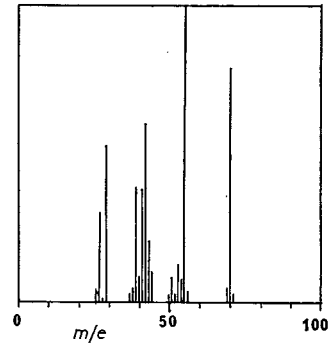




Fig. 6. Mass spectrum of peak 7 after background subtraction and normalisation.

Fig. 7. Mass spectrum of peak 18 after background subtraction and normalisation.

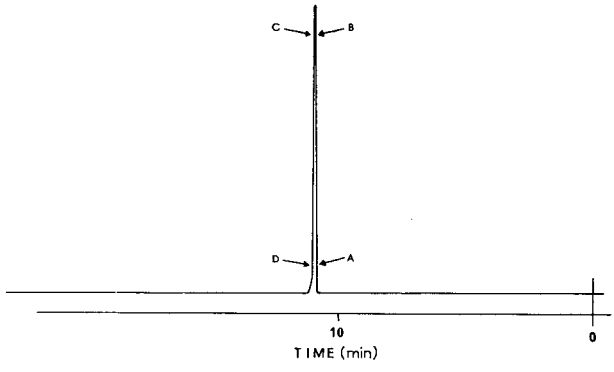



Fig. 8. TIC elution curve for toluene showing points at which mass spectra (Table II) were recorded.

at *m/e* 39 and 91 was taken as a measure of spectrum bias, and it was concluded that the scan speed used in our experiments was sufficient (Table II).

The use of a highly efficient column with a non-selective phase seems appropriate, since some closely related isomers which give similar mass spectra can be distinguished chromatographically and their structures confirmed when the separation principle

TABLE II

RATIO OF ION INTENSITIES IN MASS SPECTRUM OF TOLUENE AT DIFFERENT POINTS ON GC ELUTION CURVE

| *Points on the elution curve*[a] | *Ratio of ion intensities at m/e 39 and 91* |
|---|---|
| A | 0.08 |
| B | 0.15 |
| C | 0.12 |
| D | 0.10 |
| Published spectrum | 0.13–0.20 |

[a] See Fig. 8.

is known. For example, the *cis*- and *trans*-isomers of 2-butene, which give almost identical mass spectra, were differentiated in this way, while *o*-xylene was similarly distinguished from the *meta*- and *para*-isomers. On the other hand, some fractions overlap even when a capillary column with efficiency as high as 530,000 theoretical plates is used. For a more complete analysis, complementary use of an efficient polar capillary column is clearly necessary.

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CHROM. 4375

# DETERMINATION OF CREATININE IN SOUPS AND SOUP PREPARATIONS BY ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

Ion-exchange chromatography, coupled with continuous spectrophotometric monitoring in the UV region, was used to determine the total creatinine in soup preparations, dehydrated soups and tinned soups, containing meat extracts. This is an accurate method because it permits the separation of interfering substances that simulate creatinine in JAFFÉ's test. The recovery of creatinine in the soup preparations, dehydrated soups, and tinned soups amounted to 99.8 ± 0.8 %, 100.3 ± 0.9 %, and 100.2 ± 1.1 %, respectively.

## INTRODUCTION

In our previous chromatographic analysis of soup preparations[1], an unknown compound, sometimes present in a large amount, gave a peak so close to that of adenine as to interfere with its determination. From its UV absorption spectrum, JAFFÉ's test[2] and its retention time, this compound was identified as creatinine. The first aim was to separate these two compounds by a suitable choice of the molarity and the pH of the eluent buffer. It was decided to use ion-exchange chromatography to separate the creatinine in soup and soup preparations from interfering substances which sometimes introduce an element of uncertainty into HADORN's method[3], so far the only official method for the determination of the meat-extract content of these products[4]. The insufficient specificity of JAFFÉ's test had previously led others to effect this separation by ion-exchange chromatography, coupled with UV spectrophotometric determination[5–7]. However, this technique was not suitable for our purpose, so with some modifications we used the previously developed method for the determination of ribonucleotides[1]; this new method will be described below.

## EXPERIMENTAL

### *Apparatus and reagents*

The chromatographic column (30 × 0.9 cm) was packed to a height of 5 cm with the cation-exchange resin Aminex $A_5$ (Bio-Rad Labs., Richmond, Calif., U.S.A.), the spherical particles of which had a diameter of 8–12 $\mu$. The column was used in

conjuction with a Mini-Pump (Milton Roy Co., Fla., U.S.A.) and a Beckman DB-G double-beam spectrophotometer fitted with a 10-in. Beckman recorder and a microcell with a quartz window, a light path of 10 mm, and a total volume of 0.3 ml (catalogue Beckman No. 97290).

The reagents were: HCl, 2 *N*; Brockmann's III alumina; Buffer pH 4.30 = 0.3 *M* sodium acetate, adjusted to pH 4.30 ± 0.02 with glacial acetic acid; Buffer pH 3.70 = 0.3 *M* acetic acid, adjusted to pH 3.70 ± 0.02 with 40% NaOH; 1 m*M* cytosine in buffer pH 3.70; 0.5 m*M* creatinine in buffer pH 3.70. 0.1 ml/l of caprilyc acid was added to all buffers as preservative.

*Procedure*

A sample of a soup preparation, a dehydrated soup, or a tinned soup was weighed out accurately and dissolved in distilled water in such a way as to obtain a solution containing about 0.5 μmole of creatinine per ml. Whenever necessary, the fat and the insoluble components were removed by treating part of the solution with kieselguhr and by filtering it through a Whatman No. 40 paper. About 20 ml of the filtrate were collected, and exactly 10 ml were transferred with a pipette into a flat-bottomed porcelain dish. 5 ml of 2 *N* HCl were added, and the mixture was evaporated to dryness on a boiling water bath. The dry residue was taken up in 5 ml of 2 *N* HCl and again evaporated to dryness. When the residue was very dark (particularly in the case of soups), this operation was repeated for a third time. The residue was finally dissolved in exactly 10 ml of distilled water and filtered through a Whatman No. 40 paper. The filtrate was then passed through an Allihn tube (I.D. 0.9 cm), packed with 2.5 g of Brockmann's alumina. About 6 ml of the eluate were collected, and 5 ml were then transferred with a pipette into a flat-bottomed porcelain dish. 1 ml of 2 *N* HCl was added, and the solution was evaporated to dryness on a boiling water bath. The residue was dissolved in exactly 5 ml of a 1 m*M* solution of cytosine in pH 3.7 buffer, and, whenever necessary, filtered through a Whatman No. 42 paper.

About 1 ml of the filtrate was put on the chromatographic column and eluted with a pH 4.30 buffer at a rate of 120 ml/h and at a temperature of 56°. The eluate was monitored for creatinine in the UV region at 240 mμ and at a slit width of 1.5 mm.

The column needs to be regenerated only when loss of efficiency or resolution is noticed. Regeneration is carried out by passing 10 ml of 2 *N* HCl through the column and then some 1 *N* NaOH until the NaOH front, which is darker, reaches the bottom of the column. The latter is then re-equilibrated with 30–40 ml of a pH 4.30 buffer.

## RESULTS AND DISCUSSION

The data in Table I indicate that this method gives accurate and reproducible results. These results were obtained for (a) a laboratory soup preparation, (b) a commercial soup preparation, (c) a dehydrated soup and (d) a tomato soup. An accurately known amount of creatinine was added to each of these, and sample (b) also contained some meat extract, in which the creatinine had been accurately measured. The added creatinine was always recovered with a good accuracy, and the standard deviation never exceeded ± 1.1%.

Sample (d) was the most interesting because it contained some substances that simulate creatinine in JAFFÉ's test (*i.e.* give the same color when reacted with sodium

TABLE I

RECOVERY OF CREATININE ADDED TO A SOUP AND A SOUP PREPARATION

a = Soup preparation without meat extract; b = soup preparation with meat extract; c = dehydrated vegetable soup; d = tinned tomato soup.

| *Sample* | *Creatinine added (mg)* | *Creatinine found (mg)* | *Recovery (%)* |
|---|---|---|---|
| 1a | 27.00 | 27.11 | 100.4 |
| 2a | 27.00 | 26.81 | 99.3 |
| 3a | 27.00 | 26.81 | 99.3 |
| 4a | 27.00 | 26.95 | 99.8 |
| 5a | 27.00 | 27.11 | 100.4 |
| 6a | 27.00 | 26.95 | 99.8 |
| 7a | 27.00 | 27.29 | 101.1 |
| 8a | 27.00 | 26.78 | 99.2 |
| | | Average recovery | 99.9 ± 0.7 |
| 1b | 27.00 | 27.13 | 100.5 |
| 2b | 27.00 | 26.78 | 99.2 |
| 3b | 27.00 | 26.70 | 98.9 |
| 4b | 27.00 | 27.16 | 100.6 |
| 5b | 27.00 | 27.00 | 100.0 |
| | | Average recovery | 99.8 ± 0.8 |
| 1c | 56.50 | 55.93 | 99.0 |
| 2c | 56.50 | 56.44 | 99.9 |
| 3c | 56.50 | 56.73 | 100.4 |
| 4c | 56.50 | 57.12 | 101.1 |
| 5c | 56.50 | 57.01 | 100.9 |
| | | Average recovery | 100.3 ± 0.9 |
| 1d | 56.50 | 56.50 | 100.0 |
| 2d | 56.50 | 55.71 | 98.6 |
| 3d | 56.50 | 56.50 | 100.0 |
| 4d | 56.50 | 57.40 | 101.6 |
| 5d | 56.50 | 56.95 | 100.8 |
| | | Average recovery | 100.2 ± 1.1 |

picrate in an alkaline medium), so that this test gives a creatinine content for tomato soup not containing meat extract.

These interfering substances cannot be removed by passing the sample through alumina or by extracting it with diethyl ether. The present method permits the chromatographic separation of creatinine from interfering substances, since these emerge in the first 10 min of the elution, while creatinine appears only after 17 min.

The creatinine zone generally did not contain other substances absorbing in the UV region. This was confirmed analyzing a protein hydrolysate, a yeast autolysate and various fresh and dehydrated vegetables by the proposed method. In fact, it was only in the case of tomato that a peak appeared which was not well resolved from the creatinine peak. Under the present conditions, however, this peak was small and did not interfere with the analysis (*cf.* Table I), also because it was not superimposed on the creatinine peak. Furthermore, this substance has a slightly shorter retention time than creatinine and cannot be mistaken for the latter when present by itself. This can be seen from Fig. 1, showing the graphs obtained in the analysis of tomato soup with and without a meat extract, as well as in the analysis of a standard mixture.

The present method has dispensed with the ether extraction which is time-consuming, rather difficult and gives rise to inaccuracies. Our method is thus rapid, accurate and easy to carry out. The use of an internal standard, cytosine, which closely followed creatinine in the elution, means that the amount of sample placed on the column need not be accurately known.

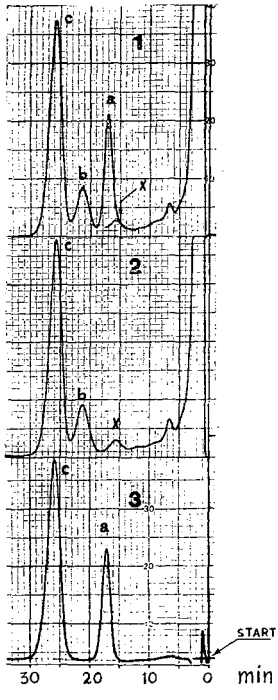


Fig. 1. (1) Chromatogram of a tomato soup with meat extract; (2) chromatogram of a tomato soup without meat extract; (3) chromatogram of a standard mixture of creatinine and cytosine. a = Creatinine; b = adenine; c = cytosine; x = unknown from tomato.

For calculation of results, the method of the ratio of peak areas using an internal standard was adopted. At creatinine concentrations of 0.2–1 $\mu$mole/ml, the calibration curve was a straight line. To obtain very accurate results, it is useful therefore to elute occasionally a standard mixture, containing cytosine in the same amount as added to the samples to be analyzed, also containing creatinine in an amount very close to what is expected to be present in the samples to be analyzed. This operation minimizes the inevitable instrumental error.

## CONCLUSIONS

The method proposed for the determination of creatinine in soup preparations and soups containing meat extracts is more accurate and less time-consuming than the methods used so far. Furthermore, it enables one to avoid the inconvenience caused by creatinine-like interfering substances which make the analysis inaccurate in the case of tomato soup.

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CHROM. 4366

# SEPARATION AND DETECTION OF GALLIC ACID AND ITS ALKYL ESTERS BY POLYAMIDE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A procedure for preparing polyamide thin layers and chromatographic solvents necessary for a distinct separation of gallic acid and its alkyl esters has been described. A clean-up procedure necessary for the separation and identification of these compounds from fatty foods was investigated. All the procedures were very simple, convenient and feasible for the analysis of these compounds and will also be applicable to the separation and identification of other gallic acid esters in fatty foods.

## INTRODUCTION

When fatty foods are oxidized or hydrolyzed under the influence of air, moisture, light and microorganisms during storage, they deteriorate because of the production of small amounts of hydroxy fatty acids, ketones, aldehydes and organic peroxides. Consequently, alteration in taste and odor of fatty foods, namely rancidity, occurs. This rancidity is primarily due to organic peroxides, hydroxy fatty acids and aldehydes. One effective and secure means of preserving fatty foods from deterioration is the addition of antioxidants in very low concentrations. Antioxidants generally available in most countries are nordihydroguaretic acid (NDGA), butylated hydroxytoluene (BHT), butylated hydroxyanisol (BHA), some alkyl esters of gallic acid, *e.g.* propyl gallate (PG) and isoamyl gallate (IAG), and ethyl protocatechuate (EPC). The addition of these antioxidants to fatty foods has found increasing use in recent years. Therefore a simple, rapid and reliable method for the separation and detection of these antioxidants from fatty foods is strongly demanded today.

Although various methods for the separation and detection of these compounds from fatty foods have been reported by many investigators, generally antioxidants are separated from fatty foods by extraction with a suitable organic solvent and are detected by thin-layer, paper and gas chromatography with or without any purification procedure; of these methods thin-layer chromatography is widely applied since it is rapid, economical and convenient.

Since SEHER[1] first succeeded in separating some antioxidant mixtures on silica

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gel thin layers, some other investigators[2–4], testing the separation and detection of various mixtures of antioxidants on silica gel or acetylated cellulose, have not obtained satisfactory results. The use of polyamide powder in TLC has been introduced by DAVÍDEK *et al.*[5,6] and by EGGER[7], and the application of this stationary phase for the separation of various natural and synthetic products has been frequently reported to date (for a review see HÖRHAMMER *et al.*[8]). This chromatographic adsorbent has also been successfully applied to column chromatography for the analysis of many groups of natural phenolic[9–16] as well as other[17] products. DAVÍDEK[18] has used polyamide layers ("loose layers") to obtain a distinct separation of gallic acid from its alkyl esters and to shorten the long development time which is inevitably required in paper chromatography. Later COPIUS-PEEREBOOM[19] succeeded in preparing firmly bound layers of polyamide, containing such a binding agent as starch or polyvinyl acetate to improve the "loose layers". He has found these layers to be useful for the separation of fat antioxidants.

However, the authors were confronted with several problems when making use of the above experiences:

(1) Testing DAVÍDEK's method[18], it proved impossible to prepare a uniform firmly bound thin layer of polyamide.

(2) Using "loose layers" great care had to be taken to avoid disturbing or damaging the thin layer, especially during solvent development and during spraying with a visualizing reagent.

(3) A good distribution and sharpness of the spots of alkyl esters of gallic acid were observed neither on "loose layers" nor on polyamide layers containing a binding agent.

The present investigation was undertaken to eliminate the above-mentioned difficulties experienced when using polyamide layers and to propose a simple and reliable method for the separation and identification of antioxidants from fatty foods.

## EXPERIMENTAL

### *Thin-layer chromatography*

*Adsorbent.* Polyamide powder (obtained from E. Merck, Darmstadt, G.F.R.) was used as the adsorbent. Before use it was washed with benzene in a centrifuge tube and thereafter with a volume of methanol equal to twice the volume of polyamide powder. It was dried at 60°.

*Reagents.* Gallic acid and six of its alkyl esters (Table I) were obtained from Tokyo Kasei Kogyo Co., Ltd. Before use they were twice crystallized from dilute ethanol. Test solutions were prepared by dissolving 10 mg of each of the compounds in 20 ml of acetone. Aliquots ($\mu$l) of this solution were used for detection and separation.

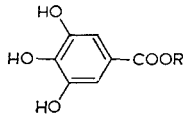
The visualizing reagent was prepared by dissolving 10 mg of analytical reagent grade 2,6-dichloroquinone-4-chlorimide (obtained from Tokyo Kasei Kogyo Co., Ltd.) in 100 ml of isopropanol.

The solvents used are listed in Table III. They were all of analytical reagent grade.

*Apparatus.* Thin-layer applicator and accessories were obtained from Yasawa Seisaku Co., Ltd. Glass plates were 20 cm × 5 cm. The chromatographic chamber had

TABLE I

STRUCTURAL FORMULAS OF GALLIC ACID AND ITS ALKYL ESTERS



| *Compound* | *R* |
|---|---|
| Gallic acid | H |
| Methyl gallate | $CH_3$ |
| Ethyl gallate | $CH_2CH_3$ |
| Propyl gallate | $CH_2CH_2CH_3$ |
| Isoamyl gallate | $CH_2CH_2CH(CH_3)_2$ |
| Lauryl gallate | $CH_2(CH_2)_{10}CH_3$ |
| Stearyl gallate | $CH_2(CH_2)_{16}CH_3$ |

a diameter of 9 cm and a height of 27 cm. The UV light source (3650 Å) was supplied by Manasulu Ultraviolet Kagaku Kogyo Co., Ltd.

*Preparation of polyamide layers.* According to the method generally used in the preparation of silica gel thin layers, glass plates were coated with a slurry composed of 15 g of polyamide powder and 50 ml of isopropanol. An applicator giving a thin layer approximately 250 $\mu$ thick was used. After drying the plates in air for about 15 min, they were further dried at 60° for 30 min and allowed to cool at room temperature. Then they were stored, until required, in a dessicator containing silica gel.

*Development and detection.* 0.5–1 $\mu$l of the test solution of gallic acid and its alkyl esters were spotted with a micropipette on the starting line 2 cm from the bottom of the plate. The plate was then placed inside a chamber containing the mobile phases to a depth of about 1 cm. Development was carried out by the ascending technique until the solvent front had travelled a distance of 10 cm from the starting line. After development the plate was dried in air, and the spots were observed under UV light before spraying with the visualizing reagent.

*Column chromatography*

*Adsorbent.* Polyamide powder (obtained from M. Woelm, Eschwege, G.F.R.) was used as the adsorbent. Before use it was treated in a manner similar to that described for TLC.

*Reagents.* Of the test compounds listed in Table I, gallic acid and stearyl gallate were used after two crystallizations from dilute ethanol.

The solvents *n*-hexane, benzene, acetonitrile, methanol and ethyl acetate were of analytical reagent grade.

*Apparatus.* The column used consisted of a glass tube, 1 cm in diameter and 20 cm in length, and was equipped with a cockstop and glass wool.

*Preparation of the polyamide column.* A slurry of polyamide powder and *n*-hexane was poured into a glass tube to make a 10-cm high column bed. A round piece of filter paper was placed on the surface of the column bed.

*Separation and detection.* A sample of fatty food containing 1 mg of both gallic acid and its alkyl esters was weighed into a centrifuge tube, and 30 ml of acetonitrile were added. The mixture was shaken to extract the antioxidants and then centrifuged at 3000 r.p.m. The supernatant was then decanted into a 100-ml flask; an additional 20 ml of acetonitrile were added to the sediment and the same extraction procedure was repeated. All the supernatants were combined in the flask and evaporated to dryness at 40–45° under reduced pressure. After the residue had been dissolved in 20 ml of benzene, 30 ml of *n*-hexane and small amounts of anhydrous sodium sulfate were added to the solution. The mixture was allowed to stand for 30 min and was then transferred to the polyamide column by means of a pipette. The flask containing the anhydrous sodium sulfate was rinsed thrice with small portions of benzene, and the washings were also transferred onto the column. Before the benzene solution was eluted from the column, the residue in the flask was rinsed two to three times with small portions of ethyl acetate–methanol (1:4), and the washings were also applied on the column. The flow rate was maintained at approximately 2 ml/min. The effluent was collected upon application of the ethyl acetate–methanol mixture on the column. About 30 ml of effluent were collected in a 100-ml flask and evaporated to dryness at 40–45° under reduced pressure. The residue was dissolved in small amounts of methanol. The solution was applied to polyamide plates for TLC, as already described. An outline of the above purification procedure is given in Fig. 1.

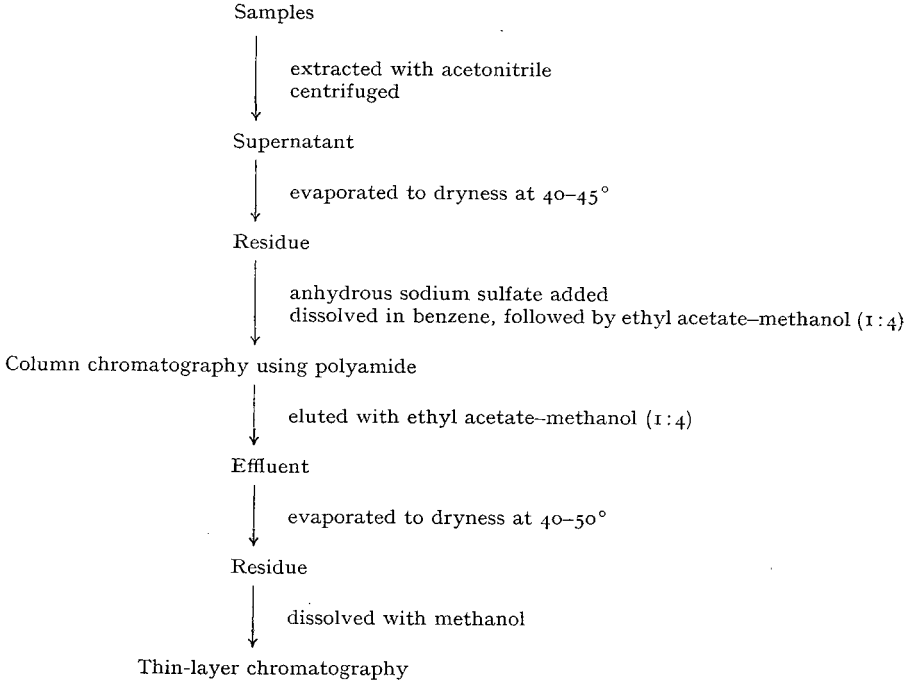



Fig. 1. Purification of gallic acid and its alkyl esters from fatty foods.

TABLE II

$R_F$ VALUES OF GALLIC ACID AND ITS ALKYL ESTERS OBTAINED ON POLYAMIDE THIN-LAYER PLATES USING SOLVENT SYSTEMS 1–13

| *No.* | *Solvent system* | *Gallic acid* | *Methyl gallate* | *Ethyl gallate* | *Propyl gallate* | *Isoamyl gallate* | *Lauryl gallate* | *Stearyl gallate* |
|---|---|---|---|---|---|---|---|---|
| 1 | Carbon tetrachloride | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 2 | Ethyl acetate | 0.0 | 0.03 | 0.03 | 0.04 | 0.06 | 0.05 | 0.06 |
| 3 | Methanol | 0.48 | 0.58 | 0.60 | 0.60 | 0.61 | 0.46 | 0.31 |
| 4 | Ethanol | 0.36 | 0.53 | 0.57 | 0.57 | 0.62 | 0.59 | 0.53 |
| 5 | Isopropanol | 0.12 | 0.20 | 0.31 | 0.33 | 0.39 | 0.41 | 0.40 |
| 6 | Benzene–methanol (3:1) | 0.13 | 0.45 | 0.53 | 0.56 | 0.67 | 0.84 | 0.89 |
| 7 | Carbon tetrachloride–ethanol (3:2) | 0.13 | 0.39 | 0.46 | 0.50 | 0.60 | 0.76 | 0.86 |
| 8 | Carbon tetrachloride–isopropanol (3:2) | 0.06 | 0.23 | 0.29 | 0.32 | 0.44 | 0.64 | 0.74 |
| 9 | Carbon tetrachloride–ethyl acetate–acetic acid (25:5:1) | 0.07 | 0.30 | 0.36 | 0.42 | 0.52 | 0.83 | 0.96 |
| 10 | Carbon tetrachloride–ethanol–acetic acid (16:4:1) | 0.01 | 0.36 | 0.41 | 0.54 | 0.67 | 0.87 | 0.96 |
| 11 | Carbon tetrachloride–isopropanol–acetic acid (40:2:2) | 0.04 | 0.17 | 0.25 | 0.30 | 0.43 | 0.72 | 0.90 |
| 12 | Benzene–methanol–acetic acid (25:4:1) | 0.05 | 0.23 | 0.29 | 0.40 | 0.52 | 0.71 | 0.84 |
| 13 | Carbon tetrachloride–isopropanol–formic acid (40:6:2) | 0.06 | 0.25 | 0.34 | 0.39 | 0.53 | 0.81 | 0.89 |

RESULTS AND DISCUSSION

*Polyamide thin-layer plates*

When the plates, coated with commercial polyamide powder, were used for development with solvent systems containing such a polar solvent as acetic acid or formic acid, the thin layers became so fragile that cracking and peeling appeared in all areas of the plates, particularly on the solvent front. In order to prevent these difficulties, commercial polyamide powder was thoroughly washed with both benzene and methanol before preparation of the thin-layer plates.

*Separation and detection of gallic acid and its alkyl esters on polyamide thin layers*

As seen from the $R_F$ values in Table II, when using solvent systems 1–5, gallic acid and its alkyl esters were not clearly separated. However, it was observed, when using solvents 3 and 4, that the $R_F$ values of isoamyl, lauryl and stearyl gallate decreased as the length of the carbon chain increased. Such a tendency was also found by COPIUS-PEEREBOOM[19] when he analyzed some alkyl esters of gallic acid on a starch-bound polyamide layer using a solvent system consisting of methanol–acetone–water (60:20:20).

Gallic acid and its alkyl esters separated considerably better in solvent systems 6–8. The distribution and sharpness of spots on the thin-layer chromatograms were in general better than when using carbon tetrachloride–ethanol (7:3), which had been recommended by DAVÍDEK[18]. It was observed, however, that the spots of propyl and ethyl gallate did not separate from each other.

Solvent systems 9–13 contain formic or acetic acid. The distribution and sharpness of spots using solvent systems 9–11 were the best thus far obtained. It was found, however, that the separation between isoamyl and propyl gallate was not sufficient. On the other hand, as shown in Fig. 2, gallic acid and its alkyl esters were distinctly separated with round spots using solvent system 13. In addition, the distribution and sharpness of spots on the chromatogram using solvent system 12

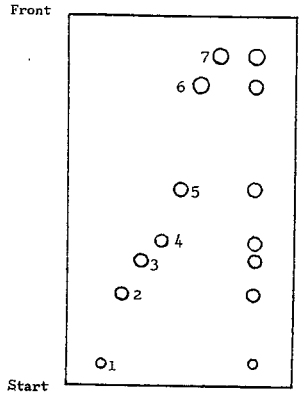



Fig. 2. Chromatogram of gallic acid and its alkyl esters and a mixture of them on polyamide thin layers. Solvent system: carbon tetrachloride–isopropanol–formic acid (40:6:2). Development time: 210 min. Temperature: 20 ± 1°. 1 = gallic acid; 2 = methyl gallate; 3 = ethyl gallate; 4 = propyl gallate; 5 = isoamyl gallate; 6 = lauryl gallate; 7 = stearyl gallate.

were similar to those using solvent system 13. Development time required 190 min in solvent system 12 and 210 min in solvent system 13 at 20 ± 1°.

*Relationship between the $R_F$ values of gallic acid and its alkyl esters and the alcohol content in solvent systems 12 and 13*

As the best distribution and sharpness of spots was obtained using solvent systems 12 and 13, the question was posed whether any amount of methanol or isopropanol in two solvent systems is suitable for separation. It was found that the compounds tested were distinctly separated with round spots when the methanol content was about 13–19 vol.% in system 12 and when the isopropanol content was about 12–16 vol.% in system 13. In addition, the sharpness of all the spots was superior in solvent system 13 to that in solvent system 12.

*Sensitivity test for the detection of gallic acid and its alkyl esters on polyamide thin layers*

When spotting 0.1, 0.2, 0.3 and 0.5 μg of the compounds on a thin layer and then developing it with solvent system 12, each spot was detected both by spraying with the visualizing reagent and under UV light. UV light produced dark spots and the visualizing reagent produced grayish-green spots.

TABLE III

MINIMUM AMOUNTS OF GALLIC ACID AND ITS ALKYL ESTERS DETECTED ON POLYAMIDE THIN-LAYER PLATES UNDER UV LIGHT OR WITH A VISUALIZING REAGENT

Solvent system: benzene–methanol–acetic acid (25:4:1). The color observed with the visualizing reagent was grayish-green in all cases. Absorption was observed in all cases.

| *Compound* | $R_F$ *value* | *Amount detected (μg)* | |
|---|---|---|---|
| | | *Visualizing reagent* | *UV light (3650 Å)* |
| Gallic acid | 0.05 | 0.1 | 0.3 |
| Methyl gallate | 0.23 | 0.1 | 0.3 |
| Ethyl gallate | 0.29 | 0.1 | 0.3 |
| Propyl gallate | 0.34 | 0.1 | 0.3 |
| Isoamyl gallate | 0.43 | 0.1 | 0.3 |
| Lauryl gallate | 0.71 | 0.1 | 0.5 |
| Stearyl gallate | 0.84 | 0.1 | 0.5 |

The results, shown in Table III, indicate that detection using the visualizing reagent is more sensitive than under UV light. This is clearly attributable to the fact that the thin layer itself has a considerable UV absorption. The detection limit for the compounds was 0.1 μg with the visualizing reagent and under UV light it was 0.3 μg for gallic acid and methyl, ethyl, propyl and isoamyl gallate and 0.5 μg for stearyl and lauryl gallate. It is assumed that low sensitivities of stearyl and lauryl gallate might be dependent mainly upon any dispersion in their spots which might be brought about by the long development time and their higher $R_F$ values.

*Purification of gallic acid and its alkyl esters from fatty foods*

When gallic acid and its alkyl esters, which were separated from fatty foods by extraction with acetonitrile only, were detected on polyamide plates, the $R_F$ values

were considerably variable because of such interfering materials in the extract as lipids, tocopherols and fat-soluble pigments. Thus polyamide column chromatography, previously reported by us[20], was applied to remove these extraneous substances.

Judging from the $R_F$ values of the compounds on polyamide thin layers, elution of gallic acid and stearyl gallate on the polyamide column was examined by the following process. One milliliter of a test solution, prepared by dissolving 10 mg of stearyl gallate in 10 ml of benzene, was applied to a polyamide column and eluted with a mixture of ethyl acetate–methanol (1:4). Every 2 ml of the effluent were collected in a small test tube immediately after loading the solvent onto the column, and each sample was transferred to a 10-ml flask for evaporation at 40–45° under reduced pressure. Each residue was dissolved in 10 ml of methanol to measure the absorbance of stearyl gallate of $\lambda_{max} = 275$ m$\mu$ with 1-cm quartz cells using methanol as a blank.

The behavior of gallic acid on a polyamide column was examined in a procedure similar to that used for stearyl gallate. However, the test solution for the column was prepared by dissolving gallic acid in ethyl acetate, and the absorbance of each effluent fraction was measured at $\lambda_{max} = 270$ m$\mu$.

Each elution pattern of the two compounds obtained by this procedure is shown in Fig. 3. It may be observed from this figure that each 1 mg of gallic acid and stearyl gallate on the polyamide column is completely eluted when the effluents have reached about 20 ml.

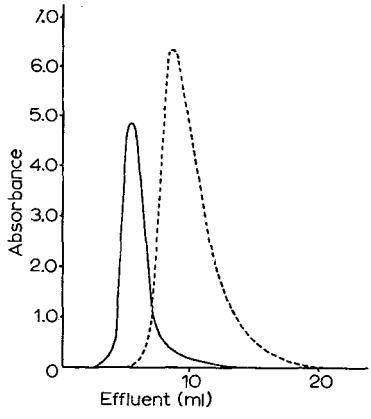



Fig. 3. Elution patterns on a polyamide column of stearyl gallate and gallic acid with methanol–ethyl acetate (4:1) as the solvent system.

*Separation and identification of gallic acid and its alkyl esters from fatty foods*

The separation and identification of gallic acid and its alkyl esters from beef tallow, lard or olive oil, in which each compound was added at a concentration of 100 p.p.m., were carried out according to the procedure described in EXPERIMENTAL. It was found that the $R_F$ values of these compounds on polyamide thin layers were in good agreement with those of the standard substances.

Such a method is also applicable to the analysis of gallic acid and its alkyl esters in any other fat-rich foods.

## CONCLUSIONS

The uniform and firmly bound thin layers of polyamide powder were easily prepared by using the polyamide powder treated with both benzene and methanol. It was found in this investigation that the polyamide thin layers were suitable for the analysis of gallic acid and its alkyl esters.

By the ascending development with one of two solvent systems containing an organic acid, solvent systems 12 and 13, gallic acid and its alkyl esters were distinctly separated with round spots on polyamide thin layers. In particular, it is worth insisting that some alkyl esters of gallic acid, carbon chains of which differ by one carbon atom, were separated without overlapping.

In the comparative sensibility test for the detection of gallic acid and its alkyl esters on polyamide thin layers under UV light and by spraying with the visualizing reagent, the detection limit under UV light was 0.3 $\mu$g for gallic acid and methyl, ethyl, propyl and isoamyl gallate and 0.5 $\mu$g for stearyl and lauryl gallate. By spraying with the visualizing reagent, 0.1 $\mu$g of these compounds was detected.

The simultaneous identification of gallic acid and its alkyl esters from some fatty foods was successfully carried out by extraction with acetonitrile and purification of the extract on the polyamide column and then detection on polyamide thin layers.

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CHROM. 4370

# CHROMATOGRAPHY OF AROMATIC HYDROCARBONS ON IMPREGNATED LAYERS

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SUMMARY

Using silica gel layers impregnated with compounds having electron-acceptor properties, compared with non-impregnated layers a decrease in $R_F$ values of aromatic hydrocarbons occurs when the same solvent is used. The $R_F$ values do not correspond with the values of solid complexes of hydrocarbons with the relevant acceptors. They vary with the concentration of impregnating agents and, thus, are an indication of a modification in adsorption properties of the silica gel layer. The effect of impregnating layers when using a solvent of electron-acceptor properties is very limited. Chloranil and pyromellitic dianhydride form colored complexes with aromatic hydrocarbons and can be used for detection on impregnated layers with acceptor compounds which do not form colored complexes with the hydrocarbons.

## INTRODUCTION

Aromatic hydrocarbons as donors of $\pi$ electrons are capable of forming donor–acceptor complexes (EDA) with substances having electron-acceptor properties. This finding has been used by various authors for separation of polycyclic aromatic hydrocarbons on thin layers. Until now, the following methods have been used:

(1) a plate already prepared is impregnated with a complex-forming agent;

(2) a complex-forming agent is added to the adsorbent during preparation of the plate;

(3) a plate already prepared without a complex-forming agent is developed in a system containing the complex-forming agent.

FRANCK-NEUMANN AND JÖSSANG[1] were the first to point out the possibility of using the EDA complexes in thin-layer chromatography. They separated polycyclic aromatic hydrocarbons on layers impregnated with *sym.*-trinitrobenzene, whereas polynitroaromatic substances were developed on non-impregnated layers in anthracene-containing systems. BERG AND LAM[2] added a small amount of complex-forming agent to the aluminium oxide and silica gel used as adsorbents. For separation of aromatic hydrocarbons, KESSLER AND MÜLLER[3] used a saturated solution of picric acid as a developing system. Thin-layer chromatography was also used for the study of EDA

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complexes of other $\pi$ donors, *e.g.* aromatic amines on layers impregnated with polynitroaromatic compounds[4–6].

In the present study the effect is examined of impregnating commercially available silica gel plates with tetracyanoethylene and some acceptors from the group of halogen quinones, aromatic anhydrides, N-methylated cyclic ureids and bile acids, not yet used for separation of aromatic hydrocarbons. The study also included the caffeine previously examined[2]. The polynitro substances as complex-forming agents were not studied here, since a considerable amount of work dealing with these substances already exists[1–6]. As developing systems simple solvents with relatively clear-cut electron-acceptor (tetrachloroethylene) and electron-donor (di-*n*-butyl ether) properties were used.

## EXPERIMENTAL

### *Chemicals*

The origin and purity of the aromatic hydrocarbons studied and the methods used for their purification are listed in Table I. As solvents, predistilled di-*n*-butyl ether and tetrachloroethylene were used.

Tetramethyluric acid (TMUA) used for impregnation of the layers was prepared synthetically[7]. Caffeine PhBs 2, chloranil (Lachema p.a.) and tetracyanoethylene (TCNE, Lachema, pure) were purified by a repeated vacuum sublimation. Pyromellitic dianhydride (PMDA, Fluka, pure) and desoxycholate sodium (Léčiva Praha) were not purified prior to use.

TABLE I

AROMATIC HYDROCARBONS INVESTIGATED

| *No.* | *Hydrocarbon* | *Origin* | *Purity* | *Purification method* |
|---|---|---|---|---|
| 1 | Pentamethylbenzene | Synthetized after ref. 17 | | Crystallization from ethanol |
| 2 | Hexamethylbenzene | Synthetized after ref. 17 | | Crystallization from ethanol |
| 3 | 1-Methylnaphthalene | B.D.H. Great Britain | not given | — |
| 4 | 2-Methylnaphthalene | E. Merck | for synthesis | Distillation |
| 5 | 2,3-Dimethylnaphthalene | Koch-Light | purum | — |
| 6 | 2,6-Dimethylnaphthalene | Koch-Light | purum | — |
| 7 | Acenaphthene | Schering | purum | — |
| 8 | Acenaphthylene | B.D.H. Great Britain | not given | — |
| 9 | Fluorene | Loba-Chemie | purum | Recrystallization from xylene |
| 10 | 2.3-Benzofluorene | Schuchardt | purum | — |
| 11 | Fluoranthene | Schuchardt | not given | Crystallization from ethanol |
| 12 | 20-Methylcholanthrene | Koch-Light | purum | — |
| 13 | Biphenyl | Chemko, Strážske | not given | — |
| 14 | *m*-Terphenyl | Chemko, Strážske | not given | — |
| 15 | Anthracene | Lachema | for analysis | — |
| 16 | Phenanthrene | Lachema | | Zone refining |
| 17 | Chrysene | Loba-Chemie | purum | — |
| 18 | Pyrene | Koch-Light | purum | Sublimation |
| 19 | 3.4-Benzopyrene | Fluka | puriss. | — |
| 20 | 1.2,5.6-Dibenzanthracene | Koch-Light | purum | — |
| 21 | 1.2,3.4-Dibenzopyrene | Koch-Light | purum | — |
| 22 | 1.2,4.5-Dibenzopyrene | Koch-Light | purum | — |
| 23 | 3.4,9.10-Dibenzopyrene | Koch-Light | purum | — |
| 24 | Anthanthrene | Koch-Light | purum | — |

*J. Chromatog.*, 45 (1969) 278–283

TABLE II

$R_F$ VALUES OF AROMATIC HYDROCARBONS WITH VARIOUS ACCEPTORS

| *No.* | *Hydrocarbon* | *Di-n-butyl ether* | | | *Tetrachloroethylene* | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | — | *0.05 M TMUA* | *Satd. solution TMUA* | — | *0.05 M Caffeine* | *Satd. solution Caffeine* | *Satd. solution PMDA* | *Satd. solution chloranil* |
| 1 | Pentamethylbenzene | front | 0.96 | 0.90 | 0.74 | 0.76 | 0.84 | 0.74 | 0.72 |
| 2 | Hexamethylbenzene | front | 0.95 | 0.90 | 0.74 | 0.75 | 0.83 | 0.69 | 0.73 |
| 3 | 1-Methylnaphthalene | front | 0.85 | 0.75 | 0.73 | 0.73 | 0.76 | 0.71 | 0.76 |
| 4 | 2-Methylnaphthalene | front | 0.88 | 0.78 | 0.74 | 0.75 | 0.77 | 0.70 | 0.78 |
| 5 | 2,3-Dimethylnaphthalene | front | 0.83 | 0.67 | 0.75 | 0.74 | 0.74 | 0.60 | 0.67 |
| 6 | 2,6-Dimethylnaphthalene | front | 0.87 | 0.71 | 0.76 | 0.75 | 0.76 | 0.64 | 0.67 |
| 7 | Acenaphthene | front | 0.82 | 0.66 | 0.75 | 0.74 | 0.73 | 0.64 | 0.66 |
| 8 | Acenaphthylene | front | 0.72 | 0.52 | 0.70 | 0.69 | 0.63 | 0.46 | 0.62 |
| 9 | Fluorene | front | 0.80 | 0.65 | 0.68 | 0.69 | 0.66 | 0.61 | 0.60 |
| 10 | 2.3-Benzofluorene | 0.71 | 0.48 | 0.24 | 0.39 | 0.57[a] | 0.11[a] | 0.43 | 0.38 |
| 11 | Fluoranthene | 0.70 | 0.36 | 0.13 | 0.43 | 0.51[a] | 0.13[a] | 0.37 | 0.36 |
| 12 | 20-Methylcholanthrene | 0.68 | 0.32 | 0.11 | 0.39 | 0.53[a] | 0.11[a] | 0.34 | 0.35 |
| 13 | Biphenyl | front | 0.92 | 0.84 | 0.71 | 0.72 | 0.76 | 0.71 | 0.72 |
| 14 | *m*-Terphenyl | 0.94 | 0.89 | 0.77 | 0.62 | 0.62 | 0.69 | 0.68 | 0.64 |

[a] In di-*n*-butyl ether.

TABLE III

$R_F$ VALUES OF AROMATIC HYDROCARBONS WITH VARIOUS ACCEPTORS

| *No.* | *Hydrocarbon* | *Di-n-butyl ether* | | | | | *Tetrachloroethylene* | | |
|---|---|---|---|---|---|---|---|---|---|
| | | — | *0.05 M TMUA* | *Satd. solution TMUA* | *0.05 M Caffeine* | *Satd. solution Caffeine* | — | *Satd. solution PMDA* | *Satd. solution chloranil* |
| 1 | Anthracene | 0.69 | 0.49 | 0.26 | 0.55 | 0.42 | 0.45 | 0.46 | 0.46 |
| 2 | Phenanthrene | front | 0.68 | 0.44 | 0.66[a] | 0.59[a] | 0.71 | 0.51 | 0.60 |
| 3 | Chrysene | 0.67 | 0.32 | 0.12 | 0.47 | 0.27 | 0.40 | 0.36 | 0.37 |
| 4 | Pyrene | 0.70 | 0.34 | 0.13 | 0.51 | 0.32 | 0.46 | 0.36 | 0.43 |
| 5 | 3.4-Benzopyrene | 0.68 | 0.20 | 0.05 | 0.43 | 0.18 | 0.43 | 0.38 | 0.34 |
| 6 | 1.2,5.6-Dibenzanthracene | 0.68 | 0.28 | 0.07 | 0.44 | 0.21 | 0.37 | start | 0.32 |
| 7 | 1.2,3.4-Dibenzopyrene | 0.67 | 0.15 | 0.03 | 0.37 | 0.12 | 0.37 | 0.28 | 0.36 |
| 8 | 1.2,4.5-Dibenzopyrene | 0.65 | 0.12 | 0.03 | 0.34 | 0.10 | 0.39 | 0.26 | 0.34 |
| 9 | 3.4,9.10-Dibenzopyrene | 0.61 | 0.14 | 0.03 | 0.33 | 0.12 | 0.37 | 0.28 | 0.31 |
| 10 | Anthanthrene | 0.66 | 0.14 | 0.04 | 0.33 | 0.13 | 0.43 | 0.26 | 0.34 |

[a] In tetrachloroethylene.

*Chromatography*

Benzene solutions of aromatic hydrocarbons (kept in darkness) were applied to 150 × 150 mm reflex silica gel foils Silufol (Kavalier, n.p.) without a luminescence indicator. Prior to use, the plates were impregnated by spraying as follows:

(1) 0.05 *M* and saturated aqueous solution of TMUA;
(2) 0.05 *M* and saturated aqueous solution of caffeine;
(3) saturated solution of PMDA in ethyl acetate;
(4) saturated solution of chloranil in chloroform;
(5) 0.05 *M* aqueous solution of sodium desoxycholate;
(6) 0.05 *M* TCNE solution in acetone.

After chromatography aromatic hydrocarbons were detected by spraying the plate with a saturated solution of chloranil in chloroform. The color of the spots could be intensified by heating the plates to about 110°. Substances No. 10–12, 15 and 17–24 could also be observed as fluorescent spots under UV light. For comparison of $R_F$ values, solid complexes of pyrene with TMUA[8], PMDA[9] and TCNE[10] have been prepared.

## RESULTS AND DISCUSSION

Concentration of the acceptor compound in solution used for impregnation shows a marked effect on $R_F$ values of aromatic hydrocarbons, as is evident from Tables II and III. These changes in $R_F$ values are evidence of the altered adsorption properties of a layer, which behaves like a new type of adsorbent. The solid EDA complexes of pyrene listed under EXPERIMENTAL behave similarly to pyrene itself on non-impregnated layers. Due to the effect of solvent and adsorbent, a disintegration of the relatively weak donor–acceptor bond occurs[4]. As impregnating agents PMDA, chloranil, TMUA and caffeine, the last of these has been recommended[2], gave good results. When sodium desoxycholate was used, no effect was observed with aqueous solutions which were found suitable as mobile phase for separation by paper chromatography[11]. $R_F$ values of the aromatic hydrocarbons obtained on plates impregnated with tetracyanoethylene were not reproducible. TCNE is often used as a detection agent for aromatic compounds[12–14], but later its limited effectiveness was reported[15]. On the layers impregnated with chloranil and PMDA lasting color stains are achieved after application of aromatic hydrocarbons (Table IV). For detection purposes, however, spots on plates impregnated with chloranil are more suitable; yellow-colored spots occurring mostly on plates impregnated with PMDA give less contrast when compared with spots on plates impregnated with chloranil.

The $R_F$ values obtained on non-impregnated layers are influenced by the adsorption forces which increase, as a rule, with the number of aromatic rings in a molecule, regardless of their arrangement[16]. On layers impregnated with acceptor compounds, the situation is similar, since an increasing number of rings in a molecule of an aromatic hydrocarbon is also accompanied by a decrease in their ionization potential. The stability of the EDA bond is proportional to the difference between the electron affinity of the acceptor and the ionization potential of the donor.

The solvent has also a considerable effect on the quality of separation. Tetrachloroethylene causes considerably smaller differences than di-*n*-butyl ether in $R_F$ values of aromatic hydrocarbons on both impregnated and non-impregnated plates,

TABLE IV

COLORS OF $\pi$ COMPLEXES OF CHLORANIL AND PYROMELLITIC DIANHYDRIDE WITH AROMATIC HYDROCARBONS

| *No.* | *Hydrocarbon* | *Color of $\pi$ complex with chloranil* | *Color of $\pi$ complex with PMDA* |
|---|---|---|---|
| 1 | Pentamethylbenzene | brownish red | yellow |
| 2 | Hexamethylbenzene | reddish violet | light yellowish brown |
| 3 | 1-Methylnaphthalene | greyish brown | yellow |
| 4 | 2-Methylnaphthalene | brown | yellow |
| 5 | 2,3-Dimethylnaphthalene | brown | yellow |
| 6 | 2,6-Dimethylnaphthalene | grey | yellow |
| 7 | Acenaphthene | bluish grey | orange |
| 8 | Acenaphthylene | brown | yellow |
| 9 | Fluorene | brownish violet | yellow |
| 10 | 2.3-Benzofluorene | dark brownish grey | yellowish brown |
| 11 | Fluoranthene | brown | light yellow |
| 12 | 20-Methylcholanthrene | greenish yellow | grey |
| 13 | Biphenyl | yellowish brown | light yellow |
| 14 | *m*-Terphenyl | yellowish brown | light yellow |
| 15 | Anthracene | greenish blue | reddish pink |
| 16 | Phenanthrene | brown | yellow |
| 17 | Chrysene | brownish grey | yellowish brown |
| 18 | Pyrene | dark green | light red |
| 19 | 3.4-Benzopyrene | greenish yellow | grey |
| 20 | 1.2,5.6-Dibenzanthracene | grey | yellowish brown |
| 21 | 1.2,3.4-Dibenzopyrene | greenish yellow | yellowish brown |
| 22 | 1.2,4.5-Dibenzopyrene | greenish yellow | greyish brown |
| 23 | 3.4,9.10-Dibenzopyrene | greenish yellow | yellowish green |
| 24 | Anthanthrene | yellow | light green |

since with an excess of the acceptor solvent the effect of the complex-forming agent from the impregnated plate can only be shown to a lesser degree.

It is impossible to attain such a homogeneous distribution of the complex-forming agent on the plate by the spray-impregnation method as by the addition of the complex-forming agent to the adsorbent during preparation of the plates[2]. A satisfactory reproducibility ($R_F$ value $\pm 0.05$), however, indicates that the former method is also suitable for practical separation of aromatic hydrocarbons.

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CHROM. 4372

# THIN-LAYER CHROMATOGRAPHY OF NUCLEIC ACID BASES, NUCLEOSIDES, NUCLEOTIDES AND RELATED COMPOUNDS

## IX. QUANTITATIVE ANALYSIS BY *IN SITU* REFLECTANCE SPECTROSCOPY*

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SUMMARY

Five nucleo derivatives have been chosen for quantitative *in situ* determination by reflectance spectroscopy after two-dimensional separation from a complex mixture by thin-layer chromatography on cellulose. The use of an internal standard to compensate for fluctuations in the chromatographic procedure resulted in an improvement in the reproducibility of data by about 3–4% relative standard deviation. Average accuracies obtained with the method in the analysis of an artificial mixture ranged between 5.1 and 4.0 %. The use of a linear calibration curve of substance to standard ratio plotted *versus* square root of concentration proved most advantageous. The use of one set of calibration curves for the analysis of mixtures on various days gave an average error of about 16% but was tremendously timesaving. The method was successfully applied to the analysis of nucleo derivatives in an extract of cartilage red bone marrow.

## INTRODUCTION

Reports on the reflectance spectroscopic evaluation of nucleo derivatives have been previously communicated[1–4]. Time studies revealed no significant changes, either with regard to peak shift or intensity of maxima, over a 24-h period[3]. Shifts of maxima over an analytically useful concentration range were $\pm 2$ nm (*cf.* ref. 3).

In view of these facts, it was decided to work out a quantitative *in situ* reflectance spectroscopic method for some nucleo derivatives with the use of the Zeiss

* For part VIII *cf.* ref. 3.
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chromatogram spectrophotometer. The method was to be applied to the determination of nucleo derivatives in biological materials.

## EXPERIMENTAL

Chromatographically pure hypoxanthine, uridine, uracil, inosine and thymine have been used for the preparation of stock solutions in 0.04% v/w NaOH. In many cases adenine has been added as internal standard. The plates were coated with Cellulose MN-300 (Macherey, Nagel & Co., Düren, G.F.R.), purified according to ref. 5. All solvents used were of reagent grade.

Samples and standards were applied with 5-$\mu$l capillaries (Microcaps, Drummond Scientific Co. Ltd., Broomall, Pa., U.S.A.). Two-dimensional chromatography was carried out according to a previously described method[5]. The spots were viewed under an UV lamp (Camag Ltd., Muttenz, Switzerland) at 254 nm and marked on the backside of the plate with a grease pencil. Reflectance measurements were made with the Zeiss chromatogram spectrophotometer (Zeiss, Oberkochen, G.F.R.). The chromatographic peaks were evaluated by planimetry with a planimeter manufactured by Ott Ltd., Kempten, Bavaria, G.F.R.

## RESULTS AND DISCUSSION

### *Calibration curves*

The concentration–reflectance relationships of the 16 nucleo derivatives qualitatively investigated in the previous communication[3] have been studied in this proj-

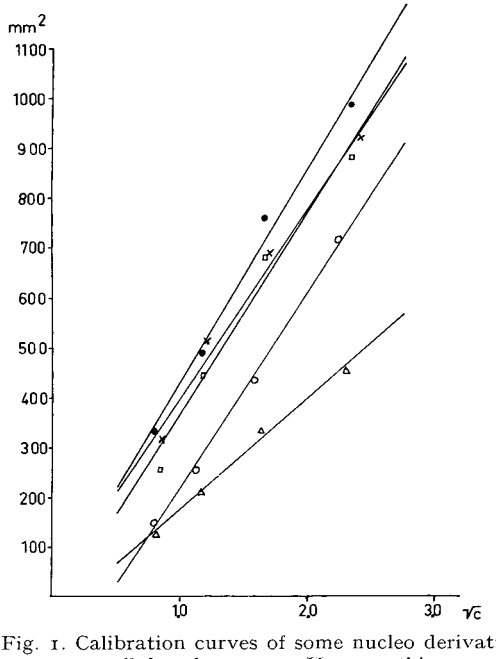


Fig. 1. Calibration curves of some nucleo derivatives determined by *in situ* reflectance spectroscopy on cellulose layers. ●, Hypoxanthine; ×, thymine; □, xanthine; △, nicotinamide; ○, AMP-3′.

ect. Fairly linear calibration curves were obtained with peak areas plotted *versus* square root of concentration ($\sqrt{c}$). A few examples of these calibration plots are given in Fig. 1. The curves have been computed statistically and correlation coefficients ranging between 0.980 and 0.994 have been obtained.

*Internal standards*

KLAUS[6] has introduced the use of an internal standard in one-dimensional thin-layer chromatography of bands, to compensate for fluctuations inherent in the chromatographic procedure. Such an internal standard would be even more desirable in two-dimensional chromatography and the advantage of using an internal standard for the problem at hand was clearly demonstrated with hypoxanthine (Table I). Three micrograms of uracil and adenine were added as internal standards to 3 $\mu$g of hypoxanthine. Chromatography was carried out in the usual way once one in a set of 6 and once of 12 analyzed on the Zeiss instrument. Improvements in reproducibility between 3 and 4 % relative standard deviation were observed with the use of both uracil and adenine as internal standards. Since uracil is itself a component of the biological samples of interest, it was decided to use adenine which is not present and does not interfere with any of the naturally occurring nucleo derivatives on the chromatogram.

TABLE I

A COMPARISON OF THE REPRODUCIBILITY OF DATA WITH AND WITHOUT THE USE OF INTERNAL STANDARDS

| | *Hypo-xanthine* | *Uracil* | *Adenine* | *Hypoxanthine/ uracil* | *Hypoxanthine/ adenine* |
|---|---|---|---|---|---|
| Mean and standard deviation[a] ($n = 6$) | 752 ± 69 | 790 ± 65 | 780 ± 75 | 0.96 ± 0.069 | 0.96 ± 0.05 |
| % Rel. st. dev. | 9.1 | 8.3 | 9.6 | 6.3 | 5.1 |
| Mean and standard deviation ($n = 12$) | 700 ± 70 | 760 ± 81 | 740 ± 60 | 0.924 ± 0.066 | 0.948 ± 0.065 |
| % Rel. st. dev. | 10.0 | 10.7 | 8.1 | 7.1 | 6.9 |

[a] St. dev. $= s = \sqrt{\frac{\Sigma(M - \bar{M})^2}{(n-1)}}$.

*Analysis of a synthetic mixture*

Chromatographic separation has been carried out as usual[5]. The chromatogram, as well as the corresponding chromatographic peaks, are shown in Fig. 2. (Arrows mark the scan direction.) For quantitative work, the peak evaluation method also shown in Fig. 2 proved to be the best of a number of techniques tested, particularly for not completely resolved double peaks.

Calibration curves of ratios (substance/adenine) plotted *versus* concentration showed the usual bent shape (Fig. 3). Reasonably linear curves were obtained, with the origin generally differing somewhat from zero, if the ratios were plotted *versus* the square root of concentration (Fig. 3). Both calibration curves were used for evaluation purpose. The linear plots gave generally somewhat better results. The results of this analysis are presented in Table II. The total time per analysis is two days.

This includes chromatographic separation on one day and evaluation of data by the internal standard method on the second. Actual working time for one technician is about

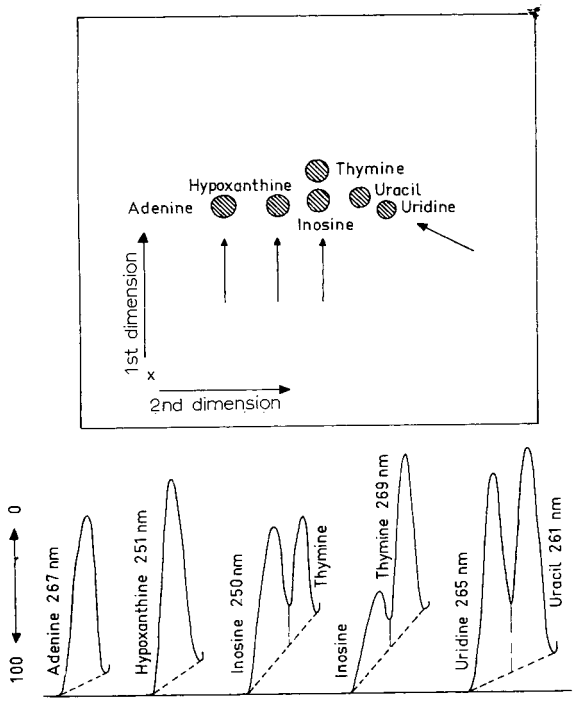


Fig. 2. Thin-layer chromatogram and corresponding chromatographic peaks. Scanning speed, 7.5 cm/min; recorder speed, 8 cm/min. Arrows mark the direction of scan.

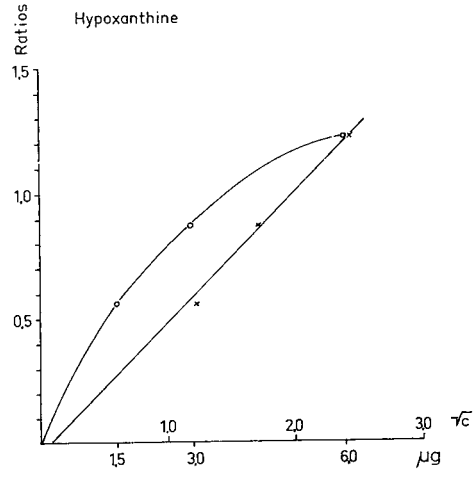


Fig. 3. Calibration curves of peak area ratios hypoxanthine/adenine plotted *versus* concentration (○—○) and *versus* square root of concentration (×—×) of hypoxanthine.

TABLE II

ANALYSIS OF A KNOWN ARTIFICIAL MIXTURE

| *Compound* | *Amount present* (μg/*spot*) | *Amount found*[a] | | *Percent deviation* | |
|---|---|---|---|---|---|
| | | *Conc.* | $\sqrt{c}$ | *Conc.* | $\sqrt{c}$ |
| Hypoxanthine | 4.12 | 4.56 | 4.26 | 10.7% | 3.4% |
| Inosine | 3.90 | 4.01 | 4.04 | 2.8 | 3.6 |
| Thymine | 4.35 | 4.06 | 4.11 | 6.7 | 5.5 |
| Uridine | 3.83 | 3.80 | 3.56 | 0.7 | 7.0 |
| Uracil | 3.60 | 3.43 | 3.65 | 4.7 | 1.4 |
| | | | Average deviation | 5.12% | 4.02% |

[a] Average of three independent analyses.

TABLE III

THE USE OF ONE SET OF CALIBRATION CURVES FOR THE ANALYSIS OF MIXTURES ON VARIOUS DAYS

| *Compound* | *Amount present* (μg) | *Mixture* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *1* | | *3* | | *1* | | *2* | |
| | | *Calibration curves set* | | | | | | | |
| | | *2* | | *3* | | *3* | | *3* | |
| | | *Amount found* | | *Amount found* | | *Amount found* | | *Amount found* | |
| | | (μg)/$\sqrt{c}$ | *Conc.* | (μg)/$\sqrt{c}$ | *Conc.* | (μg)/$\sqrt{c}$ | *Conc.* | (μg)/$\sqrt{c}$ | *Conc.* |
| Hypoxanthine | 4.12 | 5.48 | 5.3 | 4.97 | 4.75 | 5.06 | 4.8 | 4.24 | 4.50 |
| Percent error | | 33.0 | 28.6 | 20.6 | 15.3 | 22.8 | 16.5 | 2.9 | 9.2 |
| Inosine | 3.9 | — | — | 4.45 | 4.6 | — | — | 3.03 | 3.00 |
| Percent error | | — | — | 14.1 | 18.0 | — | — | 22.3 | 23.1 |
| Thymine | 4.35 | 5.15 | 5.25 | 5.15 | 5.25 | 3.8 | 3.63 | 3.28 | 3.18 |
| Percent error | | 18.4 | 20.7 | 18.4 | 20.7 | 12.6 | 16.5 | 24.6 | 26.9 |
| Uridine | 3.83 | — | — | 4.00 | 4.15 | — | — | 2.82 | 2.85 |
| Percent error | | — | — | 4.4 | 8.3 | — | — | 26.4 | 25.6 |
| Uracil | 3.6 | 3.6 | 3.45 | 2.72 | 2.68 | 3.76 | 3.63 | 3.53 | 3.45 |
| Percent error | | 0.2 | 4.2 | 24.4 | 25.6 | 4.4 | 0.8 | 1.9 | 4.2 |
| Average percent error for one analysis | | 17.1 | 17.8 | 16.4 | 17.6 | 13.3 | 11.3 | 15.6 | 17.8 |
| | | | | | | Total average percent error | | 15.6 | 16.2 |

6–8 h. Nine standards of three different concentrations and three samples were chromatographed on twelve plates in the same chromatographic tank. Number of plates and separation time would remain the same no matter how many components are being determined in the mixture, which makes the method timewise even more attractive.

TABLE IV

RESULTS OF ANALYSIS OF THREE BATCHES OF CARTILAGE EXTRACT (RUMALON)

| *Compound* | *Batch 3 (MK 4087)* | | | | *Batch 2*[a] *(MK 4072)* | | *Batch 1*[a] *(MK 4048)* | |
|---|---|---|---|---|---|---|---|---|
| | $\sqrt{c}$ (μg) | | *Conc.* (μg) | | $\sqrt{c}$ (μg) | *Conc.* (μg) | $\sqrt{c}$ (μg) | *Conc.* (μg) |
| | *St. dev.*[b] | % *St. dev.* | *St. dev* | % *St. dev.* | | | | |
| Hypoxanthine | 3.63 ± 0.36 | 10 | 3.62 ± 0.42 | 11.5 | 1.35 | 1.20 | 2.82 | 2.75 |
| Inosine | 0.51 ± 0.26 | 51 | 0.46 ± 0.17 | 37 | 0.26 | 0.23 | 0.25 | 0.28 |
| Uridine | 1.23 ± 0.23 | 18.7 | 1.24 ± 0.29 | 23.4 | 0.96 | 0.95 | 0.49 | 0.45 |
| Uracil | 5.18 ± 0.36 | 6.9 | 4.77 ± 0.45 | 9.5 | 1.04 | 1.10 | 1.80 | 1.77 |

[a] Results are obtained from one analysis for each batch.
[b] Standard deviations are calculated from six analyses.

The use of one set of calibration curves for the determination of mixtures analyzed on different days was also investigated (Table III). An average percent error of around 16% was found compared to around 4 and 5% error with standards chromatographed simultaneously with every determination (Table II). The timesaving factor is however so enormous that the latter method may have some merit for certain applications.

*Analysis of biological material*

Cartilage red bone marrow extract (Rumalon, Robapharm Ltd., Basle, Switzerland) was examined for these components. The same chromatographic procedure was used[5], and a complete spotchart and data on a preliminary investigation of this system were given earlier[1]. After freeze-drying the various batches, 5:1 dilutions were made and analyzed similar to the artificial mixture. The reproducibility of hypoxanthine in Rumalon was checked. A relative standard deviation of 605 $mm^2 \pm 37$ or 6.1% was found. This is in the order of magnitude of reproducibilities observed with the artificial mixture.

Results obtained from three batches of Rumalon are presented in Table IV. The high percentage of relative standard deviation for inosine is due to the low concentration of this component, which at a dilution ratio of 5:1 approaches a detection limit. Extracts of higher concentration are hard to handle. From Table IV it can be seen that the differences in concentration of hypoxanthine and uracil in the three batches are significant. Differences of inosine are not significant and differences of uridine between batches number 1 and 2 are barely significant. The use of 4 times 2 standards of 4 different concentrations for the calibration curve proved somewhat more advantageous than the use of 3 times 3 standards of only 3 different concentrations.

## CONCLUSIONS

Reflectance spectroscopy can be used as a means of quantitative determination of nucleo derivatives in biological systems. Even though the method has been applied only to a small group of such compounds, it could well be extended to all the nucleo derivatives tested earlier and essentially to all stable and ultraviolet-active organic systems. In the case of Rumalon, the method can be adopted as a semiroutine analysis for periodic checks of the nucleo derivative content of the various extraction batches, prior to entering the pharmaceutical processing stage.

## ACKNOWLEDGEMENTS

R. W. FREI is thankful to Robapharm Ltd., Basle for financial support and available facilities to carry out this research. Part of this project was supported by a grant of the National Research Council of Canada.

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CHROM. 4367

# PAPER ELECTROPHORESIS AND CHROMATOGRAPHY OF URANIUM(VI) IN CHLORIDE–ORGANIC SOLVENT MEDIA

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SUMMARY

The electromigration of uranium(VI) using paper as carrier and its chromatographic behaviour in hydrochloric acid (0.5–5 *M*) and metal chloride solutions containing varying amounts of ethanol or acetone is reported. The influence of the concentration of the ligands, as well as that of the organic solvents on the complex formation, and the significance of the solvation state of the uranyl species, as well as the nfluence of the potential gradient applied, are discussed.

## INTRODUCTION

The first report on electromigration in uranyl chloride solutions was given by DITTRICH[1], who found that uranium(VI) moved to the cathode. BACHELET *et al.*[2] observed cationic motion of uranium(VI) in mixtures of HCl and NaCl of high concentration applying potential gradients of 5–50 V/cm. Cationic migration was observed in 0.1 *M* solutions of HCl, KCl and $NH_4Cl$ by MUKERJEE[3] and in 0.1 *M* HCl by MOJUMDAR[4].

This paper describes an investigation on the electromigration of uranium(VI) in hydrochloric acid and metal chloride solutions containing ethanol or acetone, dependent on the ligand concentration and on the content of the organic solvent of the solution, as well as on the voltage applied. The purpose of these electrophoresis studies was to obtain information about the complex formation in chloride solutions containing organic solvents. The investigation on the paper chromatographic behaviour of uranium(VI) in these systems should complete the studies.

## EXPERIMENTAL

Whatman No. 3 paper and Schleicher & Schüll 2043b Mgl were used as carriers.

Solutions of uranium(VI) were prepared from $UO_2(NO_3)_2 \cdot 6H_2O$ (Riedel de Haën A.G., Seelze b. Hannover) by repeated evaporation with conc. HCl. The chloride concentration of these solutions containing uranium (5 mg/ml) was, in each case, as high as that of the electrolyte system studied.

The technique of electrophoresis and chromatography used has been reported in a previous paper[5]. The electropherograms and chromatograms were dried at 130°. As the details of the determination of the mean migration distance and of the possibilities of quantitative interpretation of the data, as well as the widest range of voltage that can be applied have already been discussed[5], no further description will be given here.

Each system was investigated in the widest range of voltage possible.

## RESULTS AND DISCUSSION

### *Solutions containing ethanol*

If the distance of migration, $d$, is plotted against the period of electrophoresis, $t$, the resulting straight line indicates that the composition of the solution is constant during the electrophoresis. This is the case during the first 20 min in chloride systems containing ethanol. After longer periods, the evaporation of ethanol results in changes of the complex formation, and hence in a flexure in the curve; whereas in aqueous solutions the composition of the system is constant during a period of 60 min.

The slope of the $d$–$t$ curves represents the macroscopical velocity of migration, $v_i = \delta d/\delta t = z_i e_0 \xi / 6\pi\eta r_i$*. Assuming that only one kind of migration species is present in the solution, the velocity should increase in a solution of constant composition in the same relation as the applied voltage (that is 1 : 1). This is not the case in all systems, *e.g.* in 2.5 *M* HCl–80 vol. % ethanol (Fig. 1). According to the comprehensive explanations previously given[5], the existence of several differently charged migrating species should be considered in these systems, though cations and neutral species predominate in the solution.

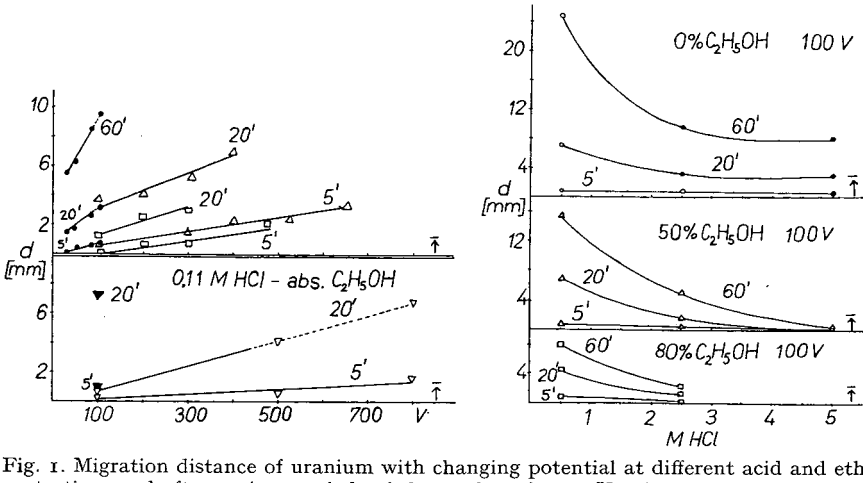



Fig. 1. Migration distance of uranium with changing potential at different acid and ethanol concentrations and after various periods of electrophoresis. 100 V voltage corresponds to a potential gradient of about 5 V/cm, 300 V to 15 V/cm, 500 V to 25 V/cm and 800 V to 40 V/cm. —●—, 2.5 *M* HCl; —△— 0.5 *M* HCl–80 vol. % ethanol; —□—, 2.5 *M* HCl–80 vol. % ethanol; —▽—, 0.11 *M* HCl–absolute ethanol. For comparison values in 0.5 *M* HCl are presented, —▼—.

Fig. 2. Migration distance with changing acid concentrations at different ethanol concentrations at a constant potential (100 V) after various periods of electrophoresis. —○—, 0% ethanol; —△—, 50 vol. % ethanol; —□—, 80 vol. % ethanol; —●—, extrapolated values.

* $z_i e$ corresponding to ionic charge; $\xi$ = field strength; $\eta$ = viscosity; $r_i$ = ionic radius.

The migration distance and velocity depend on the position of the equilibrium of complex formation and on the ratio of the rate of complex formation and migration velocity of the ions involved.

In hydrochloric acid solutions containing ethanol, uranium is predominantly present as cationic and neutral species in the concentration range studied. No information about the nature of the cations ($UO_2^{2+}$ to $UO_2Cl^+$) present can be obtained from these data, since quantitative interpretation of the results is not possible.

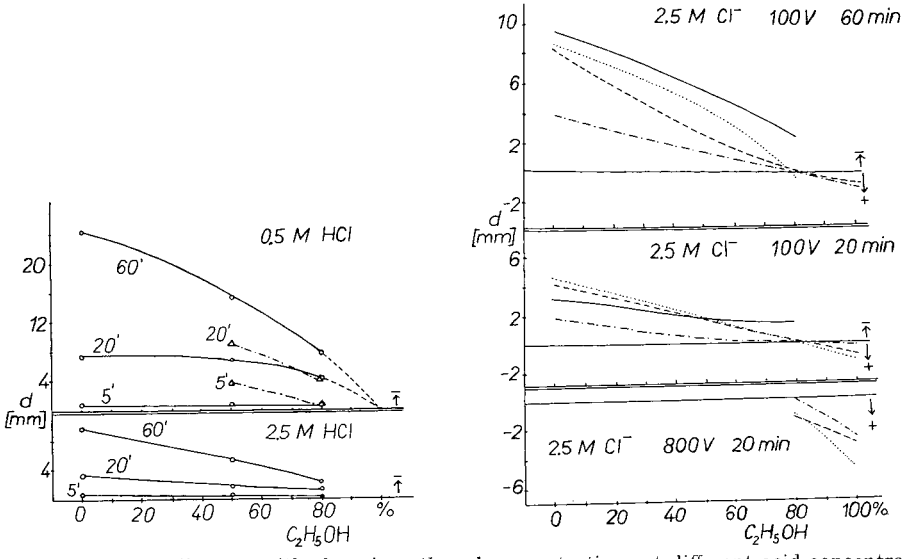



Fig. 3. Migration distance with changing ethanol concentrations at different acid concentrations and at different potentials after various periods of electrophoresis. —○—, 100 V; ···△···, 200 V.

Fig. 4. Migration distance with changing ethanol concentrations at a constant ligand concentration (2.5 *M* $Cl^-$) at different potentials after various periods of electrophoresis. ———, HCl; – – – –, LiCl; ·····, $MgCl_2$; –·–·–, $AlCl_3$.

Increasing acid concentration (Fig. 2) as well as increasing content of ethanol (Fig. 3) increase the formation of higher complexes. The higher the amount of the neutral species, the smaller the cationic migration. In 5 *M* HCl–60 vol. % ethanol solutions no migration occurs at all at 100 V. Although only overall cationic migration can be observed in the systems studied, in solutions with a high amount of ethanol, the existence of anionic species seems to be possible, as has been mentioned above.

Addition of ethanol increases the complex formation as the dielectric constant of the mixture decreases, and hence the ionic interaction increases. The cationic species are not only stabilized by a solvate shell in aqueous solutions but also in ethanol to some extent, since in solutions containing dry HCl dissolved in absolute ethanol cationic migration was observed (Fig. 1).

The complex formation is increased in the salt solutions studied (Figs. 4 and 5). In systems containing a chloride concentration of 2.5 *M* and 80 vol. % ethanol or 5 *M* [$Cl^-$] and 60 vol. % ethanol, the amount of the anionic species ($UO_2Cl_3^-$) is noticeable. In solutions in which the salt has been dissolved in pure ethanol, the amount of the anions is great, since anionic migration occurred. Usually the cationic

migration increases in the following order: $AlCl_3 < LiCl < MgCl_2 < HCl$. The explanation of these data is rather difficult since the situation in solutions of the same $Cl^-$ concentration is complicated by the influence of the varying pH value, viscosity, dielectric constants and activity coefficients. (The electrode compartments contained 50 ml of salt and 10 ml of acid solution.) The pH of the salt solutions is lower than that of the acid systems, and hence hydrolysis and polymerization yielding higher uranium aggregates might be possible. There is a possibility that in acid solutions the anions $UO_2Cl_3^-$ and the hydronium ions form ion pairs, that is neutral units, whereas the ionic association in salt solutions, especially if higher charged cations are involved, is less probable.

*Solutions containing acetone*

The composition of aqueous acid solutions containing acetone is constant during the first 12–13 min of electrophoresis. In these systems the capillary effect is also more significant.

Increasing acid and acetone concentrations increase the complex formation.

The voltage dependence of the migration[5] indicates that in 2.5 and 5 *M* solutions of considerable acetone concentration differently charged species, cations and anions, are present. The inversion of the migration direction with increasing voltage in 2.5 *M* HCl–60 vol. % acetone solutions, for instance, is due to the high amount of the anionic complexes ($UO_2Cl_3^-$) in these solutions (Fig. 7). In solutions of 60 vol. % acetone, cations and neutral species predominate in about 0.5–1 *M* HCl, whereas in 4–5 *M* HCl predominantly anions are present (Fig. 6). As expected, the inversion of the migration direction occurs in solutions of an acid concentration (Fig. 6) or of an acetone content (Fig. 7) which are lower, the higher the voltage applied. Anionic migration can be clearly observed in all systems containing no or only small amounts of "free" water (*e.g.* 5 *M* HCl–60 vol. % acetone, 2.5 *M* HCl–80 vol. % acetone). (It should be mentioned that in such media condensation reactions take place altering the viscosity,

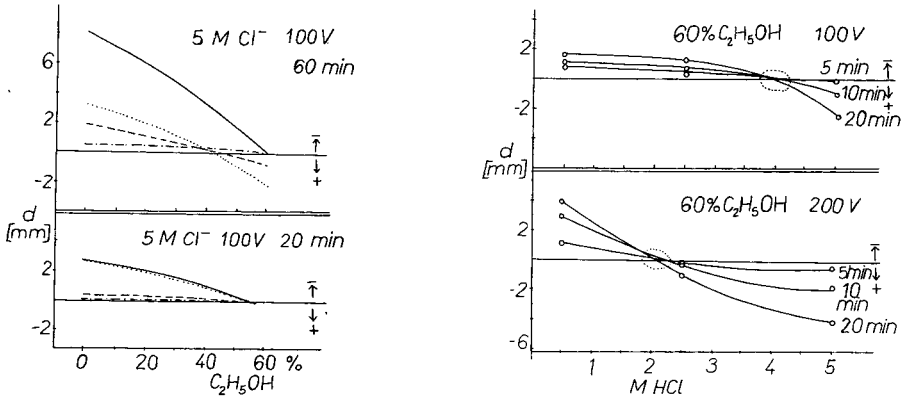
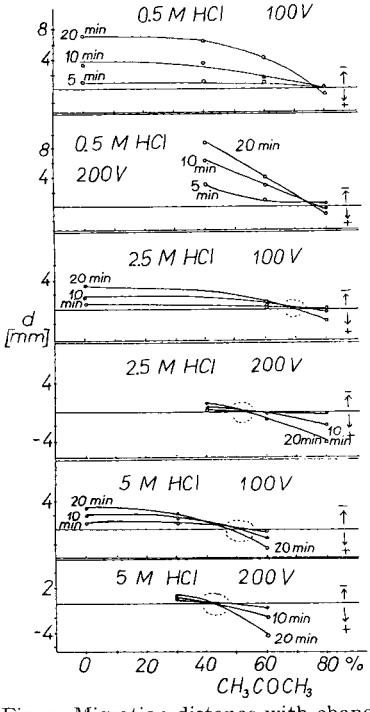



Fig. 5. Migration distance with changing ethanol concentrations at constant ligand concentration (5 *M* $Cl^-$) and at a constant potential (100 V) after various periods of electrophoresis. ———, HCl, – – – –, LiCl; · · · · · ·, $MgCl_2$; –·–·–, $AlCl_3$.

Fig. 6. Migration distance with changing acid concentrations at a constant acetone concentration (60 vol. %) at different potentials after various periods of electrophoresis.

the composition and hence the dielectric constants of the solutions and the tendency to complex formation.) In these solutions only one kind of migrating species, that is anions, exists.



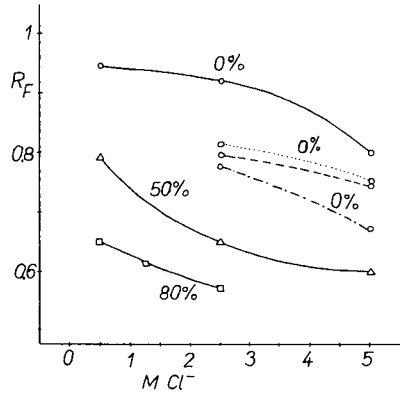




Fig. 7. Migration distance with changing acetone concentrations at different acid concentrations and at different potentials after various periods of electrophoresis.

Fig. 8. $R_F$ values with changing ligand concentrations at various ethanol concentrations. ———, HCl; – – – –, LiCl; · · · · · ·, $MgCl_2$; –·–·–, $AlCl_3$.

Concerning the complex formation and hence the migration in the systems containing ethanol or acetone, the discrepancy observed may be due to the different solvation tendency of the solvents studied. Ethanol, but probably not acetone, is able to stabilize the uranyl cations by a solvate shell. This would explain the increased complex formation in acetone solutions.

*Paper chromatographic behaviour*

Data on paper chromatographic behaviour are presented in Figs. 8–11. Increasing ligand concentrations as well as increasing concentrations of organic solvents decrease the $R_F$ values in all systems in which, on the one hand, overall cationic migration or no movement and, on the other hand, an increase in the distribution on anion exchange resins[6] can be observed. This decrease in mobility is due to the increased formation of neutral complexes. The $R_F$ values increase in systems in which overall anionic migration and a decrease in the distribution occur. This increase in the mobility can be explained by an increase in the amount of the ionic species in the solution.

In any case, the higher the amount of the ionic species in solution—regardless of whether cations or anions are involved—the higher the $R_F$ value. The ions seem to prefer the mobile phase. A minimum of the curve indicates that formation of neutral complexes and subsequently that of anions take place.

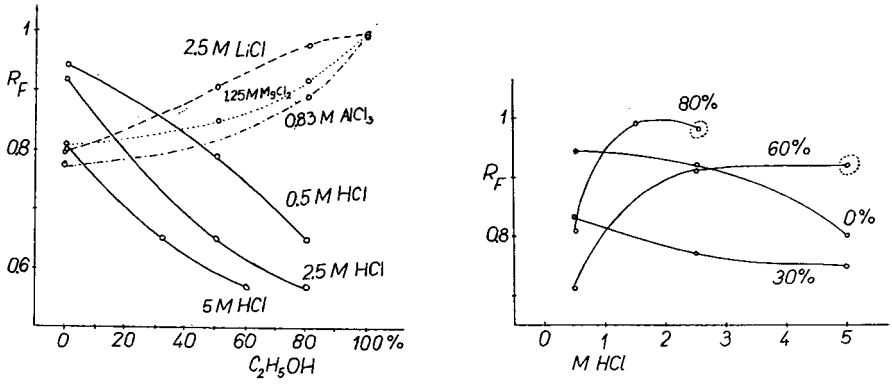



Fig. 9. $R_F$ values with changing ethanol concentrations at various ligand concentrations. ———, HCl; – – – –, LiCl; ·····, $MgCl_2$; –·–·–, $AlCl_3$.

Fig. 10. $R_F$ values with changing acid concentrations at various acetone concentrations. ◎, systems which do not contain any "free" water.

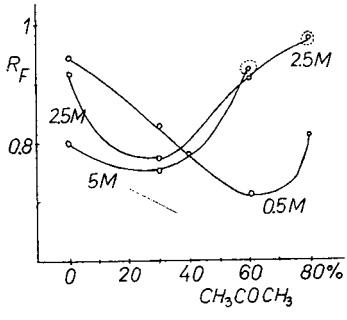



Fig. 11. $R_F$ values with changing acetone concentrations at various acid concentrations. ◎, systems systems which do not contain any "free" water.

All data obtained from acid solutions containing ethanol or acetone agree with the results on anion exchange of SCHNEIDER[6]. There is a fairly good agreement between the data obtained from aqueous acid solutions and the results of JØRGENSEN and MARCUS. According to JØRGENSEN[7] in 1 *M* HCl $[UO_2Cl(H_2O)_n]^+$ is present and according to MARCUS[8] in 0.5–4 *M* HCl $UO_2Cl^+$ and in 4–6 *M* HCl $UO_2Cl_2$ exists. It should be emphasized that the distance as well as the direction of migration depends greatly on the voltage applied; and therefore, for instance, cationic motion can occur at 100 V even in a solution in which the neutral species predominate!

The chromatographic data agree with the results reported in the literature[9,10].

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CHROM. 4361

# ION EXCHANGE IN FUSED SALTS

## IV. ION-EXCHANGE PROPERTIES OF CRYSTALLINE ZIRCONIUM PHOSPHATE IN MOLTEN $NaNO_3$–$KNO_3$ AT 450°

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SUMMARY

The chemical stability of crystalline zirconium phosphate in molten $KNO_3$ and $NaNO_3$ has been determined. It was found that ion-exchange experiments can be carried out, without appreciable decomposition of the material, up to 350° or 500° with the H form or the salt form of the exchanger, respectively. Forward and reverse ion-exchange isotherms for Na/K exchange in molten $NaNO_3$–$KNO_3$ mixtures at 450° show that contrary to what is found in aqueous solution, no appreciable hysteresis occurs. The isotherm curves show two well-defined vertical portions, each being related to the coexistence of two immiscible phases, one transforming into the other. The shape of the isotherm curves also indicates that $Na^+$ is always preferred to $K^+$ over the whole range of the solvent composition.

## INTRODUCTION

In previous papers[1–3] ion exchange of alkali metal ions in molten nitrates has been investigated by using amorphous zirconium phosphate as exchanger. Crystalline zirconium phosphate seems a more suitable exchanger to obtain better insight into the ion-exchange phenomena occurring in molten salt media. In fact, with this material instead of the amorphous, it is possible to follow structural changes occurring during the ion-exchange process by X-ray analysis[4–6]. On the other hand, ion-exchange experiments on molten salt media at high temperature also seem to be useful to obtain additional information on the ion-exchange mechanism of crystalline zirconium phosphate. Since crystalline zirconium phosphate has never been employed for ion-exchange studies in fused salts, preliminary research on its stability in molten sodium and potassium nitrates at various temperatures has been performed. Also the forward and reverse $Na^+$–$K^+$ exchange in molten $NaNO_3$–$KNO_3$ mixtures at 450° has been investigated over the whole range of the ionic composition of the solvent.

## EXPERIMENTAL

### *Chemicals*

All reagents (ERBA RP products) were dried at 110° for 24 h, mixed in proper proportions and then dried again at 110°. Dimethylsulphone (DMS) was supplied by K & K Laboratories; crystalline zirconium phosphate in the sodium form (ZPNa) was obtained by titrating the hydrogen form (ZPH) prepared according to a procedure described in a previous paper[5]; ZPK was obtained from ZPNa as reported by TORRACCA[6]. The anhydrous sodium and potassium forms of the exchanger were obtained by vacuum drying at 160° and 110°, respectively.

### *Apparatus and procedures*

The experiments were performed in an electrical furnace, the temperature of which was controlled by a Ni–Cr thermocouple and SAE 136 thermoregulator at $\pm 5°$.

Several ZPNa (or ZPK) samples (1 g) were added to 50 g of $NaNO_3$–$KNO_3$ mixtures, at various compositions, contained in a Pyrex cylinder provided with a sintered-glass disk (G 4) at the bottom. Each sample was left for a given time at the operating temperature, vacuum filtered at 450°, cooled and then washed several times with fused DMS at 160° to eliminate the small amount of nitrates adhering to the surface of the exchanger even after careful filtration*. Finally the excess of DMS was removed by washing with acetone at room temperature.

Isotherm curves were obtained by determining the ionic equivalent fraction of potassium ($\bar{X}_K$) in the exchanger at the various melt compositions. The equivalent fraction of potassium in the melt ($X_K$) before and after the equilibrium was assumed to remain constant, since the number of mequiv. of alkali counter-ions in 1 g of exchanger is negligible with respect to the number of mequiv. contained in 50 g of fused solvent.

### *Analytical procedures*

400–500 mg of the exchanger were dissolved in 20 ml of 1 *M* hydrofluoric acid, and distilled water was added to 100 ml. The alkali metal ions were determined with an E.E.L. flame photometer. Orthophosphate and zirconium ions were determined as reported in ref. 7.

X-ray diffraction patterns were obtained with copper radiation (Cu K$\alpha$, $\lambda =$ 1.542 Å).

## RESULTS AND DISCUSSION

### *Stability of crystalline ZP in fused alkali nitrates*

Crystalline ZPH exhibits a good thermal stability, condensation of its acid groups to pyrophosphate starting at about 320°–350° and reaching to completeness only at about 600°[4].

Some preliminary experiments in $NaNO_3$ at 350° showed that the hydrogen of the exchanger can be easily substituted by $Na^+$. During the exchange, nitrogen

* During the washing with DMS ion-exchange reactions seem to be excluded, since it was shown experimentally that the composition of the alkali nitrates dissolved in DMS was equal to that of the solvent.

dioxide was evolved from the melt, the displaced $H^+$ reacting with $NO_3^-$ according to the schematic reaction:

$$2H^+ + 2NO_3^- = H_2O\uparrow + N_2O_4\uparrow + \tfrac{1}{2}\,O_2\uparrow \qquad (1)$$

By percolating fused $NaNO_3$ over ZPH, full conversion to ZPNa was obtained. It must be noted that direct conversion of the hydrogen to the sodium form in fused salts is not possible for the amorphous ZP (ref. 3), owing to its lower thermal stability (condensation to pyrophosphate starts at about 180°). When ZP is converted in salt form, condensation of phosphate groups cannot occur, and the exchanger can be heated at very high temperatures without decomposition. The chemical stability of crystalline ZP was thus checked by contacting for different times 1 g of ZPNa (or ZPK) with 50 g of fused $NaNO_3$ (or $KNO_3$) at various temperatures.

Both crystalline ZPNa and ZPK were found to be very stable in molten alkali nitrates until about 500°. Above this temperature phosphates were released to the solvent, their amount increasing with the contact time and temperature (see Table I). For this reason, all the ion-exchange experiments were performed at temperatures lower than 500°.

TABLE I

DEGREE OF HYDROLYSIS OF ZPK IN MOLTEN $KNO_3$ FOR DIFFERENT TIMES AND TEMPERATURES

| *Temperature (°C)* | *Time (days)* | *Degree of hydrolysis*[a] |
|---|---|---|
| 450 | 30 | < 1% |
| 550 | 1 | ~ 5% |
| 550 | 2 | ~ 7% |
| 550 | 3 | ~ 10% |
| 600 | 1 | ~ 10% |
| 650 | 1 | ~ 20 ÷ 30% |
| 700 | 1 | 80 ÷ 100% |

[a] Expressed as: $\dfrac{\text{number of mmoles of phosphate released}}{\text{total number of mmoles of phosphate in the exchanger}} \times 100$

*Ion-exchange rate*

Since the equilibration time could be dependent on the process under consideration and on the ionic composition of the melt, the time necessary to reach equilibrium for some ionic fractions of the melt was determined before determining isotherm curves.

At $X_K = 0.25$, 0.75 and 0.90, equilibrium was reached in less than two days for both processes, while longer times (about 4 days) were required for the $\overline{Na}^+$–$\overline{K}^+$* process at $X_K = 0.50$. It was noted that for $X_K = 0.95$, equilibrium was not reached even after 18 days (Fig. 1). However, if the sample was contacted with the melt for a longer time, the first interplanar distance of the exchanger was increased from 8.7 Å (pure ZPK) to 11.3 Å, and nitrates were found in the exchanger even after prolonged washing with DMS. This phenomenon can be explained assuming that, for very long equilibration times, the melt can penetrate the exchanger. Therefore, to be

* The convention $\overline{Na}^+$–$\overline{K}^+$ and $\overline{K}^+$–$\overline{Na}^+$ represents $Na^+$ replacing $K^+$ in the exchanger and *vice versa*.

sure of the equilibrium and to avoid invasion phenomena, the contact time of the experiments was 10 days.

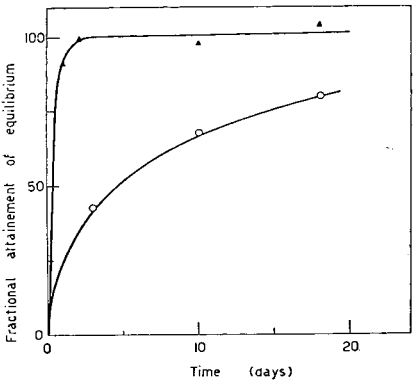


Fig. 1. Ion-exchange rate of crystalline zirconium phosphate in molten $NaNO_3$–$KNO_3$ ($X_K$ = 0.95) at 450°. ○, $Na^+$ displaces $K^+$ from ZPK; ▲, $K^+$ displaces $Na^+$ from ZPNa.

*Ion-exchange isotherms*

Owing to the stability of the salt forms of crystalline ZP at high temperature, it was possible to study the $\overline{Na}^+$–$\overline{K}^+$ exchange in molten $NaNO_3$–$KNO_3$ mixtures at 450° over the whole range of composition of this solvent (m.p. of $NaNO_3$, 314°; m.p. of $KNO_3$, 347°). Further, to ascertain if the ion-exchange hysteresis found for crystalline ZP in aqueous solution at room temperature[6] also occurs at 450°, the $\overline{K}^+$–$\overline{Na}^+$ isotherm was determined.

Fig. 2b shows the results obtained at 450° in fused salts while, for comparison, in Fig. 2a the same isotherms obtained in aqueous solution[6] are reported. In molten nitrates forward and reverse isotherms are practically the same; therefore no appreciable ion-exchange hysteresis takes place at 450°, contrary to what was found at room temperature.

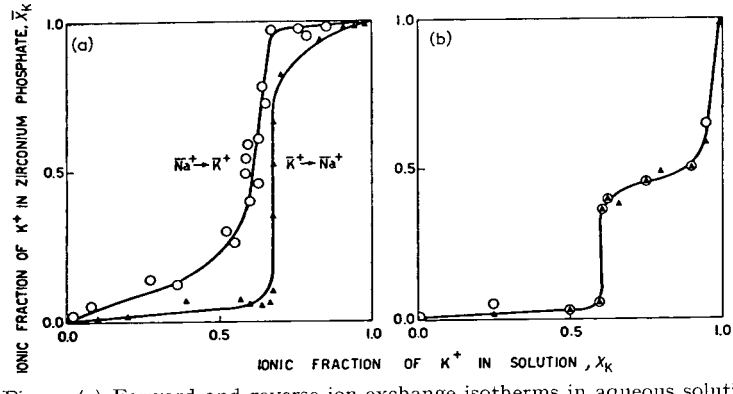


Fig. 2. (a) Forward and reverse ion-exchange isotherms in aqueous solution showing hysteresis. ▲, $K^+$ displaces $Na^+$ from ZPNa dried at room temperature; ○, $Na^+$ displaces $K^+$ from ZPK dried at room temperature. (b) Forward and reverse ion-exchange isotherms in molten $NaNO_3$–$KNO_3$ mixtures at 450°. ○, $Na^+$ displaces $K^+$ from ZPK; ▲, $K^+$ displaces $Na^+$ from ZPNa. (Values for $\bar{X}_K$ at $X_K$ = 0.95 are taken after 18 days of equilibration.)

Another difference between the behavior of crystalline ZP in aqueous solution and in molten nitrates can be seen from Fig. 2. While in aqueous solution $Na^+$ is preferred only at low $X_K$ values, in molten nitrates the isotherm curve lies entirely below the diagonal of the isotherm plane; therefore $Na^+$ is always preferred to $K^+$ over the whole range of the exchanger composition. This result agrees with what is obtained for $\overline{Na}^+$–$\overline{K}^+$ exchange in the amorphous ZP[2].

In molten nitrates the isotherm curve shows two vertical portions in which the exchanger exhibits different $\overline{X}_K$ values for a same $X_K$ value. In aqueous solution the vertical part of a given isotherm of the crystalline ZP has been related to the coexistence of two immiscible phases, one transforming into the other[6].

X-ray diffraction patterns showed that two diffraction maxima at 7.9 Å and 8.7 Å, respectively, were present in the vertical portion at $X_K = 0.95$ of the isotherm $\overline{Na}^+$–$\overline{K}^+$. The relative intensities of these peaks were found to vary with time and hence with the degree of the exchanger conversion (Fig. 3).

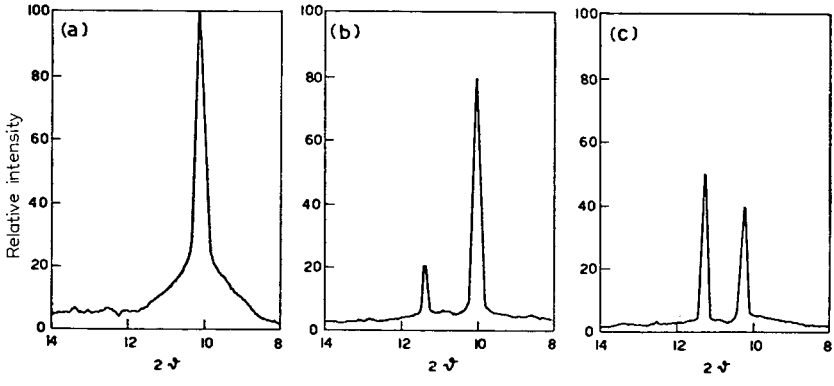


Fig. 3. Modification of X-ray diffraction pattern of crystalline ZPK after 2 and 10 days of equilibration with $NaNO_3$–$KNO_3$ mixture ($X_K = 0.95$ at 450°). (a) pure ZPK, $\overline{X}_K = 1$; (b) 2 days of equilibration, $\overline{X}_K = 0.83$; (c) 10 days of equilibration, $\overline{X}_K = 0.70$.

Two diffraction maxima at 7.7 Å and 7.9 Å were found also in the vertical portion at $X_K = 0.61$ of the isotherms $\overline{K}^+$–$\overline{Na}^+$ and $\overline{Na}^+$–$\overline{K}^+$.

Taking into account that the first interplanar distances of pure ZPNa and pure ZPK are 7.6 Å and 8.7 Å, respectively*, the shape of the isotherm curve $\overline{K}^+$–$\overline{Na}^+$ can be explained as follows. From $X_K = 0.0$ to $X_K \simeq 0.6$ the exchanger strongly prefers sodium ion; therefore $K^+$ content increases slowly. At $X_K \simeq 0.6$ two immiscible phases, having the approximate compositions $Zr(Na_{0.95}K_{0.05}PO_4)_2$ and $Zr(Na_{0.6}K_{0.4}PO_4)_2$, respectively, are found.

The composition of the melt remains fixed until the exchanger is completely converted to the latter form. From $X_K \simeq 0.6$ to $X_K \simeq 0.9$, $Zr(Na_{0.6}K_{0.4}PO_4)_2$ becomes richer in $K^+$ until at $X_K = 0.95$ two immiscible phases having the approximate compositions $Zr(Na_{0.5}K_{0.5}PO_4)_2$ and $Zr(Na_{0.02}K_{0.98}PO_4)_2$, respectively, are formed

* ZPNa and ZPK obtained from the nitrate melts at 450° exhibit the same X-ray diffraction patterns as ZPNa and ZPK obtained from aqueous solution and heated at 450°.

again. Thus the isotherm curve shows another vertical portion at $X_K = 0.95$ until pure ZPK is obtained.

Similar considerations can also be made for the isotherm $\overline{Na}^+$–$\overline{K}^+$.

The interplanar distances were determined at room temperature in this work. Therefore to establish for certain the phase transformations occurring at 450°, determinations with a high-temperature X-ray camera, at present not available in our laboratory, will be necessary.

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## Notes

CHROM. 4371

### The use of high-efficiency packed columns for gas–solid chromatography

### I. The complete separation of $^{14}N_2$–$^{15}N_2$

In the last few years, several isotopic pairs were separated in our laboratory, either by means of partition or by adsorption gas chromatography[1,2]. All these studies were carried out with glass capillary columns. The good results obtained for some isotopic systems[3] encouraged us to try gas chromatographic separation of isotopes on a semipreparative scale. This paper reports the first results in this direction. The system investigated is $^{14}N_2$–$^{15}N_2$, a partial separation of which was already obtained using glass capillary adsorption columns[4].

It is well known that the isotope effect in systems other than deuterium-substituted compounds is appreciable only at very low temperature. Moreover, the capacity ratio of the substance eluted must have a value around 10 in order to use the column efficiency completely. These conditions were achieved by using Graphon, a partially graphitized carbon black kindly furnished by Cabot Corp., as adsorption medium and working at 77°K. In order to avoid peak tailing, a slight deactivation was necessary even at room temperature. This was accomplished by treating the adsorbent with a small amount of squalane (1‰ w/v), distributed on the surface in a very thin and uniform layer. Nitrogen was strongly retained at the temperature of liquid nitrogen so that a further deactivation was necessary. This was done by using a mixture of CO and $H_2$ as carrier gas. CO was more strongly adsorbed and acted as a mobile deactivator. Experiments were made to test oxygen, argon and methane as deactivators, but the results obtained were unsatisfactory.

A Carlo Erba Model AtC/f gas chromatograph equipped with a thermal conductivity cell was used. The apparatus was modified for low-temperature work by connecting the cell to the column, fitted in a large dewar container with two narrow copper tubings of negligible volume. The gas line was also modified to allow mixing of the

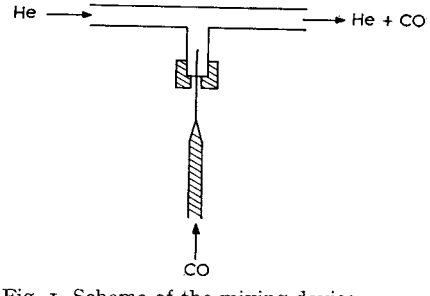


Fig. 1. Scheme of the mixing device.

carrier gas and the deactivating gas before entering the reference arm of the cell and the column. Mixing was effected by inserting in the carrier gas line a steel needle connected through a capillary to the cylinder of the deactivating gas. The scheme of the mixing device is shown in Fig. 1. The flow of the two gases was monitored by two rotametters before the mixing point, while the total flow rate was measured at the end of the column.

Columns were made of copper tubings each 15 m long. They were packed separately, tested and sealed together to the desired length of 60 m. The total number of theoretical plates, measured on the nitrogen peak was 45 000 with a linear gas velocity of 1.6 cm/sec where the minimum HETP was obtained. Such a low value of the linear gas velocity can be explained by the fact that at low temperature the diffusion is low so that the minimum of the Van Deemter curve is shifted towards the low velocities.

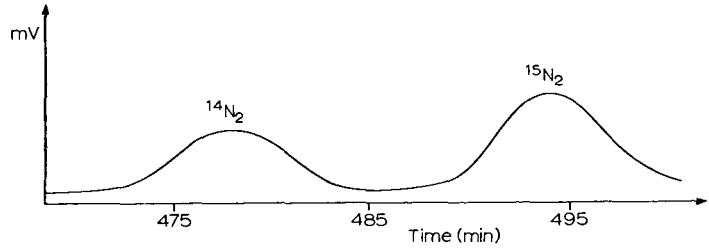


Fig. 2. Gas chromatographic separation of nitrogen isotopes. Column, 60 m × 4 mm I.D.; temp., 77°K; inlet pressure, 1.7 atm; flow rate, 75 ml/min.

Under these conditions the chromatogram shown in Fig. 2 was obtained. The sample injected was 0.2 cc. Resolution was 1.2 and the ratio of corrected retention volumes was 1.03, which shows that under our conditions an unusually large isotope effect takes place.

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CHROM. 4373

## Zum Einfluss der stationären Phase auf die Retentionsindizes isomerer Alkane

Die Retentionsindizes isomerer Alkane hängen, wenn auch nur in geringem Masse, von der Natur der stationären Phase ab. Im folgenden soll diese Abhängigkeit als Funktion der molekularen Parameter der Alkane untersucht werden.

In einer früheren Mitteilung[1] konnte gezeigt werden, dass die Retentionsindizes isomerer Alkane an Squalan als stationäre Phase eine Funktion der Zahl $n_3$ der durch drei C–C-Einfachbindungen getrennten Kohlenstoffatome und des quadratischen Mittelwertes des Radius $R$ eines Moleküls sind (s. Fig. 1 und Tabelle I).

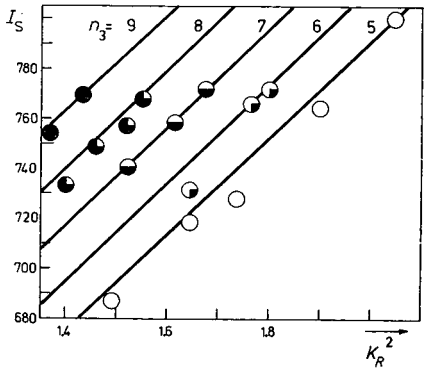



Fig. 1. Die Abhängigkeit der Retentionsindizes isomerer Octane an Squalan von $n_3$ und $K_R^2 = \overline{R^2}/a_1^2$. Die eingezeichneten Geraden entsprechen der Gl.(1).

Der quadratische Mittelwert des Radius ist dem mittleren Trägheitsmoment des Moleküls proportional und lässt sich für bestimmte Molekülmodelle einfach berechnen[1,2]; die Werte für die Alkane $C_6$ bis $C_8$ sind in Tabelle I zusammengestellt. Für die Retentionsindizes isomerer Alkane an Squalan (S) gilt (s. Fig. 1):

$$I_S = 100n + A_S(n_3 - n_{3(0)}) + B_S(K_R^2 - K_{R(0)}^2) \quad (1)$$

Hierbei sind $n$ die Zahl der Kohlenstoffatome des Alkans und $K_R^2 = \overline{R^2}/a_1^2$ der auf die Länge $a_1$ einer C–C-Einfachbindung bezogene quadratische Mittelwert des Radius. $A_S$ und $B_S$ sind empirisch zu bestimmende Konstanten, durch den Index o werden die Werte der entsprechenden unverzweigten Alkane gekennzeichnet.

Es zeigt sich, dass bei Benutzung anderer stationärer Phasen (P) ebenfalls der durch Gl.(1) gegebene Zusammenhang besteht; die Konstanten haben jedoch andere Werte:

$$I_P = 100n + A_P(n_3 - n_{3(0)}) + B_P(K_R^2 - K_{R(0)}^2) \quad (2)$$

Um den Einfluss der stationären Phase auf die Retentionsindizes besser erfassen zu können, ist es zweckmässig, die Differenz der Retentionsindizes $\Delta I$ für die beiden

TABELLE I

DIE AUF DIE LÄNGE EINER C–C-EINFACHBINDUNG BEZOGENEN QUADRATISCHEN MITTELWERTE DER RADIEN, DIE ZAHL DER DURCH DREI C–C-BINDUNGEN GETRENNTEN KOHLENSTOFFATOME, UND DIE RETENTIONSINDIZES DER ISOMEREN ALKANE $C_6$ BIS $C_8$ (BEI 25°)

Temperatur: 25°.

| *Substanz* | $K_R^2$ | $n_3$ | *Retentionsindizes* | | |
|---|---|---|---|---|---|
| | | | $I_{Sq}$ | $I_{OD-1}$ | $I_{DMS}$ |
| *n*-6 | 1.4132 | 3 | 600 | 600 | 600 |
| 2M-5 | 1.2510 | 3 | 569.1 | 568.6 | 567 |
| 3M-5 | 1.1965 | 4 | 582.8 | 581.5 | 585 |
| 2,2M-4 | 1.0370 | 3 | 534.6 | 532.9 | 531 |
| 2,3M-4 | 1.0895 | 4 | 565.1 | 563.4 | 567 |
| *n*-7 | 1.7271 | 4 | 700.0 | 700.0 | 700 |
| 2M-6 | 1.5672 | 4 | 666.1 | 665.5 | 664 |
| 3M-6 | 1.4865 | 5 | 675.3 | 673.9 | 677 |
| 3Ä-5 | 1.4059 | 6 | 684.4 | 684.6 | 691 |
| 2,2M-5 | 1.3288 | 4 | 624.2 | 622.9 | 620 |
| 2,3M-5 | 1.3273 | 6 | 669.1 | 666.9 | 675 |
| 2,4M-5 | 1.4074 | 4 | 629.2 | 628.0 | 624 |
| 3,3M-5 | 1.2487 | 6 | 655.3 | 642.3 | 660 |
| 2,2,3M-4 | 1.1701 | 6 | 635.9 | 632.7 | 640 |
| *n*-8 | 2.0449 | 5 | 800.0 | 800.0 | 800 |
| 2M-7 | 1.8913 | 5 | 764.5 | 763.8 | 763 |
| 3M-7 | 1.7984 | 6 | 771.9 | 769.8 | 774 |
| 4M-7 | 1.7673 | 6 | 766.9 | 765.3 | 769 |
| 3Ä-6 | 1.6744 | 7 | 771.9 | 769.2 | 777 |
| 2,2M-6 | 1.6464 | 5 | 718.1 | 716.2 | 714 |
| 2,3M-6 | 1.6142 | 7 | 758.6 | 755.8 | 764 |
| 2,4M-6 | 1.6449 | 6 | 730.8 | 728.7 | 729 |
| 2,5M-6 | 1.7377 | 5 | 727.8 | 726.2 | 723 |
| 3,3M-6 | 1.5230 | 7 | 740.4 | 738.3 | 744 |
| 3,4M-6 | 1.5525 | 8 | 768.1 | 765.5 | 778 |
| 2M-3Ä-5 | 1.5214 | 8 | 757.6 | 755.1 | 767 |
| 3M-3Ä-5 | 1.4306 | 9 | 769.4 | 765.3 | 782 |
| 2,2,3M-5 | 1.4010 | 8 | 733.2 | 729.9 | 741 |
| 2,2,4M-5 | 1.4931 | 5 | 687.1 | 685.4 | 681 |
| 2,3,3M-5 | 1.3698 | 9 | 754.4 | 749.8 | 768 |
| 2,3,4M-5 | 1.4612 | 8 | 748.8 | 749 | 758 |

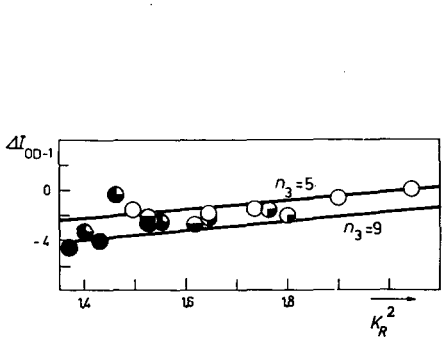
stationären Phasen S und P als Funktion von $n_3$ und $K_R^2$ zu untersuchen. Aus den Gl.(1) und (2) folgt:

$$\Delta I = I_P - I_S = (A_P - A_S)(n_3 - n_{3(0)}) + (B_P - B_S)(K_R^2 - K_{R(0)}^2)$$

$$= A_D(n_3 - n_{3(0)}) + B_D(K_R^2 - K_{R(0)}^2) \quad (3)$$

Präzise Messungen der Retentionsindizes sämtlicher isomerer Alkane $C_5$ bis $C_8$ bzw. $C_9$ an verschiedenen stationären Phasen liegen von TOURRES[3] an Squalan und von DIEDEREN[4] an Octadecen-1 und Dimethylsulfolan vor (s. Tabelle I). Da die Retentionsindizes geringfügig von der Temperatur abhängen, wurden — um die Messungen beider Autoren vergleichen zu können — die Werte von TOURRES auf eine Temperatur von 25° extrapoliert.

In den Fig. 2 und 3 sind für die isomeren Octane und in Fig. 4 für die isomeren Hexane und Heptane die Differenzen der Retentionsindizes an den stationären Phasen Octadecen-1 und Squalan (Fig. 2) bzw. Dimethylsulfolan und Squalan (Fig. 3 und 4) als Funktion von $K_R^2$ — mit $n_3$ als Parameter — dargestellt. In allen Fällen ergibt sich der durch Gl.(3) beschriebene lineare Zusammenhang zwischen $\Delta I$, $n_3$ und $K_R^2$. Die Aufspaltung, d.h. die Konstante $A_D$, wird umso grösser, je grösser das Dipolmoment der stationären Phase ist. Analoge Untersuchungen[5] über Flüssigkeitsgemische, die isomere Alkane enthalten, haben gezeigt, dass diese Aufspaltung auf die Induktionskräfte, deren Grösse durch das Dipolmoment der einen Phase und der — nur von $n_3$ und nicht von $K_R^2$ abhängenden — Polarisierbarkeit der isomeren Alkane bestimmt wird, zurückzuführen ist.



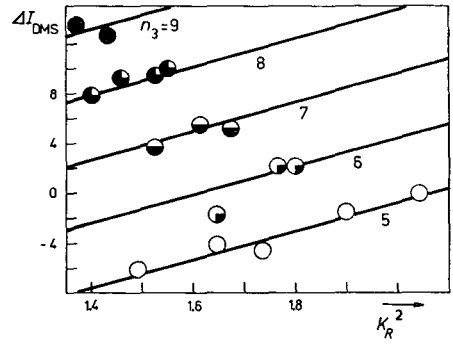




Fig. 2. Die Abhängigkeit der Differenz der Retentionsindizes $\Delta I_{OD-1}$ der isomeren Octane an den stationären Phasen Octadecen-1 und Squalan von $n_3$ und $K_R^2$. Die eingezeichneten Geraden entsprechen der Gl.(3).

Fig. 3. Die Abhängigkeit der Differenz der Retentionsindizes $\Delta I_{DMS}$ der isomeren Octane an den stationären Phasen Dimethylsulfolan und Squalan von $n_3$ und $K_R^2$. Die eingezeichneten Geraden entsprechen der Gl.(3).

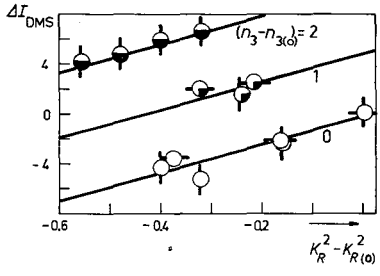



Fig. 4. Die Abhängigkeit der Differenz der Retentionsindizes $\Delta I_{DMS}$ der isomeren Hexane (—○—) und Heptane (○) an den stationären Phasen Dimethylsulfolan und Squalan von $n_3$ und $K_R^2$. Die eingezeichneten Geraden entsprechen der Gl.(3).

Die Konstanten $A_D$ und $B_D$ haben für die isomeren Alkane $C_6$ bis $C_8$ folgende Werte:

| | $A_D$ | $B_D$ |
|---|---|---|
| Octadecen-1–Squalan | −0.40 | − 3.47 |
| Dimethylsulfolan–Squalan | 5.13 | −11.28 |

Aus den Gleichungssystemen (1) und (2) bzw. (1) und (3) kann man $K_R^2$ eliminieren:

$$I_P = 100n + a_P(n_3 - n_{3(0)}) + c_P(I_S - 100n) \quad (4)$$

bzw.

$$\Delta I = I_P - I_S = a(n_3 - n_{3(0)}) + \gamma(I_S - 100n) \quad (5)$$

Zwischen den Konstanten der Gln.(1–5) bestehen noch folgende Beziehungen:

$$\begin{aligned} a_P &= A_P - A_S \frac{B_P}{B_S} & c_P &= \frac{B_P}{B_S} \\ a &= A_D - A_S \frac{B_D}{B_S} & \gamma &= \frac{B_D}{B_S} \end{aligned} \quad (6)$$

Trägt man die an einer (stark polaren) Phase gemessenen Retentionsindizes isomerer Alkane als Funktion der an einer zweiten (unpolaren oder schwach polaren) Phase gemessenen Retentionsindizes ($I_S$–$I_P$-Diagramm) auf, so erhält man ein System paralleler Geraden, deren Abstände durch $n_3$ bestimmt werden. Das prinzipiell gleiche Ergebnis erhält man, wenn man $\Delta I$ als Funktion von $I_S$ (oder $I_P$) aufträgt (s. Fig. 5).

Aus den an einer unpolaren (oder schwach polaren) und einer stark polaren stationären Phase gemessenen Retentionsindizes kann man in einem $I_S$–$I_P$-Diagramm unmittelbar die isomeren Alkane in Gruppen mit gleichen Werten von $n_3$ aufgliedern. Da, wie Fig. 1 am Beispiel der isomeren Octane zeigt, der Retentionsindex von isomeren Alkanen mit gleichen Werten von $n_3$ eine eindeutige Funktion von $K_R^2$ ist, ist es möglich, durch die Messung der Retentionsindizes an zwei stationären Phasen unterschiedlicher Polarität die isomeren Octane eindeutig zuzuordnen. Die quadratischen Mittelwerte der Radien höherer molekularer isomerer Alkane unterscheiden sich in einigen Fällen nur wenig, so dass dann nicht in allen Fällen eine eindeutige Zuordnung allein aus den Messungen der Retentionsindizes an zwei stationären Phasen möglich ist.

Fasst man in einem $I_S$–$I_P$-Diagramm Verbindungen zusammen, die jeweils die gleiche Anzahl primärer, sekundärer usw. Kohlenstoffatome enthalten, so liegen

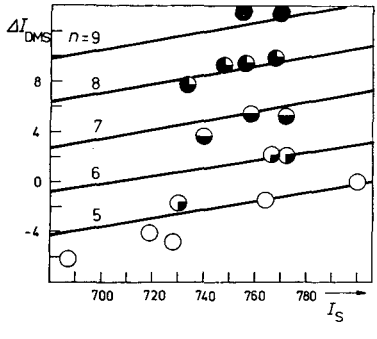



Fig. 5. Die Abhängigkeit der Differenz der Retentionsindizes $\Delta I_{DMS}$ der isomeren Octane an den stationären Phasen Dimethylsulfolan und Squalan vom Retentionsindex an Squalan.

diese Punkte, wie WALRAVEN *et al.*[6] gezeigt haben, auf dachziegelartig angeordneten parallelen Geraden. Man kann dieses Verhalten verstehen, wenn man berücksichtigt, dass die von WALRAVEN u.a. eingeführte Code ($C_{WLK}$) eindeutig von den Parametern $n_3$ und $K_R^2$ abhängt. In Fig. 6 sind jeweils isomere Octane mit gleicher Anzahl

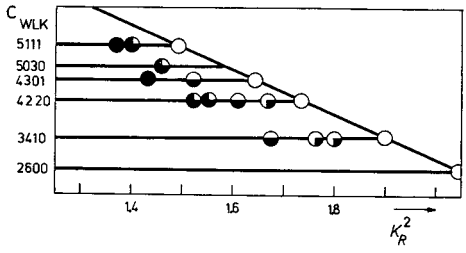



Fig. 6. Die Abhängigkeit der Code ($C_{WLK}$) von $n_3$ und $K_R^2$. In der Code geben die Ziffern jeweils die Zahl der primären, sekundären usw. Kohlenstoffatome an.

primärer, sekundärer usw. Kohlenstoffatome, also Verbindungen, die die gleiche Code ($C_{WLK}$) haben, zusammengefasst und als Funktion von $K_R^2$ aufgetragen. Die (willkürliche) Ordinateneinteilung wurde so gewählt, dass die Werte von $C_{WLK}$ für Verbindungen mit $n_3 - n_{3(0)} = 0$ eine lineare Funktion von $K_R^2$ sind. Wie Fig. 6 zeigt, lässt sich $C_{WLK}$ in erster Näherung in der Form

$$C_{WLK} = C_{WLK(0)} + A_C(n_3 - n_{3(0)}) + B_C(K_R^2 - K_{R(0)}^2) \quad (7)$$

darstellen, wobei $C_{WLK(0)}$, $A_C$ und $B_C$ empirisch zu bestimmende Konstanten sind. Es ist damit möglich, aus dem Gleichungssystem (1), (2) und (7) die Parameter $n_3$ und $K_R^2$ zu eliminieren. Man erhält damit in Analogie zu den obigen Gleichungen

$$I_P = I_{P(0)} + \gamma_C (I_S - 100n) + \lambda_C C_{WLK} \quad (8)$$

$I_{P(0)}$, $\gamma_C$ und $\lambda_C$ sind wiederum empirisch zu bestimmende Konstanten. Dies ist der analytische Ausdruck für die von WALRAVEN u.a. (s. oben) experimentell gefundenen Zusammenhänge.

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CHROM. 4364

## Direct hydrogenation of unsaturated fatty acids on the support of the gas–liquid chromatographic column

When qualitatively identifying fatty acids separated by GLC analysis, extrapolations from plots of relationships between the logarithms of specific elution volumes and the number of carbon atoms, polar and nonpolar phases, the number of double bonds, Kovats' indices, etc. can be used. To supplement data on qualitative identification of unsaturated and saturated fatty acids in a mixture, the so-called discrimination chromatogram, *i.e.* a new GLC analysis of the sample after its chemical treatment by bromination, oxidation or hydrogenation, can be used.

LANDOWNE AND LIPSKI[1] analyzed mixtures of unsaturated and saturated fatty acids using bromination. They first carried out a GLC analysis of an untreated sample and then compared the results with those of a GLC analysis of derivatives of unsaturated fatty acids after bromination. CARTONI *et al.* used bromination for the separation of stereoisomers of 9:10-octadecenoic acid on an open tubular capillary GLC column[2]. JAMES AND WEBB[3] used an oxidative method for distinguishing unsaturated and saturated fatty acids, and GUNSTONE AND SYTER[4] used the oxidative method for determination of the structure of some unsaturated fatty acids. FARQUHAR *et al.* introduced GLC separation of unsaturated and saturated fatty acids before and after hydrogenation in a microdevice using platinum as catalyst[5]. SMITH[6] applied this method for qualitative and quantitative analyses of fatty acid in milk fat by GLC, for example. Bromination and/or hydrogenation in combination with GLC analysis of fatty acids as described above are introduced by BURCHFIELD AND STORRS[7] in the chapter *Subtraction methods* of their monograph. Microhydrogenation of unsaturated fatty acids on filter paper with the use of palladium as catalyst was described by KAUFMANN AND CHOWDHURY[8]; the combination of this method with GLC analysis of fatty acid mixtures was published by KOMAN[9].

In the present paper, direct hydrogenation of unsaturated fatty acids by filling the GLC column with palladium as catalyst precipitated on Celite is described, and the separations are compared with results achieved on the column without catalyst.

*Experimental*

*Preparation of columns.* Two identical aluminium columns 2 m long and 4 mm in diameter were packed with 12.4 g of Celite having a particulate diameter of 0.12–0.15 mm. Celite was floated, acid washed[10], silanized with dichlorodimethylsilane[11] and coated with 20% DEGS which was prepared according to ref. 12. At the inlet of one column, 250 mg of Celite with freshly precipitated palladium were added.

*Preparation of the catalyst.* Palladium was precipitated and applied as described in refs. 8, 13 and 14, dealing with distinguishing "critical pairs" of unsaturated and saturated fatty acids by partition paper chromatography. In our case, unsilanized Celite was used as a support and promotor of the catalyst. The precipitation of palladium was carried out under the following conditions. 6.4 ml of a 10% solution of palladium dichloride and 10 ml of a freshly prepared mixture of 80 ml of 20% KOH and 20 ml of 40% formaldehyde were added to 1.0 g of unsilanized Celite. Then the Celite was washed for 10 min with a 5% solution of acetic acid, and the acid was

removed by a five-fold washing with water. The Celite with palladium precipitated in this way was dried for 30 min at 90°, and 250 mg were applied to the inlet of one column.

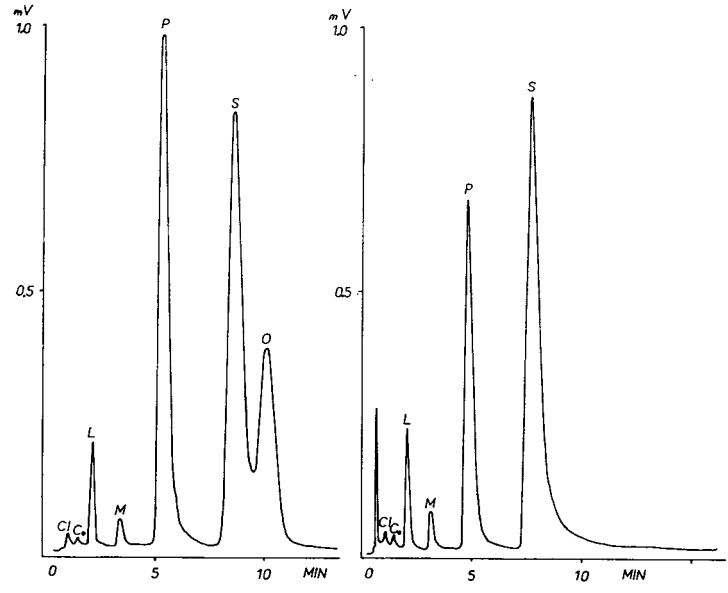


Fig. 1. Chromatograms of mixtures of fatty acids separated on columns without (left-hand side) and with palladium (right-hand side). L = lauric ($C_{12:0}$); M = myristic ($C_{14:0}$); P = palmitic ($C_{16:0}$); S = stearic ($C_{18:0}$); and O = oleic ($C_{18:1}$) acid.

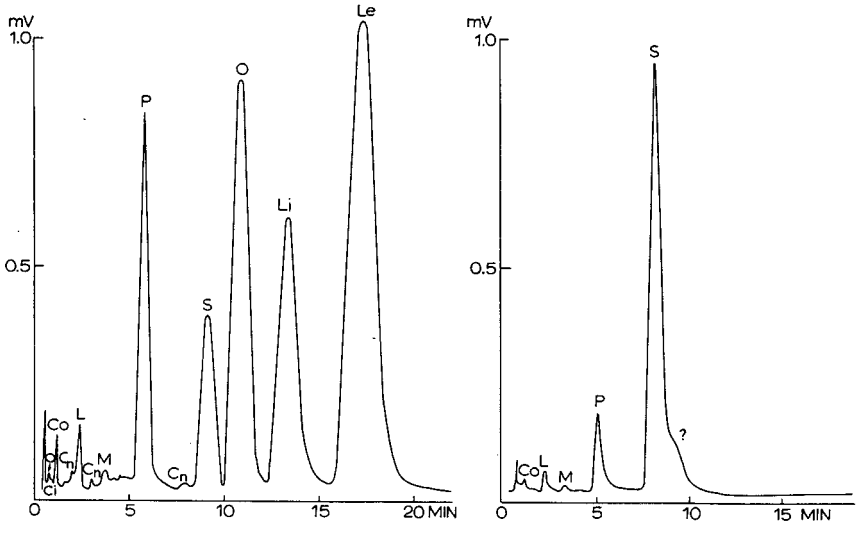


Fig. 2. Chromatograms of mixtures of fatty acids separated on columns without (left-hand side) and with palladium (right-hand side). P = palmitic ($C_{16:0}$); S = stearic ($C_{18:0}$); O = oleic ($C_{18:1}$); Li = linoleic ($C_{18:2}$); and Le = linolenic ($C_{18:3}$) acid.

*Chromatographic conditions*. Before use both prepared columns were conditioned for 24 h at 220°. For conditioning, as well as for the GLC analysis of fatty acid mixtures, hydrogen as carrier gas was used. Optimal values of the hydrogen flow (65 ml/min) and the optimal column temperature (197°) were determined experimentally. For the assay 0.1–0.35 $\mu$l of fatty acid mixtures, a temperature of 295° at the injection port of the gas chromatograph (type CHROM 3*) and isothermic conditions were used.

*Results and discussion*

The results of the GLC separations of some natural fatty acid mixtures on the columns with and without palladium, presented in Figs. 1–3, indicate a complete

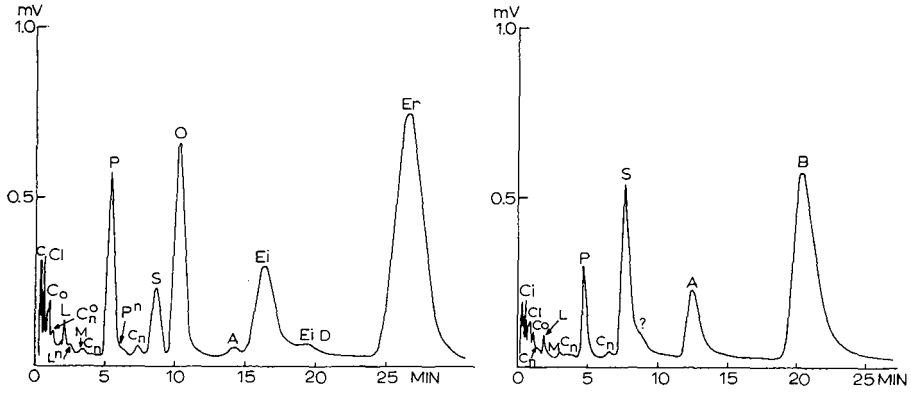


Fig. 3. Chromatograms of mixtures of fatty acids separated on columns without (left-hand side) and with palladium (right-hand side). P = palmitic ($C_{16:0}$); S = stearic ($C_{18:0}$); O = oleic ($C_{18:1}$); A = arachidic ($C_{20:0}$); Ei = eicosenoic ($C_{20:1}$); EiD = eicosadienoic ($C_{20:2}$); Er = erucic ($C_{22:1}$); and B = behenic ($C_{22:0}$) acid.

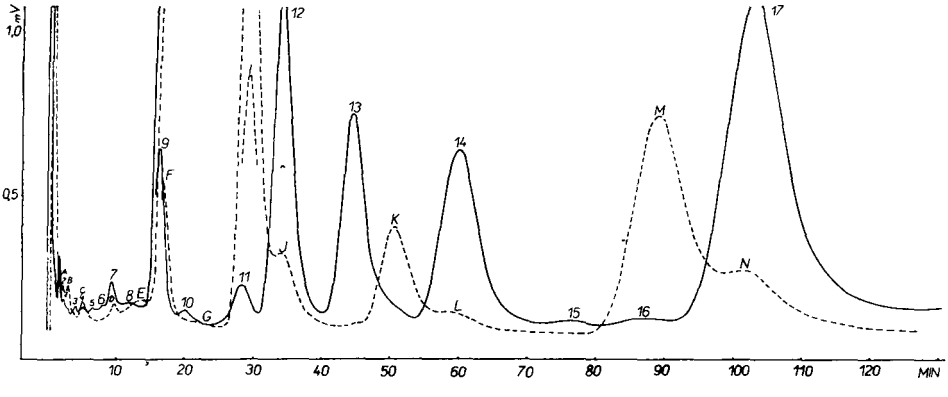


Fig. 4. Chromatograms of a mixture of fatty acids: part of sample unhydrogenated (full line) and part pre-hydrogenated on paper before GLC analysis according to ref. 9 (dotted line). Individual peaks: F ≡ 9 = palmitic acid ($C_{16:0}$); I ≡ 11 = stearic acid ($C_{18:0}$); J ≡ 12 = oleic acid ($C_{18:1}$); 13 = linoleic acid ($C_{18:2}$); K = arachidic acid ($C_{20:0}$); L ≡ 14 = eicosenoic acid ($C_{20:1}$); 15 = eicosadienoic acid ($C_{20:2}$); M ≡ 16 = behenic acid ($C_{22:0}$); N ≡ 17 = erucic acid ($C_{22:1}$)**.

* Manufactured by Laboratorní přístroje n.p. Praha, Czechoslovakia.

** In all figures the peaks in front of palmitic acid belong to short-chain minority fatty acids of the analysed mixtures.

conversion of unsaturated fatty acids into the corresponding saturated ones. An example of a GLC analysis of a fatty acid mixture before and after microhydrogenation, according to ref. 9, is presented in Fig. 4. Comparison of Figs. 3 and 4 shows that the hydrogenation of unsaturated fatty acids is quantitative on the column with the catalyst, while the unsaturated fatty acids from the same mixture hydrogenated on filter paper prior to the GLC analysis did not react completely.

It is apparent from the results that the application of the described direct hydrogenation of unsaturated fatty acids on the GLC column with palladium can be recommended as a supplement for qualitative identification of unsaturated and saturated fatty acids in mixtures.

When the column with palladium is already prepared and GC equipment with a dual column system is used, the time required for obtaining a discrimination chromatogram under the conditions described for hydrogenation is reduced to the time required for injection of the sample only.

The direct hydrogenation of unsaturated fatty acids on the GLC column was verified by numerous examples using known natural fatty acid mixtures and gave a good reproducibility. It was successfully used for qualitative and quantitative determinations of fatty acids, *e.g.* in the citrinin producer *Penicillium notatum* during cultivation[15].

The activity of palladium which was prepared and used at the beginning of these experiments has remained unchanged for two years.

*Note*

Recently we have found that the hydrogenation presented herein can be simplified further by placing 250 mg of the catalyst prepared as described above into a "microreactor" which can be connected to the front of any GC column.

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CHROM. 4362

## Gas chromatography of gases emanating from the soil atmosphere

In different denitrification studies, many investigators[1-3] have studied the composition of the soil atmosphere. It is desirable to develop a method suitable for separation and analysis of oxygen, nitrogen, carbon dioxide, nitric oxide, nitrous oxide and nitrogen dioxide. The present studies were undertaken to separate the gases by gas chromatography at ambient temperature.

*Experimental*

A method for the gas chromatographic analysis of a sample of soil atmosphere was developed using a three-column system and one detector. The columns used in three stages are Carbowax on glass beads[4], Porapak Q[5] and molecular sieve[4] respectively. The details of the construction and operation of the three columns are as follows: the first is a 1 ft. × 1/8 in. O.D. stainless-steel column, externally placed and filled with 0.5 % Carbowax 1500 on 60–80 mesh silanized glass beads; the second is a 18 ft. × 3/16 in. O.D. stainless-steel column packed with Porapak Q 80–100 mesh and activated before use at 230° for 1–2 h; the third is a 3 ft. × 1/4 in. O.D. stainless-steel column packed with 0.2–0.5 mm molecular sieve 5 A and activated at 220° for 24 h.

This three-column system is operated in series so that the sample enters the first external column, flows to the second Porapak Q column and passes to the third molecular sieve column.

The detector is a Gow Mac thermal conductivity cell with W2X filaments and is operated by a bridge current of 250 mA. The detector signal output is fed to a 1 mV recorder. Helium was used as carrier gas at a flow rate of 50 ml/min.

*Results and discussion*

The first external column is placed in a bath of liquid air. At low temperature this column retains $NO_2$, NO, $CO_2$ and $N_2O$. The remaining gases ($O_2$ and $N_2$) flow to the second and third columns. The Porapak Q column gives a composite peak of

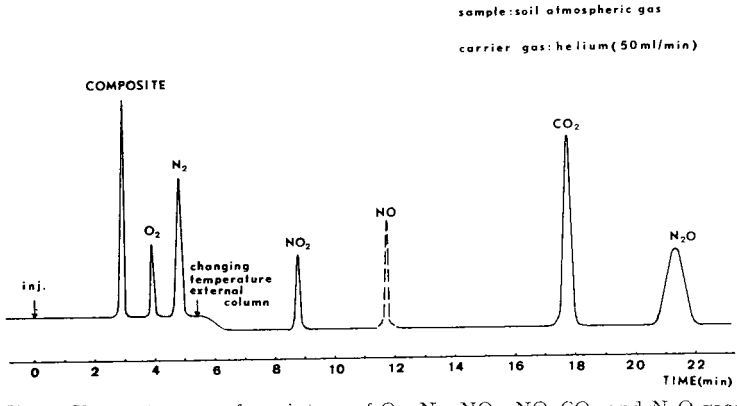


Fig. 1. Chromatogram of a mixture of $O_2$, $N_2$, $NO_2$, NO, $CO_2$ and $N_2O$ gases. Sample, soil atmospheric gas; carrier gas, helium (50 ml/min).

$O_2$ and $N_2$, and the molecular sieve column separates them. After $O_2$ and $N_2$ have been detected, the temperature of the first column is increased to a high temperature by boiling water so that $NO_2$, NO, $CO_2$ and $N_2O$ flow to the Porapak Q column, where they are separated.

However, it is impossible to have oxygen and nitric oxide together in one sample because, in the presence of oxygen, nitric oxide is immediately oxidized to nitrogen dioxide[6].

By changing the external column from low to ambient temperature the baseline is also changed; but after some time it will adjust itself.

Using helium at a flow rate of 50 ml/min, the column system gives, as shown in Fig. 1, a good separation for all the gases.

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CHROM. 4376

## An inexpensive and efficient pyrolysis unit for the analysis of picloram and other herbicides by thermal decomposition

Recently we found that picloram (4-amino-3,5,6-trichloropicolinic acid) could be quantitatively analyzed by electron-capture gas chromatography via thermal decarboxylation[1] rather than by esterification[2-4]. The decarboxylation technique has many advantages over the esterification method. For example, picloram in water, soil, or forage can be easily determined. This method should be applicable to other benzenoid herbicides and pesticides which have carboxyl of ester groups.

Commercial pre-column pyrolysis units are generally expensive and some expose the sample to a metallic heating element, thus making them incompatible with electron-capture detectors. There is, therefore, a need for an inexpensive and efficient pyrolysis unit which is specifically constructed for this purpose and which will not impair normal use of the gas chromatograph. We have constructed such a unit; its adaption to a Barber Coleman Model 5630 gas chromatograph fitted with an electron-capture detector is described below.

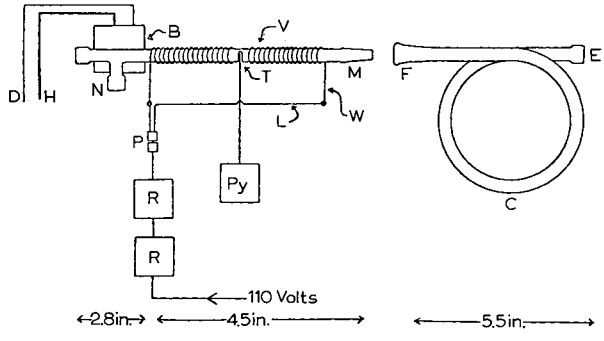


Fig. 1. Schematic of pyrolysis unit. D = thermocouple lead from chromatograph; H = cartridge-heater lead from chromatograph; N = nitrogen and injection inlet "tee"; B = injection heater block; P = male and female 110-volt plug; R = rheostat; V = vycor tube; T = thermocouple; Py = pyrometer; L = insulated copper lead; W = nichrome wire lead; M = male vycor ground-glass joint; F = female vycor ground-glass joint; C = 6-ft. spiral glass column; E = sample exit to electron-capture detector.

The following modifications were made to the chromatograph and column: the aluminum injection port heater was removed and the thermocouple leads were rerouted to the exterior through a hole in the extreme lower left-hand corner of the voltage control plate. A 6-ft. spiral glass column was cut 4.75 in. from the injector tip, and a female vycor ground-glass (10/30) joint connected to the column through a graded seal.

The pyrolysis unit (Fig. 1) was constructed by joining the nitrogen and injector inlet "tee" (N) to a vycor tube that was 4.5 in. long and 5 mm in inside diameter. As much soft glass as possible was eliminated between the "tee" and the vycor tube. A male ground vycor (10/30) joint (M) was connected to the other end of the vycor tube.

The aluminum block heater was installed on the inlet "tee" and covered with asbestos board (0.25 in. thick) which was held in place by copper wire. A thermocouple well was made in the center of the vycor tube and a Chromel–Alumel thermocouple inserted and held in place with asbestos putty. The tube was then uniformly wound with 26-gauge nichrome wire, each wind being separated by a wind of asbestos string. Three layers of asbestos string were then wrapped around the tube for insulation. The nichrome wire leads were connected to a 110-volt plug through 3-ft. lengths of insulated copper wire.

The pyrolysis unit was inserted approximately one-third of the way through the injection port and connected to the column, without grease via a male–female joint (M,F). The connection was secured with a No. 18 ball-and-socklet clamp. The portion of the pyrolysis tube exposed to the exterior was covered with fiberglass insulation. The rheostats connected in series were used to control pyrolysis temperatures. The pyrolysis unit was supported by a 3-finger clamp fastened to the exterior of the chromatograph.

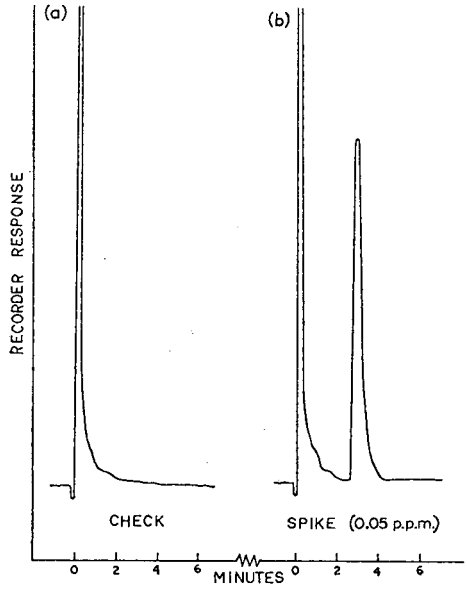


Fig. 2. Representative chromatograms of the decarboxylation of picloram obtained with the pyrolysis unit described herein. a = solvent; b = solvent plus picloram (0.5 $\mu$g per ml).

We have found that this unit, using vycor chips as an inert contact material, gave excellent results[1]. Due to the selectivity of this procedure, chromatograms are free from impurity peaks (Fig. 2) thus, making this technique ideal for trace analysis of herbicides in samples of high organic content. A detailed procedure employing thermal decarboxylation will be presented at a later date.

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CHROM. 4386

## Thin-layer chromatographic separation of Δ-fac(N) and Δ-mer(N) isomers of tris(S-(+)-α-alaninato)cobalt (III)

α-Amino acids, which are bidentate ligands, coordinate around $Co^{3+}$ in either $\Lambda(C_3)$ or $\Delta(C_3)$ absolute configuration (PIPER's notation[1]), while the unsymmetrical character of these ligands leads to geometrical isomerism. Many of the stereochemical questions are connected with both the rapid and the efficient separation of possible isomers. In the case of S-(+)-α-alanine, four isomers are known[2,3]: Λ,Λ-fac(N) and Λ,Δ-mer(N). From these, the Λ-fac(N) isomer is quite insoluble in water and the Λ-mer(N) one is only sparingly soluble. In the present note we describe the separation of geometrical isomers of Δ configuration with the axial disposition of $CH_3$ groups (k'k'k' arrangement of chelate rings, ob conformation with the C–C chelate axes oblique to the $C_3$ axis of rotation[4] )(Fig. 1).

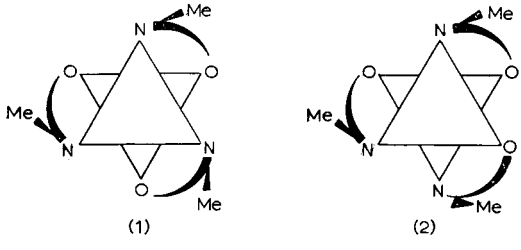


Fig. 1. Λ-Fac(N) (1) and Δ-mer(N) (2) isomers of Co(S-(+)-α-alaninate)$_3$. Projection around $C_3$ axis of rotation.

*Results*

From the data given in Table I the following conclusions can be drawn. The best separation was achieved when the water content varied from 30 to 40 %. The $R_F$ values of the fac(N) isomer decrease with the decreasing polarity of the solvent systems used. Even though the polarity of isopropanol (based on dielectric constants[5])

TABLE I

$R_F$ VALUES OF $\Delta$-fac(N) AND $\Delta$-mer(N) ISOMERS OF Co(S-(+)-$\alpha$-ALANINATE)$_3$

| *Isomer* | *Water content in the alcohol used* (%) | |
|---|---|---|
| | *30* | *40* |
| | *Ethanol* | |
| fac(N) | 0.55 | 0.70 |
| mer(N) | 0.71 | 0.75 |
| | *n-Propanol* | |
| fac(N) | 0.38 | 0.57 |
| mer(N) | 0.57 | 0.73 |
| | *Isopropanol* | |
| fac(N) | 0.43 | 0.56 |
| mer(N) | 0.67 | 0.81 |

is lower than that of *n*-propanol, higher $R_F$ values were obtained in the former case and thus steric factors must be taken into account. Differences in solvation of $CH_3$ groups cannot be considered, because both isomers have their alkyl groups in the axial position. As in other octahedral cobalt(III) complexes, the mer(N) isomer (usually called *trans* or $\alpha$) has higher $R_F$ values than the fac(N) one[6,7]. Because both isomers differ only in their symmetry, we assume that separation occurs on account of the difference in dipole moments. Actually it was observed that in the systems with the more polar stationary phase, for the compounds with the lower dipole moment higher $R_F$ values were obtained[8]. Dipole moment differences further lead to different adsorbability, which would be greater for fac(N) isomers. This was demonstrated by chromatography on different adsorbents, where $R_F$ values of fac(N) isomers decreased in the order cellulose > silica gel > alumina, while $R_F$ values of mer(N) isomers were almost constant (see Table II).

TABLE II

$R_F$ VALUES OF $\Delta$-fac(N) AND $\Delta$-mer(N) ISOMERS OF Co(S-(+)-$\alpha$-ALANINATE)$_3$ ON DIFFERENT SORBENTS

Solvent system: *n*-propanol–*n*-butanol–water (4:3:3).

| *Isomer* | *Cellulose*[a] | *Silica gel*[b] | *Alumina*[c] |
|---|---|---|---|
| fac(N) | 0.35 | 0.29 | 0.20 |
| mer(N) | 0.49 | 0.49 | 0.44 |

[a] Bělá p. Bezděz, Č.S.S.R.
[b] MN-G, Macherey, Nagel & Co.
[c] Reanal, Hungary.

Using 70 % isopropanol as solvent, we found that $\Delta$-fac(N) and $\Delta$-mer(N) isomers are formed initially (detectable after less than 5 min) in the reaction of $Co(OH)_3$ with S-(∓)-, or RS-α-alanine, respectively. The same isomers were also detected in the reaction of $[Co(NH_3)_6]Cl_3$ with the same ligands, although according to the literature[2,9] this method would give only insoluble $\Lambda$-fac(N)-Co(S-, or RS-α-alaninates)$_3$. These results, which will be published in more detail elsewhere, are supported by the fact that in Co(α-amino-acidate)$_3$ complexes little stereospecificity occurs.

*Experimental*

Geometrical isomers of Co(S-(+)-α-alaninate)$_3$ were prepared according to DENNING AND PIPER[3] and characterized on the basis of elemental analysis, electron absorption spectra ($\Delta$-fac(N): $\lambda_{max}$ = 375 and 520 μm, $\varepsilon$ = 150 and 188; $\Lambda$-mer(N): $\lambda_{max}$ = 375 and 535 μm, $\varepsilon$ = 95 and 60), and specific rotation ($\Delta$-fac(N): $[\alpha]_D = -986°$; $\Lambda$-mer(N): $[\alpha]_D = -655°$).

Chromatography was carried out on silica gel coated (MN-G, Macherey, Nagel & Co.) glass plates (10 × 20 cm). Ten microlitres of an approximately $1 \cdot 10^{-4}$ *M* solution of isomers were applied on the starting line (1 cm from the lower edge of the plates). The plates were developed to a height of 10 cm. Detection was made with $Na_2S$.

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CHROM. 4374

## Thin-layer chromatography of some substituted esters and diamides of malonic acid on silica gel

In the synthesis of some pharmaceuticals in which malonic acid esters were used as intermediates, it was necessary to conduct some purity control tests. Though substituted malonic acid esters have mostly been studied by GC[1,2], not much TLC[3] work seems to have been published. Therefore it was interesting to know the TLC behaviour of mono- and disubstituted esters and also diamides of malonic acid.

The substituted malonic acid esters were prepared by alkylation. Diamides could be synthesized through aminolysis of the corresponding esters. The nonreactive ethylbutylmalonic acid ester was converted via the Na derivative of *m*-phenylenediamine to the di(*m*-amino)anilide of ethylbutylmalonic acid according to the process of STERN[4].

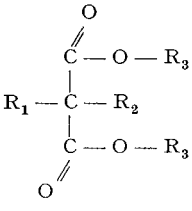
*Experimental*

*Materials.* 10 $\mu$g of each substance were applied on a thin-layer plate pre-coated by Woelm with Silica Gel F 254/366. After equilibration ascending chromatography was carried out at 20° in standard glass tanks. The solvent systems used were: (I) cyclo-hexane–ether–toluene (3:2:1); (II) benzene–ethyl acetate–ether (8:3:2); (III) benzene–methanol (5:1).

*Visualization.* The thin-layer plates were viewed under UV light at 254 m$\mu$. Not all substances quenched the fluorescence. Only diamides could be identified by this method. For this purpose the plate was put in an iodine chamber before examining it

TABLE I

COLOURS AND $R_F$ VALUES OF MALONIC ACID ESTERS



| $R_1$ | $R_2$ | $R_3$ | $R_F$ value in solvent system I | Colour of the spots |
|---|---|---|---|---|
| $-C_4H_9$ | $-H$ | $-C_2H_5$ | 0.53 | deep violet |
| $-C_2H_5$ | $-C_2H_5$ | $-C_2H_5$ | 0.54 | yellowish |
| $-C_2H_5$ | $-H$ | $-C_2H_5$ | 0.47 | deep violet |
| $-C_2H_5$ | $-C_4H_9$ | $-C_2H_5$ | 0.57 | yellowish |
| $-C_2H_5$ | $-C_2H_5$ | $-CH_3$ | 0.44 | yellowish |
| $-C_3H_7$ | $-H$ | $-C_2H_5$ | 0.46 | deep violet |
| $-CH_3$ | $-H$ | $-C_2H_5$ | 0.30 | deep violet |
| $-CH_2-CH=CH_2$ | $-CH_2-CH=CH_2$ | $-C_2H_5$ | 0.48 | yellowish |
| $-Br$ | $-C_4H_9$ | $-C_2H_5$ | 0.49 | yellowish |
| $-H$ | $-H$ | $-C_2H_5$ | 0.26 | deep violet |

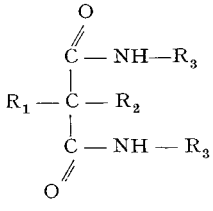
under UV light. For the mono- and disubstituted esters of malonic acid, a solution of Bromocresol Green/Bromophenol Blue and potassium permanganate[5] was tested as a spraying reagent. In this case, the spots were neither so defined nor was the colour so stable. Moreover, this spraying reagent was not sensitive enough. A better method of visualization was applied. The thin-layer plate was placed in an iodine tank for 1–2 min and then immediately sprayed with Bromocresol Purple[6]. The spots could be seen in normal light.

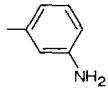
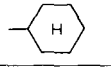
*Results*

With Bromocresol Purple, monosubstituted esters gave blue whereas the disubstituted esters showed yellowish blue spots. On the basis of these colours, one can differentiate between the mono- and disubstituted malonic acid esters. The results have been recorded in Tables I and II.

TABLE II

$R_F$ VALUES OF MALONIC ACID DIAMIDES



| $R_1$ | $R_2$ | $R_3$ | *Solvent system* | $R_F$ *value* |
|---|---|---|---|---|
| H | $-C_4H_9$ | $-C_6H_5$ | II | 0.15 |
| H | $-C_2H_5$ | $-C_6H_5$ | II | 0.45 |
| $-C_2H_5$ | $-C_4H_9$ |  | II | 0.61 |
| $-C_4H_9$ | –H |  | III | 0.60 |

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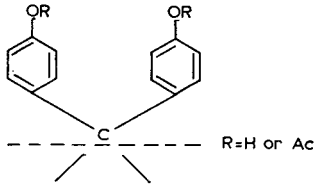
## Thin-layer chromatographic investigations of some synthetic compounds having laxative properties

In the past few years, synthetic laxatives have been more widely used in pharmacy than those derived from plants. (The reasons are too obvious to be discussed here.) 4,4'-Dihydroxydiphenylisatin or its acetyl derivatives, well-known for their laxative properties, and a few other related substances are studied herein. Quality control was necessary at various stages of production, and thin-layer chromatography (TLC) proved to be a very suitable method for this purpose. Because very few publications[1–4] have appeared in this field, a systematic investigation of such substances by TLC was considered worthwhile. In all these compounds, the dihydroxy- (or diacetoxy-)diphenyl group was a common feature, as shown below.




*Experimental*

Thin-layer plates approx. 0.25 mm thick were prepared manually[5] or using an applicator. The adsorbent used was Silica Gel GF Woelm TLC. The plates could be used after drying at room temperature for about 24 h. TLC plates of Woelm precoated with Silica Gel F 254/366 could be used with equal success.

All substances, except phenolphthalein, were synthesized. While thiazolyl derivatives (compounds 8–11) were prepared according to GEIGER *et al.*[6], 4,4'-dihydroxydiphenyl-pyrid-2-yl-methane and its diacetyl derivative were synthesized according to SEEGER AND KOTTLER[7].

The following solvent systems were employed: (I) chloroform–acetone (1:1); (II) chloroform–cyclohexane–ethyl methyl ketone (1:1:1); (III)chloroform–benzene–ethyl methyl ketone (1:1:1); (IV) carbon tetrachloride–cyclohexane–ethyl methyl ketone (1:1:1); (V) carbon tetrachloride–benzene–ethyl methyl ketone (1:1:1); (VI) chloroform–benzene–acetone (1:1:1).

The solvent front was allowed to advance 10 cm from the starting line. After development, the plates were dried and then viewed at 254 mμ under an UV lamp. The separated substances showed up as dark spots against a greenish fluorescent background. Table I shows the $R_F$ values of various substances on plates prepared manually.

*Discussion*

In these experiments, it was found that for compounds 1–7 and compounds 8–11 solvent systems VI and II, respectively, proved to be most suitable. The separations were equally good whether the thin-layer plates were prepared manually or using an applicator or whether TLC plates of Woelm precoated with Silica Gel

TABLE I

| *No.* | *Substances* | *Solvent systems* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *I* | *II* | *III* | *IV* | *V* | *VI* |
| 1 | Phenolphthalein | 0.80 | 0.39 | 0.61 | 0.28 | 0.64 | 0.57 |
| 2 | Diacetoxyphenolphthalein | 0.93 | 0.75 | 0.86 | 0.58 | 0.82 | 0.79 |
| 3 | 4,4′-Dihydroxydiphenyl-pyrid-2-yl-methane | 0.76 | 0.14 | 0.19 | 0.13 | 0.22 | 0.35 |
| 4 | 4,4′-Diacetoxydiphenyl-pyrid-2-yl-methane | 0.94 | 0.59 | 0.65 | 0.51 | 0.57 | 0.74 |
| 5 | 4,4′-Dihydroxydiphenylisatin | 0.66 | 0.15 | 0.20 | 0.10 | 0.27 | 0.29 |
| 6 | 4,4′-Diacetoxydiphenylisatin (Diphesatin) | 0.91 | 0.50 | 0.61 | 0.35 | 0.57 | 0.69 |
| 7 | 4,4′-Diacetoxydiphenyl-N-acetoxyisatin (Trisatin) | 0.94 | 0.83 | 0.92 | 0.74 | 0.88 | 0.86 |
| 8 | 4,4′-Diacetoxydiphenyl-4-methylthiazol-2-yl-methane | 0.84 | 0.65 | 0.79 | 0.49 | 0.72 | 0.85 |
| 9 | 4,4′-Dihydroxy-3,3′-dimethyldiphenyl-4,5-dimethylthiazol-2-yl-methane | 0.69 | 0.40 | 0.53 | 0.27 | 0.44 | 0.63 |
| 10 | 4,4′-Dihydroxydiphenyl-4-ethyl-5-methyl-thiazol-2-yl-methane | 0.66 | 0.35 | 0.49 | 0.23 | 0.42 | 0.59 |
| 11 | 4,4′-Dihydroxydiphenyl-4-methyl-5-ethyl-thiazol-2-yl-methane | 0.61 | 0.30 | 0.42 | 0.20 | 0.36 | 0.53 |

F 254/366 were used. $R_F$ values on plates prepared manually were slightly higher than on precoated plates or on those prepared using an applicator. Acetyl derivatives showed $R_F$ values greater than those of their corresponding hydroxyl compounds. The increasing number of acetyl groups on the molecule enhanced the corresponding $R_F$ values.

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CHROM. 4383

## A technique for improved thin-layer chromatography of phospholipids

Although many systems utilizing thin-layer chromatography are available for separations of phospholipids by thin-layer chromatography, it has been very difficult to get good separations of a complex mixture of phospholipids on a single plate[1,2]. The present communication details methodology for improved resolution of components of such mixtures without prior laborious column separations before application to thin-layer plates.

In 1963, HORROCKS[3] reported several modifications of the stationary phase which improved phospholipid separation on thin-layer chromatograms. One of these was the addition of 20 ml of a saturated solution of sodium borate to 40 ml of water which was mixed with 30 g of Silica Gel G and applied to the thin-layer plate. This modification of the stationary phase was reported by these workers to give improved separation of phospholipids with a one-dimensional system of chloroform–methanol–ammonium hydroxide (65:24:4). In addition, several two-dimensional systems for the separation of phospholipids with various proportions of chloroform–methanol–ammonium hydroxide are also available in the literature[1,4,5]. The modification proposed in this paper involves the use of a stationary phase of Silica Gel G buffered with borate buffer at pH 8. Thirty grams of Silica Gel G were suspended in 60 ml of borate buffer (0.02 *M* sodium tetraborate, 3 ml; 0.02 *M* boric acid, 100 ml) and were made fresh each time plates were prepared. After the plates had been spread and dried at room temperature for several hours, they were activated at 100° for 1 h. The plates then could be stored over dessicant until use. Before being used, the plates had to be reactivated at 100° for 1 h.

Two solvent systems were tested for the separation of phospholipids on these plates, *i.e.* a one-dimensional system consisting of chloroform–methanol–water (65:25:4) and a two-dimensional system previously described by SKIDMORE AND ENTENMAN[4]. For the first dimension a system consisting of chloroform–methanol–7 *N*

TABLE I

$R_F$ VALUES FOR TWO SYSTEMS FOR SEPARATION OF PHOSPHOLIPIDS

Adsorbent: SGG = Silica Gel G (E. Merck). SGG (borate) = Silica Gel G made up with borate buffer at pH 8. Solvent systems: I = Chloroform–methanol–7 *N* ammonium hydroxide, in the ratios (a) 60:35:5 and (b) 35:60:5. II = Chloroform–methanol–water (65:25:4).

| *Phospholipids* | *Two-dimensional system* | | | | *One-dimensional system* | |
|---|---|---|---|---|---|---|
| | *SGG* | | *SGG (borate)* | | *SGG* | *SGG (borate)* |
| | *Ia* | *Ib* | *Ia* | *Ib* | *II* | *II* |
| Cardiolipin | 0.91 | 0.94 | 0.91 | 0.95 | 0.92 | 0.91 |
| Phosphatidic acid | 0.77 | 0.71 | 0.73 | 0.71 | — | — |
| Phosphatidyl ethanolamine | 0.65 | 0.60 | 0.60 | 0.60 | 0.83 | 0.75 |
| Phosphatidyl serine | 0.20 | 0.47 | 0.14 | 0.15 | 0.40 | 0.35 |
| Phosphatidyl choline | 0.36 | 0.36 | 0.35 | 0.30 | 0.60 | 0.53 |
| Phosphatidyl inositol | 0.30 | 0.64 | 0.17 | 0.66 | 0.30 | 0.24 |
| Lysophosphatidyl choline | 0.14 | 0.09 | 0.09 | 0.09 | 0.23 | 0.13 |

ammonium hydroxide (60:35:5) was used, while for the second dimension the system was chloroform–methanol–7 *N* ammonium hydroxide (35:60:5). With both solvent systems the buffered borate plates gave improved resolution of the phospholipid with less streaking and diffusion. This improved resolution enables one to separate quantitatively up to 200 $\mu$g of each of seven phospholipids. Total phospholipids were detected by immersing the entire plate in a closed container with iodine crystals and iodine vapors. Ninhydrin spray was used to detect amino nitrogen groups[6], and acid molybdate for phosphate[7], anthrone and diphenylamine[6] for glycolipid and Chargaff's reagent for choline[6].

Table I shows the $R_F$ values (distance of center of spot from starting point divided by distance of solvent front from starting point) of seven phospholipids separated by the two-dimensional system and six phospholipids separated by the one-dimensional system. Figs. 1 and 2 show the comparison of thin-layer plates with the two-dimensional system only.

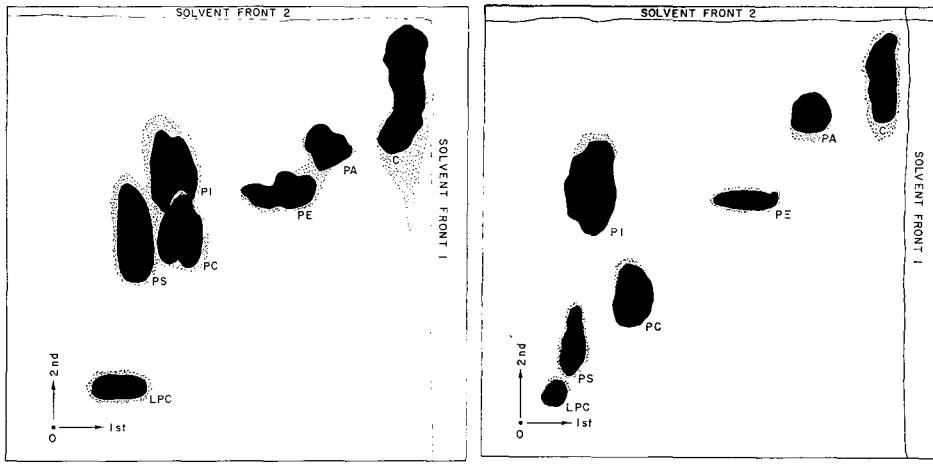


Fig. 1. Thin-layer chromatogram of phospholipid standards on Silica Gel G. The developing solvent in the first direction was chloroform–methanol–7 *N* ammonium hydroxide (60:35:5) and in the second, the ratio of the same solvent was 35:60:5. O = origin; LPC = lysophosphatidyl choline; PS = phosphatidyl serine; PC = phosphatidyl choline; PI = phosphatidyl inositol; PE = phosphatidyl ethanolamine; PA = phosphatidic acid; and C = cardiolipin.

Fig. 2. Thin-layer chromatogram of phospholipid standards on Silica Gel G in borate buffer at pH 8. The developing solvent in the first direction was chloroform–methanol–7 *N* ammonium hydroxide (60:35:5) and in the second, the ratio of the same solvents was 35:60:5. For abbreviations, see the legend to Fig. 1.

It may be seen from Table I that the plates buffered at pH 8 with borate buffer give better separation than those not buffered with both solvent systems for phosphatidyl serine, phosphatidyl inositol, and phosphatidyl choline. As shown on the tracings of the thin-layer plates (Figs. 1 and 2), much less diffusion of the spots occurs under conditions of borate buffering. This was confirmed by doing a recovery experiment. Samples of 200 $\mu$g of each phospholipid were applied to these plates and the phospholipids separated by the two-dimensional system. The spots were recovered

from the plates and analyzed for lipid[8] and phosphorus[9]. Calculation revealed that 95 % of the starting material was recovered.

An additional benefit of this method is that the complex mixture of phospholipids extracted from bacteria could be separated without the use of previous column separation and on a single plate. Other procedures required prior purification of the extract before adequate resolution could be obtained on the plates.

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CHROM. 4348

## Determination of inorganic radioiodide in $^{131}$I-labelled compounds by means of thin-layer chromatography

Organic compounds labelled with a radioiodide for use in medical diagnosis or therapy generally contain inorganic radioiodide as their main impurity. According to current regulations, the radioiodide content should never exceed 5 %.

Several techniques are used for the determination of inorganic radioiodide, *viz.* paper chromatography, paper electrophoresis, thin-layer chromatography, precipitation, thin-layer electrophoresis, etc. Several authors, pharmacopeias, Atomic Energy Commissions and commercial firms such as Squibb, Abbott, Amersham, Hoechst, etc. have published on this topic[1–10].

Using thin-layer chromatography[11–12], we have devised a method for separating radioiodide from organic molecules[13–16]. We have tried to reduce the development time of the chromatogram and looked for readily available solvents for use with most of these compounds; 1 *N* hydrochloric acid was found to be the most suitable.

Chromatograms were prepared by us on glass plates using Silica Gel G (Merck); the chromatogram was developed at ambient temperature and the substances localized by chemical and autoradiographic means. Quantitative determinations were made with a model 7200 Packard Radioscanner, which is capable of detecting 0.5 % radioiodide concentrations.

*Experimental*

Ascending chromatography was carried out on Silica Gel G (Merck), 250 $\mu$, using 1 $N$ HCl as the solvent. The development was 15 min. The $R_F$ value of the radioiodide was 0.98–1.00.

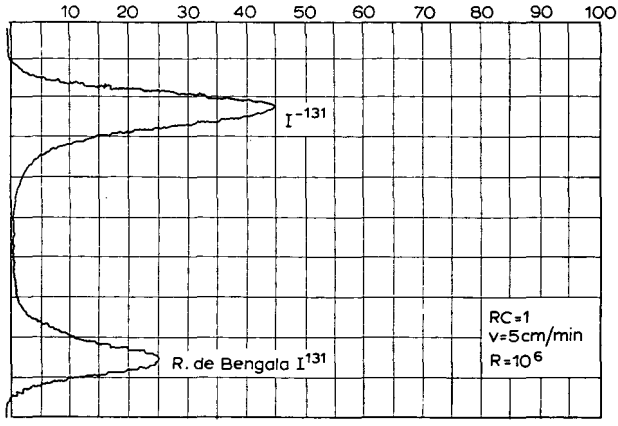
Table I lists the $^{131}$I-labelled organic compounds chromatographed with this solvent and the corresponding $R_F$ values.

Fig. 1 shows the clear separation of the labelled substances from the inorganic radioiodides.

TABLE I

$R_F$ VALUES OF $^{131}$I-LABELLED SUBSTANCES

| *No.* | *$^{131}$I-labelled substance* | *$R_F$ value* |
|---|---|---|
| 1 | *p*-Aminohippuric acid | 0.0 |
| 2 | Diphenylhydantoin | 0.0 |
| 3 | "Hypaque" sodium | 0.0 |
| 4 | Hippuran | 0.25 |
| 5 | "Urografin" | 0.0 |
| 6 | Albumin | 0.0 |
| 7 | Rose Bengal | 0.0 |
| 8 | "Biligrafin" | 0.0 |
| 9 | "Alilinulina" | 0.0 |
| 10 | Insulin | 0.0 |
| 11 | TSH | 0.0 |
| 12 | Sodium iodothalamate | 0.04 |
| 13 | "Diprocon" | 0.36 |
| 14 | Bromosulphalein | 0.0 |
| 15 | Iododeoxyuridine | 0.0 |
| 16 | Chlorambucil | 0.0 |
| 17 | Iodoform | 0.0 |
| 18 | $\gamma$-Globulin | 0.0 |
| 19 | Congo Red | 0.0 |
| 20 | "Cystokon" | 0.0 |
| 21 | "Telepaque" | 0.0 |
| 22 | Iodoantipirine | 0.0 |
| 23 | Fibrinogen | 0.0 |
| 24 | Bilirubin | 0.0 |
| 25 | Biliverdine | 0.0 |
| 26 | H.G.H. | 0.0 |




The method is considered suitable for rapid control procedures at the plant and in situations where it is not easy to obtain various chromatographically pure solvents.

*Note*

When the experiments are repeated under the same conditions but using glass fibre (ITLC), the displacement times are reduced from 15 min to 3 min for the same distance.

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CHROM. 4360

## Ion-exchange adsorption of radionuclides by ferrocyanide molybdate

A number of compounds containing ferrocyanide show selective adsorption of radionuclides, especially of $^{137}Cs$ (refs. 1–19) and some, therefore, have been used for selective adsorption of cesium from a mixture of radionuclides. BAETSLÉ *et al.* investigated the relationship between the Mo/Fe ratio in "ferrocyanide molybdate (FeMo)" and the mixing ratio of the starting materials. They clarified the crystallographic structure, and they also determined the ion-exchange behavior and some column characteristics of FeMo for several radionuclides[20–23].

In work on the preparation of zinc ferrocyanide, it was found that the composition and ion-exchange behavior of a ferrocyanide were greatly dependent on the mixing ratio of the starting materials. This tendency was also observed in the preparation of nickel or copper ferrocyanide. In order to confirm the existence of such correlations among the compounds containing ferrocyanide, we prepared three varieties of FeMo by varying the mixing ratio of the starting materials. One of them (No. 3) was found to be hardly soluble in water, permitting adsorption of radionuclides in aqueous media.

In the present paper the following studies are reported: (1) comparison of the solubility or adsorption behavior of three adsorbents, (2) relationship between the distribution coefficient of No. 3 and nitric acid concentrations, (3) variation of adsorption behavior of No. 3 with or without addition of concentrated salts, (4) adsorption behavior of several radionuclides for No. 3 in sea water, and (5) isolation of $^{137}Cs$ from sea water containing $^{85}Sr$, $^{144}Ce$, $^{137}Cs$, etc. through the column studies.

*Experimental*

*Preparation of adsorbent.* Three varieties of adsorbents were prepared by adding 0.2 *M* $Na_2MoO_4$–0.4 *M* HCl to 0.2 *M* $Na_4Fe(CN)_6$–0.4 *M* HCl in the mole ratios of 0.1,1, and 8 to 1, respectively. The resulting precipitates were numbered in series: adsorbent No. 1, No. 2 and No. 3. The adsorbents were ground, dry-sieved through a 200–300 mesh sieve, and used for batch equilibrium studies. Adsorbent No. 3 sieved through a 50–100 mesh sieve, was used for column studies. The adsorbents were analyzed by the method reported by BAETSLÉ *et al.*[21].

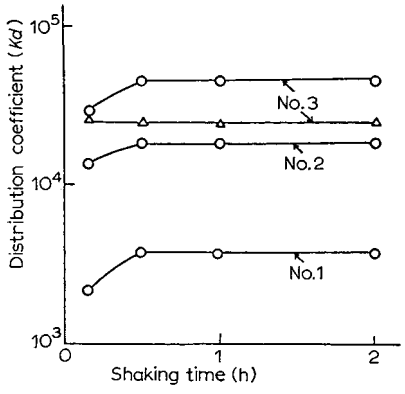
*Batch equilibrium studies.* Distribution coefficients (ml/g), $K_d$, of radionuclides were determined by batch equilibrium studies. 0.1 g of adsorbent was weighed in a glass-stoppered test tube to which 5 ml of a desired solution containing a radionuclide were added. After a given period of agitation at 25.0 ± 0.5°, the mixture was centrifuged at 8000 *g* for 1 min and the radioactivity in 2 ml of the supernatant was counted using a conventional well-type scintillation counter.

*Column studies.* Seven grams of adsorbent were slurried with about 70 ml of 0.1 *M* nitric acid and poured into a conventional glass column (I.D. 1 cm), pulled to a tip and plugged with glass wool at the outlet. The resultant bed was usually 10 cm high. Adsorption and elution were run at a flow rate of 5 ml/min.

All reagents used in the experiment were of reagent grade.

*Results and discussion*

*Comparison of three adsorbents.* The Mo/Fe ratio of adsorbent No. 3 was 3.4,



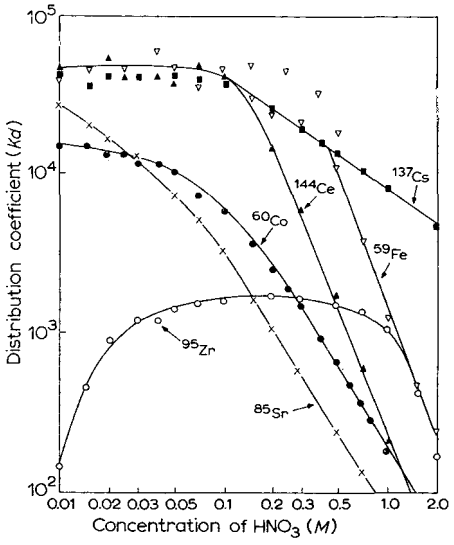




Fig. 1. Dependence of $K_d$ values on the shaking time. O—O, $K_d$ values in dioxane–water (1:1); △—△, $K_d$ values in water.

Fig. 2. Dependence of $K_d$ values for several radionuclides on the concentrations of nitric acid.

which is lower than that reported by BAETSLÉ *et al.*[21]. No. 1 and 2, which were water soluble, could not yield the reliable analytical values. No. 3 was hardly soluble in water. The solubilities of No. 1 and 2 were decreased by adding an equal volume of dioxane to the aqueous phase. The $K_d$ values increased when the shaking time was increased, with the equilibrium attained at 30 min (*cf.* Fig. 1). At equilibrium, the $K_d$ values decreased in the following order: No. 3 > No. 2 > No. 1. Since No. 3 was hardly soluble in water, it was used for the subsequent batch and the column studies. In an aqueous medium, the adsorption equilibrium for $^{137}Cs$ was attained at 10 min.

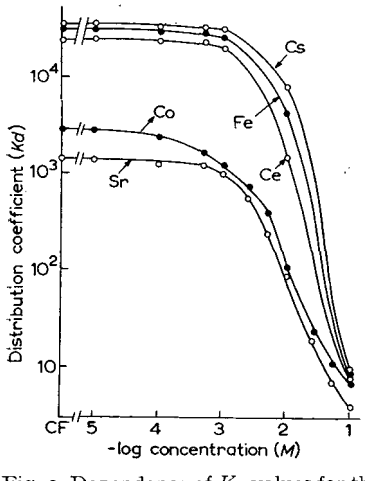



Fig. 3. Dependence of $K_d$ values for the radionuclides on their concentrations in the initial solution. CF = radionuclides without addition of the carriers.

Subsequent batch equilibrium studies were, therefore, carried out by shaking for 10 min.

*Distribution coefficients of No. 3 and nitric acid concentrations.* The dependence of the $K_d$ values for $^{137}Cs$, $^{59}Fe(III)$, $^{144}Ce(III)$, $^{60}Co$, $^{85}Sr$ and $^{95}Zr(IV)$ on the concentrations of nitric acid is shown in Fig. 2. The individual points are an average value of 4–6 determinations. The slope is −1 for Cs/H, −2 for Co(II)/H and Sr(II)/H, and −3 for Fe(III)/H and Ce(III)/H over the concentration range from around 0.1 to 2 *M* nitric acid. The observed slopes of $^{137}Cs$ and $^{85}Sr$ are almost consistent with those reported by HUYS AND BAETSLÉ[20]. The decrease in $K_d$ values for $^{95}Zr(IV)$ observed for the lower range of nitric acid concentration might be due to the hydrolysis in the solution. Fig. 3 shows the dependence of $K_d$ values for the radionuclides on their concentrations in the initial solution. The $K_d$ values for the radionuclides (carrier-free) remain almost unchanged over the concentration range not exceeding ion-exchange capacity, indicating that the observed $K_d$ values of the radionuclides are due to their own ion-exchange affinities. In the concentration range from around 0.1 to 2 *M* nitric acid, the dependence in Fig. 2 suggests that these radionuclides are adsorbed by an ion-exchange mechanism.

TABLE I

INFLUENCE OF THE ADDITION OF CONCENTRATED SALTS ON THE DISTRIBUTION COEFFICIENTS

| *Nuclide* | *Distribution coefficient* | | | |
|---|---|---|---|---|
| | *0.1 M* $HNO_3$ | *0.1 M* $HNO_3$ + *0.5 M* $NH_4NO_3$[a] | *0.1 M* $HNO_3$ + *0.5 M NaCl*[a] | $HNO_3$ + *sea water*[b] |
| $^{59}Fe$ | $3 \times 10^4$ | $7 \times 10^2$ | $2 \times 10^3$ | $1 \times 10^3$ |
| $^{60}Co$ | $5 \times 10^3$ | $2 \times 10^2$ | $4 \times 10^2$ | $1 \times 10^2$ |
| $^{65}Zn$ | $3 \times 10^4$ | $2 \times 10^3$ | $3 \times 10^3$ | $4 \times 10^3$ |
| $^{85}Sr$ | $2 \times 10^3$ | $< 1$ | 5 | 4 |
| $^{95}Zr$ | $1 \times 10^3$ | $2 \times 10^2$ | $5 \times 10^2$ | $7 \times 10$ |
| $^{106}RuNO$ | | | 7 | 5 |
| $^{144}Ce$ | $4 \times 10^4$ | $< 1$ | $2 \times 10$ | $1 \times 10$ |
| $^{137}Cs$ | $5 \times 10^4$ | $3 \times 10^2$ | $8 \times 10^3$ | $5 \times 10^3$ |

[a] Dissolution of $NH_4NO_3$ or NaCl in 0.1 *M* $HNO_3$ to make 0.5 *M* solutions.
[b] Addition of $HNO_3$ to sea water up to a molar concentration of 0.1.

*Distribution coefficients of No. 3 in concentrated salt solutions.* Table I shows the variation in $K_d$ values for several radionuclides on addition of concentrated salts to 0.1 *M* nitric acid. The $K_d$ values for $^{85}Sr$ or $^{144}Ce$ decrease markedly on addition of these salts to a much larger extent than expected from mutual exchange of ammonium or sodium ions. The reason for these decreases probably lies partly in the interaction of $^{85}Sr$ or $^{144}Ce$ and the concentrated salts. The extent of the decrease in $K_d$ values on addition of sodium chloride is less than that on addition of ammonium nitrate. Such a minor decrease in sodium chloride may be due to the fact that the ion-exchange affinity of the adsorbent for sodium ions is lower than that for ammonium ions.

In the case of sea water, $K_d$ values for $^{59}Fe$, $^{60}Co$ and $^{137}Cs$ are considerably higher than those for $^{85}Sr$, $^{106}RuNO$ and $^{144}Ce$. The $K_d$ values for these radionuclides in sea water are nearly equal to those in 0.5 *M* sodium chloride. The concentration of sodium chloride in sea water corresponds to about 0.5 *M* (ref. 24). In sea water,

the amount of sodium chloride is dominant over all the other components: magnesium, calcium, potassium, strontium, aluminum, organic materials, etc. The finding that the $K_d$ values for the radionuclides in sea water are nearly coincident with those in 0.5 $M$ sodium chloride may be explained by the fact that the radionuclides in extreme minority are governed by an ion-exchange property of the ferrocyanide molybdate without suffering any disturbance from the coexisting materials in large quantity. As stated above, it is noteworthy that the $K_d$ values for $^{85}Sr$ or $^{144}Ce$ decreased markedly on addition of the concentrated salts. Such a decrease could not be observed when using any other compounds containing ferrocyanide such as potassium, cobalt, nickel, zinc, sodium, or potassium zinc ferrocyanide. It may, therefore, be considered to be the characteristic adsorption behavior of the ferrocyanide molybdate.

*Isolation of $^{137}Cs$ from a mixture of radionuclides in sea water.* When 1 l of sea water spiked with $^{85}Sr$, $^{144}Ce$ and $^{137}Cs$ was passed through a column (1 × 10 cm), the spiked $^{137}Cs$ was quantitatively adsorbed by the column and a large portion of $^{85}Sr$ and $^{144}Ce$(90 %) passed through. The $^{85}Sr$ and $^{144}Ce$ retained were eluted with 50 ml of 1 $M$ ammonium nitrate–0.1 $M$ nitric acid solution. The adsorbed $^{137}Cs$ was eluted by 4 $M$ ammonium nitrate–4 $M$ nitric acid solution (Fig. 4). The maximum activity

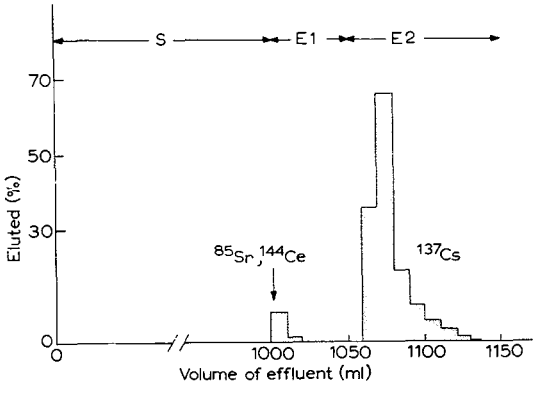



Fig. 4. Isolation of $^{137}Cs$ in sea water containing $^{85}Sr$, $^{144}Ce$ and $^{137}Cs$. S = sea water containing $^{85}Sr$, $^{144}Ce$ and $^{137}Cs$; E1 = 1 $M$ $NH_4NO_3$–0.1 $M$ $HNO_3$; E2 = 4 $M$ $NH_4NO_3$–4 $M$ $HNO_3$. Column, 1 × 10 cm, 50–100 mesh.

was obtained at 30 ml of the effluent. From the whole effluent of 100 ml, $^{137}Cs$ was recovered quantitatively. The $\gamma$-ray spectra characterization showed that the $^{137}Cs$ effluent was not contaminated by $^{85}Sr$ and $^{144}Ce$. $^{59}Fe$ and $^{60}Co$ were added to the above-mentioned sea water which was allowed to pass through the column, followed by the elution of $^{85}Sr$ and $^{144}Ce$ by the same procedure as mentioned above. $^{59}Fe$ and $^{60}Co$ along with $^{137}Cs$ adsorbed by the column were eluted with 4 $M$ ammonium nitrate–4 $M$ nitric acid solution. When these radionuclides were eluted immediately after adsorption, $^{137}Cs$ was recovered by about 100 %, $^{59}Fe$ by 60 % and $^{60}Co$ by 70 %. After standing for more than one day, recovery of $^{137}Cs$ remained the same, but a large portion of the adsorbed $^{59}Fe$ and $^{60}Co$ could not be eluted. Such poor recoveries may be ascribed to an isotopic exchange or an isotopic displacement occurring between the adsorbent and $^{59}Fe$ or $^{60}Co$. Behavior of $^{106}RuNO$ toward the column was similar

to that of $^{144}Ce$ or $^{85}Sr$, *i.e.*, the large portion was not adsorbed and the adsorbed fraction was capable of eluting with 1 *M* ammonium nitrate–0.1 *M* nitric acid solution. Behavior of $^{95}Zr$ in the form of zirconium fluoride was also identical to that of $^{144}Ce$, $^{85}Sr$ or $^{106}RuNO$, provided that it was applied in the form of zirconium fluoride.

The authors are indebted to Dr. MASAMI IZAWA for his invaluable advice and encouragement. We are also indebted to Mr. YASUSHI OAMI for his cooperation in carrying out this study.

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## Book Reviews

CHROM. 4323

Chromatography, edited by D. R. BROWNING, a volume in *Instrumental Methods Series*, McGraw-Hill, Maidenhead, 1969, 151 pp., price 32 s.

The editor has endeavoured, in about 150 pages, to provide an introduction to chromatography for students and practising chemists. Seven authors have contributed eight chapters, entitled principles of chromatography (11 pages), column chromatography (18), paper chromatography (16), thin-layer chromatography (28), gas chromatography (30), ion-exchange chromatography (16), zone electrophoresis (16) and gel permeation chromatography (9).

Most topics which are relevant to the practice of chromatography are included, though the depth of treatment varies considerably. The most disappointing omission from the book is any mention of the techniques and potential of the new high-speed high-efficiency column chromatography; space is, however, found for zone electrophoresis.

Most chapters give, in their different ways, a reasonable, elementary introduction to their subject. The chapter on thin-layer chromatography gives a lot of information about commercial sources of equipment and materials whereas the other chapters, to their detriment for a novice audience, give little.

The main impression the reviewer gained from this book was of a lack of editorial control. This shows up in the variations in treatment of the different topics noted above, the absence of uniformity over, for example, application of the different techniques (some chapters deal with this explicitly, others only incidentally). Compare too the detail of the TLC chapter with the brief, well-written but rather advanced discussion of gel permeation. Other minor irritations occur like the appearance of wrong initials for famous chromatographers in the references sections; why should the TLC chapter alone exclude author initials from its references?

Altogether, this is a disappointing book, badly edited and not particularly readable nor likely to induce enthusiasm in beginners.

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CHROM. 4266

*Electrophoresis* by D. J. SHAW, Academic Press, London and New York, 1969, 144 pp., price 35 s.

In the words of the author, "This book presents an introduction to the principles, methods and applications of the various forms of electrophoresis (micro-, moving boundary and zone) and a short account of the related phenomena of electro-osmosis and streaming potential". To the general reader the author has succeeded in his aim but the specialist and potential specialist will wish to peruse more comprehensive texts.

Interest for readers of this Journal is restricted mainly to the thirty-two pages devoted to zone electrophoresis, but despite such restricted coverage in terms of space, the chapter on the technique forms an excellent and reasonably up-to-date introductory essay. Users of zone electrophoresis will, by their wider interests, profit by perusal of much of the rest of the book, particularly with regard to those sections concerned with the application of microelectrophoresis to the study of cell surfaces and colloid stability.

For a book concerned with the wider view of electrophoresis, the section concerned with practical applications of particle electrophoretic studies is too restricted to be of value. With the technological importance of the fields mentioned, particularly electrophoretic deposition, the book would have been richer by the inclusion of a full chapter devoted to such applications.

The book is attractive in format with generous use of clear, easy to follow figures, and has adequate subject and author indexes.

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CHROM. 4384

*Purification of Inorganic and Organic Materials — Techniques of Fractional Solidification*, edited by MORRIS ZIEF, M. Dekker, New York, 1969, 318 pp., price $ 17.75.

Readers of this journal are likely to be specialists in the field of gas chromatography and will no doubt turn first to Chapter 6 on gas–liquid chromatography. If they feel, on reading it, that they have learned little, this is an indication of their own expertise and no reflection at all on the competence of ROY A. KELLER, who has produced an admirable monograph within his allotted space.

The book is not, of course, intended for the expert in gas chromatography, nor for that matter in any of several refined analytical techniques that form the subject of its first few chapters. It is, in fact, exactly what its title suggests, an account of fractional solidification techniques and these are exemplified by their application to a

dozen or so organic and inorganic compounds. Although these are substances of considerable intrinsic importance (they include, for example, anthracene, indium antimonide, alkali halides and benzoic acid) it is the chapters on general methods which are likely to be of the greatest utility.

The book is really an extension of *Fractional Solidification* by M. ZIEF AND W. WILCOX, which includes some basic theory on solidification processes and the monographs in the present volume contain only a minimum of mathematics. The purification methods described are mostly based on zone melting and three of them are primarily of interest for the novel heating methods employed (radio-frequency, electron beam and gas discharge). For the organic chemist, the chapters on the purification of aromatic amines and of dienes, and those on the purification of organic compounds (one of them on a kilogram scale) are of considerable value and the excellent account of the "Freezing Staircase" technique by C. P. SAYLOR deserves special mention.

In the introduction to Chapter 1, C. L. GRANT rightly points out that the demand for materials of high purity is necessarily accompanied by a need for methods of detecting and measuring impurities at very low levels and appropriately, the other chapters in the first part of the book deal with such methods. Most of them are spectrometric but they also include gas chromatography, differential thermal analysis and electrical resistance ratio measurement.

To the gas chromatographer the most stimulating chapter is probably that on the concentration of flavours at low temperatures. The technique consists in extracting the material under investigation into a solvent such as very pure benzene and concentrating the sample by re-using solvent in a pure crystalline form. The combination of this procedure with gas chromatography is obviously a powerful tool in the study of natural flavourings.

The book concludes appropriately with a chapter on the choice of containers for pure materials.

To sum up, this book is a useful source of practical information on the purification of fusible organic and inorganic solids and in conjunction with its predecessor should find a place in every laboratory concerned with the preparation of highly pure materials. The editor is to be congratulated on the high standard and clarity of his contributors and the book is well printed and produced.

*May and Baker Ltd., Dagenham (Great Britain)* G. A. P. TUEY

# News

## Meetings

IUPAC-SYMPOSIUM "CYCLOADDITION REACTIONS"

A IUPAC-Symposium on *Cycloaddition Reactions* will take place in Munich, Germany, September 7–10, 1970. The following plenary lecturers have accepted the invitation of the programme committee (R. GOMPPER, R. HUISGEN, J. SAUER):

P. D. BARTLETT, Harvard University (Cambridge, U.S.A.), *Some Borderline Cases in Cycloaddition.*

H. BESTIAN, Farbwerke Hoechst AG, (Frankfurt/M., G.F.R.), *Cycloadditionen mit Sulfonylisocyanaten.*

C. S. FOOTE, University of California (Los Angeles, U.S.A.), *Mechanisms of Addition of Singlet Oxygen to Olefins and Other Substrates.*

G. M. J. SCHMIDT, Weizman Institute (Rehovoth, Israel), *Photodimerisationen im festen Zustand.*

P. S. SKELL, Pennsylvania State University (University Park, Pa. U.S.A.), Topic follows.

N. J. TURRO, Columbia University (New York, U.S.A.) *Cycloaddition Reactions of Carbonyl Compounds Possessing High Energy Content.*

G. WILKE, Max-Planck-Institut für Kohleforschung (Mülheim/Ruhr, G.F.R.), *Cycloadditionen unter dem Einfluss von Übergangsmetallen.*

R. B. WOODWARD, Harvard University (Cambridge, Mass., U.S.A.), *Orbital Symmetry Correlations in Cycloadditions.*

Colleagues interested to submit a discussion paper or to take part in the Symposium should ask for further material until December 31, 1969 at Dr. W. FRITSCHE, Gesellschaft Deutscher Chemiker, 6000 Frankfurt/M., Postfach 119075 (G.F.R.).

ANALYTICA 70

*Internationale Fachausstellung für Biochemische Analyse—29. April bis 2. Mai 1970.*

Im Frühjahr 1968 fand erstmals in München eine Internationale Fachausstellung für biochemische Analyse—die ANALYTICA 68—statt. Etwas über 5000 Fachleute besuchten diese Fachausstellung, an der sich 90 Aussteller aus neun Ländern Europas beteiligten. Gleichzeitig fand eine internationale Tagung Biochemische Analytik statt, zu der 800 Teilnehmer aus 17 Ländern Europas und aus Übersee nach München kamen.

Auf Anregung der Aussteller, die zum weitaus überwiegenden Teil mit dem Ergebnis ihrer Beteiligung an der ANALYTICA 68 zufrieden waren, haben sich der Fachbeirat der ANALYTICA und die MMG für eine Wiederholung dieser Fachveran-

staltung im zweijährigen Turnus entschlossen. Die ANALYTICA 70 findet nunmehr vom 29. April bis 2. Mai 1970 statt. Gleichzeitig mit der ANALYTICA 70 wird wiederum eine wissenschaftliche Tagung unter dem Thema *Biochemische Analytik* durchgeführt, auf der international anerkannte Wissenschaftler sprechen werden. Neben Referaten und Praktika zu den einzelnen Themen, wie beispielsweise, Trennung von Zellkomponenten, Bedeutung und Messen von Ultrakurzzeitreaktionen in der Biochemie und Mikrospektrophotometrie, ist auch ein Symposium zum Thema *Analytische Probleme in der Pharmakokinetik* vorgesehen.

Der Rahmen der Biochemischen Analytik ist allerdings wesentlich weiter gefasst. Er reicht z.B. von der täglichen Arbeit im Krankenhauslabor über die Erforschung der Wirkung und des Stoffwechsels der Arzneimittel bis zur Arbeit an neuen Erkenntnissen über die biochemischen Grundlagen des Lebens. Alle dazu notwendigen Reagenzien und Geräte bis zu den Grossgeräten und Computern werden auf der Fachausstellung ANALYTICA 70 zu finden sein. Die Aussteller werden wie bei der vorhergehenden Veranstaltung im Jahre 1968 wieder an ihren Geräten mit den in der biochemischen Analytik tätigen Wissenschaftlern ausführliche Fachgespräche führen können.

Auskunft über die Fachausstellung ANALYTICA 70: Münchener Messe- und Ausstellungsgesellschaft mbH., 8 München 12, Theresienhöhe 13, Postfach 200, B.R.D. Telefon: (0811) 76711, Telex: 05-212086, TA: AMEG.

## Manufacturers' Literature

The Gas-Chrom Newsletter, May 1969, was received some little time ago but contains some pertinent information on the $BF_3$–propanol esterification kit, a simple hydrogenator, pretested OV-225 on Gas-Chrom Q, and amine analysis using Pennwalt 223. Mention is also made to the book published by Dr. C. W. GEHRKE and coworkers on amino acid analysis.

*Pesticide analysis*

An information bulletin *Analytical Liquid Chromatography, The Useful Approach to Pesticide Analysis*, by D. F. HORGAN, W. A. DARK AND K. J. BOMBAUGH has been published by Waters Associates. The paper describes the liquid chromatography separation techniques for a variety of pesticides. Detectability, resolution, and effect of sample load in residue analysis are also discussed.

*Polymer analysis*

The proceedings of the *5th and 6th International Gel Permeation Seminars* are now available. These volumes discuss the theory, practical applications and computer resolution of the gel permeation technique.

For further information apply to the publisher under reference No. Chrom. N-246.

CHROM. 4404

# ANALYSIS OF α-HYDROXY KETONES BY GAS CHROMATOGRAPHY

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(Received September 25th, 1969)

SUMMARY

α-Hydroxy ketones, acetoin and 3-hydroxy-2-pentanone have been isolated from an alcohol–water solution as their 2,4-dinitrophenylhydrazones, and the latter converted in acid solution to the corresponding vicinal diketones by water–steam distillation. Analysis of the diketones formed has been effected by head-space gas chromatography of the distillate by means of an electron-capture detector.

## INTRODUCTION

The closely related compounds, diacetyl (2,3-butanedione), acetoin (3-hydroxy-2-butanone), 2,3-butanediol and 2-aceto-2-hydroxylactic acid, appear in fermentation solutions, in addition to smaller amounts of their next higher homologues. Gas chromatographic methods have been developed for the analysis of diketones[1–3], glycols[4] and 2-aceto-2-hydroxycarboxylic acids[5] but gas chromatography has only occasionally been used for the analysis of α-hydroxy ketones[6].

The analysis of acetoin by gravimetric[7–9] and colorimetric[10] methods has been based upon the formation of coloured metallic salts of dimethylglyoxime after the oxidation of acetoin to diacetyl, and other colorimetric methods rely upon the colour reaction between diacetyl and creatine[11,12], or between diacetyl and chromotropic acid[13]. The estimated quantity of acetoin is the sum of the α-hydroxy ketones present.

A method is presented here for the analysis of acetoin and 3-hydroxy-2-pentanone, separately, by gas chromatography, after the isolation of these compounds from an alcohol–water solution as 2,4-dinitrophenylhydrazones.

## MATERIAL AND METHODS

### *Preparation of 2,4-dinitrophenylhydrazones of α-hydroxy ketones*

The 2,4-dinitrophenylhydrazone of acetoin was prepared both from the dimeric acetoin (research chemical, Aldrich Chemical Co., Inc., Milwaukee 10, Wisc., U.S.A.) and 2-acetolactic acid (synthesised by the KRAMPITZ's method[14]). In the former case, 100 mg of crystals of dimeric acetoin were first washed with diethyl ether and after drying, were dissolved in a small amount of water. For precipitation, 100 ml of 2,4-dinitrophenylhydrazine (analar, BDH Ltd., Poole, Great Britain) reagent solution (2.5 g of reagent/1000 ml 2 *N* HCl) were added. The solution was saturated with

$Na_2SO_4 \cdot 10\ H_2O$ (guaranteed reagent, E. Merck AG., Darmstadt, Germany) to minimise the solubility of the hydrazone; after the solution had been allowed to stand at 4° overnight, the precipitate formed was filtered, washed with water, and recrystallised twice from an alcohol–water (1:1) mixture (m.p. 107°).

For the preparation of the acetoin hydrazone from 2-acetolactic acid, the ester derivative of the latter, 2-aceto-2-acetoxypropionic acid ethyl ester, was synthesised by the KRAMPITZ's method[14]. A sample of 100 mg of the ester was dissolved in 14 ml of 0.1 *N* NaOH solution, and hydrolysed at 40° for 1 h. The liberated acetolactic acid was decarboxylated by means of 10 ml of 9 *M* $H_2SO_4$ solution, and the solution was kept at 40° for 2 h and then neutralised with solid $NaHCO_3$ (guaranteed reagent, E. Merck AG., Darmstadt, Germany). The precipitation and recrystallisation of 2,4-dinitrophenylhydrazone of acetoin were effected as described above (m.p. 106°).

The 2,4-dinitrophenylhydrazone of 3-hydroxy-2-pentanone was prepared from the ethyl ester of 2-aceto-2-acetoxybutyric acid synthesised by KRAMPITZ's method (*cf.* ref. 5); the same procedures as above then being employed, *viz.* hydrolysation, decarboxylation, precipitation and recrystallisation (m.p. 98°).

*Isolation of 2,4-dinitrophenylhydrazones of α-hydroxy ketones from diluted alcohol–water solution by adsorption*

In cases where the concentrations of α-hydroxy ketones are no more than some tens of milligrammes per litre or less, the isolation of their hydrazones by precipitation is difficult, because of the solubility of the α-hydroxy ketone hydrazones. The adsorption method[15] developed earlier, with some slight modification, can be used. A model solution of acetoin and 3-hydroxy-2-pentanone was prepared from the synthesised 2-aceto-2-hydroxycarboxylic acid derivatives mentioned above. For this purpose, $10^{-1}$ mmole (20.2 mg) of 2-aceto-2-acetoxypropionic acid ethyl ester, and $2.5 \cdot 10^{-2}$ mmole (5.4 mg) of 2-aceto-2-acetoxybutyric acid ethyl ester were dissolved in 4 ml of 0.1 *N* NaOH solution, and hydrolysed at 40° for 1 h. After acidification with 2.5 ml of 9 *M* $H_2SO_4$, the solution was kept at 40° for 2 h during which time, the acetohydroxy acids were decarboxylated. The volume of the solution was adjusted to 10 ml with water, and used as the base solution of α-hydroxy ketones. A sample of 1 ml of the base solution (containing $10^{-2}$ mmole, or 0.88 mg of acetoin and $2.5 \cdot 10^{-3}$ mmole, or 0.25 mg of 3-hydroxy-2-pentanone) was dissolved in 100 ml of 5 % ethanol–water mixture; in another parallel experiment 50 μl of 2,3-butanediol (puriss., Fluka AG., Buchs, Switzerland) was added to clarify its possible influence upon the analysis of α-hydroxy ketones. The solutions of 2,4-dinitrophenylhydrazine reagent and concentrated hydrochloric acid (guaranteed reagent, E. Merck AG., Darmstadt, Germany) were added in the proportions of 100 ml and 30 ml respectively. The solutions were mixed with a magnetic stirrer overnight at 4°. For precipitation of the excess of reagent present, the solutions were neutralised with solid $NaHCO_3$, and the precipitate formed was filtered off. For the isolation of α-hydroxy ketone hydrazones from the solutions, 3 g of activated carbon (Dargo Grade G-60, Atlas Chemical Industries, Inc., Wilmington, Del., U.S.A.) was added to the solutions and mixed with a magnetic stirrer for 1 h. Carbon was filtered in a glass filter crucible on a layer of 2–4 mm of Hyflo-Super-Cel (a Celite product, Johns-Manville, Lompoc, Calif., U.S.A.) and washed with 200 ml of 94.5 wt. % ethanol (pure, Rajamäki Factories of the Finnish State Alcohol Monopoly, Rajamäki, Finland), and 200 ml of water.

*Conversion of 2,4-dinitrophenylhydrazones of α-hydroxy ketones to diketones*

For conversion of the α-hydroxy ketone hydrazones adsorbed on the carbon to the corresponding diketones, the carbon was transferred into a micro steam-distillation apparatus and 4 ml of a mixture (1:1) of water and concentrated $H_2SO_4$ (guaranteed reagent, E. Merck AG., Darmstadt, Germany), and 100 mg of 2-oxoglutaric acid (for biochemistry, E. Merck AG., Darmstadt, Germany) were added. The mixture was distilled with water–steam until the amount of distillate was 100 ml. During the distillation, the α-hydroxy ketones were liberated and oxidised to the corresponding vicinal diketones; these were collected in the distillate.

Experiments to convert α-hydroxy ketone hydrazones to diketones were also made with pure (precipitated and recrystallised) 2,4-dinitrophenylhydrazones of acetoin and 3-hydroxy-2-pentanone. A solution of these hydrazones was made by dissolving $10^{-1}$ mmole (26.8 mg) of acetoin hydrazone and $2.5 \cdot 10^{-2}$ mmole (7.0 mg) of 3-hydroxy-2-pentanone hydrazone in 40 ml of a mixture (1:1) of concentrated sulphuric acid and water. A part of this solution (4 ml) was transferred into the micro steam-distillation apparatus mentioned above, and distilled with water–steam in the presence of 2-oxoglutaric acid (100 mg) until the amount of distillate was 100 ml.

*Analysis of the steam distillates*

During the distillation of a mixture of activated carbon and sulphuric acid solution, traces of $SO_2$ can be formed, therefore 200 μl of acetaldehyde (for synthesis, E. Merck AG., Darmstadt, Germany) was added to each distillate (100 ml) and mixed in thoroughly for the liberation of diketones (*cf.* ref. 3) in the distillate possibly converted to non-volatile sulphites. 24 ml vessels (Fig. 1) were filled to the top with the steam distillates. The flasks were provided with magnetic stirrers, and sealed with membranes of silicone rubber. Each flask was connected to an empty flask by an injection needle and a silicone rubber capillary tube, and each pair, filled and empty, was placed in a water bath at 40°. After the flask had stood for 15 min in the bath, 10 ml of air was injected into the filled flask (Fig. 1). The solution in the flasks was

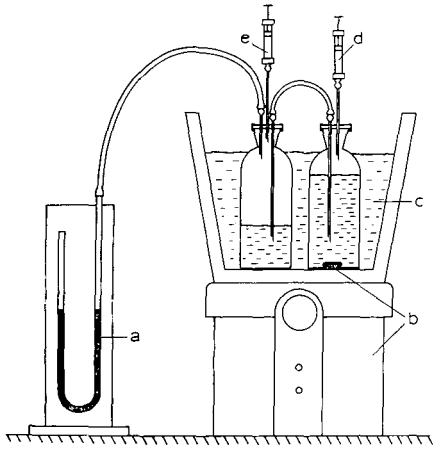


Fig. 1. Sampling system. a = Water manometer; b = magnetic stirrer; c = constant temperature bath; d = syringe for head-space sampling; e = syringe for pressure regulation.

stirred magnetically for another 15 min, and 2-ml aliquots of the gas phase in the head space of the sampling flask (Fig. 1) were taken. The samples were analysed gas chromatographically by a Perkin-Elmer F 11 apparatus. To preclude condensation, the sampling syringe was warmed up 40° before sampling.

*Conditions of analysis.* Electron capture detector, sensitivity for air peak 10 and for diketone peaks 5; column length 2 m (glass), internal diameter 3 mm, filling ma-

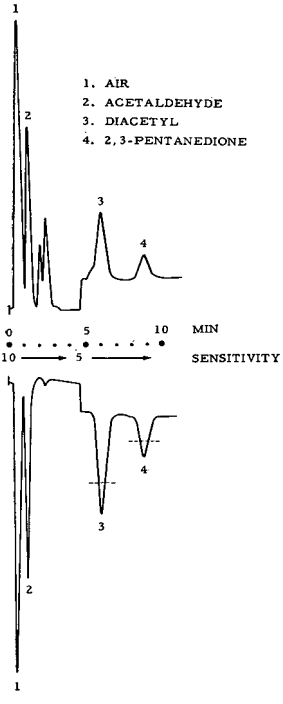


Fig. 2. Head-space gas chromatograms of the steam distillates of acetoin and 3-hydroxy-2-pentanone hydrazones adsorbed on carbon from the solution (upper) and of the equivalent reference mixture of the corresponding pure hydrazones (lower).

terial (two liquid phases) approx. 30 cm packing of Celite 545 + diglycerol (20 wt. %) and approx. 165 cm packing of Celite 545 + 1,2,3-tri(2-cyanoethoxy)propane (TCP) (20 wt. %); carrier gas, nitrogen (99.999 %), inlet pressure 0.7 kp/cm$^2$, flow rate 52 ml/min.

## RESULTS AND DISCUSSION

Fig. 2 reproduces two gas chromatograms. The upper one illustrates vicinal diketones produced during the steam distillation of α-hydroxy ketone hydrazones isolated from the solution by adsorption on carbon, and the lower the vicinal diketones produced during the steam distillation of pure recrystallised hydrazones of α-hydroxy ketones. If the adsorption of α-hydroxy ketone hydrazones from the solution on the carbon, their subsequent separation from the carbon, and the conversion to diketones

during the distillation had been complete, the corresponding diketone peaks in both chromatograms should have been equal in size. To facilitate comparison, the peak heights in the upper chromatogram are indicated by horizontal dotted lines crossing the corresponding peaks for the same components in the reference, lower chromatogram. It can be seen that the peak heights of the diketones in the upper chromatogram are about three quarters of those in the lower chromatogram, and consequently the isolation and conversion procedures are not quite quantitative; nevertheless the method gives reproducible results, and the peak heights change in relation to the change in concentration of α-hydroxy ketones. The two extra peaks, between the peaks of acetaldehyde and diacetyl in the upper chromatogram, are produced by influence of activated carbon upon the sulphuric acid during the distillation.

2,3-Butanediol in the solution studied did not interfere with determination of the α-hydroxy ketones; the chromatogram was identical with that illustrated in the upper chromatogram in Fig. 2.

Preliminary experiments have indicated that the method is suitable for the study of fermentation solutions and beverages.

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# APPLICATION OF A COLOUR REACTION FOR THE IDENTIFICATION OF METHACRYLATE MONOMER AND POLYMER

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SUMMARY

The colour reaction described produces conclusive positive and negative results for the methacrylate and acrylate esters, respectively. However, the positive result is not restricted to the methacrylate esters but is applicable to other compounds examined, *i.e.* esters, amides, aromatic compounds, olefins, containing the $CH_2=$ $=C(CH_3)-$ structure or the $-CH=C(CH_3)-$ structure, where a conjugated situation with another double bond occurs.

The colour reaction is readily applied as a gas chromatographic effluent test but the limits of detection of the effluent tests are considerably greater than those of an acceptable spot test and identification of minor compounds on pyrolysis is not possible with this or other effluent tests.

## INTRODUCTION

A colour reaction to distinguish polymers of methyl methacrylate from polyacrylates has been briefly noted by MANO[1]. The test as described requires the depolymerisation of approximately 0.5 g of polymer in a tube protected against loss of the volatile degradation products. The monomer was diluted with several volumes of concentrated sulphuric acid and gently heated to obtain a pale yellow coloured solution. To the cooled solution was added half its volume of water and then zinc dust. A blue colour immediately develops if methacrylates are present. This colour can be layered to chloroform, the yellow colour not being extracted. When sodium nitrite replaces zinc the same colour is obtained.

This test has been used with some success with polymers of *n*-alkyl methacrylates which depolymerise substantially to monomer although some anomalous behaviour has been observed, while attempts at using the reaction as a gas chromatographic elution procedure have been less successful.

In this paper the general applicability of this reaction with a variety of unsaturated compounds experienced in polymers has been examined while the limits of detection for use on an effluent test procedure in gas chromatography have been determined.

## EXPERIMENTAL

The reaction has been conducted essentially as described by MANO. Pyrolysis was carried out carefully in a long test tube (6 × 3/8 in.) which acts as an air condenser such that possible absorption on filter paper was avoided.

The test was carried out in 2 × 1/8 in. micro test tubes using one drop of pyrolysis product and two drops of concentrated nitric acid. Heating was rarely required, a yellow-brown colouration normally being obtained on mixing. Zinc dust was replaced by a crystal of sodium nitrite, which was added to the acidic solution. In many cases a pale green or blue colouration was obtained and layering in two drops of added chloroform was essential to ensure that a positive test was obtained.

Gas chromatography was carried out using an F & M Model 810 R-19-29 Gas Chromatograph employing simultaneous thermal conductivity and flame ionisation detection equipped with a Leeds and Northrup twin-channel 0–1 mV Speedomax Recorder. Pyrolysis was carried out using a Philips Curie Point Pyrolyser. The following operating conditions were used:

Columns: matched each 10 ft. × 1/4 in. O.D. aluminium packed with 10% methyl silicone polymer SE-30 on 60–80 mesh acid-washed Celite 560.

Injection temperature: 200°.

Carrier gas: helium, inlet pressure 40 p.s.i., flow rate 60 ml/min.

Thermal conductivity detector: maintained at 200° with a bridge current of 200 mA.

Column temperature: isothermal operation at 100°.

## RESULTS AND DISCUSSION

While only the *n*-alkyl methacrylate polymers depolymerise to yield substantial yields of monomer the majority of polymers do produce some detectable monomer on degradation of such a large-sized sample. As degradation is necessary the principal studies have been concerned with monomers; many of the commercially available acrylic esters have been examined and the reactions observed are shown in Table I. The reactions of a selection of unsaturated compounds which have structures similar to that of the methacrylate esters are included in Table I.

A strong positive test was observed with all of the methacrylate esters, while conclusive negative results were observed with the acrylate esters and with other monomers not possessing a methyl group on the α-carbon atom.

Several other compounds containing the structure $CH_2=C(CH_3)-$ were examined and each produced a positive result, *i.e.* methacrylamide, α-methylstyrene, isopropenyl acetate, isobutylene, diisobutylene and 2-methyl-1-pentene.

Saturated derivatives of the methacrylate esters, the isobutyrates, *i.e.* methyl isobutyrate, *n*-butyl isobutyrate and isobutyric acid, yield respectively a green colour not extracted by chloroform, a completely negative yellow solution and a blue colour not extracted by chloroform.

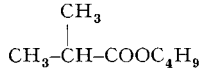
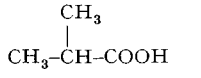
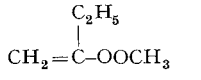
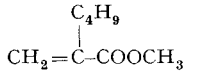
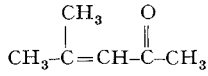
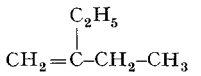
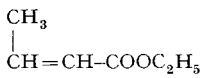
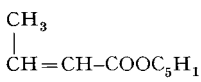
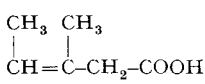
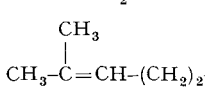
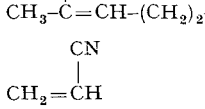
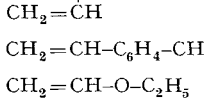
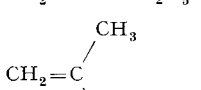
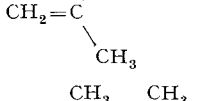
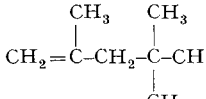
Replacement of the α-methyl group of the methacrylate ester by a chain, *i.e.* methyl α-ethylacrylate and methyl α-*n*-butylacrylate resulted in a negative test as did an olefin of similar structure, *i.e.* 2-ethyl-1-butene which produced a blue colour that was not extractable.

TABLE I

COLOUR REACTIONS OF UNSATURATED COMPOUNDS

| *Compound* | *Structure* | *Colour observed with sodium nitrate* | *Extracted colour* | *Result* |
|---|---|---|---|---|
| Acrylate[-] | $CH_2=CH-COOR^a$ | | | |
| Methyl ester | | yellow | yellow | neg. |
| Ethyl ester | | yellow | yellow | neg. |
| Isopropyl ester | | yellow | yellow | neg. |
| *n*-Propyl ester | | yellow | yellow | neg. |
| *n*-Butyl ester | | yellow | yellow | neg. |
| Isobutyl ester | | yellow | yellow | neg. |
| 2-Ethylhexyl ester | | yellow | yellow | neg. |
| $\beta$-Ethoxyethyl ester | | yellow | yellow | neg. |
| Glycidyl ester | | yellow | yellow | neg. |
| 2-Cyanoethyl ester | | yellow | yellow | neg. |
| Acrylic acid | | yellow | yellow | neg. |
| Methacrylates | $CH_2=C(CH_3)-COOR^a$ | | | |
| Methyl ester | | blue | blue | pos. |
| Ethyl ester | | blue | blue | pos. |
| *n*-Propyl ester | | blue | blue | pos. |
| *n*-Butyl ester | | blue | blue | pos. |
| Isobutyl ester | | green | blue | pos. |
| *n*-Pentyl ester | | blue | blue | pos. |
| *n*-Hexyl ester | | blue | blue | pos. |
| *n*-Nonyl ester | | blue | blue | pos. |
| Decyl ester | | blue | blue | pos. |
| Allyl ester | | blue | blue | pos. |
| Hydroxyethyl ester | | blue | blue | pos. |
| Hydroxypropyl ester | | blue | blue | pos. |
| Methacrylic acid | | blue | blue (fades) | pos. |
| Miscellaneous | | | | |
| Acrylamide | $CH_2=CH-CONH_2$ | yellow | yellow | neg. |
| Methacrylamide | $CH_2=C(CH_3)-CONH_2$ | blue | blue | pos. |
| Styrene | $C_6H_5-CH=CH_2$ | yellow | yellow | neg. |
| $\alpha$-Methylstyrene | $C_6H_5-C(CH_3)=CH_2$ | green | blue | pos. |
| Vinyl acetate | $CH_3COO-CH=CH_2$ | yellow | yellow | neg. |
| Isopropenyl acetate | $CH_3COO-C(CH_3)=CH_2$ | green | blue-green | pos. |
| 2-Methyl-1-pentene | $CH_2=C(CH_3)-(CH_2)_2-CH_3$ | green | blue | pos. |
| Methyl isobutyrate | $CH_3-CH(CH_3)-COOCH_3$ | green | yellow | neg. |

TABLE I (*continued*)

| *Compound* | *Structure* | *Colour observed with sodium nitrate* | *Extracted colour* | *Result* |
|---|---|---|---|---|
| *n*-Butyl isobutyrate |  | yellow | yellow | neg. |
| Isobutyric acid |  | blue | yellow | neg. |
| Methyl α-ethylacrylate |  | yellow | yellow | neg. |
| Methyl α-butylacrylate |  | yellow | yellow | neg. |
| 4-Methyl-pent-3-en-2-one |  | blue | blue | pos. |
| 2-Ethyl-1-butene |  | blue | yellow | neg. |
| Ethyl crotonate |  | yellow | yellow | neg. |
| Isoamyl crotonate |  | green | yellow | neg. |
| 3-Methyl-2-butenoic acid |  | | | |
| 6-Methyl-hept-5-en-2-one |  | yellow | yellow | neg. |
| Acrylonitrile |  | yellow | yellow | neg. |
| Divinyl benzene |  | yellow | yellow | neg. |
| Ethyl vinyl ether |  | yellow | yellow | neg. |
| Isobutylene |  | green | green | pos. |
| Diisobutylene |  | blue | green | pos. |

[a] Appropriate substituent.

With the methyl group on the $\beta$-carbon atom, *i.e.* ethyl and isoamyl crotonate, yellow and green solutions were obtained but the colours were not transferred into chloroform. With a methyl group on each side of the double bond, *i.e.* 3-methyl-2-butenoic acid a green colour was observed but was not transferred into chloroform. 6-Methyl-hept-5-en-2-one with two methyl groups on the chain ends results in a yellow solution while with 4-methyl-pent-3-en-2-one a positive result was obtained.

The results indicate that a positive reaction is not restricted to the methacrylate esters but also can be obtained with the other compounds examined that contain the $CH_2=C(CH_3)-$ structure, *i.e.* ester, amide, aromatic compound and olefin. The possible anomaly with 4-methyl-pent-3-en-2-one may be explained by the presence of a conjugated system between the olefinic double bond and the carbonyl group, while with the two similar compounds, *i.e.* 3-methyl-2-butenoic acid and 6-methyl-hept-5-en-2-one, one and two methylene groups separate the two double bonds. The crotonates are similar in structure to the anomalous compound in that a conjugated system occurs but these produce negative results. The $\alpha$-carbon atom of the olefinic double bond with the crotonates is not completely substituted and is apparently of importance as all of the compound examined that produce positive results are of this structure.

The limit of detection as an effluent test was determined firstly by injection of methyl methacrylate monomer into the chromatograph. The effluent collector consisted of an 1/8 in. Swagelok nut screwed on to the exit of the thermal conductivity detector and fitted with a silicone rubber septum through which passed a glass capillary tube. Capillaries made from syringe needles of electroplated steel were unsuitable as minor reaction with the nitric acid reagent produced a green colour. Using the micro tubes as receivers a good positive test was obtained with 0.25 $\mu$l while an inconclusive result was obtained with 0.20 $\mu$l. Pyrolysis of samples of polymethyl methacrylate and poly-*n*-butyl methacrylate equivalent to 0.25 $\mu$l, *i.e.* 250 $\mu$g, similarly produced a satisfactory test. To achieve reproducible pyrolysis chromatograms small sample size is essential. The use of approximately 10 $\mu$g of acrylic polymers deposited on the ferromagnetic wire by evaporation of a solution has been shown to be very satisfactory[2] and it is apparent that application of the effluent test with routine pyrolysis with these quantities of sample is not possible. Examination of a larger sample of the pyrolysis residue may be successfully conducted.

Effluent colour reactions for detection of the principal types of compounds have been reported by WALSH AND MERRITT[3]. The sensitivity, *i.e.* minimum detectable amount, varied between 20 and 100 $\mu$g and it is apparent that while effluent tests are often of considerable value, their sensitivity does not approach that of the classical spot tests, where a limit of 5 $\mu$g is expected.

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CHROM. 4399

# AUTOMATION IN DER GASCHROMATOGRAPHIE

# ENTWICKLUNG EINES GERÄTS ZUR AUTOMATISCHEN GASCHROMATOGRAPHISCHEN BESTIMMUNG VON HERBIZID- UND INSEKTIZID-RÜCKSTÄNDEN UND -FORMULIERUNGEN

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(Eingegangen am 25. September 1969)

SUMMARY

*Automation in gas chromatography. Development of an apparatus for the automatic gas chromatographic determination of herbicide and insecticide residues and formulations*

A new all-glass system for the automated transport of samples into a gas chromatograph is described. It is especially suitable for the determination of thermally unstable substances and consists of a heated evaporation chamber directly connected to the gas chromatographic column. Samples are fed into the system through a horizontally attached side arm. Microliter quantities of sample solutions in hexane or ethyl ether are applied to glass capillary tubes 7 mm in length and fed into the side arm after evaporation of solvent. About 70 samples can be transported discontinuously into the evaporation chamber by pushing a metal pin which moves with an outside magnet. Samples of formulations and residues of triazin-herbicides and halogenated hydrocarbons have been analyzed automatically using several specific gas chromatographic detectors.

## EINLEITUNG

Die Gaschromatographie mit extrem empfindlichen, molekülspezifischen Detektoren wie Mikrocoulometer[1], Coulson Leitfähigkeitsdetektor[2,3] und Phosphordetektor[4] ist heute die wichtigste Methode zur Bestimmung von Herbizid- und Insektizid-Rückständen. Auch die Reinheitsbestimmung von Wirksubstanzen sowie die Gehaltskontrolle von Formulierungen erfolgt überwiegend gaschromatographisch unter Anwendung von Flammionisations- und Wärmeleitfähigkeitsdetektor.

Für den Routinebetrieb müssen die Geräte möglichst rationell, am besten sogar während der Nacht eingesetzt werden, was eine automatische Eingabe der Analysenmuster voraussetzt. Die kommerziell erhältlichen automatischen Injektion-Systeme sowie die von CHAMBERLAIN *et al.*[5] und MENINI UND NORYMBERSKI[6] zur Steroidbestimmung verwendeten Geräte haben den Nachteil, dass die zu bestimmenden Sub-

stanzen, auf feine Drahtnetz-Zylinder aufgezogen, in den Einspritzblock gelangen. Thermisch bzw. gegen Metalle empfindliche Substanzen erleiden dabei erhebliche Zersetzung. Ausserdem sind die für den automatischen Betrieb umgebauten Geräte nicht mehr oder nur schlecht für manuelle Einspritzung verwendbar. Wir haben deshalb ein Allglas-System zur automatischen Eingabe von Analysenmustern in einen Gaschromatographen entwickelt, bei dem die zu bestimmenden Substanzen erst im Detektor mit Metall in Berührung kommen und bei dem eine wartungsfreie Analyse während der Nacht möglich ist. Bei nichtautomatischem Betrieb kann weiterhin manuell eingespritzt werden.

## APPARATIVES

### *Geräte*

Tabelle I enthält die Geräte zur Gaschromatographie.

TABELLE I

GERÄTE ZUR GASCHROMATOGRAPHIE

| | |
|---|---|
| Gaschromatographen | Doppelkolonnen-Instrumente Aerograph Modell 1700 |
| Detektoren | Aerograph Flammionisationsdetektor |
| | Dohrmann Verbrennungsofen mit T 300 Microcoulometer-Titrationszelle und C 200 Coulometer |
| | Coulson Leitfähigkeitsdetektor Modell 90-80-60 mit CIC Pyrolyzer Modell 121 |
| | CIC Leitfähigkeitsdetektor-Zelle Modell 80 |
| | CIC Wechselstrombrücke Modell 90 |
| | CIC Wasseraufbereitungssystem Modell 60 |
| | Quarz-Verbrennungsrohr mit Nickel- und Strontiumhydroxyd-Füllung |
| Säulen | Glas, Durchmesser 1/4 in., Länge 1 m |
| Kugelschliffe | Pyrex-Glas, KS 13/5, Hochglanz, bis 5 atü dicht |
| Säulen-Füllungen | 0.5% XE-60 auf Chromosorb G, 60/80 mesh |
| | 2% AFL auf Chromosorb G, 60/80 mesh |
| Schreiber | Honeywell 1 mV |
| Integrator | Aerograph Digital Integrator Modell 480 |
| Schaltuhren | Schleicher microlais 0–60 min |
| | Schleicher microlais 0–60 sec |
| Temperatur-Regler | Jumo |
| Antriebsmotoren | Escap 20 AR 601 |

### *Apparatur zur Probeneingabe*

Das System zur automatischen Probeneinführung (Fig. 1 und 2) besteht aus einem 20 cm langen, vertikal an der Frontplatte eines Gaschromatographen befestigten Verdampfungsraum (1) (Pyrex-Glasrohr, i.D. 15 mm). Dieser wird aussen durch einen Eisen–Konstantan-Wiederstand (2) geheizt und über ein Thermoelement (3) mit Temperaturregler konstant auf der Temperatur des Einspritzblocks (10) gehalten. Zur Temperaturkontrolle wird ein zweites Thermoelement (7) direkt auf die freie "accessory"-Position der Temperaturskala des Geräts geschaltet. Der Verdampfungs-

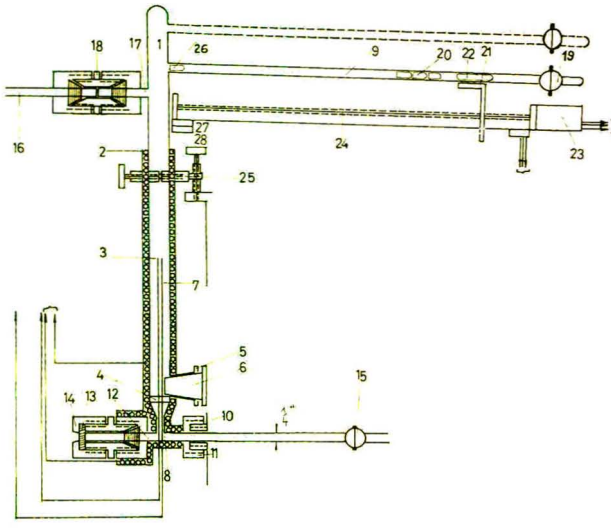



Fig. 1. Apparat zur automatischen Eingabe von Analysenmustern in den Gaschromatographen. 1 = Verdampfungsraum; 2, 3, 7 = Heizung und Temperaturregler; 4 = Glasfritte; 5, 6 = Schlifföffnung mit Teflon-Stopfen; 8, 12 = geheiztes Glas-T-Stück; 9 = Glasrohr zur Probeneingabe; 10 = Einspritzblock; 11, 14, 18 = Befestigungsschrauben; 13 = Septum; 15, 19 = Hochglanz-Kugelschliffe; 16, 17 = Zuleitungsrohr für Trägergas; 20, 26 = Glasgefässe zur Aufnahme der Proben; 21 = Magnet; 22 = Metallstift; 23 = Transportmotor; 24 = Schraubengewinde; 25 = Halterung; 27, 28 = End- und Start-Schalter.

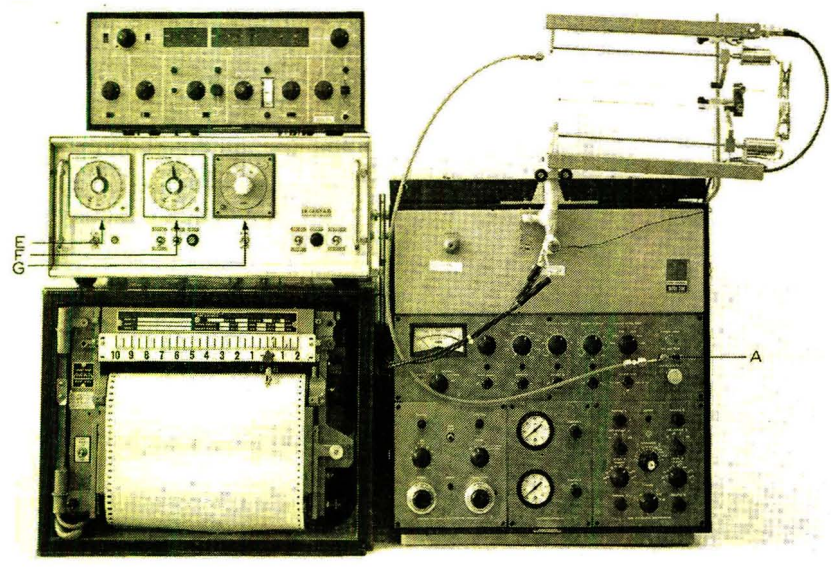



Fig. 2. Gesamtansicht der Apparatur zur automatischen Gaschromatographie. A = Gas accessory loop; E = Schaltuhr 0–60 min; F = Schaltuhr 0–60 sec; G = Temperaturregler für Verdampfungsraum.

raum verengt sich unten auf einen äusseren Durchmesser von 1/4 in. und endet in einem waagrecht angeschmolzenen T-Stück (i.D. 1/16 in., ä.D. 1/4 in.). Der längere Arm des T-Stückes (8) wird von aussen durch den auf 6.5 mm aufgebohrten Einspritzblock des Gaschromatographen hindurchgeschoben, mit einem nachträglich angeschmolzenen Kugelschliff (15) direkt auf die Säule aufgesetzt und mit einer Imperialschraube (11) befestigt. Der 3 cm lange, aus dem Einspritzblock herausragende Teil des T-Stücks (12) wird mit einer 1/4 in. Swagelokschraube (14) und einem Teflonkegel abgedichtet und durch ein Septum (13) verschlossen. Durch dieses können Proben manuell eingespritzt werden.

Am oberen Ende des Verdampfungsraumes ist seitlich ein 2 cm langes Glasrohr (17) (Pyrex, 1/4 in. a.D.) angeschmolzen. Es ist über eine Swagelok-Kupplung (18) und zwei Teflonkegel mit einem Nylonschlauch (16) verbunden. Die normalerweise im Injektorblock mündende Trägergaszuleitung wird vorher am sog. "Gas accessory loop" des Gaschromatographen (Fig. 2, A) abgezweigt und über den Nylonschlauch (16) in den Verdampferraum (1) geleitet. Ein zweites, 30 cm langes, horizontales Glasrohr (9) (3.5 mm i.D.) dient zur Aufnahme der Analysenproben-Behälter (20, 26). Diese bestehen aus genau 7 mm langen, einseitig zugeschmolzenen Glasröhrchen von 2 mm a.D.

5 $\mu$l-Aliquote der Analysenmuster in Hexan-Lösung werden jeweils mit Hilfe einer Hamilton Mikrospritze in die Röhrchen gebracht. Nach dem Verdunsten des Lösungsmittels können diese (insgesamt *ca.* 35 Stück) durch den Kugelschliff (19) eingebracht werden. Nach den Proben wird zuletzt ein glasüberzogener Metallstift (22) eingeführt. Es ist wichtig, dass der Schliff (19) mit einer Schraubklemme fest angezogen wird.

*Transport- und Steuereinrichtung*

Eine einfache, auf einem Stativ am GC montierte Vorrichtung sorgt für den automatischen Transport der Proberöhrchen (20) in die Verdampfungskammer (1). Parallel zu dem die Proben enthaltenden Glasrohr (9) bewegt sich über ein Gewinde (24) ein kleiner Magnet (21) angetrieben von dem Elektromotor (23). Bei der über die Schaltuhren E und F (Fig. 2 und 3) regulierbaren Bewegung zieht der Magnet den Metallstift im Glas mit sich und dieser schiebt dabei die Proberöhrchen vor sich her, so dass nach jeder Fortbewegung des Magneten um 7 mm ein Röhrchen auf die Glasfritte (4, Fig. 1) der Verdampfungskammer fällt. Die Substanz verdampft, wird vom Trägergas in die Säule gespült und von einem der genannten Detektoren registriert. Die Detektorsignale werden vom Schreiber aufgezeichnet und vom Integrator in "integral count"-Einheiten zusammen mit den Retentionszeiten des entspr. Peaks ausgedruckt.

Durch Anschmelzen eines zweiten, horizontalen Glasrohres analog (9) und Montage einer zweiten Transportvorrichtung analog (23, 24) kann die Kapazität des Systems auf 70 Analysenmuster pro Nacht verdoppelt werden. Nachdem der Magnet (21) die ganze Länge des Gewindes durchlaufen hat wird gleichzeitig mit dem Endschalter (27) für den ersten Motor der Startschalter (28) für den zweiten Motor betätigt, der den Probentransport im oberen Glasrohr besorgt.

Für den automatischen Nachtbetrieb der Apparatur werden normalerweise folgende Bedingungen gewählt: Im Abstand von 20 min gibt die Schaltuhr E (Fig. 2 und 3) je einen Startimpuls an Drucker und Null-Reset des Integrators, an die Schaltuhr F (Fig. 2 und 3) und an einen Motor (23, Fig. 1; M, Fig. 3). Der

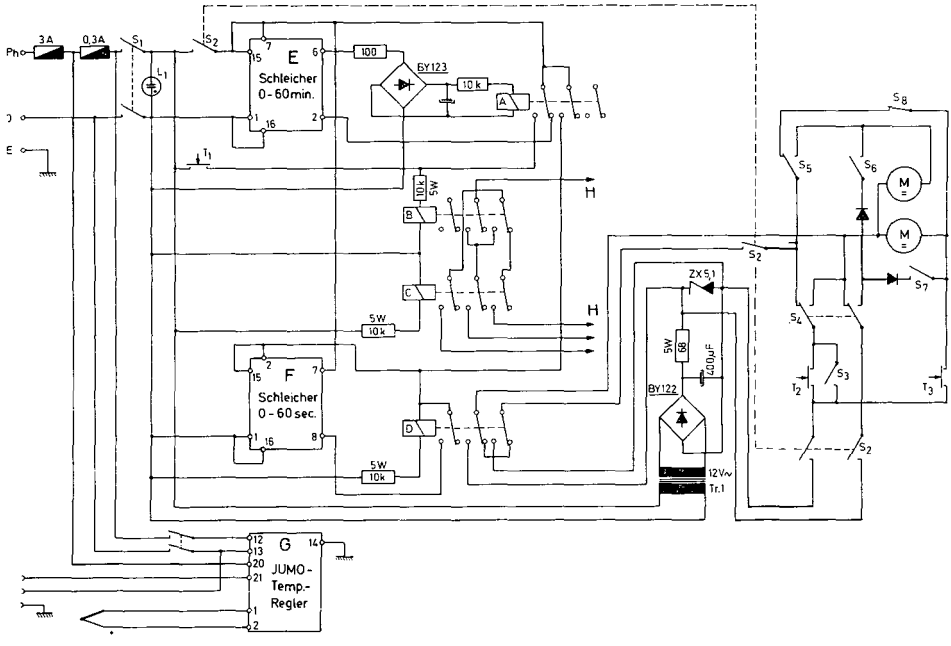



Fig. 3. Transport- und Steuer-Einheit. E = Schaltuhr 0–60 min; F = Schaltuhr 0–60 sec; G = Temperaturregler für Verdampfungsraum; H = Leitungen zum Integrator; M. = Transportmotoren.

Motor beginnt zu laufen und bewegt innerhalb von 28 sec (diese Zeit wurde empirisch ermittelt) den Magneten (21, Fig. 1) um 7 mm vorwärts, so dass das vorderste Proberöhrchen (26, Fig. 1) in den Verdampfungsraum fällt. Nach 28 sec schaltet die Uhr F den Motor wieder ab. Das Zählwerk des Integrators beginnt bei jedem Startimpuls von Null an zu integrieren, so dass die Retentionszeiten bei jeder Probeneingabe direkt ausgedruckt werden. Ausserdem druckt der Integrator bei jedem Startimpuls von E den Buchstaben "N" aus, was die Zuordnung der Flächen zu den entspr. Peaks auf dem Schreiberpapier wesentlich erleichtert.

Kleine Störpeaks können durch Anheben der "Reizschwelle" des Integrators (Integral reject) ausgeschaltet werden. Ausserdem verhindert ein "Integrator delay" die Registrierung unerwünschter Peaks jeder Grösse während einer gewissen, wählbaren Zeitspanne. Dadurch bleiben die vom Integrator ausgedruckten Daten übersichtlich und können mühelos ausgewertet werden.

## ERGEBNISSE

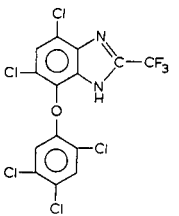
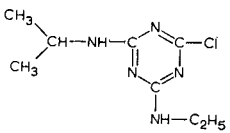
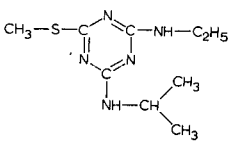
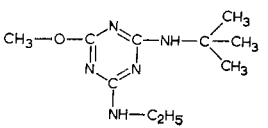
Als Testsubstanzen verwendeten wir verschiedene Agrochemikalien—Wirkstoffe der Firma J. R. Geigy A.G., Basel, teils von Handelsprodukten, teils von Produkten im fortgeschrittenen Stadium der Entwicklung.

Tabelle II enthält eine Zusammenstellung der untersuchten Substanzen mit Angabe der jeweiligen gaschromatographischen Bedingungen. Die Wirkstoffe wurden als Reinsubstanzen, in Formulierungen und als Rückstände in Erde untersucht.

TABELLE II

ZUSAMMENSTELLUNG DER UNTERSUCHTEN WIRKSUBSTANZEN

FID = Flammionisationsdetektor; MC = Mikrocoulometerdetektor; CLD = Coulson Leitfähigkeitsdetektor.

| *Code Nr. und 'common name'* | *Strukturformel* | *Anwendungsgebiet* | *Gaschromatographische Bedingungen* | | | | |
|---|---|---|---|---|---|---|---|
| | | | *Säulenfüllung* | *Säulentemp. (°C)* | *Temp. Inj.-block (°C)* | *Detektor* | *Ret. Zeit (min)* |
| GS 24802 |  | | XE-60 0.5% WS auf Chromosorb G | 200 | 220 | 220° FID | 7.0 |
| G 30027 Atrazin |  | Herbizid | AFL 2% auf Chromosorb G | 195 | 225 | 240° FID MC CLD | 8.3 |
| G 34162 Ametryn |  | Herbizid | AFL 2% auf Chromosorb G | 195 | 225 | FID 240° | 17.2 |
| GS 14259 |  | Herbizid | AFL 2% auf Chromosorb G | 195 | 225 | FID 240° | 8.2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| GS 13529 |  | Herbizid | AFL 2% auf Chromosorb G | 195 | 225 | FID<br>CLD<br>240° | 9.0 |
| GS 18622 |  | Herbizid | AFL 2% auf Chromosorb G | 195 | 225 | FID<br>240° | 7.2 |
| GS 13528 |  | Herbizid | AFL 2% auf Chromosorb G | 195 | 225 | FID<br>240° | 13.2 |
| GS 14260 |  | Herbizid | AFL 2% auf Chromosorb G | 195 | 225 | FID<br>CLD<br>240° | 20.4 |
| Aldrin |  | Insektizid (interner Standard) | AFL 2% auf Chromosorb G | 195 | 225 | FID<br>240° | 26.0 |

TABELLE III

MITTELWERTE VON RETENTIONSZEITEN UND PEAKFLÄCHEN SOWIE DEREN RELATIVE STANDARDABWEICHUNGEN ($s_{rel}$) BEI ZEHNFACHER GASCHROMATOGRAPHISCHER BESTIMMUNG VERSCHIEDENER MENGEN GS 24802 NACH METHYLIERUNG

Detektor: FID.

| *Eingegebene Substanzmenge* ($\mu g$) | *Manuelle Einspritzungen* | | | | *Automatische Probeneingabe* | | | |
|---|---|---|---|---|---|---|---|---|
| | *Retentionszeit* (*sec*) | $s_{rel}$ (%) | *Peakfläche* (*Integral counts*) | $s_{rel}$ (%) | *Retentionszeit* (*sec*) | $s_{rel}$ (%) | *Peakfläche* (*Integral counts*) | $s_{rel}$ (%) |
| 0 | — | | 0 | | — | | 0 | |
| 0.5 | 352 | 0.52 | 32 070 | 2.8 | 458 | 3.4 | 31 830 | 2.8 |
| 1 | 355 | 0.33 | 68 090 | 2.3 | 460 | 3.2 | 66 101 | 1.3 |
| 2 | 354 | 0.45 | 137 251 | 1.5 | 464 | 3.0 | 133 141 | 1.3 |

Tabelle III zeigt Peakflächen und Retentionszeiten bei manueller Einspritzung bzw. automatischer Eingabe verschiedener Mengen GS 24802. Diese Wirksubstanz lässt sich gaschromatographisch am besten nach Methylierung mit Dimethylsulfat bestimmen, und ist thermisch so empfindlich, dass sie sich in einer nach MENINI UND NORYMBERSKI[6] entwickelten Apparatur sofort auf den Metallzylindern, die zur Probeaufnahme verwendet werden, im Einspritzblock zersetzt. Gerade bei dieser empfindlichen Substanz war der Vorteil unseres Allglas-Systems klar ersichtlich.

Der statistische Vergleich der Standardabweichung in Tabelle III durch eine F-Prüfung am 95%-Niveau zeigt, dass sich die Reproduzierbarkeiten der Analysenwerte bei automatischer Probeneingabe und bei manueller Einspritzung nicht signifikant unterscheiden. Die durch ungleichmässiges Fallen der Proberöhrchen in den Verdampfungsraum auftretenden Streuungen in der Retentionszeit sind zwar signifikant grösser als bei manuellem Betrieb (95% Vertrauensgrenzen), jedoch durchaus tolerierbar.

Bei automatischer Eingabe der Proben in den Einspritzblock verdampfen die Substanzen langsamer als bei manueller Einspritzung in Lösung durch das Septum. Dies äussert sich in einer geringen Verbreitung der Peaks und einer Verlängerung der Retentionszeit.

Tabelle IV enthält die Wirksubstanzgehalte einiger Triazin-Herbizid-Formulierungen, ermittelt durch eine klassische Titrationsmethode, durch normale gaschromatographische Bestimmung mit internem Standardzusatz und durch automatische Analyse im Nachtbetrieb, wobei im letzteren Fall jedes Muster vierfach bestimmt wurde.

Diese Gegenüberstellung zeigt die Eignung des Systems für ein wichtiges neues Anwendungsgebiet. Die Anwesenheit mehrerer technischer Wirkstoffe derselben Substanzklasse in einer Formulierung macht die Gehaltsbestimmung der Einzelkomponenten mit klassischen elektrochemischen Methoden sehr schwierig, mit automatisch betriebener Gaschromatographie dagegen wesentlich einfacher, spezifischer und zuverlässiger.

Der Einsatz des automatischen Probeneingabesystems für die Analyse von Herbizid-Rückständen in Erde war von besonderem Interesse, da bei diesen Untersuchungen in unseren Laboratorien grosse Musterserien anfallen.

TABELLE IV

MITTELWERTE UND RELATIVE STANDARDABWEICHUNGEN BEI GASCHROMATOGRAPHISCHER UND TITRIMETRISCHER GEHALTSBESTIMMUNG VERSCHIEDENER TRIAZIN-FORMULIERUNGEN

GC automatisch: vierfache Bestimmung; GC manuell und Titration: Doppelbestimmung.

| *Wirksubstanzen der Formulierung* | *Prozent Wirksubstanzgehalt* | | | |
|---|---|---|---|---|
| | *GC automatisch* | $s_{rel}$ (%) | *GC manuell* | *Titration* |
| Atrazin | 24.5 | 1.3 | — | 24.0 |
| Ametryn | 52.1 | 1.2 | — | 52.9 |
| Ametryn | 24.2 | 0.85 | | 24.2 |
| GS 14259 | 2.47 | 3.9 | 2.30 | 2.50 |
| GS 13529 | 2.33 | 9.5 | 2.40 | 2.51 |
| GS 18622 | 4.04 | 7.6 | 4.80 | — |
| GS 13528 | 7.97 | 4.3 | 7.50 | — |

Für die Analyse von Erdproben, die auf Rückstände meist mehrerer Triazin-Herbizide untersucht werden müssen bewährte sich der Einsatz des Coulson Leitfähigkeitsdetektors zur gaschromatographischen Endbestimmung. Dieser Detektor erlaubt als einziger (neben dem für Rückstandsanalysen nicht brauchbaren FID) den Nachweis von Chlor-Methoxy- und- Methylmercapto-Triazinen in einem Arbeitsgang. Die Automation gerade dieses gaschromatographischen Systems erschien daher für das Rückstandslabor besonders lohnend.

Die Daten der Tabelle V zeigen eine Versuchsreihe zur Prüfung der Reproduzierbarkeit der Peak-Flächen bei manueller Einspritzung und bei Einsatz der Automatik am Coulson Leitfähigkeitsdetektor.

TABELLE V

GASCHROMATOGRAPHISCHE BESTIMMUNG VON TRIAZIN-HERBIZIDEN MIT COULSON LEITFÄHIGKEITS-DETEKTOR

Durchschnittsgehalte und relative Standardabweichungen berechnet aus jeweils fünf Wiederholungen.

| | *Manuelle Einspritzungen* | | | *Automatische Probeneingabe* | | |
|---|---|---|---|---|---|---|
| | *100 ng Atrazin* | *Mischung je 100 ng* | | *100 ng Atrazin* | *Mischung je 100 ng* | |
| | | *Atrazin* | *GS 14260* | | *Atrazin* | *GS 14260* |
| Durchschnittsfläche (Integral counts) | 65 640 | 59 952 | 55 921 | 38 841 | 39 996 | 29 335 |
| Relative Standardabweichung (%) | 4.1 | 2.5 | 1.2 | 3.7 | 1.3 | 2.2 |

Tabelle VI enthält Rückstandsdaten mehrerer Triazine in Erde. Die Extrakte wurden einerseits durch manuelle Einspritzung und Auswertung mit Coulson Leitfähigkeitsdetektor, andererseits automatisch im Nachtbetrieb bestimmt. Die Einstellung des Integrators ist hier etwas kritisch. Bei Peaks mit Schwanzbildung muss die Kurvensteigung, bei der die Integration der Fläche beendet sein soll ziemlich klein

TABELLE VI

MANUELLE UND AUTOMATISCHE GASCHROMATOGRAPHISCHE BESTIMMUNG VON TRIAZIN-RÜCKSTÄNDEN IN ERDE MIT COULSON LEITFÄHIGKEITSDETEKTOR

$\bar{x}$ = Mittelwert.

| *Muster Nr.* | *Herbizide (p.p.m.)* | | | | | |
|---|---|---|---|---|---|---|
| | *Automatische Bestimmung* | | | *Manuelle Bestimmung* | | |
| | *Atrazin* | *GS 13529* | *GS 14260* | *Atrazin* | *GS 13529* | *GS 14260* |
| 1 | 0.50 | | 0.08 | | | |
| | 0.40 | | 0.10 | 0.34 | | 0.05 |
| | 0.50 | | 0.13 | | | |
| | $\bar{x} = 0.47$ | | $\bar{x} = 0.10$ | | | |
| 2 | | 0.18 | 0.02 | | | |
| | — | 0.22 | 0.02 | | 0.30 | 0.03 |
| | | 0.14 | 0.03 | | | |
| | | $\bar{x} = 0.18$ | $\bar{x} = 0.02$ | | | |
| 3 | 0.48 | | | | | |
| | 0.45 | | | 0.56 | | |
| | 0.42 | | | | | |
| | $\bar{x} = 0.45$ | | | | | |
| 4 | 0.60 | | | | | |
| | 0.60 | | | 0.55 | | |
| | 0.63 | | | | | |
| | $\bar{x} = 0.61$ | | | | | |

vorgewählt werden, da sonst der letzte Teil des Peaks im Bereich der flachen negativen Kurvensteigung nicht mitintegriert wird.

Tabelle VII enthält eine Gegenüberstellung von Atrazin-Rückstandswerten in Erde, die gaschromatographisch mit Microcoulometerdetektor sowohl manuell wie automatisch bestimmt wurden. Bei Verwendung des Microcoulometerdetektors ist zu beachten dass die Elektrolytflüssigkeit in der Messzelle während der Nacht z.T. verdampft, was die Empfindlichkeit des Systems ändert. Durch Kühlung der Titrierzelle von aussen kann der Verlust an Elektrolytflüssigkeit verringert werden. Zur Sicherheit wird nach fünf Proben unbekannten Gehalts je ein "fortified sample", also eine Blindprobe mit genau bekanntem Wirkstoffzusatz mitanalysiert.

## RATIONALITÄTS-BETRACHTUNG

Bei manuellem Betrieb eines Gaschromatographen erreicht eine Arbeitskraft im Maximum 30 Einspritzen pro Tag, was einer Gesamtkapazität von etwa 20 Mustern entspricht. Die Vorbereitung von 70 Proben entspr. etwa 50 Analysenmustern für den automatischen Betrieb erfordert etwa zwei Arbeitsstunden, ebenso die Zuordnung und Auswertung der ausgedruckten Daten. Selbst wenn jedes einzelne Muster doppelt bestimmt wird, erreicht man insgesamt eine beträchtliche Rationalisierung der Gaschromatographie bei grösserer Sicherheit der Einzelergebnisse.

TABELLE VII

GASCHROMATOGRAPHISCHE BESTIMMUNG VON ATRAZIN-RÜCKSTÄNDEN IN ERDE MIT MICROCOULOMETERDETEKTOR

| *Muster Nr.* | *Automatischer Bestimmung* | *Manuelle Bestimmung* |
|---|---|---|
| 1 | 0.35 | 0.33 |
| 2 | 4.3<br>4.2 | 4.5 |
| 3 | 6.6<br>7.2 | 8.8 |
| 4 | 1.2<br>1.2 | 1.6 |
| 5 | 11.0<br>11.0 | 12.1 |
| 6 | 3.3<br>3.2 | 3.8 |
| 7 | 1.2<br>1.1 | 1.3 |
| 8 | 0.35<br>0.25 | 0.30 |
| Blindmuster 1 | <0.02 | <0.02 |
| Blindmuster 2 | <0.02 | <0.02 |
| Blindmuster + Zusatz 0.1 p.p.m. | 0.07 | 0.12 |

ZUSAMMENFASSUNG

Das beschriebene Allglas-Probeneingabesystem zur automatischen gaschromatographischen Bestimmung thermisch empfindlicher Insektizide und Herbizide besteht aus einem geheizten, durch den Einspritzblock direkt mit der Säule verbundenen Verdampfungsraum mit seitlichem Ansatz zur Aufnahme der Analysenmuster. Mikroliter-Aliquote der Proben in Hexan oder Äther werden in 7 mm lange, einseitig zugeschmolzene Glasröhrchen eingefüllt. Nach dem Verdampfen des Lösungsmittels lassen sich etwa 70 Glasröhrchen in die Apparatur einführen. Der Probentransport in den Verdampfungsraum erfolgt diskontinuierlich durch Schub eines Metallstiftes der von einem aussen angebrachten Magneten bewegt wird. Analysen von Formulierungen und Rückständen von Triazin-Herbiziden und halogenierten Kohlenwasserstoffen wurden unter Verwendung verschiedener z.T. spezifischer Detektoren automatisch durchgeführt.

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CHROM. 4388

# CHROMATOGRAPHY OF N-METHYLCARBAMATES IN THE GASEOUS PHASE

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SUMMARY

Gas–liquid chromatography was used to successfully separate certain pesticidal phenyl N-methylcarbamates from their phenolic moieties. A slightly polar 3 % OV-17 liquid phase was utilized and compared with a mixed liquid phase polarity column, 1.5 % SE-30 and 2 % Carbowax 20M. Retention times, carbamate to phenol ratios, peak symmetry values and height equivalent to a theoretical plate values are reported as functions dependent on temperature. Small amounts of breakdown products were observed with a majority of the compounds at 180° on the OV-17 column.

## INTRODUCTION

The toxic potential of the older pesticides has been carefully documented[1–4]. The increased use of carbamates as pesticides during the past ten years has stimulated the need for toxicologic, pharmacologic and environmental pollution studies of these compounds so that toxicity due to acute and long term exposure can be rationally treated. To undertake the above mentioned studies it is necessary to have a sensitive and precise analytical method. The investigation reported in this paper is a continuation of the work undertaken by STROTHER[5] to develop successfully a sensitive, yet simple gas–liquid chromatographic (GLC) procedure which permits rapid separation and identification of intact phenyl N-methylcarbamates.

Two different type columns were prepared for this study. One column contained OV-17 (Applied Science Labs.) and the other was a mixed liquid phase column containing 1.5 % SE-30 (non-polar) and 2 % Carbowax 20M (polar). These columns were studied to determine if the methylcarbamates could be chromatographed with little loss due to decomposition on the column. Previous attempts at developing a liquid phase to accomplish this with such a wide range of pesticidal compounds has been unsuccessful. Although GLC has been accomplished with some carbamates, STROTHER[5] chromatographed phenyl N-methylcarbamate, 3-methylphenyl N-methylcarbamate and 3,5-dimethylphenyl N-methylcarbamate with less than 15 % decomposition. However, in most cases decomposition results when these carbamates are chromatographed. WISNEWSKI[6] has chromatographed Carbaryl utilizing an F & M Model 402

gas chromatograph with flame ionization detectors. The alternative criterion with most compounds up to this point has been identification of the corresponding phenol[7,8] or chemical alteration of the compound and then subjection to gas chromatography, of which the silylation procedures developed by FISHBEIN AND ZIELINSKI[9] are a good example. Chromatography of simple N-methylcarbamates and some therapeutically useful nitrogen-unsaturated carbamates has also been developed[10–17]. The major limitations for intact chromatography are probably thermal instability[18] and column substrate interaction. In the present investigation these two problems were minimized for some carbamates in the 3 % OV-17 column.

## MATERIALS AND METHODS

The carbamates utilized in this experiment were:

(1) Banol (6-chloro-3,4-dimethylphenyl N-methylcarbamate)
(2) Bayer 37344 (4-methylthio-3,5-dimethylphenyl N-methylcarbamate)
(3) Bayer 39007 (O-isopropoxyphenol N-methylcarbamate)
(4) Bayer 42696 (3-(dimethylamino)-4-methylphenyl N-methylcarbamate)
(5) Bayer 50282 (4-(diallylamino)-3,5-dimethylphenyl methylcarbamate)
(6) Carbaryl (1-naphthyl N-methylcarbamate)
(7) HRS 1422 (3,5-diisopropylphenyl N-methylcarbamate)
(8) HRS 9485 (O-(alloxy)phenyl N-methylcarbamate)
(9) Matacil (4-(dimethylamino)-3-methylphenyl N-methylcarbamate)
(10) 3-Methylphenyl N-methylcarbamate
(11) Phenyl N-methylcarbamate
(12) Zectran (4-(dimethylamino)-3,5-dimethylphenyl N-methylcarbamate)

The carbamates were from the following sources: compound 1 was supplied by the Upjohn Co., compounds 2–5 and 10 by Chemagro Corp., compound 7 by Hooker Chemical Co. and compound 8 by Hercules Powder Co.; compounds 10 and 11 were synthesized by the method of BENSON AND GAJAN[19]. The phenol moiety of the carbamates, when not supplied by the manufacturer, was obtained by hydrolysis of the respective carbamates.

## EXPERIMENTAL

The 3% w/w OV-17 (low polar) liquid phase was prepared on 100–200 mesh Chromosorb AW-DMCS high performance support. The packing was placed in a 4 ft. × 5 mm U-shaped glass tube and conditioned for 72 h at 190°, for 60 h at less than 87 ml $N_2$/min and then for 12 h at 87 ml $N_2$/min.

The second column was a mixture of 1.5% SE-30 (non-polar) and 2% Carbowax 20M (polar) liquid phases on 80–100 mesh Gas-Chrom Q. It was conditioned at 215° for 24 h at less than 80 ml $N_2$/min and then for 12 h at 84.7 ml $N_2$/min.

The instrument utilized was a Barber-Colman Series 5000 Dual Gas Chromatograph, employing flame ionization detectors. The detector was maintained at 250° and the injection port at 260°.

Experimental variables such as percent breakdown, carbamate to phenol ratio, peak symmetry values and height equivalent to a theoretical plate (HETP) values were examined as a function of temperature with the flow rate being held constant.

The flow rate during the experiment through the OV-17 column was 88 ml $N_2$/min. The flow rate through the SE-30 column was 100 ml $N_2$-min.

Equimolar solutions (0.226 *M*) of all the carbamates were prepared fresh and the standard injection volume used was 1 $\mu$l. The calculation of retention times was made from the solvent front to maximum peak height. A polarity test was performed on both columns by injecting a mixture of ethanol, methyl ethyl ketone, cyclohexane and benzene in a 40:20:5:10 volume relationship at 72°.

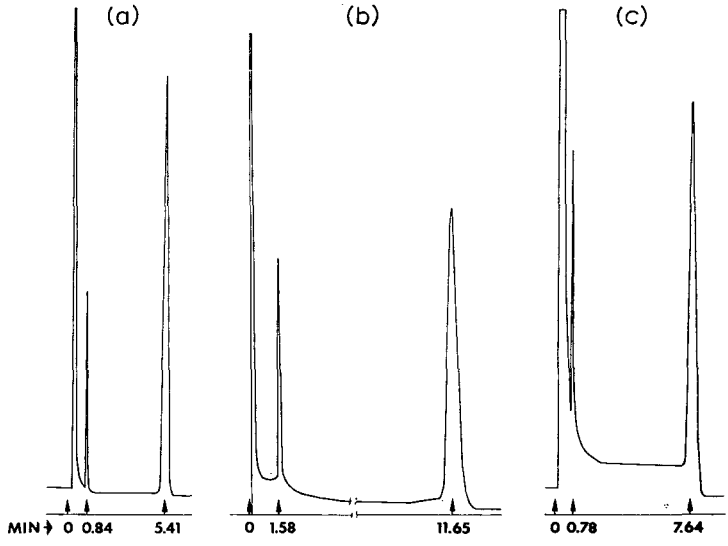


Fig. 1. Separation of (a) 4-dimethylamino)-3-methylphenyl N-methylcarbamate (Matacil), (b) 4-(dimethylamino)-3,5-dimethylphenyl N-methylcarbamate (Zectran), and (c) O-(alloxy)phenyl N-methylcarbamate (HRS 9485).

## DISCUSSION

On the basis of the elution characteristics of the polarity mixture it is felt that the SE-30 liquid phase was slightly polar since it eluted ethanol first and then methyl ethyl ketone but could not separate cyclohexane from benzene. The OV-17 column was somewhat more polar than the SE-30 column and was unable to distinguish methyl ethyl ketone from cyclohexane.

General separation characteristics of the OV-17 column agreed with the results of STROTHER[5] and FISHBEIN AND ZIELINSKI[18]. Longer retention times were observed with thio derivatives (Mesurol) than with ring N-substituted methylcarbamates (Zectran, Matacil). Thermal instability seemed to be greater with the non-N-substituted methylcarbamates (Carbaryl, Banol). The data seem to indicate that there was column substrate interaction especially at lower temperatures with the 1.5% SE-30 and 2% Carbowax 20M column. Oxygen-substitution (Bayer 39007) seemed to result

in shorter retention times than with the ring N-substituted methylcarbamates (Bayer 50282 and 42696) or the non-N-substituted methylcarbamates (Carbaryl, Banol). Lastly, the absence of the methyl group on the number five position of the ring on Matacil tended to decrease residence on the OV-17 column when compared to Zectran which has a methyl at carbon 5 of the ring and is otherwise similar to Matacil. For the retention time of each specific carbamate see Table I. Retention times given in Table I are relative to 3-methylphenol at each specified temperature for each column.

The retention times of all carbamates chromatographed on the SE-30 column were shorter than those on the OV-17 column. This substantiates the polarity experiment previously described. Since the SE-30 column is somewhat less polar, it has less affinity for the carbamate, which is then eluted more quickly and thus has a shorter retention time.

The efficiency of separation by the liquid phases of both columns is shown in Tables II and III. On the OV-17 column at 180° the percent breakdown varied between 4 and 66% with eight of the twelve compounds showing less than 15% breakdown. Fig. 1 is an example of the chromatograms obtained. A closer examination of the percentage breakdown values demonstrates the practicality of using GLC in the identification of phenyl N-methylcarbamates. Recovery of the intact carbamate was good in most cases. HRS 1422 was chromatographed and 95.2 % was recovered intact. Values for other compounds were: 3-methylphenyl N-methylcarbamate, 96 %; Zectran, 91.4 %; Matacil, 92.3 %; HRS 9485, 90.6 % and Baygon, 91.2 %. On the mixed phase column only two compounds showed less than 15 % breakdown at 215°. In Table III HETP values were calculated for each carbamate at each temperature to gain a picture of the efficiency of the whole column. Calculations were based on the method of ETTRE[21]. The HETP values correspond best for similar compounds at 190° on the OV-17 column. Matacil and Zectran are closely related (Zectran having a methyl group at position 5 on the ring) and had similar HETP values, *viz.* 5.71 and 5.91, respectively. HETP values for unsubstituted N-methylcarbamates were similar, *viz.* HRS 9485, 5.91; Carbaryl, 5.88; and Banol, 5.91. The latter data indicate that 190° is the most efficient temperature for the column to operate at. For the SE-30 column the most efficient temperature was 205°. The conclusion is based upon the close HETP values of Matacil (0.48), Zectran (0.44) and Bayer 42696 (0.43).

Another column characteristic or parameter of column efficiency is peak symmetry, which provides a measure of the degree to which the system used limits the realization of good column performance. A quantitative picture of the excellent peak symmetry obtained with the OV-17 column is given in Table II. Calculations were made according to the method of DAL NOGARE AND CHIU[20]. Seven of the twelve carbamates had very good peak symmetry with little trailing as indicated by $A_s$ values of +1.05 or less. Compounds 3, 7 and 10 had near perfect symmetry at 190° as indicated by an $A_s$ value of 1.00. The square of the asymmetry value, $(A_s)^2$, can be used to estimate approximately how the system in use limits the realization of good column performance. To gain knowledge concerning the limiting effects of secondary absorption and instrumental precision by the OV-17 column for efficient chromatography of each carbamate, $(A_s)^2$ values were calculated and specific values for each carbamate are listed in Table IV. For example, at 205° Zectran has an $(A_s)^2$ value of 1.02, which indicates that the OV-17 column is 2% less efficient than should be. Asymmetry values with the SE-30 column varied from +1.18 to +4.42. Marked

TABLE I

RETENTION TIMES OF CARBAMATES ON OV-17 AND ON SE-30 + 2% CARBOWAX 20 M COLUMNS AT THREE DIFFERENT TEMPERATURES

Retention times given in minutes relative to 3-methylphenol.

| | *OV-17* | | | *SE-30 + Carbowax 20 M* | | |
|---|---|---|---|---|---|---|
| | *180°* | *190°* | *205°* | *190°* | *205°* | *215°* |
| 6-Chloro-3,4-dimethylphenyl N-methylcarbamate (Banol) | 33.35 | 24.68 | 19.66 | 0.0 | 0.0 | 0.0 |
| 4-(Methylthio)-3,5-dimethylphenyl methylcarbamate (Mesurol) | 44.90 | 54.78 | 41.55 | 0.0 | 0.0 | 0.0 |
| O-Isopropoxyphenyl N-methylcarbamate (Baygon) | 16.30 | 12.85 | 10.52 | 12.87 | 7.36 | 7.84 |
| 3-Dimethylamino)-4-methylphenyl N-methylcarbamate (Bayer 42696) | 30.13 | 16.34 | 26.17 | 15.91 | 13.50 | 13.68 |
| 4-(Diallylamino)-3,5-dimethylphenyl methylcarbamate (Bayer 50282) | 131.10 | 57.73 | 62.75 | 0.0 | 0.0 | 0.0 |
| 1-Naphthyl N-methylcarbamate (Carbaryl) | 63.33 | 50.24 | 47.31 | 0.0 | 0.0 | 0.0 |
| 3,5-Diisopropylphenyl N-methylcarbamate (HRS 1422) | 33.13 | 16.32 | 18.52 | 12.87 | 10.93 | 11.70 |
| O-(Alloxy)phenyl N-methylcarbamate (HRS 9485) | 30.88 | 18.63 | 14.79 | 6.80 | 2.66 | 3.00 |
| 4-(Dimethylamino)-3-methylphenyl N-methylcarbamate (Matacil) | 33.13 | 24.76 | 18.66 | 0.0 | 13.48 | 14.92 |
| 3-Methylphenyl N-methylcarbamate | 5.02 | 3.05 | 3.93 | 5.03 | 3.26 | 3.57 |
| Phenyl N-methylcarbamate | 8.00 | 6.90 | 6.51 | 6.63 | 4.67 | 5.14 |

TABLE II

PERCENTAGE BREAKDOWN AND CARBAMATE TO PHENOL RATIO OF CARBAMATE AT THREE DIFFERENT TEMPERATURES

| | *OV-17* | | | | | | *SE-30 + Carbowax 20 M* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *180°* | | *190°* | | *205°* | | *190°* | | *205°* | | *215°* | |
| | *% breakdown* | *C/P* | *% breakdown* | *C/P* | *% breakdown* | *C/P* | *% breakdown* | *C/P* | *% breakdown* | *C/P* | *% breakdown* | *C/P* |
| Banol | 35.3 | 1.83 | 57.70 | 0.73 | 68.9 | 0.45 | — | — | — | — | — | — |
| Bayer 37344 | 66.1 | 0.51 | 90.6 | 0.07 | 88.3 | 0.13 | — | — | — | — | — | — |
| Bayer 39007 | 13.5 | 6.42 | 21.9 | 3.55 | 17.7 | 4.66 | 26.1 | 2.83 | 44.2 | 1.26 | 41.2 | 1.42 |
| Bayer 42696 | 8.81 | 10.32 | 50.6 | 0.97 | 63.4 | 0.58 | 6.9 | 0.44 | 68.8 | 0.45 | 36.1 | 1.77 |
| Bayer 50282 | 11.3 | 11.91 | 56.3 | 0.97 | 74.3 | 3.60 | — | — | — | 2.23 | — | 1.61 |
| Carbaryl | 66.8 | 7.95 | 80.0 | 0.77 | 88.9 | 0.34 | — | — | — | — | — | — |
| HRS 1422 | 4.8 | 0.50 | 8.2 | 0.25 | 8.5 | 0.15 | 38.8 | — | 11.5 | — | 12.3 | — |
| HRS 9485 | 9.4 | 19.97 | 16.3 | 11.27 | 15.9 | 10.74 | 23.9 | 0.63 | 68.5 | 7.67 | 40.7 | 8.15 |
| Matacil | 7.7 | 9.63 | 50.6 | 5.14 | 21.9 | 5.32 | 29.9 | 3.19 | 29.9 | 0.46 | 38.2 | 1.46 |
| 3-Methylphenyl N-methylcarbamate | 4.0 | 24.0 | 46.3 | 8.25 | 10.8 | [a] | 88.9 | 0.13 | 22.5 | 3.44 | [a] | — |
| Phenyl N-methylcarbamate[a] | — | — | — | — | — | — | 70.8 | 0.36 | — | — | — | — |
| Zectran | 8.6 | 10.5 | 15.1 | 5.66 | 18.6 | 4.37 | 55.8 | 0.79 | 35.8 | 1.82 | 15.0 | 5.68 |

[a] The phenol peak was very small and in the solvent front. Accurate measurement was not possible.

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TABLE III

PEAK ASYMMETRY, $A_s$, AND SQUARED PEAK ASYMMETRY, $(A_s)^2$, VALUES FOR EACH CARBAMATE AS A FUNCTION OF TEMPERATURE

Calculations of the peak asymmetry values are based on DAL NOGARE AND CHIU[20].

| | *OV-17* | | | | | | *SE-30 + Carbowax 20 M* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *180°* | | *190°* | | *205°* | | *190°* | | *205°* | | *215°* | |
| | $A_s$ | $(A_s)^2$ | $A_s$ | $(A_s)^2$ | $A_s$ | $(A_s)^2$ | $A_s$ | $(A_s)^2$ | $A_s$ | $(A_s)^2$ | $A_s$ | $(A_s)^2$ |
| Banol | 1.08 | 1.17 | 1.06 | 1.12 | 1.13 | 1.28 | 0 | — | 0 | — | 0 | — |
| Bayer 37344 | 1.07 | 1.15 | 1.20 | 1.40 | 1.25 | 1.56 | 0 | — | 0 | — | 0 | — |
| Bayer 39007 | 1.31 | 1.72 | 1.00 | 1.00 | −1.05 | 1.10 | — | — | 1.45 | 2.10 | 1.24 | 1.54 |
| Bayer 42696 | 1.02 | 1.04 | 1.13 | 1.28 | 1.02 | 1.04 | 1.18 | 1.39 | 1.45 | 2.10 | 1.23 | 1.52 |
| Bayer 50282 | 1.02 | 1.04 | 1.02 | 1.04 | 1.11 | 1.23 | 0 | — | 0 | — | 0 | — |
| Carbaryl | 1.14 | 1.30 | 1.23 | 1.51 | 1.05 | 1.10 | 0 | — | 0 | — | 0 | — |
| HRS 1422 | 1.01 | 1.02 | 1.00 | 1.00 | 1.40 | 1.96 | 1.46 | 2.13 | 1.63 | 2.66 | 1.48 | 2.19 |
| HRS 9485 | 1.01 | 1.02 | 1.07 | 1.15 | 1.17 | 1.37 | 1.61 | 2.59 | 2.78 | 7.73 | 4.42 | 19.54 |
| Matacil | 1.03 | 1.06 | 1.14 | 1.30 | 1.04 | 1.08 | 1.19 | 1.42 | 1.32 | 1.74 | 1.43 | 2.05 |
| 3-Methylphenyl N-methyl-carbamate | 1.03 | 1.06 | 1.00 | 1.00 | −1.15 | 1.32 | 1.43 | 2.05 | 1.55 | 2.40 | 1.20 | 1.44 |
| Phenyl N-methylcarbamate | — | — | — | — | — | — | 1.10 | 1.21 | 1.10 | 1.21 | 1.25 | 1.56 |
| Zectran | 1.03 | 1.06 | 1.15 | 1.32 | 1.01 | 1.02 | 1.14 | 1.30 | 1.43 | 1.30 | 1.25 | 1.56 |

TABLE IV

HETP VALUES OF EACH CARBAMATE AS A FUNCTION OF TEMPERATURE

HETP units are in min/mm/ft. Calculations are based on ETTRE[21].

| | *OV-17* | | | *SE-30 + Carbowax 20 M* | | |
|---|---|---|---|---|---|---|
| | *180°* | *190°* | *205°* | *190°* | *205°* | *215°* |
| Banol | 7.25 | 5.91 | 6.17 | — | — | — |
| Bayer 37344 | 3.59 | 4.13 | 6.65 | — | — | — |
| Bayer 39007 | 5.31 | 4.57 | 4.46 | — | 0.27 | 0.31 |
| Bayer 42696 | 6.27 | 2.70 | 8.30 | 0.32 | 0.43 | 0.51 |
| Bayer 50282 | 9.52 | 2.90 | 6.68 | — | — | — |
| Carbaryl | 8.85 | 5.88 | 8.94 | — | — | — |
| HRS 1422 | 6.23 | 5.18 | 4.44 | — | 0.26 | 0.32 |
| HRS 9485 | 11.06 | 5.91 | 6.37 | 0.12 | 0.03 | 0.05 |
| Matacil | 6.23 | 5.71 | 6.47 | 0.26 | 0.48 | 0.57 |
| 3-Methylphenyl N-methylcarbamate | 5.11 | 1.52 | 4.95 | — | 0.46 | — |
| Phenyl N-methylcarbamate | 2.48 | 1.79 | 1.80 | — | 0.45 | — |
| Zectran | 7.11 | 5.59 | 6.34 | 0.15 | 0.44 | 0.38 |

trailing of peaks on the SE-30 column was observed as exemplified by HRS 9485 with an $A_s$ value of +4.42. Trailing may possibly be due to the higher polarity of the Carbowax 20M liquid phase.

Human liver *in vitro* metabolism studies utilizing Matacil were being carried on concurrently by STROTHER in the laboratory. Ether extracts from the human liver biopsy specimens incubated with Matacil were injected onto both columns to attempt separation and identification of suspected metabolites. The SE-30 column proved unsuccessful in separation or identification of any components of the ether extract. However, the OV-17 column separated the mixture and successful identification of the N-hydroxymethyl derivative has been verified by means of cochromatography with standards and simultaneous injection of standard and unknown, which results in an estimable increase in peak height at the retention time of the known standard. The OV-17 liquid phase promises to be an important tool in identification of carbamate metabolites. The metabolites of Zectran can all be separated from a mixture with very little decomposition. Tables V and VI give retention times at 180° for Carbaryl and 190° for Zectran of the various metabolite standards injected onto the column. However, purification of the ether extract by thin-layer chromatography will be

TABLE V

RETENTION TIMES OF CARBARYL METABOLITES ON OV-17 AT 180°

The concentration of all solutions was 5 mg/ml. The standard injection volume was 1 $\mu$l. The detector temperature was 260°, the injection port temperature 250° and the flow rate 88 ml $N_2$/min.

| *Compound* | $T_R$ *(min)* |
|---|---|
| 1-Naphthyl N-methylcarbamate | 30.4 |
| 4-Hydroxy-1-naphthyl methylcarbamate | 3.80 |
| 5-Hydroxy-1-naphthyl methylcarbamate | 24.2 |
| 1-Naphthol | 4.71 |

TABLE VI

RETENTION TIMES OF ZECTRAN METABOLITES ON OV-17 AT 190°

The concentration of all solutions was 5 mg/ml. The standard injection volume was 1 $\mu$l. The detector temperature was 260°, the injection port temperature 250° and the flow rate 88 ml $N_2$/min.

| *Compound* | $T_R$ (*min*) |
|---|---|
| Zectran | 11.7 |
| 4-(Dimethylamino)-3,5-dimethylphenol | 1.58 |
| 4-(Methylformamido)-3,5-dimethylphenyl methylcarbamate | 9.94 |
| 4-(Formamido)-3,5-dimethylphenyl methylcarbamate | 13.98 |
| 4-(Methylamino)-3,5-dimethylphenyl methylcarbamate | 18.80 |
| 4-Amino-3,5-dimethylphenyl methylcarbamate | 20.4 |

needed to remove unwanted materials that also chromatograph with retention times similar to some of the metabolites for definite identification from the incubation mixture.

Both columns have been in operation for two months and there is no indication that their ability to separate carbamates has diminished as a function of time, as has been previously reported by STROTHER with the SE-30 and QF-1 columns.

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CHROM. 4392

# SEPHADEX LH-20 COLUMN SEPARATION OF THYROIDAL IODOAMINO ACIDS

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SUMMARY

A solvent system consisting of low-boiling organic solvents and ammonium hydroxide effectively separated iodoamino acids of the thyroid on Sephadex LH-20 gel filtration columns. The iodoamino acids were eluted from the column in a sequence unrelated to their molecular weights suggesting that adsorptive properties of the dextran gel rather than gel exclusion effects governed the elution volume of the compounds.

The eluted iodoamino acids were recovered in a pure form by low-temperature evaporation of the solvents at reduced pressure. Good recovery of iodoamino acids from the column was obtained and reproducibility of column performance was satisfactory.

## INTRODUCTION

Various methods have been described for the separation of mixtures of thyroidal iodoamino acids by filter paper and thin-layer chromatography[1–5], ion exchange column chromatography[6,7], gas-liquid chromatography[8–10] and by Sephadex gel filtration columns[11–14].

Each of these techniques has certain advantages as well as disadvantages in terms of sample size needed, requirements for the preparation of the samples or derivatives for separation, speed of separation, complexity of equipment involved and recovery of the compounds of interest.

Gel filtration column procedures with an automatic fraction collector have the advantage of requiring little attention during the separation procedure and are reproducible with adequate recovery of the compounds from the columns. While proteins generally separate on gel filtration columns according to their molecular weights, crosslinked dextran gels often show strong adsorptive properties for aromatic substances in some cases favoring their separation[15,16]. Compounds such as thyroidal iodoamino acids also have been shown to be reversibly adsorbed to Sephadex gel filtration column beds[11,17,18].

Procedures for the separation of iodoamino acids on Sephadex G-25 gel filtration

columns using buffered salt solutions, dilute sodium hydroxide or higher boiling organic solvents have been described[12–14]. With such eluting mixtures, recovery of the separated compounds for further analysis or use was complicated by the presence of salt or alkaline residues when the pooled peaks from the column containing the compounds of interest were taken to dryness.

Sephadex LH-20 has been reported to be stable in organic solvents[17] and has been used as a desalting step in a purification procedure for the analysis of serum iodoamino acids[19]. We have developed a solvent system composed of ethyl acetate, methanol and aqueous ammonium hydroxide which effectively separated thyroidal iodoamino acids on Sephadex LH-20 gel filtration columns.

## EXPERIMENTAL

### *Reagents*

All reagents used were analytical grade. The eluting system for the Sephadex LH-20 column was a mixture of ethyl acetate–methanol–2 *N* aqueous ammonium hydroxide (400:100:40).

Solutions of thyroxine ($T_4$), triiodothyronine ($T_3$), diiodotyrosine (DIT) and monoiodotyrosine (MIT) (Calbiochem, Los Angeles, Calif.) were used at a concentration of 5 mg/ml in 1% methanolic $NH_4OH$ (methanol–concentrated ammonium hydroxide (99:1)) to standardize the Sephadex LH-20 column.

### *Iodine assay*

The iodine content of the samples was determined by a chloric acid wet ashing procedure previously described[20]. It is important that iodine assays on samples dissolved in the ethyl acetate–methanol–$NH_4OH$ eluting mixture or in the 1% methanolic $NH_4OH$ solvent be done on aliquots which have been dried completely prior to digestion with the chloric acid reagent: *methanol reacts violently with hot chloric acid.*

### *Thin-layer chromatography*

The thin-layer chromatographic procedure was described previously by us[4].

### *Preparation of gel filtration columns*

Sephadex LH-20, particle size 25–100 $\mu$ (Pharmacia, Uppsala, Sweden) was suspended in the ethyl acetate–methanol–$NH_4OH$ elution mixture. It was allowed to settle briefly and, after discarding the fines by decantation, was equilibrated in the solvent for 4 h. An 0.8 cm diameter glass column fitted with a sintered glass disc and a stopcock to control the flow rate was attached to a reservoir filled with the eluting solvent mixture. Glass ball and socket joint column connectors were used since the solvent was found to extract ultraviolet-absorbing materials from rubber or plastic connectors.

The equilibrated Sephadex was poured into the reservoir and with stirring was allowed to flow into the column in which it was packed by gravity flow to a column height of 60 cm. The solvent was permitted to flow through the gel bed in the column for 16 h before use. Between separation runs the solvent mixture was passed through the packed column continuously to maintain equilibrium conditions in the gel bed.

A fraction collector (LKB-Produkter AB, Sweden) was used to collect 4 ml

fractions of the column effluent at an elution rate of 0.5 ml/min during separation procedures. A complete separation of iodoamino acids required the collection of 100–110 × 4 ml fractions from the column.

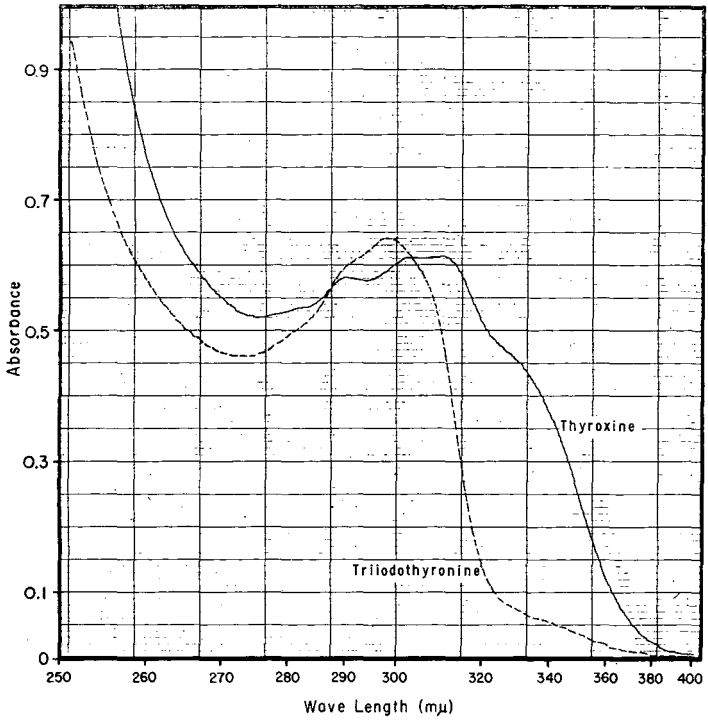


Fig. 1. Absorbance spectra of pure thyroxine and triiodothyronine in ethyl acetate–methanol–$NH_4OH$. 1.5 × $10^{-4}$ *M* solutions.

*Spectrophotometric characteristics of iodoamino acids*

A Beckman Model DK-2 recording spectrophotometer was used to obtain spectral absorbance curves of the pure iodoamino acids. The sequence of elution of the pure compounds from the column was monitored at 297 and 310 mμ using a Beckman Model DU spectrophotometer. Absorbance measurements were done on each 4 ml fraction collected from the column.

The spectrophotometric absorbance characteristics of the iodoamino acids at a concentration of 1.5 × $10^{-4}$ *M* in the eluting solvent system are shown in Fig. 1 for $T_4$ and $T_3$ and for MIT and DIT in Fig. 2. The molar extinction coefficient and the ratio of absorbance at 297 and 310 mμ of each iodoamino acid in the ethyl acetate–methanol–$NH_4OH$ solvent is listed in Table I.

*Sephadex LH-20 column separation of a pure iodoamino acid mixture*

In preliminary studies, 0.5 ml of each pure iodoamino acid standard solution (5 mg/ml in 1% methanolic $NH_4OH$) was chromatographed individually on a Sephadex LH-20 gel filtration column. The compound was eluted from the column with

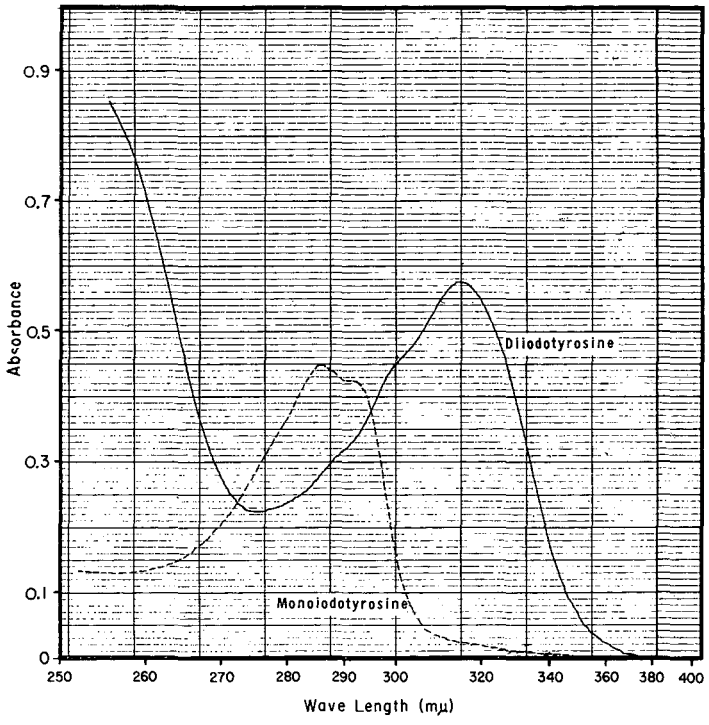


Fig. 2. Absorbance spectra of pure diiodotyrosine and monoiodotyrosine in ethyl acetate–methanol–$NH_4OH$. 1.5 × $10^{-4}$ *M* solutions.

TABLE I

SPECTROPHOTOMETRIC CHARACTERISTICS OF PURE IODOAMINO ACIDS

Molar extinction coefficient (1 cm light path) at 297 mμ in the ethyl acetate–methanol–$NH_4OH$ eluting solvent.

| *Compound* | *Extinction* | *Ratio 297/310 mμ* |
|---|---|---|
| Thyroxine | 3900 | 0.956 |
| Triiodothyronine | 4253 | 1.276 |
| Diiodotyrosine | 2720 | 0.744 |
| Monoiodotyrosine | 2053 | 9.626 |

the ethyl acetate–methanol–$NH_4OH$ eluting mixture to determine the effluent volume at which it emerged from the column. The column effluent was monitored spectrophotometrically.

After defining the elution pattern for each individual iodoamino acid, a mixture of the four iodoamino acids was prepared (5 mg of each compound per ml of 1% methanolic $NH_4OH$). An 0.5 ml aliquot of the standard mixture was applied to the column bed, allowed to penetrate the upper surface of the gel and the iodoamino acids were eluted from the Sephadex LH-20 column with the ethyl acetate–methanol–

$NH_4OH$ eluting mixture. After spectrophotometry, iodine analyses were done on small aliquots of each 4 ml fraction. The elution pattern of the standard iodoamino acid mixture from the Sephadex LH-20 column is shown in Fig. 3. The line connecting the solid circles represents the 297 m$\mu$ absorbance while the dotted line connecting the triangles is the iodine content ($\mu$g/ml) of each fraction. Fractions composing the areas under each peak were pooled and taken to dryness *in vacuo* at less than 40° and the dried samples were redissolved in a small volume of 1% methanolic $NH_4OH$. In most cases, spectrophotometric studies revealed no appreciable differences between the reconstituted pooled peaks and the original pure samples. One lot number of Sephadex LH-20 (different from the batch used for the major portion of this study) appeared to produce an unexplained spectral shift when DIT was eluted from the gel bed.

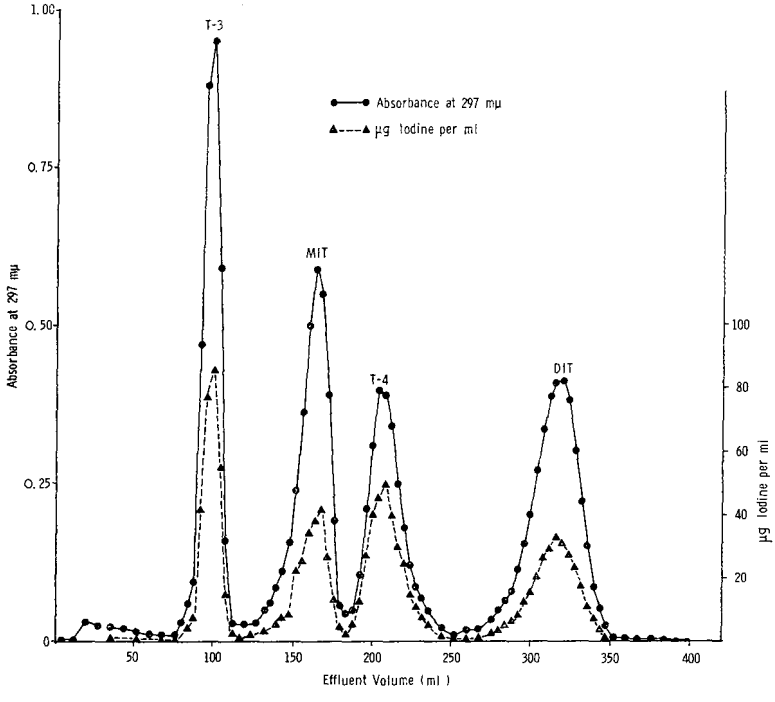


Fig. 3. Elution pattern from a Sephadex LH-20 gel filtration column of a mixture of pure iodoamino acids. Solid line = absorbance at 297 m$\mu$. Dotted line = $\mu$g iodine per ml.

Thin-layer chromatographic analysis of the reconstituted pooled peaks revealed single spots traveling to positions occupied by known pure iodoamino acid standards with no evidence of cross contamination of a pooled peak by adjacent peaks.

*Sephadex LH-20 column separation of $^{131}I$-labeled rat thyroid iodoamino acids*

The thyroid gland was removed from a normal rat that had been injected with $^{131}I$ 24 h prior to sacrifice. The gland, dissected free from connective tissue, was hydrolyzed enzymatically with Pronase[21] and subjected to a preliminary purification

on a Dowex-50 cation exchange resin column[4]. This procedure was used to remove inorganic iodide and lipids from the enzymatic digest.

A small volume (0.5 ml, equivalent to about 4 mg of thyroid) of the purified Pronase digest was placed on the Sephadex LH-20 column for separation of the $^{131}$I-labeled iodoamino acids. Radioactivity in each 4 ml fraction eluted from the column was measured in a well crystal gamma counter. The results of this experiment are given in Fig. 4. The radioactivity in each peak eluted from the column was calculated as a percentage of the total radioactivity from the column. The values were: $T_3 = 2\,\%$, $T_4 = 10\,\%$, MIT $= 32\,\%$, and DIT $= 55\,\%$.

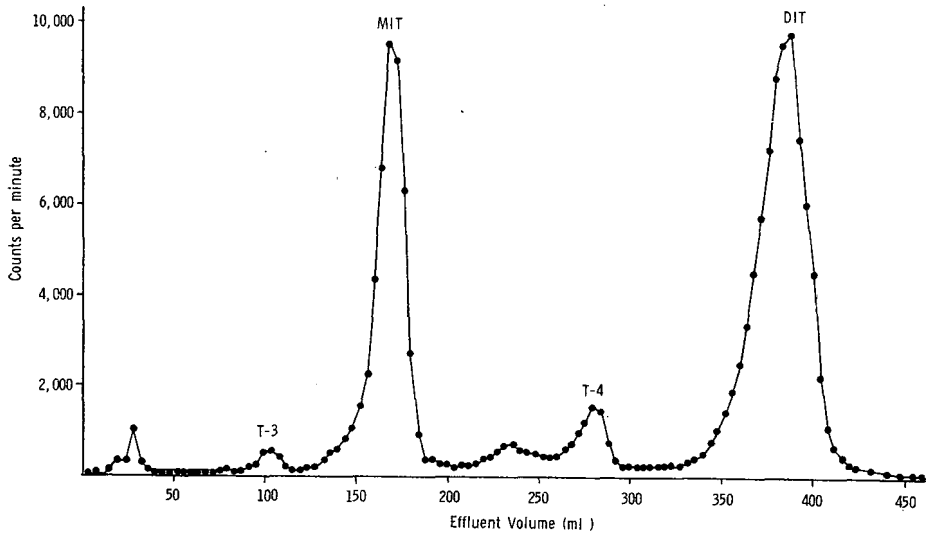


Fig. 4. Elution pattern from a Sephadex LH-20 gel filtration column of $^{131}$I-labeled iodoamino acids from a rat thyroid hydrolysate.

*Recovery of iodoamino acids from Sephadex LH-20 columns*

To evaluate the recovery of iodoamino acids from Sephadex LH-20 columns, the standard solution of each iodoamino acid was assayed for total iodine content. In a series of five replicate column runs for each compound, 0.5 ml of the standard solution was placed on the column bed. Following elution of the sample from the column, fractions composing the peak area were pooled, taken to dryness *in vacuo* and reconstituted in a small volume of 1% methanolic $NH_4OH$. Iodine analyses of the reconstituted pooled peaks from the columns were done and the results are given in Table II. These values, expressed as percentage of iodine in the sample applied to the column, show recovery of from 86.9 to 98.7%.

*Reproducibility of Sephadex LH-20 columns*

In using the LH-20 Sephadex columns for the separation of iodoamino acids, the performance of the column was checked frequently by placing 0.5 ml of the standard iodoamino acid mixture on the column bed and eluting the compounds from the column with the ethyl acetate–methanol–$NH_4OH$ mixture. The absorbance at 297 m$\mu$ for each fraction collected was measured. The sum of the absorbance in the fractions

TABLE II

RECOVERY OF IODOAMINO ACIDS FROM SEPHADEX LH-20 COLUMNS

| *Compound* | *Total iodine applied to column (mg)* | *Total iodine from column (mg)*[a] | *Recovery (%)*[a] |
|---|---|---|---|
| Triiodothyronine | 1.206 | 1.054 ± 0.0188 | 87.4 ± 1.56 |
| Thyroxine | 1.240 | 1.224 ± 0.0562 | 98.7 ± 4.53 |
| Monoiodotyrosine | 0.916 | 0.873 ± 0.0340 | 95.3 ± 3.71 |
| Diiodotyrosine | 1.241 | 1.079 ± 0.0198 | 86.9 ± 1.60 |

[a] Mean ± standard deviation; $n = 5$.

TABLE III

SEPHADEX LH-20 COLUMN REPRODUCIBILITY

| *Compound* | *Absorbance of compound as % of total 297 mμ absorbance in mixture applied to column*[a] | *Absorbance in pooled peak as % of total 297 mμ absorbance eluted from column*[b] |
|---|---|---|
| Triiodothyronine | 26.1 | 25.3 ± 2.05 |
| Thyroxine | 20.2 | 20.9 ± 1.91 |
| Monoiodotyrosine | 27.6 | 25.6 ± 1.18 |
| Diiodotyrosine | 26.0 | 28.1 ± 1.49 |

[a] Calculated from theoretical values for each compound.
[b] Mean ± standard deviation; $n = 24$.

composing the peak for each compound was expressed as a percentage of the total absorbance eluted from the column. The mean percentage distribution of absorbance for 24 different column separations of the standard iodoamino acid mixture is given in Table III. For comparative purposes, the absorbance of each compound expressed as percentage of the total 297 mμ absorbance in the mixture applied to the column also has been listed in Table III. These are theoretical calculations based on the known spectrophotometric absorbance values for each iodoamino acid. The standard deviations for the mean values as well as the agreement with the theoretical distribution values indicate good reproducibility of column performance.

## RESULTS AND DISCUSSION

The spectral absorbance curves of Figs. 1 and 2 and the results in Table I illustrate that evaluation of possibly useful solvent systems for Sephadex LH-20 column separation of iodoamino acids may be done conveniently by monitoring the column effluent spectrophotometrically. It was found that the type of solvent used noticeably affects the absorbance characteristics of iodoamino acids. An increased ethyl acetate concentration in the solvent system decreased $T_4$ absorbance and shifted the peak absorbance to a lower wavelength. Ethyl acetate had little effect on the $T_3$ absorbance characteristics. High concentrations of ethyl acetate in the solvent

system depressed DIT absorbance and caused a small shift in the absorbance peak to a lower wavelength. The 310 mμ absorbance of MIT is sharply reduced by increasing the ethyl acetate content of the solvent mixture.

Observations of the spectral absorbance characteristics in the ethyl acetate–methanol–$NH_4OH$ eluting mixture led to the selection of 297 mμ as the optimal wavelength for the most sensitive monitoring of iodoamino acid elution from Sephadex LH-20 columns. Calculation of the ratio of absorbance at 297 and 310 mμ was helpful in identifying each iodoamino acid as it emerged from the gel bed (Table I). These considerations were useful in preliminary work in which a number of different solvents and combinations were evaluated.

The eluting solvent mixture finally selected is a useful one which permits complete separation of the iodoamino acids on Sephadex LH-20 columns although more effective solvent systems undoubtedly exist. The ethyl acetate–methanol–$NH_4OH$ eluting mixture can be evaporated easily at low temperature *in vacuo*. This property offers the advantage of facilitating recovery of the iodoamino acids in the column effluent free from the eluting solvent mixture.

It was found that increasing the water content of the eluting mixture resulted in a more rapid elution of DIT and $T_4$ from the column bed. When excessive water was included in the eluting solvent mixture, the DIT and $T_4$ peaks were eluted with the MIT peak.

Fig. 3, an elution pattern of a pure iodoamino acid mixture, illustrates the type of separation obtainable. Sharp peaks appear with good separation of each compound from the other components of the iodoamino acid mixture. Each of the iodoamino acids can be recovered uncontaminated by adjacent peaks, as shown by thin-layer chromatographic studies on the pooled fractions representing each elution peak from the column.

The elution sequence of the iodoamino acids from the Sephadex LH-20 column is not directly related to the molecular weight of the compounds although in conventional gel filtration, compounds are usually eluted in decreasing order of molecular size. Retardation of phenolic and other types of aromatic molecules on Sephadex gel filtration columns has been described previously[11,17,18] and it is likely that the adsorptive properties of the dextran gel for iodoamino acids may create a favorable condition for their separation on Sephadex LH-20 columns.

The iodine content of the molecule also appears to be a factor which affects the elution sequence of iodoamino acids. In the case of the thyronine iodoamino acids, the compound with the higher iodine content is retarded on the column. The same effect is seen with the tyrosine derivatives in which MIT is eluted from the column more rapidly than DIT. It is also possible that the elution sequence of the iodoamino acids is a function of the solubility of the compounds in the eluting solvent mixture.

Fig. 3 also indicates that the progress of the Sephadex LH-20 column separation of iodoamino acids can be monitored by chemical analysis of the iodine content in each fraction of the column effluent. Good correspondence exists between the 297 mμ absorbance and the iodine content of the fraction. Spectrophotometric absorbance at 297 mμ is a better procedure for monitoring the column effluent since the procedure is non-destructive and the entire fraction can be recovered for further use. However, effluent concentrations of the iodoamino acids must be in the range of $1.0–1.5 \times 10^{-4}$ *M* or greater in order to obtain meaningful 297 mμ absorbance readings. Iodine

analysis of the column effluent fractions can be done at much lower iodoamino acid concentrations but this results in a loss of some of the sample as a consequence of the analytical procedure.

Radioactivity measurements can also be used to follow the elution of iodo amino acids from the Sephadex LH-20 gel filtration columns if radioactive compounds are available. Separation of biologically labeled iodoamino acids from a $^{131}I$-labeled rat thyroid tissue hydrolysate is shown in Fig. 4 which illustrates the separation of radioactive peaks. The nature of the small radioactive peak between MIT and $T_4$ is unknown but has been observed in several hydrolysates of $^{131}I$-labeled rat thyroids. The percentage distribution of the radioactivity in the iodoamino acid peaks is in good agreement with other reports[4,5].

Good recovery of pure iodoamino acids from Sephadex LH-20 gel filtration columns was obtained (Table II). These recovery values were based on a calculation of the iodine content in the eluted, pooled fractions composing the compound expressed as a percentage of iodine in the pure sample applied to the column bed. Although somewhat lower recovery of DIT and $T_3$ iodine compared to $T_4$ and MIT recovery suggests the possibility of some deiodination of DIT and $T_3$ on the gel bed, no appreciable amount of inorganic iodide has been located in the column effluent during fractionation of these iodoamino acid solutions.

Good reproducibility of Sephadex LH-20 column performance is indicated by the results in Table III. In these studies, the sum of the 297 m$\mu$ absorbance in the fractions composing the peak for each iodoamino acid eluted from the column was expressed as a percentage of the total 297 m$\mu$ absorbance eluted from the column in the complete separation run. The low standard deviation values for the mean percentage distribution of absorbance for each compound from the column and the agreement with the theoretical values in the standard mixture applied to the column indicates that a reproducible separation of iodoamino acids on Sephadex LH-20 columns is obtainable.

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CHROM 4407

# SEPARATION OF CONJUGATED URINARY ESTROGENS ON COLUMNS OF SEPHADEX®

## I. OPTIMIZATION OF CONDITIONS*

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SUMMARY***

Columns of Sephadex G-25, G-15 and G-10 were evaluated under a variety of conditions to determine which provided the most efficient separation of estrogens conjugated with glucuronic acid or sulfuric acid. Elution with 0.01 *M* ammonium formate of a column of Sephadex G-15 to which had been applied a complex mixture of conjugated $^{14}C$-labeled estrogens in urine provided the separation of at least nine generally distinct radioactive fractions. Similar separations have been achieved with solutions of sodium chloride as eluant but ammonium formate is preferred because of its volatility.

## INTRODUCTION

Several years ago BELING[1] used gel filtration on Sephadex G-25 with water as the eluant to separate into two major fractions (Peaks I and II) the conjugated estrogens present in late pregnancy urine. Subsequently, KUSHINSKY AND OTTERNESS[2] found that additional resolution could be achieved by using longer columns, slower flow rates and concentrated urine instead of intact urine. Primarily, the techniques provided a separation of conjugated estrogens from many non-steroidal constituents of urine but very little separation between individual or types of conjugated estrogens. More recently, BRETTHAUER AND GOLICHOWSKI[3] reported the reduction or elimination by judicious choice of eluant of the sorptive properties of Sephadex in the separation

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*** The following abbreviations are used: $E_1$ = estrone = 3-hydroxyestra-1,3,5(10)-trien-17-one; $E_2$ = estradiol = 3,17$\beta$-dihydroxyestra-1,3,5(10)-triene; $E_3$ = estriol = 3,16$\alpha$,17$\beta$-trihydroxyestra-1,3,5(10)-triene; $E_3$-3-GA = estriol-3-glucuronoside = 16$\alpha$,17$\beta$-dihydroxyestra-1,3,5(10)-trien-3-yl-$\beta$-D-glucopyranosiduronate; $E_3$-16-GA = estriol-16$\alpha$-yl-$\beta$-D-glucopyranosiduronate; $E_2$-17-GA = estradiol-17$\beta$-glucuronoside = 3-hydroxyestra-1,3,5(10)triene-17$\beta$-D-glucopyranosiduronate; $E_1$-3-$SO_4$ = estrone-3-sulfate = estra-1,3,5(10)trien-17-on-3-yl sulfate.

of phenylalanine peptides. This report and the availability of Sephadex G-15 and G-10 with the potential for separating compounds with molecular weights in the range of those of conjugated estrogens prompted us to reinvestigate the possibility of separating individual conjugated estrogens on columns of Sephadex.

A major objective of the study described here was to develop a convenient method for separating conjugated estrogens present in urine. Some effort was made to obtain separations based on molecular weight but most of the effort was directed toward developing conditions which provided the maximum number of peaks of radioactivity on plotting the elution pattern of urine containing conjugated metabolites of intravenously administered [4-$^{14}$C]estradiol.

## EXPERIMENTAL

### *Materials and methods*

Reagents and solvents, of analytical grade, were used without additional purification. Sephadex® (G-10, G-15 and G-25; Pharmacia, Piscataway, N.J.) was washed repeatedly with deionized glass-distilled water and decanted free of fine particles. Vials for liquid scintillation counting (18 × 53 mm) were purchased on special order from Acme Vial (Los Angeles, Calif.) or Owens-Illinois (Vineland, N.J.) at a cost of approximately one fifth that of standard, low-potassium vials. The same vials were used as receiving vessels in fraction collectors. Polyethylene vials (No. 6001075; Packard Instruments, Downers Grove, Ill.) were modified by cutting off the tops just above the shoulder (by means of a band saw) and enlarging the opening with a 13/16 in. drill. The polyethylene vials served as re-usable holders or carriers in the liquid scintillation counter[4].

A liquid scintillation counter (Model No. 6860, Nuclear Chicago, Des Plaines, Ill.) was used with settings optimized for the Balance Point Counting Procedure. Quenching was detected by the Channels Ratio Method and correction was made as necessary with internal standards. The scintillation fluid employed is a modification[5] of one described by BRUNO AND CHRISTIAN[6] and is particularly well suited for this type of work because of favorable characteristics with respect to both quenching and solubility.

Methanol (0.5 ml) was added to each vial before the scintillation fluid in order to facilitate solubility of the fractions to be analyzed. Under these conditions the counting efficiency for $^{14}$C was approximately 85 % with a background count of approximately 40 c.p.m. Accuracy to within 5 % was achieved with samples containing radioactivity at least four times that of the background. Background counts have not been subtracted from data shown in the Figures.

[4-$^{14}$C]Estrone, [4-$^{14}$C]estradiol and [4-$^{14}$C]estriol were purchased from Amersham/Searle (Des Plaines, Ill.). [6,7-$^{3}$H]Estradiol-17$\beta$-glucuronic acid, [6,7-$^{3}$H]-estrone, [6,7-$^{3}$H]estradiol, [6,7-$^{3}$H]estriol and [6,7-$^{3}$H]estrone-3-sulfate were purchased from New England Nuclear (Boston, Mass.). Estriol-3-glucuronide was biosynthesized using a preparation of guinea-pig liver[7] and estriol-16$\alpha$-glucuronide was biosynthesized using a preparation of human liver[8].

*Gel filtration.* A dilute slurry of gel is poured into a chromatographic column (Fischer and Porter, Warminster, Pa.) fitted with an extension tube to accommodate the excess liquid used in packing the column. When the bed has settled the excess

eluant is percolated through the column and the extension is removed. Eluant from a reservoir is percolated through the column before adding the charge. The void volume and the inner volume are determined by passing a mixture of Blue Dextran® (Pharmacia) and sodium chloride through the column and determining the elution volumes. If the Blue Dextran does not migrate down the column uniformly the column is backwashed[2] with the aid of a polyethylene or teflon tube of appropriate diameter inserted down through the column to help loosen the bed.

*Preliminary treatment of specimens of urine.* Urine was collected for 48 h from subjects who had been given 10 $\mu$Ci of [4-$^{14}$C]estradiol by i.v. injection. The urine was distilled to dryness *in vacuo* and redissolved in a volume of 0.01 *M* ammonium formate, which resulted in a solution containing at least 10,000 c.p.m./ml.

*Comparison of separation of conjugated estrogens in urine with columns of Sephadex G-25, G-15 and G-10, using water and ammonium formate as eluants*

A 1.0 ml specimen of concentrated urine containing conjugated $^{14}$C-labeled estrogens was applied to columns of Sephadex G-25, G-15 and G-10 and eluted with 0.1 *M* ammonium formate, pH 6.8. In each case the bed volume was 2 × 100 cm. The volume of eluant required to elute the radioactivity from the column of G-15 was greater than that for the column containing G-25. Although the elution volume

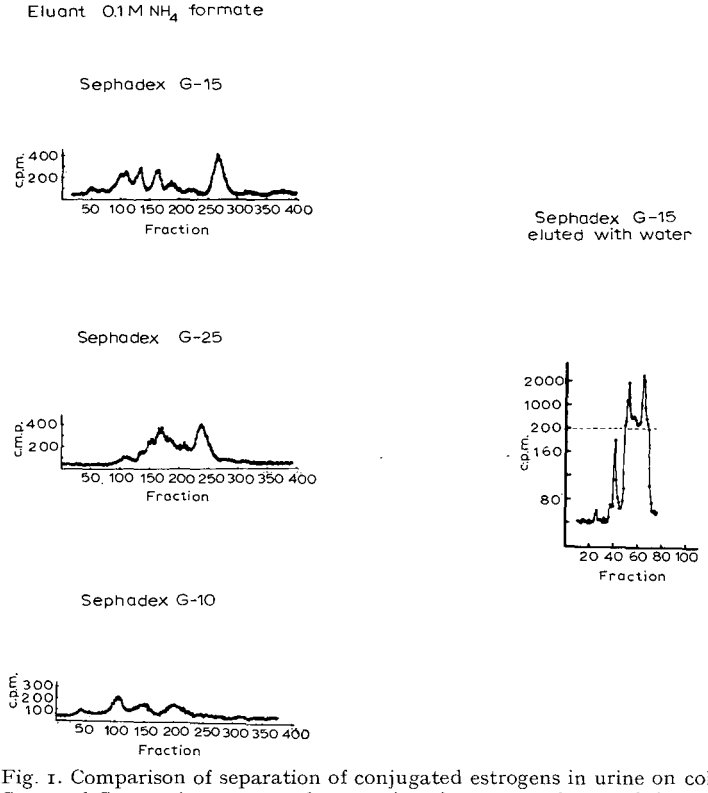



Fig. 1. Comparison of separation of conjugated estrogens in urine on columns of Sephadex G-25, G-15 and G-10, using water and ammonium formate as eluants. Column dimensions: 2 × 90 cm; charge: E.F. urine, day 1 (1.0 ml); volume per fraction: 5.0 ml.

for the column of G-10 was greatest, and in fact represents an incomplete elution because the column was stopped prematurely, the separation clearly was better with G-15 than with G-10 or G-25. The results of these studies are shown in Fig. 1, along with another in which a column of G-25 was prepared and eluted with water instead of with ammonium formate.

*Influence of salt, molarity, and pH on the pattern of elution of conjugated urinary estrogens from columns of Sephadex G-15*

A charge of conjugated urinary estrogens was chromatographed on columns of Sephadex G-15, prepared and eluted with 0.01 *M* and 0.1 *M* solutions of ammonium formate (pH 6.8), sodium chloride and ammonium hydroxide. The elution profile was similar with ammonium formate and sodium chloride as eluant. Ammonium hydroxide as eluant resulted in a smaller elution volume and a generally different pattern of elution compared with those of the other eluants. The results are summarized in Fig. 2. With $10^{-3}$ *M* eluant the resolution was considerably worse than with 0.01 *M* eluant and the recovery of radioactivity from the columns was poor at concentrations below $10^{-3}$ *M*.

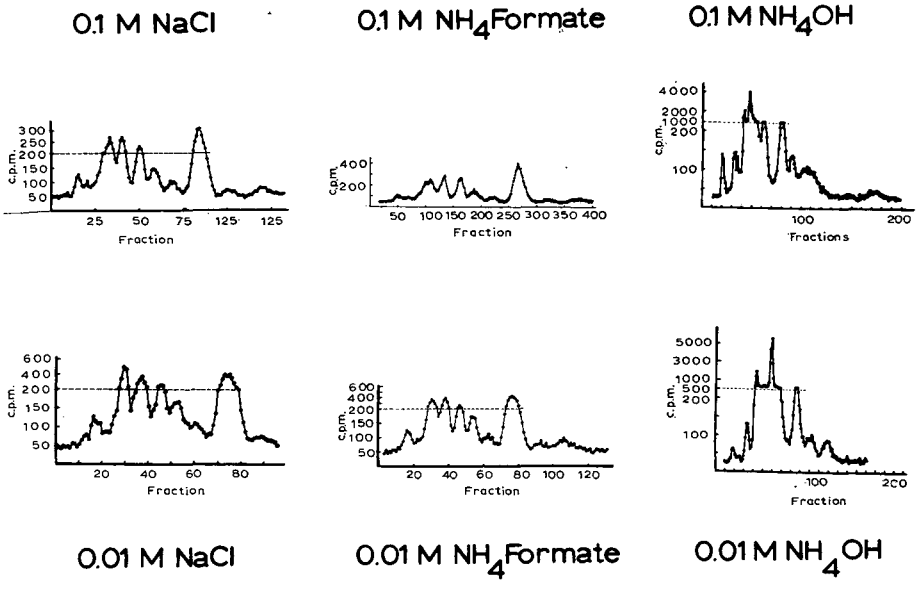



Fig. 2. Influence of salt and molarity on the pattern of elution of conjugated urinary estrogens from columns of Sephadex G-15. Column dimensions: 2 × 90 cm; charge: E.F. urine, day 1 (1.0 ml); volume per fraction: 5.0 ml (0.1 *M* ammonium formate, 0.01 *M* and 0.1 *M* ammonia) or 15 ml (sodium chloride and 0.01 *M* ammonium formate).

Other columns were prepared and eluted with 0.01 *M* ammonium formate, pH 8.7, 6.8 and 3.4. The elution volume and resolution on the columns decreased with an increase in pH. The data are summarized in Fig. 3.

*Attempt to decrease the sorptive effects of Sephadex G-15*

Three columns were packed and eluted with (a) methanol–water (80:20),

(b) pyridine (1.0 *M*), and (c) acetic acid–phenol–water (1:1:1), respectively. In each case the resolution was poor (Fig. 4) and the relatively large elution volumes preclude separations based exclusively on molecular weights. While there may be some separation based on molecular weights superimposed on the separation due to sorptive properties these columns were not pursued further because of the superior separations obtained with ammonium formate as the eluant.

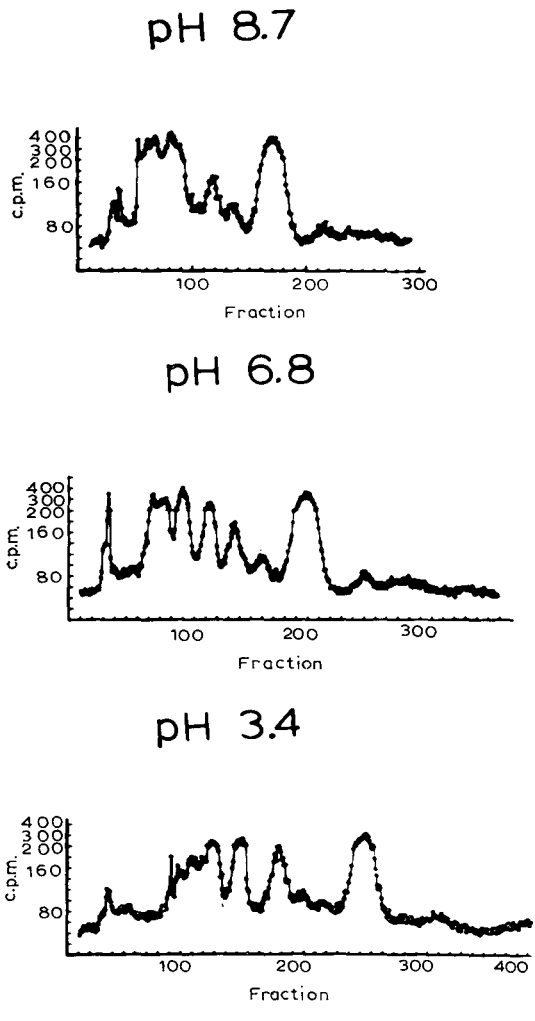
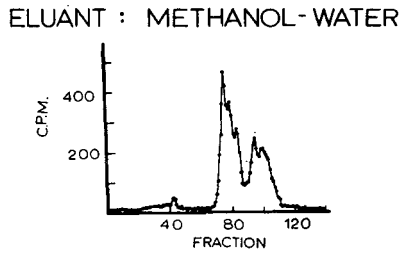
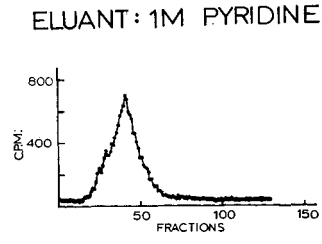



Fig. 3. Influence of pH on the pattern of elution of conjugated urinary estrogens from columns of Sephadex G-15 with ammonium formate. Column dimensions: 0.9 × 100 cm; charge: E.F. urine, day 1 (1.0 ml); eluant: 0.01 *M* ammonium formate (pH as indicated); volume per fraction: 1.7 ml.

*Capacity of columns of Sephadex G-15*

A charge of 0.2, 1.0, 5.0 and 20 ml of concentrated urine was applied to a 0.9 cm × 100 cm column and eluted with 0.01 *M* ammonium formate, pH 6.8. In



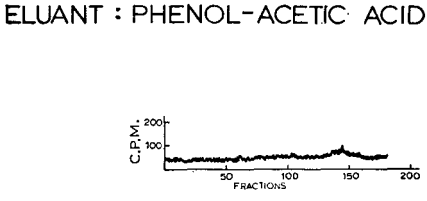




Fig. 4. Attempt to decrease sorptive effects of Sephadex G-15. Phenol-acetic acid–water (1:1:1): charge, 0.3 ml R.B. urine (2–4 h); column dimensions, 0.9 × 97 cm; volume per fraction, 5.0 ml. 1.0 *M* pyridine: charge, 0.3 ml R.B. urine (2–4 h); column dimensions, 0.9 × 45 cm; volume per fraction, 1.0 ml. Methanol–water (80:20): charge, peak I (5.0 ml) from M.S. urine; column dimensions, 2 × 88 cm; volume per fraction, 13.5 ml.

addition, a 1.0 ml charge was diluted to 20 ml with water before application to the column. The results of this study are summarized in Fig. 5. When the volume of the charge was 5 ml or less the resolution was generally constant. With a charge of 20 ml the resolution was considerably worse. The charge of 1.0 ml diluted to 20 ml yielded a similarly poor resolution, suggesting that when the volume of the charge approaches that of the void volume (27 ml) a significant deterioration of the separation occurs.

*Partial separation of conjugated urinary estrogens from other substances on the basis of weights and radioactivity*

A specimen of concentrated urine containing $^{14}C$-labeled conjugated estrogens was applied to a column of Sephadex G-15 (0.9 × 100 cm) and eluted with 0.01 *M* ammonium formate. An aliquot (one fifth) was removed for measurement of radioactivity and for determination of the weight of the residue on evaporation to dryness.

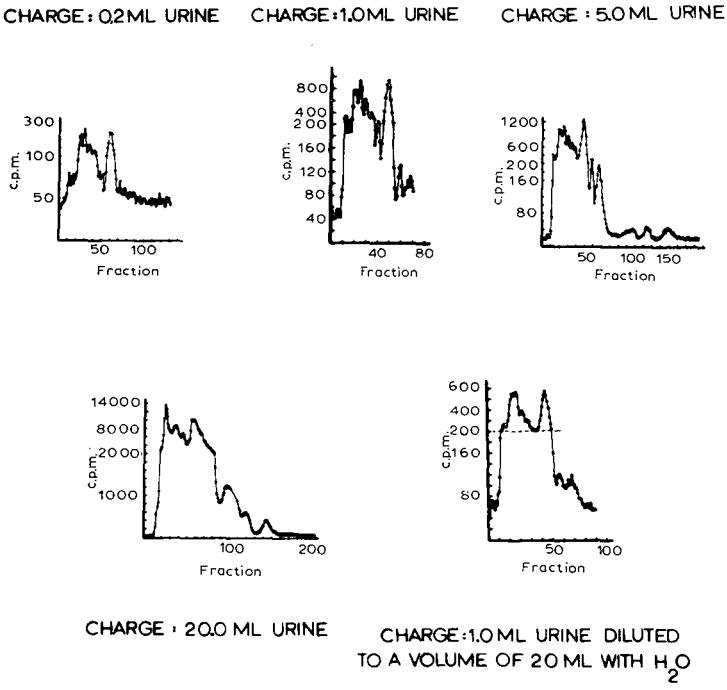



Fig. 5. Capacity of columns of Sephadex G-15. Column dimensions: 0.9 × 100 cm; charge: E.F. urine, day 1; eluant: 0.01 *M* ammonium formate; volume per fraction: 5.0 ml.

The results of this study, summarized in Fig. 6, show that most of the components which contribute significant weight are eluted before the conjugated estrogens.

*Elution volume of standards and reproducibility of columns*

In preliminary studies the elution volume of several conjugated estrogens applied to columns of Sephadex G-15 was found to vary considerably in successive

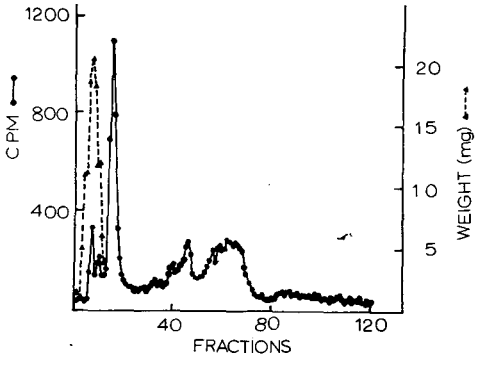



Fig. 6. Partial separation of conjugated urinary estrogens from other substances on the basis of weights and radioactivity. Column dimensions: 0.9 × 100 cm; charge: R.B. urine (4.0 ml); eluant: 0.01 *M* ammonium formate; volume per fraction: 4.3 ml.

experiments, with an apparent increase in the elution volume each time a charge was applied to the same column. To characterize the phenomenon more thoroughly two columns (0.9 × 95 cm) were prepared with fresh Sephadex G-15. A charge containing [4-$^{14}$C]estriol-16-glucuronide and [6,7-$^{3}$H]estradiol-17-glucuronide was applied to each column and eluted with 0.01 *M* ammonium formate. Using the same columns and more of the same charge the study was repeated three times. The results are summarized in Table I. Although the elution volumes increased in successive columns the ratio of the elution volumes remained constant.

TABLE I

CHANGE IN ELUTION VOLUME (ml) ON REPEATED USE OF COLUMNS OF SEPHADEX G-15 FOR SEPARATION OF PURE COMPOUNDS

Column dimensions: 0.9 × 100 cm; eluant: 0.01 *M* ammonium formate, pH 6.8; volume per fraction: 5 ml.

| *Run* | *Column 1* | | | *Column 2* | | |
|---|---|---|---|---|---|---|
| | *$E_3$-16-GA* | *$E_2$-17-GA* | *$E_3$-16-GA/ $E_2$-17-GA* | *$E_3$-16-GA* | *$E_2$-17-GA* | *$E_3$-16-GA/ $E_2$-17-GA* |
| 1 | 375 | 510 | 0.74 | 345 | 480 | 0.72 |
| 2 | 400 | 535 | 0.75 | 400 | 530 | 0.75 |
| 3 | 480 | 660 | 0.73 | 425 | 560 | 0.76 |
| 4 | > 1000 | > 1000 | | > 1000 | > 1000 | |

A large batch of used Sephadex G-15 was washed with ten bed volumes each of 1.0 *M* pyridine, 0.2 *M* formic acid, water and 0.01 *M* ammonium formate. Reference standards of [6,7-$^{3}$H]estrone-3-sulfate, [6,7-$^{3}$H]estradiol-17-glucuronide, [4-$^{14}$C]-estriol-16-glucuronide and [4-$^{14}$C]estriol-3-glucuronide were added in various combinations to different specimens of urine or to 0.01 *M* ammonium formate and the elution volumes were determined with columns containing pyridine-washed Sephadex. The results of this study, summarized in Table II, show no clear trend for a change

TABLE II

ELUTION VOLUME OF CONJUGATED ESTROGENS WITH COLUMNS OF PYRIDINE-WASHED, "USED" SEPHADEX G-15

Column dimensions: 0.9 × 47 cm; eluant: 0.01 *M* ammonium formate, pH 6.8; volume of urine, if used: 1.0 ml; volume per fraction: 3 ml.

| *Condition of Sephadex*[a] | *Type of urine added to charge* | *Elution volume (ml)* | | | |
|---|---|---|---|---|---|
| | | *$E_3$-3-GA* ($^{14}C$) | *$E_3$-16-GA* ($^{14}C$) | *$E_2$-17-GA* ($^{3}H$) | *$E_1$-3-$SO_4$* ($^{3}H$) |
| Used | Female (SH) | 66 | | | 248 |
| Used | Female (RB) | 64 | 168 | 226 | 226 |
| Used | None | 82 | | | 246 |
| Used | None | | 162 | | 242 |
| Used | None | 61 | 143 | 214 | 214 |
| Re-used | Pregnancy (MH) | | 161 | | 240 |
| Used | Pregnancy (FA) | | 163 | | |
| Re-used | None | | 152 | | 222 |
| Re-used | None | | 163 | | |

[a] See text for definition of "Used".

in elution volume in the presence or absence of urine or on re-use of the same column. It appears that pre-treatment with pyridine stabilized the system but the extent of stabilization, if real, is not known. Of the four standards tested only estrone-3-sulfate and estradiol-17-glucuronide did not separate on the columns. Two of the peaks in the elution profile of $^{14}$C-labeled conjugated estrogens in urine correspond in elution volume with those of estradiol-17-glucuronide and estriol-16-glucuronide (Fig. 7). A mixture of $^{3}$H-labeled estrone, $^{3}$H-labeled estradiol, $^{3}$H-labeled estriol and concentrated urine containing conjugated metabolites of $^{14}$C-labeled estradiol was applied to a column of Sephadex G-15 (0.9 × 100 cm) and eluted with 0.01 *M* ammonium formate. The $K_{av}$ value[9] for the free estrogens was 22.4 or greater while the $K_{av}$ value for the conjugated estrogens was between 0.97 and 10.1, thereby affording complete separation of these classes of compounds.

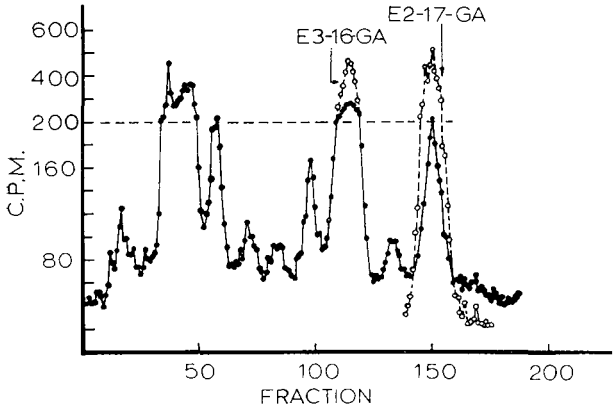



Fig. 7. Elution of synthetic radioactive standards in the presence of conjugated $^{14}$C-labeled estrogens in urine. Column dimensions: 0.9 × 100 cm; charge: E.F. urine, day 2 (0.5 ml) with and without designated standards; eluant: 0.01 *M* ammonium formate; volume per fraction: 4.5 ml.

*Effect of height of column on efficiency of separation*

A comparison was made among the separations achieved on columns of 50, 100 and 200 cm length, of a mixture of conjugated $^{14}$C-labeled estrogens in urine. The results, summarized in Fig. 8, show clearly that the best separation was obtained with the longest column.

## DISCUSSION

As part of a continuing search for better methods to separate conjugated estrogens in urine, columns of Sephadex G-25, G-15 and G-10 were evaluated under a variety of conditions to determine which provided the most efficient resolution. Particular attention was paid to the selection of conditions not likely to cause structural changes in the conjugated estrogens during the process of separation. The use of some pH values away from that at neutrality was done primarily to help understand the system and for the sake of completeness.

Nominally, gel filtration implies a technique whereby separations based on differences in molecular weight are achieved. In practice separation based exclusively

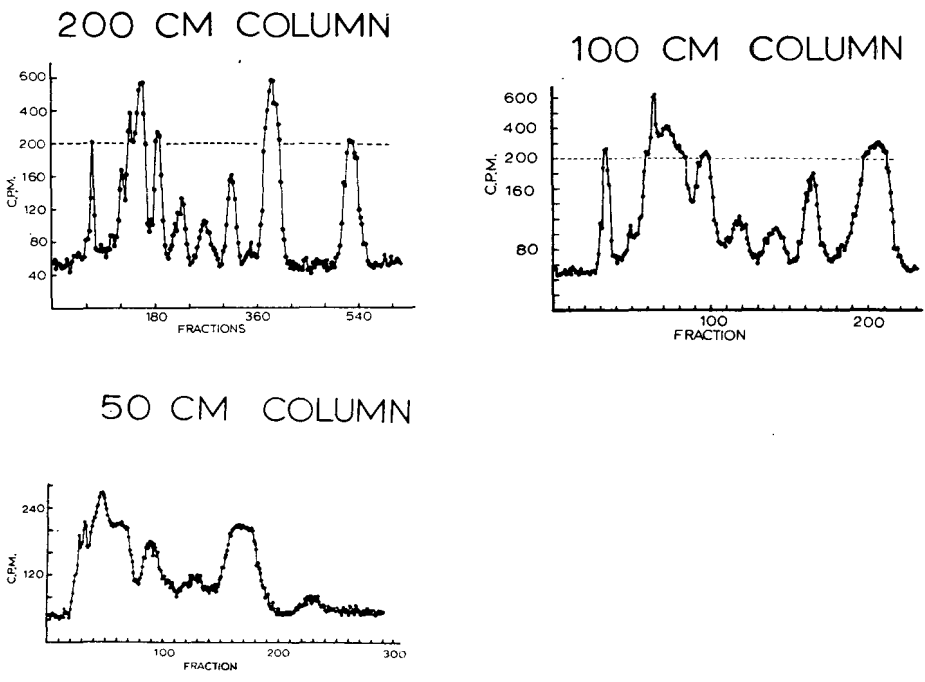



Fig. 8. Effect of height of column on efficiency of separation. Column diameter: 0.9 cm; charge: E.F. urine, day 2 (0.5 ml); eluant: 0.1 *M* ammonium formate; volume per fraction: 5.0 ml.

on molecular weight is not always achieved. Sorptive properties of the gel which are influenced by both pH and the ionic environment cause the retention of many types of relatively small molecules with potentially ionizable functional groups[3,10–17]. With some types of compounds which generally are sorbed in an unpredictable manner, careful choice of eluant has led to separations based primarily on molecular weights[3,18–20]. Other types of compounds have been resolved very effectively by empirically determining the solvent system which provides the best separation, regardless of the molecular weights. Primarily the latter approach was employed in the present investigation.

Clearly the use of some eluants such as dilute solutions of ammonium formate or sodium chloride improved the separations compared with that obtained with water as the eluant. The precise reason for the improvement is not known. From the $K_{av}$ values it is apparent that under all conditions used in this paper the separations were a result of factors other than differences in molecular weight. BELING[1] reported that pure conjugated estrogens are eluted from columns of Sephadex G-25 at close to void volume with water as the eluant. Adding even a small quantity of salt or buffer to the charge but still using water as the eluant caused the conjugated estrogens to be eluted considerably beyond the void volume. A possible explanation is that the inner matrix of the gel acts as a statically charged cage which repels the conjugated estrogens and that the charge is dissipated in the presence of inorganic ions. The changes in the sorptive properties of Sephadex with changes in the pH, in the ionic environment

or in the dielectric constant are not readily explained by classical concepts of adsorption or ion exchange. It appears that the separations which are achieved are the result of the interaction of a number of factors.

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CHROM. 4408

# SEPARATION OF CONJUGATED URINARY ESTROGENS ON COLUMNS OF SEPHADEX®

## II. SEQUENTIAL SEPARATIONS*

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SUMMARY***

Columns of DEAE-Sephadex were used to separate conjugated estrogens in urine (metabolites of [4-$^{14}$C]estradiol, administered intravenously) and reference standards. A scheme has been devised whereby many of the conjugated estrogens in urine may be separated by means of a sequence of three chromatographic procedures on columns of Sephadex G-25, DEAE-Sephadex and Sephadex G-15. The composite elution profile contains at least eighteen distinct peaks of radioactivity.

## INTRODUCTION

Many procedures for the separation of conjugated estrogens have been described. BELING[2] used gel filtration on Sephadex to separate conjugated estrogens in late pregnancy urine into two major fractions. SMITH AND KELLIE[3] showed that urate retards the elution of peak II of BELING and that peak I with appropriate pre-treatment can be subdivided into peaks IA and IB on a second column of Sephadex. Additional separation on columns of ECTEOLA cellulose and Celite 535 and by paper and thin-layer chromatography led to the indirect identification of a number of conjugated estrogens in late pregnancy urine. HAHNEL and co-workers[4,5] used DEAE-Sephadex with a gradient of sodium chloride to separate a number of estrogen sulfates and glucuronides in pregnancy urine and some reference standards. Evidence for the identity of some of the separated estrogens was based on further fractionation by means of columns of alumina and by TLC and PC. JIRKU AND LEVITZ[6] separated a number of biliary and urinary metabolites of [6,7-$^{3}$H]estrone-[$^{35}$S]sulfate by means of the following techniques: partition chromatography on columns of Celite, adsorption chromatography on alumina, gel filtration on Sephadex G-10 with aqueous methanol as eluant, high-voltage electrophoresis, PC and TLC.

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*** See preceding paper (ref. 1) for abbreviations used here.

DICZFALUSY and co-workers[7] have made extensive use of countercurrent distribution to identify conjugated estrogens in many biological fluids and in a variety of clinical and experimental situations. As more information is acquired on the large number of conjugated estrogens present in urine it becomes increasingly apparent that convenient and efficient methods are needed which will permit the separation of all the conjugated estrogens in urine. One ultimate objective of such endeavors is to assess the potential clinical significance of each of the conjugated estrogens which is present.

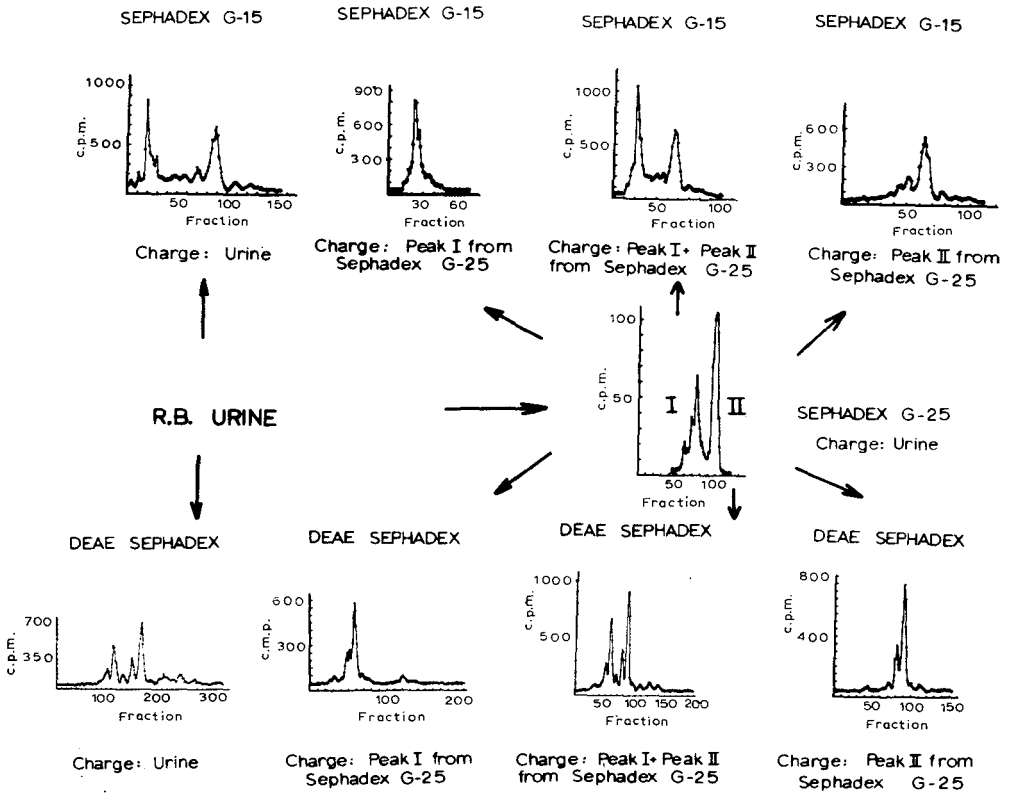


Fig. 1. Separation of conjugated estrogens in R. B. urine on columns of Sephadex G-15 and DEAE-Sephadex with and without prior separation on Sephadex G-25. Sephadex G-15 (0.9 × 100 cm): charge, peak I (0.5 ml) + peak II (0.5 ml) from Sephadex G-25 (R. B. urine); eluant, 0.01 *M* ammonium formate; volume per fraction, 5 ml. Sephadex G-25 (6.5 × 180 cm): charge, R. B. urine (150 ml); eluant, distilled water; volume per fraction, 15 ml. DEAE-Sephadex (0.9 × 100 cm): charge, peak I (0.5 ml) + peak II (0.5 ml) from Sephadex G-25 (R. B. urine); eluant, 1.0 *M* ammonium formate; volume per fraction, 3.3 ml. DEAE-Sephadex (0.9 × 100 cm): charge, peak II (0.5 ml) from Sephadex G-25 (R. B. urine); eluant, 1.0 *M* ammonium formate; volume per fraction, 3.3 ml. DEAE-Sephadex (0.9 × 100 cm): charge, peak I (0.5 ml) from Sephadex G-25 (R. B. urine); eluant, 1.0 *M* ammonium formate, volume per fraction, 3.3 ml. Sephadex G-15 (0.9 × 100 cm): charge, peak I (0.5 ml) from Sephadex G-25 (R. B. urine); eluant, 0.01 *M* ammonium formate; volume per fraction, 5.0 ml. Sephadex G-15 (0.9 × 100 cm): charge, R. B. urine (1.5 ml); eluant, 0.01 *M* ammonium formate; volume per fraction, 2.5 ml. Sephadex G-15 (0.9 × 100 cm): charge, peak II (0.5 ml) from Sephadex G-25 (R. B. urine); eluant, 0.01 *M* ammonium formate; volume per fraction, 5.0 ml. DEAE-Sephadex (0.9 × 100 cm): charge, R. B. urine (1.5 ml); eluant, 1.0 *M* ammonium formate; volume per fraction, 1.7 ml.

In the previous paper of this series[1] we described a series of studies in which conjugated estrogens in urine (metabolites of $^{14}C$-labeled estradiol, administered i.v.) and reference standards were separated on columns of Sephadex G-10, G-15, and G-25 using various solutions of salts as eluants. In this paper are described studies on the separation of conjugated estrogens in urine using columns of DEAE-Sephadex A-25, alone and in conjunction with columns of Sephadex G-25 and Sephadex G-15. The resolving power of each system was evaluated in the light of the number of peaks of radioactivity obtained, the ease of execution of the column, the capacity of the columns and the amenability of the eluates to further fractionation. As a result of these studies a convenient scheme has been devised whereby a sequence of these chromatographic separations on columns of Sephadex G-25, DEAE-Sephadex and Sephadex

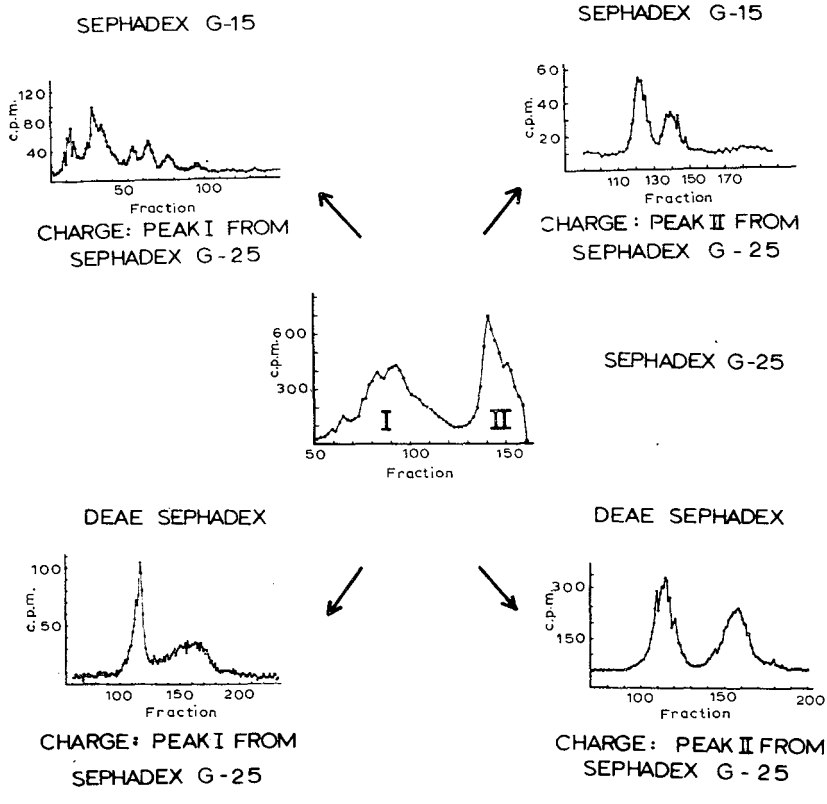


Fig. 2. Separation of conjugated estrogens in M. S. urine on columns of Sephadex G-15 or DEAE-Sephadex after prior separation on Sephadex G-25. DEAE-Sephadex (1.8 × 50 cm): charge, peak I (5.0 ml) from Sephadex G-25 (M. S. urine); eluant, linear gradient of 0.125 *M* ammonium formate (600 ml) to 0.30 *M* ammonium formate (600 ml); volume per fraction, 5.0 ml. DEAE-Sephadex (1.8 × 50 cm): charge, peak II (10 ml) from Sephadex G-25 (M. S. urine); eluant, linear gradient of 0.125 *M* ammonium formate (600 ml) to 0.30 *M* ammonium formate (600 ml); volume per fraction, 4.2 ml. Sephadex G-15 (2 × 100 cm): charge, peak I (5.0 ml) from Sephadex G-25 (M. S. urine); eluant, 0.2 *M* ammonium formate (pH 6.6); volume per fraction, 15 ml. Sephadex G-15 (2 × 100 cm): charge, peak II (5.0 ml) from Sephadex G-25 (M. S. urine); eluant, 0.2 *M* ammonium formate; volume per fraction, 3.5 ml. Sephadex G-25 (6.5 × 155 cm): charge, M. S. urine, day 2 (150 ml); eluant, distilled water; volume per fraction, 20 ml.

G-15 provides at least eighteen distinct peaks of radioactivity. The scheme and some of the experiments which led to its development are described in this paper.

## EXPERIMENTAL

*Comparison of separation of conjugated urinary estrogens using columns of Sephadex G-15, Sephadex G-25 and DEAE-Sephadex*

An aliquot of urine containing $^{14}C$-labeled conjugated estrogens (R. B. urine, day 1 plus 2) was applied to a column of DEAE-Sephadex and eluted with 1.0 *M* ammonium formate. Other aliquots were applied to columns of Sephadex G-15 and Sephadex G-25 and eluted with 0.01 *M* ammonium formate and water, respectively. The elution patterns are shown in Fig. 1. At least nine distinct peaks are discernible in the elution patterns for DEAE-Sephadex and Sephadex G-15 compared with only two major peaks for Sephadex G-25. Peaks I and II from Sephadex G-25 were concentrated *in vacuo* and applied individually and together to columns of Sephadex G-15 and DEAE-Sephadex. The results of this study are summarized in Fig. 1. The overall resolution obtained with and without preliminary fractionation on Sephadex G-25

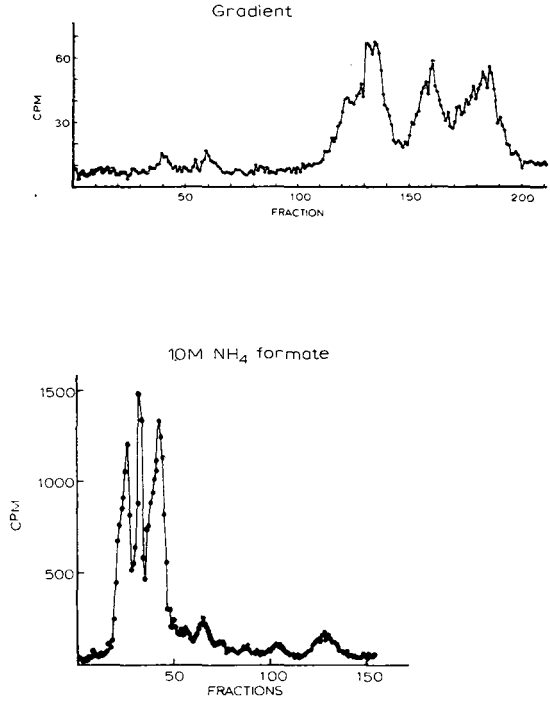


Fig. 3. Comparison of separation on a column of DEAE-Sephadex eluted with 1.0 *M* ammonium formate or with a gradient of ammonium formate. Upper part: linear gradient of 0.125 *M* ammonium formate (600 ml) to 0.25 *M* ammonium formate (600 ml): column dimensions, 1.8 × 50 cm; charge, M. S. urine (10 ml); volume per fraction, 10.5 ml. Lower part: 1.0 *M* ammonium formate; column dimensions, 0.9 × 100 cm; charge, M. S. urine (1.5 ml); volume per fraction, 4.8 ml.

was not significantly different. There is a general trend for the components of peak I to be eluted from columns of DEAE-Sephadex and Sephadex G-15 before those of peak II.

In another study a different specimen of urine (M.S.) was separated on a column of Sephadex G-25 and peaks I and II were applied separately to columns of Sephadex G-15 and DEAE-Sephadex. The eluant used with the DEAE-Sephadex column was a linear gradient of ammonium formate from 0.125 *M* to 0.30 *M* (pH 6.6). Under these conditions (gradient) there is a clear advantage to preliminary separation on Sephadex G-25 since the elution volumes of peaks I and II are nearly identical on DEAE-Sephadex. The results are summarized in Fig. 2. In another study a comparison was made between the elution pattern from columns of DEAE-Sephadex (M. S. urine) using 1.0 *M* ammonium formate and a gradient of 0.125 *M* to 0.25 *M* ammonium formate (pH 6.6). The results are shown in Fig. 3. Although the elution volumes were larger when a gradient was used the resolution obtained is not significantly better than that found without the gradient and the gradient is less convenient to use.

*Separation of conjugated urinary estrogens from the bulk of other substances on the basis of weight and radioactivity*

A specimen of concentrated urine containing $^{14}C$-labeled conjugated estrogens was applied to a column of DEAE-Sephadex (0.9 × 100 cm) and eluted with 1.0 *M* ammonium formate. Aliquots were removed for determination of the weight of the residue on evaporation to dryness. The results of this study, summarized in Fig. 4, show that there is almost a complete separation of the major weight-contributing urinary components from the conjugated estrogens. The relatively poor separation is a reflection of the large volume of eluant per fraction.

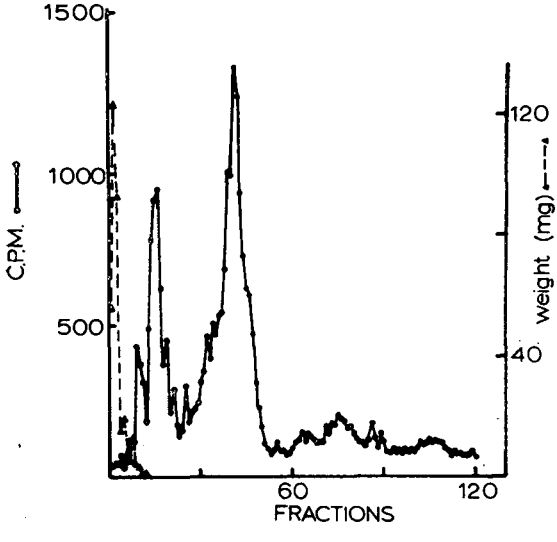


Fig. 4. Separation of conjugated urinary estrogens from the bulk of other substances on the basis of weight and radioactivity using a column of DEAE-Sephadex (0.9 × 100 cm). Charge, R. B. urine (4.0 ml); eluant, 1.0 *M* ammonium formate; volume per fraction, 3.0 ml.

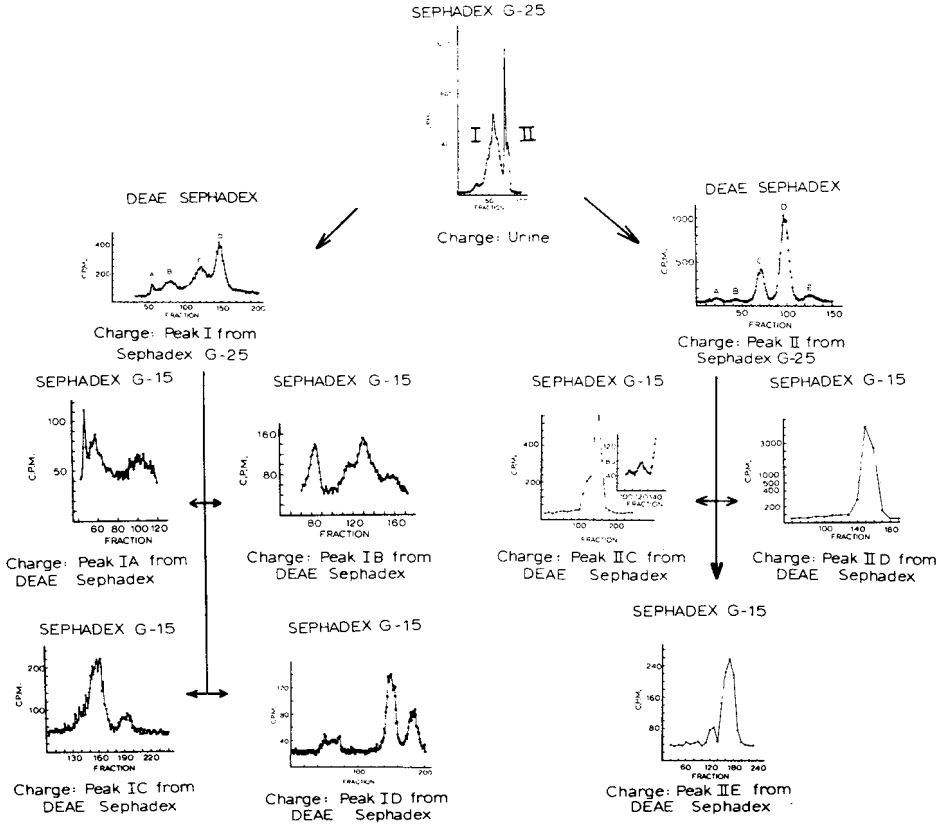


Fig. 5. Sequential separation of conjugated urinary estrogens on columns of Sephadex G-25, DEAE-Sephadex and Sephadex G-15. Sephadex G-25 (5 × 106 cm); charge, E. F. urine, days 2 + 3 (200 ml); eluant, distilled water; volume per fraction, 16 ml. DEAE-Sephadex (4 × 156 cm): charge, peak I (90 ml) from Sephadex G-25; eluant, 1.0 *M* ammonium formate; volume per fraction, 16 ml. DEAE-Sephadex (4 × 100 cm): charge, peak II (36 ml) from Sephadex G-25; eluant, 1.0 *M* ammonium formate; volume per fraction, 16 ml. Sephadex G-15 (2 × 200 cm): charge, peaks from DEAE-Sephadex (dry Sephadex G-15 added to solution of charge; mixture evaporated to dryness and applied to top of column); eluant, 0.01 *M* ammonium formate; volume per fraction, 13 ml.

*Sequential separation of conjugated urinary estrogens on columns of Sephadex G-25, DEAE-Sephadex and Sephadex G-15*

A specimen of concentrated urine containing $^{14}C$-labeled conjugated estrogens was applied to a column of Sephadex G-25 and eluted with distilled water. The two primary fractions which were obtained (peaks I and II) were reapplied, after reduction of the volume *in vacuo*, to columns of DEAE-Sephadex and eluted with 1.0 *M* ammonium formate. Peak I was resolved into four fractions (peaks IA, IB, IC and ID) and peak II into five fractions (peaks IIA, IIB, IIC, IID and IIE). Each of these fractions (except IIA and IIB which were too small for further fractionation) was applied to a column of Sephadex G-15 and eluted with 0.01 *M* ammonium formate. The elution pattern for each column is shown in Fig. 5. The scheme provided separation of at least eighteen different components as evidenced by the presence of eighteen dis-

crete or nearly discrete peaks. The pattern of IIC on Sephadex G-15 shows one peak and a pronounced shoulder which emerges as two distinct peaks when individual fractions were plotted (see inset) rather than pools of every ten fractions.

*Elution volume from DEAE-Sephadex of reference standards in the presence and absence of urine*

Four radioactive reference standards, estriol-3-glucuronide, estriol-16-glucuronide, estradiol-17-glucuronide and estrone-3-sulfate were applied to columns of DEAE-Sephadex in different combinations in the presence and in the absence of concentrated urine. The standards in the absence of urine were applied to columns with dimensions 0.9 × 50 cm and those in the presence of urine to columns with dimensions 0.9 × 100 cm. The elution volumes, summarized in Table I, are all different and each

TABLE I

ELUTION VOLUME OF REFERENCE STANDARDS IN THE PRESENCE AND ABSENCE OF URINE

Column, DEAE-Sephadex; eluant, 1.0 *M* ammonium formate. Reference standard: $E_3$-3-GA = estriol-3-glucuronide; $E_3$-16-GA = estriol-16-glucuronide; $E_2$-17-GA = estradiol-17-glucuronide; $E_1$-3-$SO_4$ = estrone-3-sulfate.

| *Column No.* | *Column dimensions (cm)* | *Charge* | *Elution volume (ml)* | | | |
|---|---|---|---|---|---|---|
| | | | *$E_3$-3-GA* | *$E_3$-16-GA* | *$E_2$-17-GA* | *$E_1$-3-$SO_4$* |
| 1 | 0.9 × 50 | Standards only | 91 | | | |
| 2 | 0.9 × 50 | Standards only | | 118 | | |
| 3 | 0.9 × 50 | Standards only | | 120 | 147 | |
| 4 | 0.9 × 50 | Standards only | 88 | 127 | | 307 |
| 5 | 0.9 × 50 | Standards only | | 127 | | |
| 6 | 0.9 × 50 | Standards only | 80 | | 151 | |
| 7 | 0.9 × 50 | Standards only | | | 160 | |
| 8 | 0.9 × 50 | Standards only | | | 156 | |
| 9 | 0.9 × 100 | Standards + urine | | 258 | | 838 |
| 10 | 0.9 × 100 | Standards + urine | | | 296 | 1100 |
| 11 | 0.9 × 100 | Standards + urine | | | | 800 |

of the standards is completely or nearly completely separated from the others. The elution volumes of the glucuronides were reproducible and were unaffected by the presence or absence of urine and appear to be related to the length of the column. There was considerably more variability in the elution volume of estrone sulfate but the elution volumes were consistently higher than those for any of the glucuronides studied.

DISCUSSION

Gel filtration on Sephadex G-25 with water as the eluant has been used to separate conjugated estrogens in urine from many other substances such as salts and inhibitors of β-glucuronidase which are present in urine[1]. The technique in addition separates completely and efficiently two major groups of conjugated estrogens on the basis of properties which are not likely to lead to separation by other chromatographic means. The capacity of the system, the relative ease of execution of the technique

and the benignity of the conditions are particularly favorable for its utilization as the first step in a scheme for the separation or isolation of conjugated estrogens in urine.

In the scheme which we have developed gel filtration on Sephadex G-25 is used as the first step and is followed by further fractionation on columns of DEAE-Sephadex and Sephadex G-15. It is apparent that under the conditions employed the factors operative in the separation of the conjugated estrogens are at least partially unique for each of the chromatographic systems which were used. Were this not so there would have been no additional resolution on sequential chromatographic manipulations. One specific example of a pair of standards which were separated on DEAE-Sephadex but not on Sephadex G-15 is estradiol-17-glucuronide and estrone-3-sulfate.

The separations achieved on columns of DEAE-Sephadex provided nine peaks of radioactivity from the components in the two peaks which were applied to the columns. In other studies, not described in the experimental section, sodium chloride was found to yield separations similar to those obtained with ammonium formate. The elution volumes were about 15 % smaller with sodium chloride as the eluant and with the DEAE-Sephadex in the chloride form. However, since sodium chloride is more difficult to remove from the eluates once the separations have been achieved, ammonium formate was the preferred eluant.

In the preceding paper of this series[1] separation of conjugated urinary estrogens on columns of Sephadex G-15 under a variety of conditions was described. In this paper the technique was extended for use in separating further fractions already separated on columns of Sephadex G-25 and DEAE-Sephadex. As a result of the combined use of all three systems at least eighteen peaks of radioactivity were apparent in the elution patterns. It seems likely that additional fractionation by TLC on silica gel would provide sufficient additional fractionation for the essentially complete separation of the major conjugated estrogens in urine for structural analysis and ultimately for potential clinical use. Although no definitive identifications have been made there is evidence based on elution volumes for the presence in urine of estradiol-17-glucuronide, estriol-16-glucuronide and estriol-3-glucuronide but no evidence for the presence of estrone-3-sulfate. Based on the elution volume of estrone-3-sulfate from columns of DEAE-Sephadex (Table I) it is possible that in the sequence of chromatographic columns where eighteen peaks of radioactivity were obtained the estrogen sulfates were not eluted from the columns and that additional peaks would have been present if the elution had been prolonged to include the small amount of radioactivity which remained on the columns.

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# GEL CHROMATOGRAPHY OF INOSITOL POLYPHOSPHATES AND THE AVIAN HAEMOGLOBIN–INOSITOL PENTAPHOSPHATE COMPLEX

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SUMMARY

Gel chromatography of inositol polyphosphates has shown that a substantial anion exclusion effect exists which is diminished, but not necessarily eliminated, by concentrations of eluant electrolyte up to 2 *M*. Under the appropriate conditions of ionic strength and pH, gel chromatography provides a useful adjunct to the established fractionation procedures for inositol phosphates. It has been successfully used to demonstrate that myoinositol 1,3,4,5,6-pentaphosphate forms a strong ionic association with pigeon and chicken haemoglobins.

## INTRODUCTION

Established methods for the fractionation of inositol polyphosphates include anion-exchange chromatography, paper chromatography, paper electrophoresis and fractional precipitation. These procedures have been reviewed by COSGROVE[1]. In addition moving paper electrophoresis[2] and thin-layer chromatography[3] have now been described.

Separations of a series of compounds by chromatography on a cross-linked dextran gel (Sephadex) have been reported for inorganic polyphosphates[4] and polynucleotides[5]. The present study records the distribution coefficients[6] ($K_d = (V_e - V_0)/V_i$, where $V_e$ is the elution volume of the solute, $V_0$ is the void volume and $V_i$ the volume of the stationary phase) of various inositol polyphosphates and some other reference compounds on a number of Sephadex gels. The variation of these $K_d$ values with ionic strength and pH is examined. The application of gel chromatography to the elucidation of the ionic nature of the pigeon and chicken haemoglobin-inositol pentaphosphate complex is described.

## EXPERIMENTAL

### *Materials*

The Sephadex (Pharmacia AB) gels and columns used are shown in Table I.

TABLE I

SEPHADEX GELS AND COLUMNS

| *Sephadex grade* | *Mesh size* ($\mu$) | *Column parameters*[a] | | | | *Sample* (*ml*) | *Fraction size* (*ml*) |
|---|---|---|---|---|---|---|---|
| | | *Length* (*cm*) | $V_t$ (*ml*) | $V_0$ (*ml*) | $V_i$ (*ml*) | | |
| G-200 | 140–400 | 36 | 1020 | 290 | 700 | 10 | 16.5 |
| G-100 | 40–120 | 78 | 770 | 260 | 470 | 8 | 14.0 |
| G-50 | 100–300 | 58 | 710 | 270 | 410 | 6 | 14.0 |
| G-25 | 100–300 | 52 | 500 | 210 | 250 | 5 | 16.5 |
| G-15 | 40–120 | 46 | 300 | 120 | 130 | 4 | 7.0 |

[a] For pH $< 13$ and eluant salt concentrations $\leqslant 2$ *M*.

The reference compounds, their methods of preparation or source and the symbols used in this paper are: myoinositol hexaphosphate[7], $IP_6$; myoinositol tripyrophosphate[7], $\pi_3\emptyset$; chicken blood myoinositol pentaphosphate[7], $IP_5$(CB); alkaline hydrolysis myoinositol pentaphosphate[2], $IP_5$(OH); myoinositol tetraphosphate[8], $IP_4$; myoinositol triphosphate[8], $IP_3$; myoinositol diphosphate[8], $IP_2$; myoinositol monophosphate[9], $IP_1$; glycerol phosphoryl myoinositol[10], GPI; myoinositol*, I; orthophosphate*, $P_i$; inorganic pyrophosphate*, $PP_i$; adenosine monophosphate*, AMP; adenosine triphosphate*, ATP; phosvitin[11], Pv; triethyl phosphate*, $Et_3P$; and fructose*, Fr.

Washed red blood cells were lysed and the haemolysates obtained by the DRABKIN procedure[12].

*Methods*

Columns were prepared and packed after equilibration with eluant, in accordance with manufacturers' directions. Lithium chloride was used as the eluant electrolyte to facilitate recovery of the ethanol-insoluble lithium phosphates[13].

The sample (200–300 $\mu$g P) was layered direct onto the filter paper or gauze disc that covered the gel surface and suitable fractions were collected. All runs were made at room temperature (20–27°). $Kd$ values were calculated by use of Blue Dextran 2000 (Pharmacia AB) and tritiated water to determine $V_0$ and $V_i$, respectively.

Aliquots from each fraction were analysed for total phosphorus[14]. Inorganic phosphorus was determined by the ascorbic acid method[15], fructose was measured by the anthrone method[16] and inositol by periodate oxidation[17]. Haemoglobin absorbance was measured at 577 nm.

The location of the inositol pentaphosphate in haemolysate fractions was determined by precipitation of the acid-soluble phosphorus as the barium salt with subsequent electrophoresis of the anions in 0.1 *M* oxalate at pH 1.5 as previously described[7].

## RESULTS AND DISCUSSION

*Anion exclusion*

The anomalous behaviour of ionic compounds of low molecular weight eluted

* Commercial products.

with water on Sephadex gels has been appreciated since the initial study of GELOTTE[6]. The nature of this effect has been greatly clarified by the work of NEDDERMEYER AND ROGERS[18], who found that irregular elution profiles of anions became symmetrical at 0.01 *M* eluant salt concentration and showed that the asymmetric profiles in distilled water could be attributed to a Donnan anion exclusion effect.

We have investigated the effect of eluant salt concentrations above that which is necessary to produce symmetrical profiles. Fig. 1 shows the results for the eluant salt molarities in the range 0.01 *M*–5 *M* for inorganic phosphate, pyrophosphate, myoinositol hexaphosphate and triethyl phosphate on Sephadex G-25.

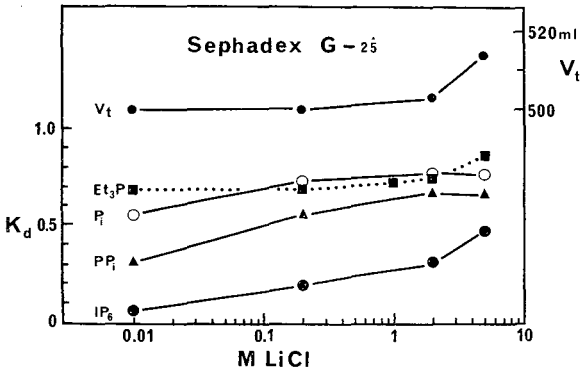


Fig. 1. Variation of the gel column ($V_t$) and distribution coefficients ($K_d$) of triethyl phosphate ($Et_3P$), inorganic phosphate ($P_i$), inorganic pyrophosphate ($PP_i$) and myoinositol hexaphosphate ($IP_6$) with the molarity of the eluant electrolyte (lithium chloride).

The uncharged molecules triethyl phosphate (Fig. 1), fructose (Table II) and myoinositol (Table III) have virtually constant $K_d$ values at eluant electrolyte concentrations of 0.01–0.2 *M*. By contrast the anions of Fig. 1 each show a marked increase in $K_d$ in this range. Iodide and sulphate anions[19] as well as polythymidylates[5] show analogous behaviour. Such results suggest the continued existence of a significant anion exclusion effect above the 0.01 *M* limit suggested by NEDDERMEYER AND ROGERS[18].

With eluant electrolyte concentrations above 2 *M* the situation becomes more complex. Both inorganic phosphate and pyrophosphate have constant $K_d$ values, but the myoinositol hexaphosphate and triethyl phosphate show increased $K_d$ values. It is not possible to decide between the anion exclusion and adsorption mechanisms in the case of the highly charged hexaphosphate but adsorption appears to be the only feasible explanation for the behaviour of the uncharged triethyl phosphate. Interpretation of the results is further complicated by an unexpected swelling of the gel matrix ($V_t$) above 2 *M* (Fig. 1).

The results from a variety of reference compounds on a range of Sephadex gels are listed in Table II and from this table it is again apparent that, for anionic compounds which are detectably excluded from the gel pores ($K_d < 0.8$) at 0.01 *M* eluant salt concentration, there is invariably an increase in $K_d$ at higher salt concentrations.

Fig. 2 shows the effect of pH on $K_d$ values in the presence of 0.1 *M* eluant salt. In the pH range 3–11 where the ionisation of carboxyl groups of the gel matrix and

TABLE II

DISTRIBUTION COEFFICIENTS OF REFERENCE COMPOUNDS ON SEPHADEX GELS

| *Grade* | *Eluant molarity* | *Distribution coefficients* ($K_d$) ± 0.05 | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *Pv* | $IP_6$ | $PP_i$ | *ATP* | $P_i$ | *Fr* | *AMP* |
| G-15 | 0.01 | 0.00 | 0.00 | 0.04 | 0.16 | 0.26 | 0.63 | 0.74 |
| | 0.20 | 0.00 | 0.06 | 0.25 | 0.35 | 0.40 | 0.65 | 1.00 |
| G-25 | 0.01 | 0.00 | 0.06 | 0.31 | 0.60 | 0.56 | 0.75 | 0.91 |
| | 0.20 | 0.00 | 0.19 | 0.56 | 0.69 | 0.73 | 0.75 | 1.06 |
| | 2.00 | 0.00 | 0.30 | 0.67 | 0.93 | 0.77 | 0.81 | 1.30 |
| | 5.00 | 0.00 | 0.47 | 0.65 | 0.88 | 0.76 | 0.79 | 1.15 |
| G-50 | 0.01 | 0.00 | 0.45 | 0.81 | 0.91 | 0.91 | 0.89 | 1.14 |
| | 0.10 | 0.00 | 0.61 | 0.83 | 0.93 | 0.89 | 0.93 | 1.14 |
| G-100 | 0.01 | 0.03 | 0.79 | 0.97 | — | 0.97 | 0.97 | 1.07 |
| G-200 | 0.01 | 0.21 | 0.90 | — | — | 0.98 | 0.98 | — |

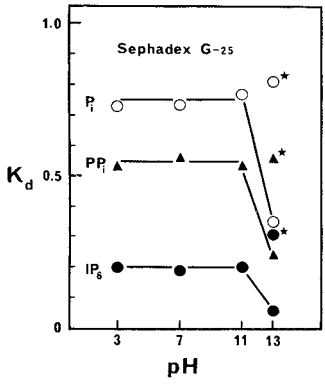


Fig. 2. Variation of the distribution coefficient ($K_d$) of inorganic phosphate ($P_i$), inorganic pyrophosphate ($PP_i$) and myoinositol hexaphosphate ($IP_6$) with the pH of the eluant electrolyte (0.1 *M* lithium chloride). Values with asterisks at pH 13 refer to an electrolyte concentration of 2 *M*.

the second dissociation of phosphate groups occurs, the incorporation of 0.1 *M* eluant salt is sufficient to suppress the expected fall in $K_d$ values caused by the repulsion of these groups.

However, under strongly alkaline conditions (pH 13), where the ionisation of hydroxyl groups in the carbohydrate gel matrix is significant, a marked anion exclusion effect becomes apparent with a striking reduction in $K_d$ values for the phosphates. If the concentration of lithium chloride in the eluant at pH 13 is now increased to 2 *M*, the $K_d$ values marked by asterisks in Fig. 2 are obtained and the anion exclusion is again substantially repressed.

*Molecular size*

DETERMANN[20] has comprehensively reviewed the various empirical relationships between $K_d$ values and molecular size parameters. In general there is an orderly

lowering of $K_d$ values as a homologous series is ascended. Table III shows such an inverse relationship between the degree of phosphorylation of the inositol ring and the $K_d$ value on two grades of Sephadex at two eluant salt concentrations. Similar results have been obtained by HOHN AND SCHALLER[5] for oligonucleotides and by OHASHI *et al.*[4] for inorganic phosphates.

TABLE III

DISTRIBUTION COEFFICIENTS OF MYOINOSITOL POLYPHOSPHATES ON SEPHADEX G-50 AND G-25

| *Grade* | *Eluant molarity* | *Distribution coefficients* ($K_d$) $\pm$ 0.05 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $IP_6$ | $\pi_3\phi$ | $IP_5(OH)$ | $IP_5(CB)$ | $IP_4$ | $IP_3$ | $IP_2$ | $IP_1$ | *GPI* | *I* |
| G-25 | 0.01 | 0.06 | 0.06 | 0.06 | 0.06 | 0.12 | 0.19 | 0.31 | 0.44 | 0.53 | 0.75 |
| | 0.20 | 0.19 | 0.19 | 0.19 | 0.19 | 0.25 | 0.33 | 0.44 | 0.59 | 0.56 | 0.75 |
| G-50 | 0.01 | 0.45 | 0.48 | 0.56 | 0.54 | 0.57 | 0.64 | 0.70 | 0.86 | 0.97 | 0.83 |
| | 0.10 | 0.61 | 0.61 | 0.62 | 0.64 | 0.68 | 0.75 | 0.79 | 0.86 | 0.97 | 0.86 |

Because both anion exclusion effects and molecular size contribute to the observed $K_d$ values in Table III no attempt has been made to establish a simple logarithmic relationship between $K_d$ and a molecular size parameter alone. Nevertheless by using a suitably calibrated column with appropriate eluant salt concentrations an unknown member of the series could be identified.

On the other hand Table III also indicates that Sephadex gel chromatography is unlikely to be of much value for separation of mixtures of closely related members of the inositol polyphosphate series which are more satisfactorily resolved by ion-exchange[1,21] and electrophoretic procedures[2].

*Application*

The major inositol polyphosphate which can be isolated from chicken blood by acidic protein precipitants is now known to be myoinositol 1,3,4,5,6-pentaphosphate[7]. Unsuccessful attempts to isolate the pentaphosphate by ultrafiltration of haemolysed red blood cells suggested that covalent or ionic linkages to some macromolecule may be present. A similar observation has been made for diphosphoglyceric acid in mammalian red blood cell haemolysates by SOLOMON *et al.*[22].

In order to examine whether or not the inositol polyphosphate was covalently or ionically linked to a macromolecule, both pigeon and chicken haemolysates were examined by gel chromatography.

The results for chicken blood haemolysate on Sephadex G-50 are shown in Fig. 3. A similar pattern was obtained for pigeon haemolysate. At 0.1 *M* eluant salt concentration and pH 7 (Fig. 3a), conditions of ionic concentration and pH which approximate to those of the red blood cell, there is a strong association of the inositol pentaphosphate with the pigeon and chicken haemoglobins. At high pH (Fig. 3b) or high eluant salt concentrations (Fig. 3c) the complex is dissociated, which indicates a strong ionic association is present under the conditions of Fig. 3a.

On Sephadex G-50 with 0.1 *M* eluant and pH 7 (Fig. 3a) the haemoglobin–inositol pentaphosphate complex is coincident with the void volume. To confirm that

the polyphosphate was associated with the haemoglobin and not some other protein, the haemolysate was examined on Sephadex G-100 under the same elution conditions. The inositol pentaphosphate and haemoglobin peaks were found to be coincident with $K_d = 0.31$.

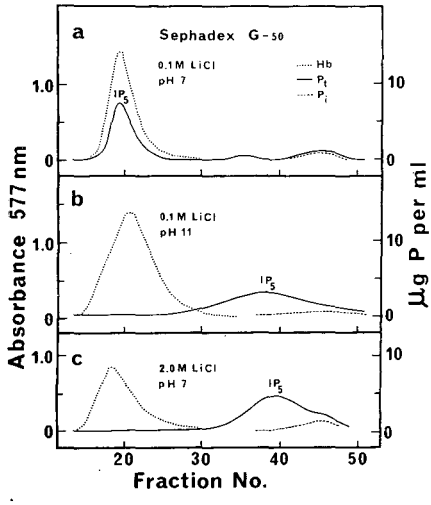


Fig. 3. (a) Assocation of the inositol pentaphosphate and chicken haemoglobin peaks at pH 7 and 0.1 *M* eluant electrolyte concentration. (b) Dissociation of the inositol pentaphosphate and haemoglobin peaks at high pH. (c) Dissociation of the complex by 2 *M* eluant electrolyte at neutral pH.

BENESCH AND BENESCH[23] have shown that the association of organic polyphosphates with both mammalian and avian haemoglobins has a considerable effect upon the oxygen affinity of the haemoglobin. In the case of diphosphoglyceric acid it is only the deoxyhaemoglobin which binds strongly to the phosphate under approximately physiological conditions. However the spectrum of the chicken and pigeon haemoglobin–polyphosphate complexes with pronounced bands at 538 and 577 nm indicates clearly that the inositol pentaphosphate binds strongly to the oxyhaemoglobin, and the binding of oxygen and inositol pentaphosphate to haemoglobin is not a mutually exclusive process as in the case of diphosphoglyceric acid[23].

ACKNOWLEDGEMENTS

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CHROM. 4385

# A GRADIENT METHOD FOR THE COUNTER-CURRENT SEPARATION OF ALKALOIDS USING A HEAVY ORGANIC PHASE

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SUMMARY

A method is proposed for the separation of all the components of a complex mixture of acids or bases by counter-current distribution with progressive variation of the pH. The function that controls the double distribution and dissociation equilibrium in the case of a weak base was investigated. Since the separation depends on the difference in the product of the dissociation constant $K_b$ and the distribution coefficient $K_r$, two possibilities are considered: the use of a lower buffer phase and an upper organic phase whose composition is progressively changed in such a way as to vary $K_r$, the bases being eluted in order of increasing $K_r \cdot K_b$, and the use of a lower organic phase with an upper buffer phase whose pH is varied from neutrality to increasingly acidic values in such a way as to extract the alkaloids in order of decreasing $K_r \cdot K_b$. With the aid of this second process, it was possible to isolate the known nine alkaloids of *Strychnos nux-vomica*, *i.e.* strychnine, $\alpha$- and $\beta$-colubrines, brucine, pseudostrychnine, pseudobrucine, icajine, vomicine, and novacine, as well as four others that had not been discovered previously. The separation of strychnine and brucine on the basis of the difference in the product $K_r \cdot K_b$ was also examined. Where this product was equal (*e.g.* for colubrines and brucine between chloroform and water), partial modification of one phase (addition of 35 % of ethyl acetate) leads to non-proportional changes in the $K_r$ values, and so permits separation.

## INTRODUCTION

The separation of basic and acidic substances by distribution at a fixed pH has had, and still has, important applications both in chromatography and in counter-current processes. Both methods have been improved by techniques (some of them patented) involving the use of different pH values. Thus columns have been prepared with zones of absorbent saturated with buffers to give a variation of the pH from the top to the bottom of the column[1], and counter-current distributions (CCD) have been carried out with buffers whose pH values varied from one test tube to the next as the stationary phase[2].

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These methods are very laborious and are not readily reproducible with the various materials to be separated. Moreover, the results are unsatisfactory in the separation of complex mixtures. They are therefore rarely used. CCD with movement of both phases in opposite directions is also rarely used, since it does not allow the the separation of mixtures of more than two or three substances simultaneously[3].

In the present article, we describe a simple method by which complex mixtures of alkaloids (more than thirteen in the case of *Strychnos nux-vomica*) can be separated with a CCD apparatus consisting of 200 tubes. We believe that this method is useful not only from the point of view of large-scale preparations but also for the isolation of the "minor alkaloids", which are present in quantities of up to 1 % with respect to the predominant components. Due to the fact that this method allows the isolation of substances present in very small quantities it may be used in structural investigations by means of mass spectrometric and X-ray methods.

Preparative chromatography does not offer acceptable solutions, both because of the difficulty of recovery and because of the frequency with which a single spot corresponds to more than one substance (the mixture of thirteen alkaloids from *Strychnos nux-vomica* chromatographed in equal quantities gives at most five or six spots depending on the mobile phase used).

In using CCD for the separation of alkaloids, it is necessary to take into account, and even to use, the difference in their p$K_a$ values (*e.g.*, strychnine 7.27, pseudobrucine 5.60 (ref. 4)), to which there should correspond a pH that differs from one case to another for a suitable distribution between immiscible solvents. Thus by variation of the two phases in the CCD apparatus, various authors[5] have separated one or two substances at a time from a mixture. The use of aqueous stationary phases having various pH values with a lighter organic phase has not given positive results[2].

In this work we used a heavier organic stationary phase, which remained the same throughout the process, with a buffered aqueous phase, the pH of which was varied discontinuously from neutrality to increasingly acidic values; this system gave selective extraction of substances from the stationary organic phase. The substances could thus be collected on emerging from the last test tube of the CCD apparatus as the pH in the reservoir tube was changed. To obtain a better understanding of the interdependence of the factors on which this method depends, the general validity of which is shown by the results obtained, it is useful to consider the following theoretical treatment. The double dissociation and distribution equilibrium, which was first discussed by IRVING AND WILLIAMS[6], is outlined below from the analytical point of view.

## THEORY

An alkaloid, as a weak base, satisfies the dissociation equilibrium

$$K_b = \frac{[OH^-]\,[B^+]}{[B\,OH]} \qquad (1)$$

According to the distribution law, the concentrations of the undissociated form in the two phases are related as follows:

$$K_r = \frac{[B\,OH]}{c_o} \qquad (2)$$

where $c_o$ = concentration in the organic phase.

The total quantity ($T$) of alkaloids is the sum of the following three terms:

$$T = c_o V_o + [\mathrm{B\,OH}] \cdot V_w + V_w \cdot [\mathrm{B^+}]$$

where $V_o$ = volume of the organic phase and $V_w$ = volume of water.

The product $[\mathrm{B\,OH}] \cdot V_w$ may be neglected in the sum, since $[\mathrm{B\,OH}] \ll [\mathrm{B^+}]$ and $[\mathrm{B\,OH}] \ll c_o$. This is because the alkaloid in the aqueous phase is mainly in the form of salt in the pH range in question. We can thus write:

$$T = c_o V_o + V_w \cdot [\mathrm{B^+}] \tag{3}$$

From eqns. (1) and (2) we find

$$[\mathrm{B\,OH}] = c_o \cdot K_r = \frac{[\mathrm{OH^-}]\,[\mathrm{B^+}]}{K_b} \tag{4}$$

From eqn. (3), if $V_w = V_o$ (volumes of the aqueous and organic phases equal), division by $V_o$ gives:

$$\frac{T}{V_o} = c_o + [\mathrm{B^+}]$$

where $T/V_o$ is the analytical concentration, which is constant, and which we shall denote by $c_t$ = total moles/l (volume of one phase). Substitution of $c_t = c_o + [\mathrm{B^+}]$ in eqn. (4) gives:

$$\frac{[\mathrm{B^+}]}{c_t - [\mathrm{B^+}]} = \frac{K_r \cdot K_b}{[\mathrm{OH^-}]} = \frac{K_r \cdot K_b [\mathrm{H^+}]}{K_w}$$

Taking logarithms and changing the sign we obtain:

$$\log \frac{c_t - [\mathrm{B^+}]}{[\mathrm{B^+}]} = \log \frac{K_w}{K_r \cdot K_b} + \mathrm{pH}$$

The expression $\log \left\{(c_t - [\mathrm{B^+}])/[\mathrm{B^+}]\right\}$ (logarithm of the reciprocal of the extraction coefficient) is thus a linear function of the pH. Fig. 1 shows the lines for the unitary values of the term $\log \left\{K_w/(K_r \cdot K_b)\right\}$ and those of strychine and of brucine. The slope is 1.

It can be seen that the concentration in the aqueous phase is equal to that in the organic phase, $c_t = 2[\mathrm{B^+}]$ and hence $\log \left\{(c_t - [\mathrm{B^+}])/[\mathrm{B^+}]\right\} = 0$, when $\mathrm{pH} = \log \left\{(K_r \cdot K_b)/K_w\right\}$.

Fig. 2 shows the concentration in the aqueous phase as a function of the pH corresponding to unitary values of the expression $\log \left\{K_w/(K_r \cdot K_b)\right\}$.

For a base having a given $K_b$, $K_r$ can be varied by variation of the organic solvent (one phase is always water). Two bases having the same value of the product $K_r \cdot K_b$ can be separated by modification of the organic phase, since the changes in the distribution coefficients $K_r$ of the two bases are generally not proportional, as can be seen in the separation of colubrines and of brucine (see later). Two alkaloids

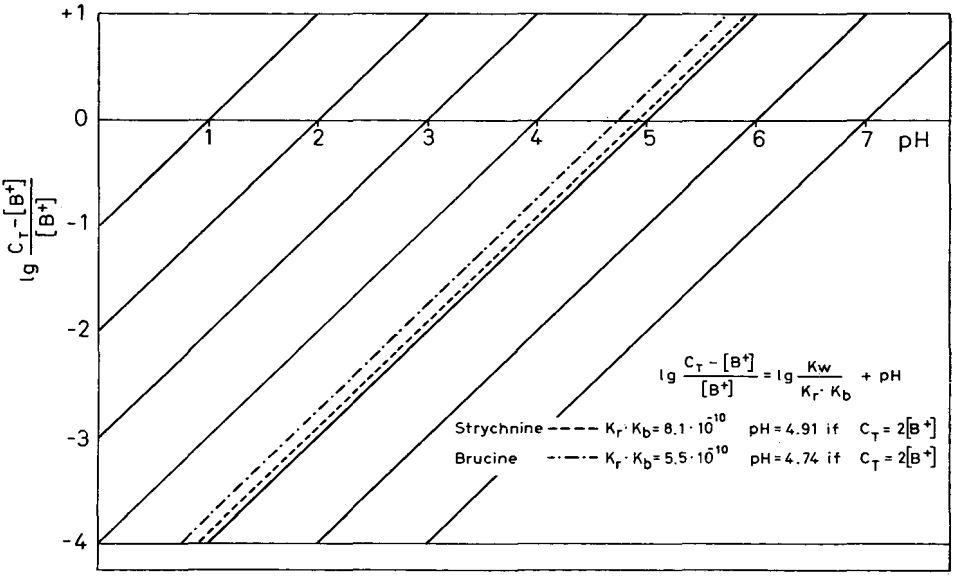


Fig. 1. Logarithm of the reciprocal of the extraction coefficient $c_t$ — $[B^+]/[B^+]$ as a function of the pH corresponding to unitary values of the term log $\{K_w/(K_r \cdot K_b)\}$.

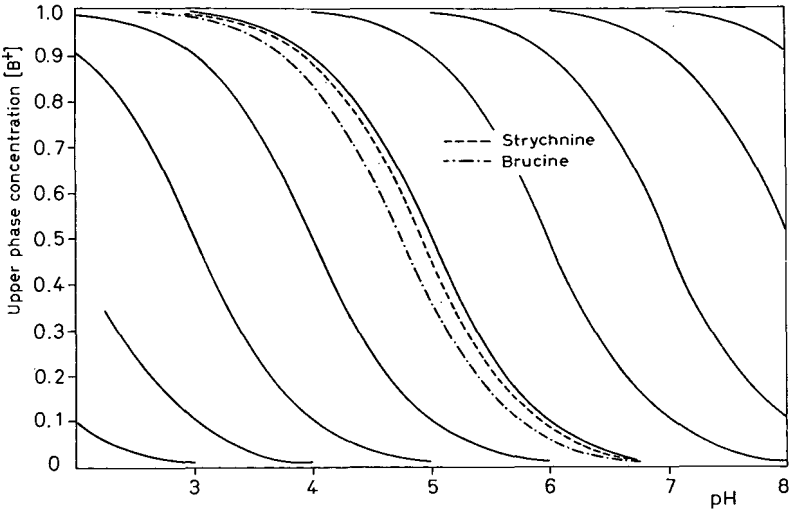


Fig. 2. Concentration of the aqueous phase $[B^+]$ as a function of the pH corresponding to unitary values of the term log $\{K_w/(K_r \cdot K_b)\}$.

having very similar values of $K_b$ can also be separated in a similar manner.The $K_b$ value of strychnine, for example, is 8% higher than that of brucine ($K_b$ of strychnine $10^{-6}$, $K_b$ of brucine $9.2 \times 10^{-7}$) (ref. 7). The value of $K_r$ between water and chloroform, as found spectrophotometrically by the present authors, is $8.1 \times 10^{-4}$ for strychnine and $6.0 \times 10^{-4}$ for brucine; the product $K_r \cdot K_b$ is therefore $8.1 \times 10^{-10}$

for strychnine and $5.52 \times 10^{-10}$ for brucine. The possibility of separation is thus further increased in the distribution, since the product $K_r \cdot K_b$ of strychnine is 32 % higher than that of brucine. Figs. 1 and 2 show the curve for strychnine and brucine.

Since the pH range must be between 7 and 3 to ensure that the alkaloids do not decompose over long periods and to guarantee the absence of emulsions, the product $K_r \cdot K_b$ must be between $10^{-7}$ and $10^{-11}$ for the countercurrent separation. Alkaloids having low basicities must therefore have a relatively high $K_r$* (unfavorable organic solvent) while a very small $K_r$ (very favorable organic solvent) is necessary for more basic alkaloids.

Complex mixtures of alkaloids can be dealt with in two ways. A stationary aqueous phase having a constant pH is used with a lighter organic phase, which is progressively varied in such a way as to decrease $K_r$ and to increase the extractive capacity for the various alkaloids, which are thus carried along in order of increasing $K_r \cdot K_b$. Alternatively, a heavier stationary organic phase may be used with a buffered aqueous phase, the pH of which varies from neutrality to increasingly acidic values in the course of the separation, so that the alkaloids are transported in order of decreasing $K_r \cdot K_b$.

The second of these procedures requires a very small volume of the solvent forming the stationary phase, which may be chloroform, dichloromethane, carbon tetrachloride, or mixtures with other solvents in a wide range of compositions such that the specific gravity is always higher than 1. The first procedure can be carried out by varying the percentages of two or more organic solvents, but requires a large volume of these solvents, and so presents the problem of their recovery and fractionation. Moreover, the lighter organic phase, by the nature of the possible constituents (ether, benzene, ethyl acetate), generally gives higher $K_r$ values than chloroform, and so makes it necessary to work in the alkaline pH range; this presents problems of stability, solubility, and emulsion formation.

In the countercurrent fractionation of the thirteen alkaloids of *Strychnos nux-vomica* in the CCD apparatus, chloroform (2 l) was used as the stationary phase with phosphate buffer of pH between 6.5 and 3.3 as the eluent. The total volume of the eluent was about 80 l for 8000 passes (200-stage Craig apparatus). Eleven clearly separated fractions were obtained, and ten of these contained only one substance each. The only mixed fraction consisted of $\alpha$- and $\beta$-colubrines and brucine, and this was further fractionated with chloroform–ethyl acetate (65:35) as the stationary phase and phosphate buffer of pH 6.2 as the mobile phase.

It is interesting to note that the $K_r$ values of these alkaloids, which had identical $K_r \cdot K_b$ values in chloroform, show distinct and non-proportional changes even when the composition of the organic phase is only partly altered.

The order in which the components appear in the CCD separation between chloroform and buffer is as follows. The alkaloids of the normal series appear first in order of increasing molecular weight (strychnine, $\alpha$- and $\beta$-colubrines, and brucine, the last three appearing together); these are followed by the alkaloids of the pseudo series (pseudostrychnine and pseudobrucine), and finally by those of the N-methyl pseudo series, again in order of increasing molecular weight (icajine, vomicine, and novacine). In the subsequent separation with chloroform–ethyl acetate (65:35) and

* Note that $K_r$ is the ratio of the concentrations of the undissociated form in the aqueous and in the organic phases.

buffer, the above order is reversed, *i.e.* brucine appears first, and is followed by $\beta$- and then by $\alpha$-colubrine.

## EXPERIMENTAL

The alkaloid mixture fractionated was the mother liquor from the crystallization of strychnine sulphate, and was supplied by Sandoz of Milan (to whom we wish to express our thanks).

The mixture was made alkaline with dilute sodium carbonate and extracted with chloroform. CCD apparatus used was a 200-stage Post apparatus, volume 10/10 ml. The aqueous phase was a 0.2 *M* phosphate buffer saturated with chloroform. Below pH 4.5, 0.2 *M* monopotassium phosphate was used with hydrochloric acid. The chloroform used as the lower phase contained 0.5 % of ethanol to prevent phosgene formation. Since water extracts ethanol from chloroform, a small quantity (< 0.1 %) of ethanol had to be added to the aqueous solution used.

Five grams of the mixture of free bases were dissolved in 16 ml of chloroform, and the solution was filtered and introduced into the first two tubes of the Craig apparatus. The volume of aqueous phase introduced in each pass was about 10 ml; the agitations for each stage were 10–12. The decantation time gradually decreased from more than 5 min at the beginning to 30–40 sec. The upper phase was collected as it left the 199th test tube. The contents of the tubes (one out of every ten) were periodically chromatographed on Silica Gel $HF_{256+366}$ with benzene–ethyl acetate–diethylamine (7:2:1) as the solvent and examined in UV light. The Dragendorff reagent can be used only after the complete removal of diethylamine at 100°. The pH of the buffer was varied from its initial value of 6.5 as indicated on the abscissa of Fig. 3, the

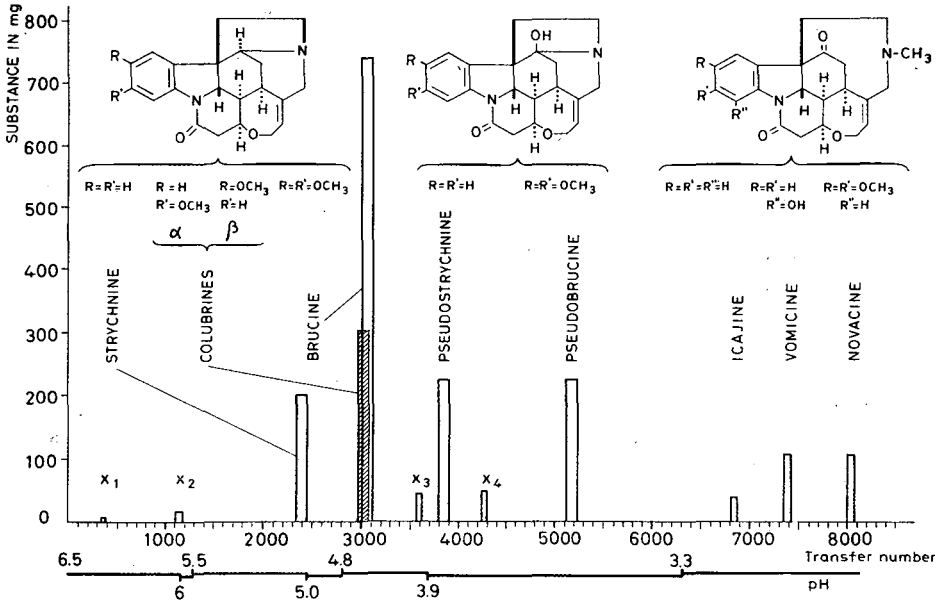


Fig. 3. Separation of *Strychnos nux-vomica* alkaloids by CCD with chloroform and buffer from pH 6.5 to 3.3.

changes being made only when the substance emerging from the 199th test tube was clearly separated from the next.

The magnitude of the pH change in the reservoir container of the apparatus depended on the ease of separation of the substances. This could be estimated from the chromatographic investigations with the aid of the curves in Fig. 2, which give the increase in the concentration in the aqueous phase as a function of the decrease in pH.

The various fractions collected were made alkaline with sodium bicarbonate and extracted with chloroform. Fraction 4, which contained the two colubrines and brucine, was further separated in the Craig apparatus with chloroform–ethyl acetate (65:35) as the stationary phase and phosphate buffer of pH 6.2 as the mobile phase. As can be seen in Table I, the brucine was separated first and collected on leaving the 199th tube, while the two colubrines were separated only after prolonged recycling. All the fractions were crystallized from ethyl acetate, (sometimes mixed with hexane). Alcohol was never used, because of the presence of pseudostrychnine and pseudobrucine, which are readily alkylated to their hydroxyl oxygens. The strychnine, α- and β-colubrines, brucine, pseudostrychnine, pseudobrucine, icajine, vomicine and novacine were identified on the basis of their mass spectra and by comparison of the IR spectra in chloroform and of the chromatographic mobilities in the following four solvent systems: methanol–chloroform (2:8); benzene–ethyl acetate–diethylamine (7:2:1); cyclohexane–chloroform–diethylamine (5:4:1); pyridine–ethyl acetate–water (11.5:75:16.5) (upper phase).

TABLE I

CCD SEPARATION OF FRACTION NO. 4 BETWEEN CHLOROFORM–ETHYL ACETATE (65:35) AND PHOSPHATE BUFFER AT pH 6.2

200-tube apparatus, volume 10/10 ml. Substance 1018 mg.

| | *Weight (mg)* | *Number of passes* | *Procedure* |
|---|---|---|---|
| Brucine | 732 | 520 | The upper phase (750 ml) was collected as it left the 199th test tube. |
| α- and β-colubrines | | 700 | The substances were not separated at the 199th test tube. |
| Recycling by junction of the 199th to the first test tube. | | | |
| α-Colubrine | 117 | 4000 | Both phases are collected between test tubes 700 (3 × 200 + 100) and 790 (3 × 200 + 190). |
| β-Colubrine | 106 | 4000 | Both phases are collected between tubes 810 (4 × 200 + 10) and 890 (4 × 200 + 90). |

The alkaloids are indicated in Fig. 3 in the order of their appearance from the CCD apparatus with chloroform and buffer (the colubrines are thus superimposed on brucine). The abscissa gives the number of passes and the pH changes, while the ordinate gives the quantity of alkaloids in each fraction. The width of the base indicates the number of passes, and hence the volume in litres (number of passes divided by 100) corresponding to the complete elution of each individual fraction. Finally, the graph also shows four alkaloids denoted by $x_1$, $x_2$, $x_3$, and $x_4$, which could not be identified with any of the known *Strychnos nux-vomica* alkaloids.

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CHROM. 4393

# ÉTUDE DES HYDROCARBURES POLYCYCLIQUES DE L'ATMOSPHÈRE

## I. PROBLÈMES NÉS DU COUPLAGE DE LA CHROMATOGRAPHIE SUR COUCHES MINCES ET DE LA CHROMATOGRAPHIE EN PHASE GAZEUSE*

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(Reçu le 21 septembre 1969)

SUMMARY

*Study of the atmospheric polycyclic hydrocarbons. Problems connected with the coupling of thin-layer chromatography and gas chromatography*

An original routine method, for the analysis of atmospheric polycyclic hydrocarbons is described, in which a thin-layer chromatographic separation is coupled with a gas chromatographic control.

## INTRODUCTION

L'étude de la pollution atmosphérique par des composés chimiques provenant des foyers domestiques et industriels, ou de la combustion incomplète des essences ou huiles lourdes a fait, ces dernières années, l'objet de nombreux travaux. Parmi ces polluants et en raison de leur potentialité carcinogène, les hydrocarbures polycycliques, aromatiques ou hétérocycliques ont été très étudiés. Il était en effet intéressant de connaître s'il existait une relation entre la distribution géographique de certaines tumeurs et la concentration atmosphérique de quelques hydrocarbures réputés cancérogènes. Pour essayer de résoudre ce problème, un certain nombre de méthodes physiques d'analyse a été utilisé. Nous distinguerons principalement des méthodes de séparation telles que la chromatographie sur couches minces, la chromatographie sur colonne et la chromatographie en phase gazeuse, et des méthodes d'identification comme la spectrographie UV, la spectrofluorométrie et la spectrographie de masse.

Les travaux les plus récents montrent que la chromatographie sur couches minces est incontestablement, parmi les techniques de séparation précitées, celle qui a été le plus employée. Il est indéniable en effet, que la diversité des adsorbants et des solvants utilisés en fait une technique relativement spécifique et aux possibilités multiples. Nous pouvons citer comme exemple,

* Ce travail a bénéficié d'une aide de la Direction des Recherches et Moyens d'Essais.

(1) La séparation des hydrocarbures appartenant à une classe déterminée: Arènes aromatiques[1]; hydrocarbures azahétérocycliques[2,3]; amines aromatiques[4]; iminohydrocarbures[4]; hydrocarbures à fonction carbonyle[4,5].

(2) La recherche, à l'intérieur du mélange total des hydrocarbures, de composés d'intérêt particulier tels que: le benzo[*a*]pyrène[6,7]; la 7H-benz[*d*,*c*]anthracène 7-one[7]; la benz[*c*]acridine[7].

Si la chromatographie sur couches minces apparaît comme une bonne technique de séparation, il faut cependant préciser qu'elle a surtout été utilisée sur des mélanges de produits synthétiques ou sur des fractions de polluants atmosphériques purifiés au préalable par des méthodes physico-chimiques. L'expérience montre en effet[1], et nous reviendrons sur ce point dans le chapitre II de cette étude, que sur un extrait benzénique total de poussières atmosphériques, un système adsorbants–solvants, aussi spécifique soit-il, ne sépare pas suffisamment les composés, pour une identification directe, à l'aide de la spectrophotométrie UV et de la spectrofluorométrie.

Le maximum d'efficacité de la chromatographie sur couches minces ne pourrait alors être atteint qu'en multipliant les systèmes chromatographiques, opérations relativement coûteuses en matériel et en temps. On ne peut donc retenir ce procédé pour une étude de routine, véritable but recherché. C'est la raison pour laquelle nous avons envisagé un couplage chromatographie sur couches minces–chromatographie en phase gazeuse. Cette dernière technique, du fait de sa grande sensibilité et de son haut pouvoir de résolution, se présente en effet comme un excellent moyen de contrôle qualitatif (indice de rétention) et quantitatif (hauteurs des pics) des chromatogrammes. La chromatographie en phase gazeuse a d'ailleurs déjà été employée mais de manière occasionnelle lors d'essais d'identification directe d'hydrocarbures présents dans l'atmosphère[8,9], dans l'analyse des fumées de cigarettes[10] ou encore dans celle des mélanges d'arènes synthétiques[11].

Pour définir avec précision les modalités de l'application d'un tel couplage, nous avons donc entrepris une étude sur un mélange de dix hydrocarbures témoins.

## MATÉRIEL ET TECHNIQUES

### *Solvants*

Les solvants, pentane, éthanol, toluène, benzène, acétone, éther, méthanol, (Prolabo, Paris, France) ont subi une bidistillation préalable et leur pureté a été contrôlée par chromatographie en phase gazeuse.

### *Témoins*

Les témoins sont au nombre de dix: anthracène, 3-méthyl-cholantrène, dibenzo-[*a*,*h*]anthracène (Eastman Organic Chemicals, Rochester 3, N.Y., U.S.A.); 9-fluorènone, xanthone, 11H-benzo[*b*]fluorène (Fluka, Buchs, Suisse); naphtalène, pyrène (Prolabo, Paris, France); benz[*a*]anthracène, 3-méthyl-cholantrène (Schuchardt, München, Allemagne); 1-méthyl-pyrène (K and K laboratories Inc., Hollywood, Calif., U.S.A.). Tous ces témoins ont été mis en solution dans le benzène à raison de 1 mg/ml.

### *Technique de chromatographie en phase gazeuse*

*Appareil.* On utilise le modèle 2500 R de Mikrotek muni de deux colonnes identiques et d'un double détecteur à ionisation de flamme.

*Colonnes.* Le support est consitué par du Chromosorb G DMCS. La phase stationaire dissoute dans le chloroforme est constituée par du SE-52. L'imprégnation est réalisée par évaporation de la solution chloroformique de phase, à température ambiante. Le taux d'imprégnation est de 4.5 % en poids par rapport au support sec. Support et phase sont soigneusement tassés dans des colonnes en inox de 1/8 de pouce et de 2 m de long.

*Conditions de chromatographie.* Les conditions de chromatographie sont les suivantes:

| | |
|---|---|
| température initiale: | 100° |
| température finale: | 290° |
| programmation de température: | 3°/min |
| atténuation: | 256 |
| gaz vecteur: | azote |
| débit: | 60 cc/min |
| température du bloc d'injection: | 250° |
| température du bloc de sortie: | 250° |
| injection: | de 1 à 4 $\mu$l |

*Technique de chromatographie sur couches minces*

*Adsorbants.* Alumine: oxyde d'aluminium G (type E) pour la chromatographie sur couches minces (E. Merck AG, Darmstadt); Cellulose acétylée: MN Cellulosepulver 300 AC (Macherey, Nagel and Co.).

*Lavage des adsorbants.* Des fractions de 20 g d'alumine ont été lavées sous agitation magnétique par 500 ml de benzène pendant 12 h, puis 500 ml d'acétone pendant 12 h, 500 ml d'éther pendant 12 h, et 500 ml de benzène pendant 12 h. Les fractions de cellulose acétylée, de 10 g, ont été lavées sous agitation magnétique, 4 fois 12 h, par le méthanol.

*Plaques.* Des plaques standards de 200 × 200 mm ont été utilisées. L'homogénéisation de 30 g d'adsorbants (20 g d'alumine + 10 g de cellulose acétylée) a été réalisée avec 80 ml d'eau dans un homogénéiseur Jouan type H 451 pendant une minute. L'épaisseur de la couche d'adsorbants a été fixée à 350 $\mu$.

*Confection des plaques.* Le système chromatographique adopté a été le suivant[1]: Adsorbants: alumine–cellulose acetylée (2:1). Solvants: 1ère dimension, pentane; 2ème dimension, éthanol–toluène–eau (17:4:4).

Trois séries de plaques ont été réalisées, (1) une série de quatre plaques faites avec les adsorbants commerciaux et sur lesquels ont migré des hydrocarbures témoins; (2) une série de quatre plaques faites dans les mêmes conditions que précédemment mais sans dépôt d'hydrocarbures; (3) une série de quatre plaques avec témoins synthétiques, confectionnées avec des adsorbants lavés selon le mode déjà indiqué.

Douze microlitres de la solution-mère, correspondant à 12 $\mu$g de chaque hydrocarbure témoin ont été déposés sur chaque plaque.

*Élution des plaques*

Sur les plaques avec adsorbants lavés ou non lavés, la zone fluorescente (portant les hydrocarbures qui ont migré), révélée en lumière UV, est eluée par le benzène à froid sous agitation magnétique. L'éluat est alors évaporé à sec, puis repris par 20 $\mu$l de benzène, avant d'être injecté dans le chromatographe. Pour les plaques ne comportant pas d'hydrocarbure témoin, une zone homologue à la précédente a été éluée et traitée de la même façon.

RÉSULTATS ET DISCUSSION

*Aspect qualitatif*

Au lieu de 10 pics de rétention normalement attendus à partir de l'éluat d'adsorbants non lavés avec témoins, apparaissent, dans la Fig. 1, 10 + *n* pics. Ces derniers qui se juxtaposent ou se superposent aux 10 témoins, et rendent le tracé difficilement interprétable, ne peuvent provenir que des impuretés des adsorbants commerciaux. La Fig. 2 vérifie cette hypothèse. Il est en effet possible par lecture différentielle des tracés 1 et 2, de déceler les 10 pics absents dans la Fig. 2, les deux tracés restant par

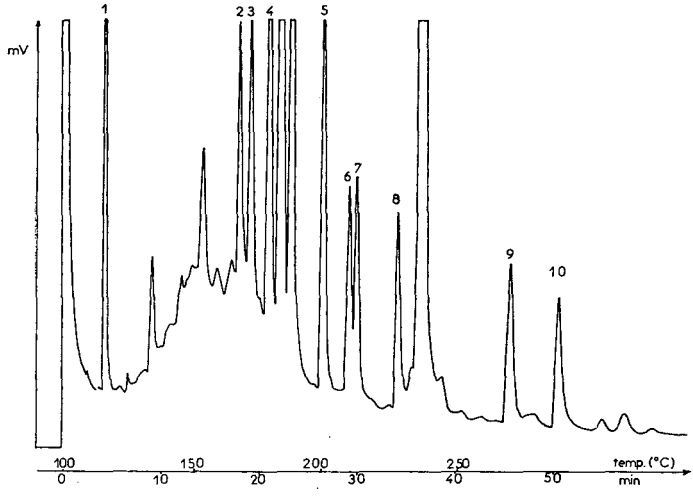


Fig. 1. Chromatogramme de l'éluat d'adsorbants non lavés avec hydrocarbures témoins. 1 = naphtalène; 2 = 9-fluorènone; 3 = anthracène; 4 = xanthone; 5 = pyrène; 6 = 11H-benzo[*b*]fluorène; 7 = 1-méthyl-pyrène; 8 = benzo[*a*]anthracène; 9 = 3-méthyl-cholantrène; 10 = dibenzo-[*a*,*h*]anthracène.

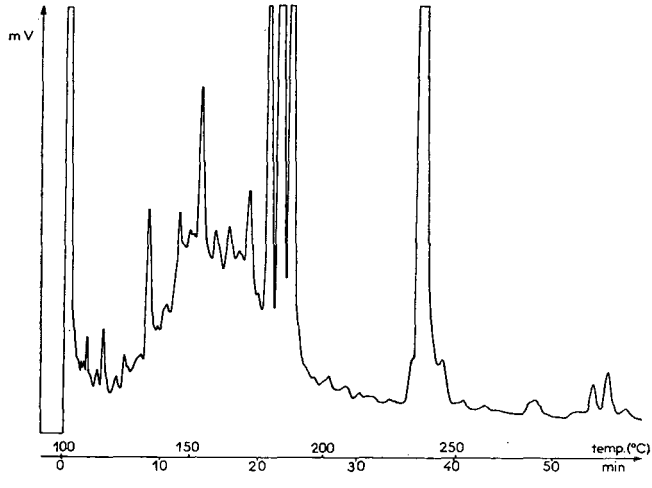


Fig. 2. Chromatogramme de l'éluat d'adsorbants non lavés sans hydrocarbures témoins.

ailleurs parfaitement superposables. Ces 10 pics correspondent bien entendu aux 10 hydrocarbures témoins. On les retrouve isolés dans la Fig. 3, où, après lavage des adsorbants—les autres conditions restant égales par ailleurs—aucun autre pic ne vient se juxtaposer à ceux des témoins.

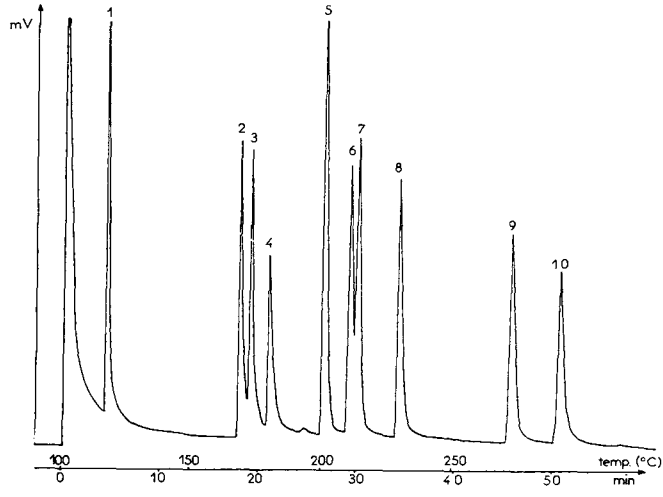


Fig. 3. Chromatogramme de l'éluat d'adsorbants lavés avec hydrocarbures témoins. (Les chiffres ont la même signification que dans la Fig. 1).

*Aspect quantitatif*

*Rentabilité de l'injection.* La rentabilité de l'injection est entachée de deux causes d'erreur: l'une due aux difficultés de la récupération de très faibles quantités d'échantillons élués; l'autre, propre à la technique chromatographique elle-même: adsorption des solutés sur le support et sur la phase.

L'incertitude dans ce cas peut être évaluée à 10 %.

*Rentabilité de l'élution.* A l'aide de la chromatographie en phase gazeuse et après courbe d'étalonnage, nous avons pu vérifier que la première élution benzénique a un rendement d'au moins 80 %. Une seconde élution entraîne moins de 5 % des échantillons.

Ces résultats montrent clairement que le couplage chromatographie sur couches minces–chromatographie en phase gazeuse est possible à condition de travailler avec des adsorbants et des solvants chromatographiquement purs.

Un tel couplage présente un intérêt certain pour l'analyse de routine. En effet, il permet en une opération rapide (extraction benzénique de poussières atmosphériques, 6 h; chromatographie sur couches minces, 2 h; chromatographie en phase gazeuse, 1 h 30) de suivre l'évolution de la pollution atmosphérique pour un site donné. Mais ce couplage n'a de valeur que dans le cas d'une analyse de routine, donc il ne peut intervenir qu'après l'identification systématique classique de chaque pic du chromatogramme correspondant au premier échantillon collecté. Dans les analyses ultérieures, les indices de rétention et les hauteurs des pics nous renseigneront sur la composition respectivement qualitative et quantitative de l'échantillon collecté. La

présence de pics inédits traduira bien entendu l'apparition de nouveaux constituants.

Il sera d'ailleurs possible, comme nous l'avons envisagé pour une prochaine étude de perfectionner cette méthode d'analyse en couplant la chromatographie en phase gazeuse avec un spectrographe de masse. Ce nouveau couplage nous permettra alors de lever toute incertitude sur la qualité du pic chromatographique et par là, de dépasser le cadre de la routine pour atteindre une technique analytique originale.

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# THIN-LAYER CHROMATOGRAPHY AND ULTRAVIOLET SPECTROPHOTOMETRY OF SULFONAMIDE MIXTURES

## A STUDY OF THE ABSOLUTE RECOVERIES

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SUMMARY

Recovery patterns were studied for individual sulfonamides separated from a mixture by thin-layer chromatography and then determined by ultraviolet spectrophotometry. To make the investigation more complete experimental conditions such as quantity of sulfonamide per spot, extracting solvent and pathlength were varied. The recoveries are never complete, but fairly constant. Factors affecting recoveries and accuracy of results are discussed.

## INTRODUCTION

Single-component sulfonamide preparations can be analyzed by spectrophotometry, colorimetry or by titration with nitrous acid[1]. The total sulfonamide content of preparations containing two or more sulfonamides can also be estimated with a fair degree of accuracy by any of the same three methods. In some special cases the individual sulfonamides can be determined by spectrophotometry[2,3] or by spectrophotometric–colorimetric procedures[4,5] without separation from mixtures. Gas chromatography has also been applied to the quantitative analysis of some sulfonamide mixtures[6,7]. Several methods of analysis of mixed sulfonamides, published in recent years, involve the estimation of the compounds separated by paper chromatography[8–13] or thin-layer chromatography[14–16]. The 15th edition of the *U.S. Pharmacopoeia*[17] and the 10th edition of the *National Formulary*[18] adopted a PC procedure for the analysis respectively of trisulfapyrimidines and sulfadiazine–sulfamerazine mixtures. These methods[17,18] were retained in the two successive editions of the *U.S. Pharmacopoeia* and the *National Formulary*. In all the PC and TLC methods mentioned[8–18] the determinative step for the estimation of the individual sulfonamides is either colorimetry or spectrophotometry. Spot size comparison allows only a rough estimation of each compound[8,15]. The colorimetric procedures used are: reaction of the sulfonamides with vanillin–hydrochloride[8], with *p*-dimethylaminobenzaldehyde[8,15] or, following diazotization, with N-(1-naphthyl)-ethylenediamine[8,10–12,14,17,18], naphthylethylenediamine chlorohydrate[13] and N,N-diethyl-

N'-(1-naphthyl)-ethylenediamine oxalate[15]. Direct spectrophotometric determinations of the separated sulfonamides have been carried out by HEINANEN *et al.*[8], OLIVARI[9], WAGNER AND WANDEL[15] and SARSUNOVA *et al.*[16]. Generally the spectrophotometric or colorimetric determinations are performed on the extracts or eluates of the developed spots, previously cut from the paper or scraped off from the thin-layer plate after being located under UV light or by spraying the chromatograms with suitable solutions. OLIVARI[9] gradually exposes the developed chromatostrips to the radiation beam of a spectrophotometer and records the total absorbance of each spot.

It is common practice, in methods based on the colorimetric or spectrophotometric examination of the extracts of PC or TLC spots, to chromatograph the compounds to be used for reference purposes under the same conditions as the sample, generally in the same paper or plate, to compensate for possible losses and other factors affecting absorbance readings. Chromatography of the standards was not found necessary by MAIENTHAL *et al.*[11] and by KUNZE AND ESPINOZA[12] in their PC procedures but must always be performed in quantitative TLC methods mainly because, as indicated by SPENCER AND BEGGS[19], compounds are apparently never completely recovered from thin-layer plates. While it seems certain that recoveries are not complete it is not too well known how reproducible they are and how they are affected by changes in experimental conditions.

This investigation originated primarily from the desirability of obtaining information on the absolute recoveries of sulfonamides chromatographed on thin-layer plates and on the reproducibility of such recoveries. The other aim of the study was to improve the accuracy of direct spectrophotometric determinations of the content of TLC spots. Spectrophotometric methods are quicker and generally more accurate than colorimetric procedures. When applied in conjunction with TLC, however, spectrophotometry has often given unsatisfactory results. WAGNER AND WANDEL[15] indicate that spectrophotometric determinations were less accurate than the ones based on colorimetry. SARSUNOVA *et al.*[16], who used only spectrophotometry, do not furnish sufficient data for an evaluation of its accuracy in this connection. BICAN-FISTER AND KAJGANOVIC[14] attempted to analyze the separated sulfonamides by spectrophotometry but were discouraged by the poor results obtained and decided in favor of a colorimetric procedure. The main reason for the reported poor accuracy of spectrophotometric determinations of compounds extracted or eluted from TLC spots is apparently the high and variable UV absorbance contributed by the adsorbent. Aqueous or alcoholic extracts of blank TLC spots, even after centrifugation or filtration through high retentive paper, have been found to have high and very erratic absorbances; see again the review part of the article of SPENCER AND BEGGS[19]. The high absorbances have been attributed by many authors to soluble absorbing substances, which could be removed by washing the adsorbent with methanol or other suitable solvent. BICAN-FISTER AND KAJGANOVIC[14] believe, however, that they are caused by colloidal-size particles suspended in the solutions. A similar conclusion was reached by SPENCER AND BEGGS[19], who succeeded in minimizing the absorbance blanks by filtering the extracts of the TLC spots through prewashed Millipore filters. In the course of the work preliminary to this investigation it was found that they can also be eliminated by reading the centrifuged aqueous or alcoholic extracts in a narrow path (0.5 cm or less). Centrifuged chloroform extracts of blank TLC spots do not have UV absorbance, even if read in 2-cm cells; for this reason this solvent

was preferred to alcohol by CIERI[20] to extract coumarins and furocoumarins from developed TLC plates.

The use of the 0.5 cm cells and the desirability of keeping high the absorbances indicated the necessity for each developed sulfonamide spot to contain 100 $\mu$g and possibly more of a sulfonamide. Separations of such quantities, rather high in TLC, were achieved by spotting the aliquots to be chromatographed over a wide area (3 cm or more). In order to make the study more extensive some experimental conditions, such as composition of the mixtures, extracting solvent and volume of extraction, were varied. Centrifugation of the extracts was preferred to Millipore filtration since it was considerably quicker. The Millipore filters have to be washed free of UV absorbing materials, an operation which could be time consuming when a large number of determinations must be carried out. The sulfonamides, whose recoveries in TLC methods are studied, are the following: sulfacetamide, sulfathiazole, sulfadiazine, sulfamerazine and sulfamethazine.

## EXPERIMENTAL

### *Apparatus*

The standard equipment for TLC of Desaga and Brinkmann was used. The Chromato-Vue apparatus was supplied by Black Light-Eastern Corp. (Long Island, N.Y.) and the spot-collecting tube (Fig. 1) by Pesce Co. (Kennett Square, Pa.).

### *Reagents and solutions*

Silica Gel H was obtained from Brinkmann Instruments Inc. (Westbury, Long Island, N.Y.) and white phosphorus for TLC from Research Specialties Co. (Richmond, Calif.). The acidic alcohol consisted of 0.4% 1 $N$ $H_2SO_4$ in 95% alcohol. The developing solvent used was: chloroform–methanol (88:12).

### *Preparation of TLC plates*

30 g of Silica Gel H and 100 mg of white phosphor were weighed in a flask. The mixture was slurried with 70 ml of 0.1 $N$ NaOH and applied to the plates to a thickness of 0.25 mm. The plates were dried in air and kept in a dust-free cabinet.

## PROCEDURE

### *Reference solutions*

For each of the sulfonamides investigated, prepare reference solutions and read UV absorbances as follows. Weigh accurately about 100 mg in a 100 ml volumetric flask. Add 5 ml of alcohol and 2 ml of strong ammonia water. Swirl to dissolve compound, fill to mark with alcohol and mix.

Dilute with alcohol to obtain a solution containing about 0.4 mg/ml. Pipet four 5 ml aliquots to separate 100 ml volumetric flasks and evaporate to dryness on a steam bath with the help of a current of air. To two of the four flasks, each containing about 2 mg of sulfonamide, add 20 ml of acidic alcohol, swirl well or warm briefly on steam bath to dissolve residue, fill to mark with acidic alcohol and mix. Record the absorbances in 0.5 cm cells from 350 to 220 m$\mu$. Average the absorbances at the maximum and use the resulting value to calculate the absorbance maximum, in 0.5 cm

cells, of an acidic alcohol solution containing 20.00 $\mu$g/ml. For sulfathiazole only, calculate, in addition to the absorbance maximum, the absorbance at 270 m$\mu$ of a solution containing 20.00 $\mu$g/ml. To the other two flasks add 20 ml 0.1 *N* NaOH, swirl well, fill to mark with 0.1 *N* NaOH, mix and record absorbances from 350 to 220 m$\mu$ in 0.5 cm cells. As previously described, calculate the absorbances, in 0.5 cm cells at the maximum near 255 m$\mu$, of a 0.1 *N* NaOH solution containing 20.00 $\mu$g/ml.

By further dilutions with alcohol prepare a solution containing about 0.1 mg/ml. Continue exactly as in preceding paragraph but read absorbances in 2 cm cells. Calculate the absorbance values, in 2 cm cells at the indicated points, of acidic alcohol and 0.1 *N* NaOH solutions containing 5.00 $\mu$g/ml. These values should agree closely with those previously determined.

*Volume delivered by micropipet*

Dilute a sulfonamide solution with alcohol to obtain a concentration of 0.5 mg/ml (solution R). Pipet two 1 ml aliquots to separate 100 ml volumetric flasks, evaporate to dryness, dissolve residue and fill to mark with 0.1 *N* NaOH. Record the UV absorbances from 350 to 220 m$\mu$ in 2 cm cells and average the absorbances at the maximum ($A_R$). With the micropipet to be used for TLC spotting, transfer six 100 $\mu$l aliquots of solution *R* to separate 10 ml volumetric flasks, evaporate to dryness, dissolve residue and fill to mark with 0.1 *N* NaOH. Record UV absorbances from 350 to 220 m$\mu$ in 2 cm cells and read absorbances at the maximum ($A_p$).

Calculate the volume in $\mu$l delivered by the micropipet by the formula:

$$V_p = 100\, A_p/A_R$$

The volume found (six determinations) was 95.95 $\pm$ 0.64 $\mu$l.

*Standard mixture No. 1*

Weigh accurately about 125 mg each of sulfacetamide (SC), sulfadiazine (SD), sulfamerazine (SM) and sulfamethazine (SH) in a 100 ml volumetric flask. Add 5 ml of alcohol and 2 ml of strong ammonia water, swirl well, fill to mark with alcohol and mix (Solution M1).

Prepare serial dilutions in alcohol to obtain a solution containing about 25 $\mu$g total sulfonamide (or 6.25 $\mu$g of each sulfonamide) per ml. Pipet three 5 ml aliquots (about 125 $\mu$g total sulfonamides) to separate 25 ml volumetric flasks and evaporate to dryness. Dissolve the residues and fill to mark with acidic alcohol, mix and read absorbances in 2 cm cells from 350 to 220 m$\mu$. Record absorbances at maximum ($_tA_a$) and calculate the percentage total sulfonamides in solution with the formula:

$$\%\ \text{Total sulfonamides} = 50{,}000\ {}_tA_a/({}_mA_{a,5,2})\,(W_t)$$

where $W_t$ is the total weight in mg of the sulfonamides taken for analysis and ($_mA_{a,5,2}$) indicates the average of the absorbances, at the maximum in 2 cm cells, of the acidic alcohol reference solutions of the four sulfonamides, each at a concentration of 5.00 $\mu$g/ml. Pipet three more 5 ml aliquots to 25 ml volumetric flasks, evaporate to dryness and continue as above but use 0.1 *N* NaOH instead of acidic alcohol and record absorbances at the maximum near 255 m$\mu$ ($_tA_b$). Similarly calculate the percentage of total sulfonamides by the formula:

$$\%\ \text{Total sulfonamides} = 50{,}000\ {}_tA_b/({}_mA_{b,5,2})\,(W_t).$$

Spot six 100 $\mu$l aliquots of solution M1 on two prepared silica gel plates, three per plate. Spot each aliquot by repeated applications over an area 3 cm wide, leaving 2 cm margins between spotting areas and at the ends. Use a current of air to dry drops between applications. Develop until the solvent front has reached the top of the plates. View developed plates under short wave UV light and circle spots with a dissecting needle, including small margins, whenever possible. The order of succession of the developed sulfonamides is shown in Fig. 2. Scrape each marked spot and transfer

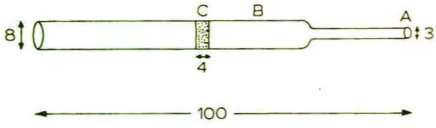


Fig. 1. Collecting tube for spots. Numbers indicate sizes in mm.

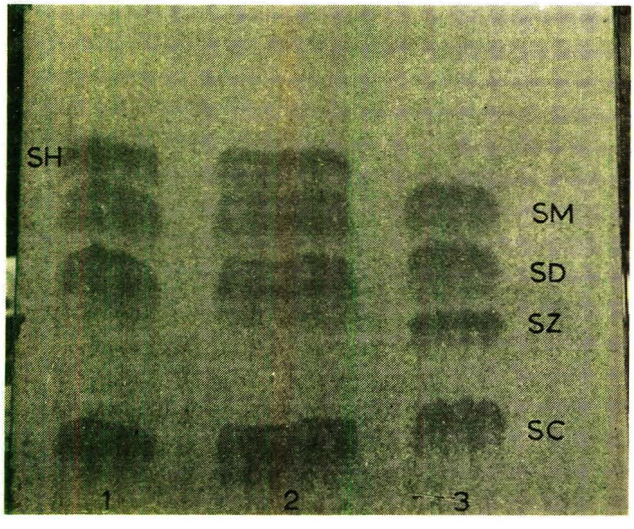


Fig. 2. Photograph of a developed chromatogram. For explanation of abbreviations, see text. Quantity of sulfonamide per spot: aliquots 1 and 3, 100 $\mu$g; aliquot 2, 200 $\mu$g.

to 25 ml volumetric flasks as follows. Attach collecting tube (Fig. 1) to a vacuum source and completely draw the content of a spot into the bulb (Fig. 1B), using the tip to loosen the adsorbent layer. Without disconnecting suction, move the collecting tube inside a flask. Remove suction and force material from tube to flask with repeated tappings. With a very gentle current of air blow into the flask any material still adhering to the tube. Identify the flasks containing the scraped spots of each aliquot. To twelve of the flasks, containing the scraped spots of three developed aliquots, add 15 ml of acidic alcohol. Stopper, shake well for 2 min, fill to mark with acidic alcohol and mix. Centrifuge 15 ml portions of the extracts in conical tubes and carefully decant about 12 ml of the clear solutions into small beakers. Read the absorbances from 350 to 220 m$\mu$ in 2 cm cells with acidic alcohol in the reference cells and record absorbances at the maximum ($_xA_a$). The letter $x$ identifies a particular sulfonamide.

Extract with 25 ml acidic alcohol and similarly centrifuge and decant into beakers six blank spots, approximately equal in size to an average developed spot. The blank spots can be scraped off at the sides of a developed plate or from an undeveloped plate. Record absorbance in 2 cm cells from 350 to 220 m$\mu$ with acidic alcohol in the reference cells. Average the six blank absorbances at 270 m$\mu$ ($_BA_a$). Calculate the percentage recovery of the individual sulfonamides with the formula:

$$\% R_x = 1250({}_xA_a - {}_BA_a) / ({}_xA_{a,5,2})(W_x)(V_p)$$

where $W_x$ is the weight in mg of the sulfonamide in the mixture, ($_xA_{a,5,2}$) the absorbance in 2 cm cells at the maximum of the sulfonamide reference solution containing 5.00 $\mu$g/ml, and $V_p$ the average volume in ml delivered by the micropipet. To the remaining twelve flasks, add 15 ml of 0.1 $N$ NaOH and continue as above always substituting 0.1 $N$ NaOH for acidic alcohol. Record absorbances at maximum near 255 m$\mu$ ($_xA_b$) and calculate percentage recovery of the individual sulfonamides by the formula:

$$\% R_x = 1250({}_xA_b - {}_BA_b) / ({}_xA_{b,5,2})(W_x)(V_p)$$

The term ($_xA_{b,5,2}$) indicates the absorbance in 2 cm cells at the maximum near 255 m$\mu$ of the sulfonamide reference solution containing 5.00 $\mu$g/ml, and $_BA_b$ is the average of six blank absorbances at 255 m$\mu$. The meaning of the other terms has already been explained. For each aliquot average the recoveries of the four sulfonamides and identify as $R_{ms}$. Calculate, also for each aliquot, the average recovery from the formulas:

$$\% R_{mc} = 10\left[\sum_{x=1}^{n} ({}_xA_a) - n({}_BA_a)\right] / n({}_tA_a)(V_p) \text{ for the alcohol solutions}$$

$$\% R_{mc} = 10\left[\sum_{x=1}^{n} ({}_xA_b) - n({}_BA_b)\right] / n({}_tA_b)(V_p) \text{ for the 0.1 } N \text{ NaOH solutions}$$

where $n$ represents the number of the sulfonamides in the mixture, four in this instance, and $V_p$ the average volume of the micropipette in ml.

*Standard mixtures Nos. 2 and 3*

Weight accurately about 200 mg each of the four sulfonamides used for preparing

TABLE I

BLANK ABSORBANCE READINGS

Blanks with 0.5 cm cells were negligible.

| *No.* | *Acidic alcohol* | | *No.* | *0.1 N NaOH* | |
|---|---|---|---|---|---|
| | *Absorbance (270 m$\mu$, 2 cm)* | *Deviations from average* | | *Absorbance (255 m$\mu$, 2 cm)* | *Deviations from average* |
| 1 | 0.046 | +0.011 | 1 | 0.029 | −0.007 |
| 2 | 0.031 | −0.004 | 2 | 0.043 | +0.007 |
| 3 | 0.033 | −0.002 | 3 | 0.020 | −0.016 |
| 4 | 0.030 | −0.005 | 4 | 0.040 | +0.004 |
| 5 | 0.034 | −0.001 | 5 | 0.047 | +0.011 |
| 6 | 0.038 | +0.003 | 6 | 0.037 | +0.001 |
| | Average 0.035 ($n = 6$) | | | Average 0.036 ($n = 6$) | |

TABLE II

COMPOSITION OF SOLUTIONS

| | *Quantity weighed (mg)* | | |
|---|---|---|---|
| | *M1* | *M2* | *M4* |
| SC | 125.7 | 200.2 | 100.9 |
| SD | 124.9 | 201.5 | 101.0 |
| SM | 124.8 | 199.9 | 99.6 |
| SH | 129.4 | 200.4 | — |
| SZ | — | — | 100.1 |
| Total | 504.8 | 802.0 | 401.6 |

TABLE III

% TOTAL SULFONAMIDES IN SOLUTION BY UV SPECTROPHOTOMETRY

| | *No.* | *Acidic alcohol* | *No.* | *0.1 N NaOH* |
|---|---|---|---|---|
| Solution M1 | 1 | 99.1 | 1 | 99.5 |
| | 2 | 100.4 | 2 | 99.1 |
| | 3 | 98.6 | 3 | 99.9 |
| | Average | 99.4 | Average | 99.5 |
| Solution M2 | 1 | 100.5 | 1 | 100.4 |
| | 2 | 100.1 | 2 | 100.5 |
| | 3 | 100.1 | 3 | 99.5 |
| | Average | 100.2 | Average | 100.1 |
| Solution M4 | 1 | 100.6 | 1 | 100.4 |
| | 2 | 99.9 | 2 | 100.1 |
| | 3 | 100.6 | 3 | 99.8 |
| | Average | 100.4 | Average | 100.1 |

solution M1 in a 100 ml volumetric flask. Dissolve in 5 ml of alcohol and 2 ml of strong ammonia water and fill to mark with alcohol (solution M2). Dilute 5.0 ml of solution M2 to 10.0 ml with alcohol (solution M3). Analyze both solutions with the same procedure used for solution M1 but transfer the scraped spots to 10 ml rather than 25 ml volumetric flasks. Centrifuge all the solution, decant about 8 ml and read the absorbances in 0.5 cm cells. The aliquots of solution M2 must be spotted over an area about 5 cm wide; consequently, only two aliquots can be spotted on one plate. Calculate percentage total sulfonamides in solution only for solution M2. For both solutions, calculate the individual recoveries of the developed sulfonamides and the $R_{ms}$ and $R_{mc}$ values with formulas similar to those given under solution M1.

*Standard mixture No. 4*

Weigh accurately about 100 mg each of sulfacetamide (SC), sulfathiazole (SZ), sulfadiazine (SD) and sulfamerazine (SM) in a 100 ml volumetric flask. Dissolve with

5 ml of alcohol and 2 ml of strong ammonia water, dilute to mark with alcohol and mix (solution M4). Analyze the solution by the same procedure as used for solutions M2 and M3.

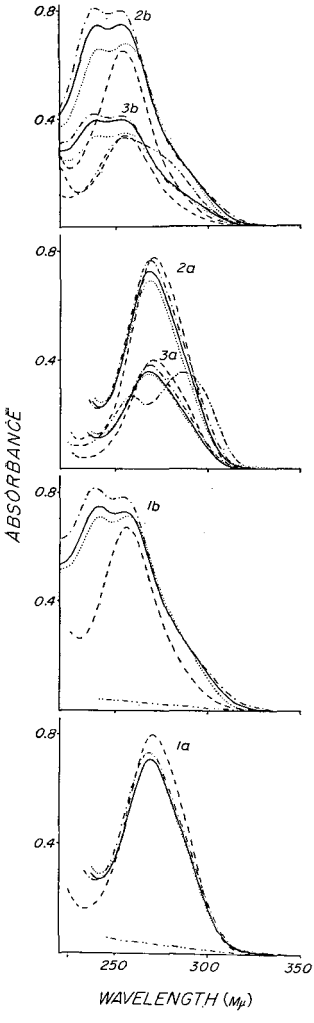


Fig. 3. UV absorbance curves of centrifuged extracts of sulfonamides separated by TLC. The letter *a* indicates acidic alcohol solution, the letter *b* 0.1 *N* NaOH solutions. Concentrations, volumes of extraction and cell paths; (1) 125 μg, 25 ml, 2 cm; (2) 200 μg, 10 ml, 0.5 cm; (3) 100 μg, 10 ml, 0.5 cm. – – – –, SC; –·–·–·–, SD; ———, SM; · · · · · ·, SH; – – · · – – · · – –, SZ; — · · — · · —, B.

## DISCUSSION

As the results of Table IV indicate, the recoveries of sulfonamides extracted from the scraped spots of developed TLC plates are never complete. Losses are generally in the 7–11% range but occasionally they are higher or lower. The recoveries of

TABLE IV

% RECOVERIES OF CHROMATOGRAPHED SULFONAMIDES

Solution M1 read in 2 cm cells, solutions M2, M3 and M4 read in 0.5 cm cells.

| *Solution* | *Aliquot* | *Extracting Solvent* | % $R_{SC}$ | % $R_{SD}$ | % $R_{SM}$ | % $R_{SH}$ | % $R_{SZ}$ | *S.D.* | % $R_{ms}$ | % $R_{mc}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| M1 | 1 | Acidic alcohol | 87.8 | 88.9 | 87.2 | 86.4 | — | 1.1 | 87.6 | 87.9 |
| | 2 | | 88.2 | 94.3 | 93.7 | 93.8 | — | 2.9 | 92.5 | 92.5 |
| | 3 | | 87.4 | 92.9 | 89.2 | 89.4 | — | 2.3 | 89.7 | 90.1 |
| | Av. | | 87.80 | 92.0 | 90.0 | 89.9 | — | 1.7 | 89.9 | 90.2 |
| | S.D. | | 0.40 | 2.8 | 3.3 | 3.7 | | | | 2.3 |
| | 4 | 0.1 *N* NaOH | 87.3 | 89.7 | 93.1 | 92.8 | — | 2.8 | 90.7 | 92.3 |
| | 5 | | 91.9 | 96.0 | 94.2 | 92.1 | — | 1.9 | 93.5 | 95.2 |
| | 6 | | 92.7 | 95.5 | 94.4 | 92.5 | — | 1.4 | 93.8 | 95.2 |
| | Av. | | 90.6 | 93.7 | 93.90 | 92.47 | | 1.5 | 92.7 | 94.2 |
| | S.D. | | 2.9 | 3.5 | 0.70 | 0.35 | | | | 1.7 |
| M2 | 1 | Acidic alcohol | 90.9 | 96.2 | 95.8 | 93.6 | | 2.4 | 94.1 | 93.9 |
| | 2 | | 89.2 | 95.5 | 94.1 | 89.1 | | 4.0 | 91.5 | 92.0 |
| | 3 | | 87.1 | 94.1 | 92.8 | 91.6 | | 3.0 | 91.4 | 90.9 |
| | Av. | | 89.1 | 95.3 | 94.2 | 91.4 | | 2.8 | 92.5 | 92.3 |
| | S.D. | | 1.9 | 1.0 | 1.5 | 2.3 | | | | 1.5 |
| | 4 | 0.1 *N* NaOH | 89.2 | 96.1 | 92.7 | 88.6 | | 3.5 | 91.7 | 91.6 |
| | 5 | | 88.5 | 95.0 | 93.5 | 90.6 | | 2.9 | 91.9 | 92.0 |
| | 6 | | 89.9 | 96.4 | 96.0 | 93.3 | | 3.0 | 93.9 | 93.8 |
| | Av. | | 89.20 | 95.83 | 94.1 | 90.8 | | 3.0 | 92.5 | 92.5 |
| | S.D. | | 0.70 | 0.74 | 1.7 | 2.4 | | | | |
| M3 | 1 | Acidic alcohol | 91.7 | 92.4 | 92.8 | 90.7 | — | 0.92 | 91.90 | 91.9 |
| | 2 | | 91.9 | 96.6 | 92.0 | 92.8 | — | 2.2 | 93.3 | 93.1 |
| | 3 | | 93.6 | 95.8 | 94.6 | 94.1 | — | 0.94 | 94.52 | 94.2 |
| | Av. | | 92.4 | 94.9 | 93.1 | 92.5 | — | 1.2 | 93.2 | 93.1 |
| | S.D. | | 1.0 | 2.2 | 1.3 | 1.7 | — | | | 1.2 |
| | 4 | 0.1 *N* NaOH | 93.9 | 95.3 | 93.7 | 94.7 | — | 0.74 | 94.40 | 94.1 |
| | 5 | | 92.2 | 98.2 | 99.7 | 93.2 | — | 3.7 | 95.8 | 95.7 |
| | 6 | | 92.5 | 96.7 | 97.8 | 93.9 | — | 2.4 | 95.2 | 95.1 |
| | Av. | | 92.87 | 96.7 | 97.1 | 93.95 | — | 2.1 | 95.2 | 94.97 |
| | S.D. | | 0.91 | 1.5 | 3.1 | 0.75 | — | | | 0.81 |
| M4 | 1 | Acidic alcohol | 89.1 | 92.2 | 93.2 | — | 91.4 | 1.7 | 91.5 | 92.2 |
| | 2 | | 91.4 | 92.5 | 94.7 | — | 90.9 | 1.7 | 92.4 | 93.2 |
| | 3 | | 88.7 | 91.7 | 93.7 | — | 90.4 | 2.1 | 91.1 | 91.6 |
| | Av. | | 89.7 | 92.13 | 93.87 | — | 90.90 | 1.8 | 91.7 | 92.33 |
| | S.D. | | 1.5 | 0.40 | 0.76 | — | 0.50 | | | 0.81 |
| | 4 | 0.1 *N* NaOH | 91.5 | 97.7 | 96.5 | — | 95.4 | 2.7 | 95.3 | 94.8 |
| | 5 | | 92.5 | 96.5 | 97.0 | — | 95.7 | 2.0 | 95.4 | 95.0 |
| | 6 | | 92.5 | 96.0 | 97.0 | — | 95.7 | 1.9 | 95.3 | 95.1 |
| | Av. | | 92.17 | 96.73 | 96.83 | — | 95.60 | 2.2 | 95.3 | 94.97 |
| | S.D. | | 0.58 | 0.87 | 0.29 | — | 0.17 | | | 0.15 |

spotted undeveloped sulfonamides were not studied in this investigation but preliminary work indicated that losses also occur and that they are slightly lower than those of the developed compounds. Attempts to increase recoveries, also made during the preparative work, were unsuccessful. The introduction of additional steps, such as washing the glass surface with a wad of cotton after removal of a spot, rinsing collection tube (Fig. 1) after transfer of a spot or performing multiple extractions of the scraped spots did not yield recoveries higher than those obtained with the simpler technique finally adopted and described in the section EXPERIMENTAL.

The reasons for the losses are not too well understood. They are probably caused, for the greater part, by a superficial loosening of silica gel particles at the spotting areas. The loosened particles scatter in air or fall when the plate is raised, carrying along the sulfonamides attached to them. Scattering of some fine silica gel particles, with small additional loss of sulfonamides, may also occur during extraction of the spots. It was often observed that, when the solvent was added, a fine mist came out of the flasks containing the silica gel spots. The possibility also exists that a small amount of sulfonamide is adsorptively retained by the silica gel and cannot be extracted but there is no definite experimental evidence to prove it. Whatever the causes for the incomplete recoveries, they seem to affect nearly equally all the sulfonamides studied. If the recoveries of the different sulfonamides for each aliquot are compared with each other, it appears that generally differences are small and not adequate to indicate a trend for higher or lower recoveries for a particular sulfonamide. Sulfacetamide is the only one that constantly shows slightly lower recoveries than the other sulfonamides in a given aliquot. The lower recoveries of sulfacetamide may be due to a partial decomposition during exposure to UV light, as suggested by previous investigators[11,12]. The recoveries of a sulfonamide in differently developed aliquots are also generally in good agreement thus indicating that the quantity of sulfonamides spotted by the micropipet is fairly constant. Unusually great losses of the amount spotted may, however, occur in the event of extensive loosening of the adsorbent layer during spotting. The occurrence of such high losses can be detected in a given aliquot by a low $R_{mc}$ value (calculated average recovery). The $R_{mc}$ values which could be computed even if the composition of the mixture is unknown and which are generally very close to the $R_{ms}$ values (actual average recovery) in this experiment varied from 87.9 to 95.7%. Deviations from these values, especially in the low side, should alert the analyst to the possibility of high errors.

Recoveries are about the same with both extracting solvents, acidic alcohol or 0.1 *N* NaOH. Both solvents are then equally suitable for the extraction of sulfonamides from TLC spots. Percentage recoveries are also independent of the quantity of sulfonamide per spot, at least in the considered range of 100–200 $\mu$g. It is consequently not necessary, in sample analysis, that the standard and sample spots contain very nearly equal quantities of sulfonamides, as long as they remain in the indicated range. Since this study was limited to the 100–200 $\mu$g range, it is not known, however, whether this constancy in percentage recovery holds outside these limits.

The absorbance blanks are apparently caused by colloidal particles remaining suspended in the centrifuged extracts of the spots and not by soluble absorbing substances present in the adsorbent. Prewashing of the Silica Gel H is consequently not necessary and does not lower absorbance blanks. These blanks are independent of the volumes of extraction and can be decreased only by decreasing the cell paths.

They are completely eliminated if 0.5 cm cells are used. If it should become necessary to use higher pathlengths, the absorbances of the sulfonamide extracts should be kept very high (0.8 or more) in order to minimize the error that may be caused by the variability of the absorbance blank.

To limit the extent of this investigation, the recoveries of sulfonamides developed on other adsorbents were not studied. Silica Gel H was preferred to Silica Gel G or other adsorbents containing a calcium sulfate binder because the spots could be more readily scraped and transferred. The white phosphorus was incorporated in the silica gel to facilitate detection of the sulfonamides under UV light. The phosphorus does not contribute any UV absorbance. The silica gel was slurried in 0.1 *N* NaOH rather than water to increase the differences in $R_F$ value between some of the sulfonamides, following a suggestion by NEW[21]. If overlapping of some of the spots still occurs in an experiment, the aliquot will have to be spotted over an area slightly wider than indicated in the method.

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CHROM. 4397

# CHROMATOGRAPHIE SUR COUCHES MINCES DE COMPOSÉS ORGANIQUES DU PHOSPHORE

## III. ANALYSE DE DIFFÉRENTS GROUPES D'ORGANOPHOSPHORÉS

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(Reçu le 23 septembre 1969)

SUMMARY

*Thin-layer chromatography of organophosphorous compounds. III. Analysis of different organophosphorous groups*

A certain number of solvent mixtures of hexane, acetone, ethyl acetate and *tert.*-butanol have been used for the separation by thin-layer chromatography of organophosphorous compounds within the following groups: phosphines, phosphites, phosphates, phosphonates, phosphinates and phosphine oxides.

## INTRODUCTION

Cette étude fait suite aux recherches déjà entreprises dans ce domaine et qui ont fait l'objet de deux publications récentes[1,2]. Les résultats présentés dans cette communication concernent la troisième catégorie des organophosphorés dans le classement que nous avons proposé[2], c'est-à-dire les composés avec même groupement phosphoré, même degré d'alkylation, arylation ou estérification mais qui diffèrent par la nature des radicaux fixés sur ce groupement phosphoré. Les séries suivantes ont été analysées: phosphites, phosphates, phosphinates, phosphonates, phosphines et oxydes de phosphines.

## PARTIE EXPÉRIMENTALE

Le couple adsorbant (silice)–solvant (hexane–acétone, 75:25), mis au point pour l'analyse des composés organophosphorés neutres de la deuxième catégorie de notre classement[2], ne convient pas à la séparation et à l'analyse à l'intérieur de tous les groupes précités.

Ce solvant donne des résultats convenables lors de l'analyse des phosphines et phosphites mais il est aisé de constater, sur la Fig. 1, qu'il convient déjà moins à l'analyse des phosphates et encore moins à celle des oxydes de phosphine. Nous avons donc modifié ce solvant pour l'adapter aux différentes séparations envisagées. La

silice (Kieselgel Merck H, HR, G etc.) reste le meilleur adsorbant pour ces chromatographies. Pour la révélation, nous utilisons toujours le réactif molybdique–perchlorique[2]. Les détails pratiques concernant l'application de cette méthode à l'analyse des organophosphorés ont été donnés dans les précédentes publications.

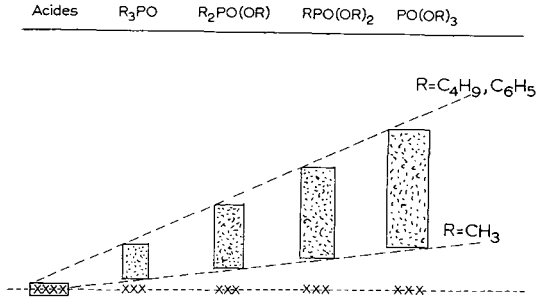


Fig. 1. Chromatographie d'organophosphorés sur couches de silice avec le solvant I: hexane–acétone (75:25).

## RÉSULTATS

Les résultats sont donnés sous forme de tableaux de $R_F$ mais nous insistons de nouveau sur la valeur relative de ces $R_F$ notamment dans le cas présent. En effet, si l'influence de la température, de la nature et de l'épaisseur des couches d'adsorbant a déjà été signalée dans la littérature et par nous-même[2], la nature des solvants a également ici une très grande influence. L'hexane et l'acétone, les principaux solvants utilisés, sont très volatils et dissolvent les graisses qui assurent l'étanchéité des cuves de développement. Il est donc très difficile, avec le matériel habituel, d'assurer un état de saturation valable et permanent dans les cuves. Nous utilisons personnellement une cuve spéciale fermée par une gorge de mercure.

Les tableaux de $R_F$ que nous donnons permettent cependant d'avoir une idée de la netteté plus ou moins grande des séparations et des possibilités offertes par la méthode.

*Groupe des phosphines et oxydes de phosphines*

Les recherches concernant ce groupe ont été menées conjointement avec les recherches sur les séparations par chromatographie des arsines et oxydes d'arsines et sur les composés homologues de l'antimoine et de l'étain[3]. Nous avons utilisé le solvant hexane–acétone (75:25) bien qu'il ne soit pas parfait pour la séparation des oxydes entre eux.

*Groupe des phosphites $(RO)_3P$*

Le solvant utilisé est le mélange hexane–acétone (75:25) (solvant I). Nous portons actuellement une grande attention à ce groupe car il est composé d'organophosphorés très instables et sensibles à l'hydrolyse. Nous utilisons la chromatographie pour étudier cette stabilité (Tableau I).

*Groupe des phosphates $(RO)_3PO$*

Le solvant le mieux adapté à la séparation des phosphates est le solvant II:

hexane–acétone–acétate d'éthyle (60:20:20). Nous montrons également dans le Tableau II l'influence de la nature de la silice.

TABLEAU I

CHROMATOGRAPHIE À 25° DES PHOSPHITES ORGANIQUES SUR COUCHE DE SILICE HR (0.25 mm) AVEC LE SOLVANT I

| *Substances* | $R_F$ |
|---|---|
| Triméthylphosphite | 0.19 |
| Triéthylphosphite | 0.35 |
| Tributylphosphite | 1.00 |
| Triphénylphosphite | 0.85 |

TABLEAU II

CHROMATOGRAPHIE À 25° DES PHOSPHATES ORGANIQUES SUR COUCHES DE SILICES MERCK, DE 0.25 mm D'ÉPAISSEUR MAIS DE NATURE DIFFÉRENTE, AVEC LE SOLVANT II

| *Substances* | $R_F$ | | | |
|---|---|---|---|---|
| | *Silice HR* | *Silice H* | *Silice G* | *Plaque DC Merck* |
| Triméthylphosphate | 0.27 | 0.28 | 0.29 | 0.21 |
| Triéthylphosphate | 0.45 | 0.51 | 0.53 | 0.37 |
| Triallylphosphate | 0.66 | 0.68 | 0.70 | 0.54 |
| Tri(*n*-)propylphosphate | 0.73 | 0.74 | 0.76 | 0.59 |
| Tri(*n*-)butylphosphate | 0.81 | 0.87 | 0.86 | 0.68 |
| Tribenzylphosphate | 0.70 | 0.69 | 0.73 | 0.55 |
| Triphénylphosphate | 0.86 | 0.89 | 0.90 | 0.73 |
| Diphénylméthylphosphate | 0.77 | 0.76 | 0.82 | 0.64 |
| $H_3PO_4$–$(RO)_2P(O)$ (OH)<br>$(RO)P(O)$ $(OH)_2$<br>R quelconque | 0.00 | 0.00 | 0.00 | 0.00 |

*Groupe des phosphonates* $(RO)_2P(O)$ $(R)$

Le solvant II, légèrement modifié dans sa composition, permet d'améliorer la séparation des phosphonates entre eux. Le solvant III hexane–acétone–acétate d'éthyle (40:15:45) convient parfaitement.

La Silice $PF_{254}$ présente l'avantage de permettre la révélation à la lumière ultraviolette de certains composés, notamment les dérivés aromatiques, sans détruire la couche d'adsorbant (Tableau III).

*Groupe des phosphinates* $R_2P(O)$ $(OR)$

Les solvants précédents ont été modifiés pour permettre une séparation acceptable, bien que nettement moins bonne que les précédentes, des phosphinates. Le solvant IV est composé d'hexane et de butanol tertiaire dans le rapport 75:25 (Tableau IV).

TABLEAU III

CHROMATOGRAPHIE À 25° DE PHOSPHONATES ORGANIQUES SUR COUCHES DE SILICE H ET $PF_{254}$, DE 0.25 mm D'ÉPAISSEUR, AVEC LE SOLVANT III

| *Substances* | $R_F$ | |
|---|---|---|
| | *Silice H* | *Silice* $PF_{254}$ |
| Diméthylphénylphosphonate | 0.41 | 0.33 |
| Diéthylphénylphosphonate | 0.56 | 0.47 |
| Di(*n*-)propylphénylphosphonate | 0.74 | 0.63 |
| Di(*n*-)butylphénylphosphonate | 0.85 | 0.75 |
| Diphénylphénylphosphonate | 0.91 | 0.92 |
| Diméthylméthylphosphonate | 0.22 | |
| Dibutylbutylphosphonate | 0.80 | |
| Diméthylbenzylphosphonate | 0.29 | |
| Diéthylbenzylphosphonate | 0.50 | |
| Diéthyl *p*-aminobenzylphosphonate | 0.10 | |
| RP(O) (OH) (OR) | 0.00 | 0.00 |
| $RP(O)(OH)_2$ | | |

TABLEAU IV

CHROMATOGRAPHIE À 25° DE PHOSPHINATES ORGANIQUES SUR COUCHE DE SILICE $PF_{254}$, DE 0.25 mm D'ÉPAISSEUR, AVEC LE SOLVANT IV

| *Substances* | $R_F$ |
|---|---|
| Méthyldiphénylphosphinate | 0.50 |
| Ethyldiphénylphosphinate | 0.58 |
| Allyldiphénylphosphinate | 0.63 |
| (*n*-))Propyldiphénylphosphinate | 0.66 |
| (*iso*-)Propyldiphénylphosphinate | 0.64 |
| (*n*-)Butyldiphénylphosphinate | 0.69 |
| Phényldiphénylphosphinate | 0.73 |
| Butyldibutylphosphinate | 0.67 |
| (RP(O) (OH) | 0.00 |

*Remarques sur les résultats obtenus*

La même séquence de migration est observée sur les chromatogrammes dans les différents groupes: $C_6H_5- > C_6H_5-CH_2- \geqslant C_4H_9- > C_3H_7- > C_3H_5- > C_2H_5- > CH_3-$.

Nous avons entrepris une étude théorique sur le comportement chromatographique de ces substances. Il nous est possible actuellement d'établir une relation linéaire entre les $R_F$ des différents organophosphorés et la somme des coefficients de Taft relatifs aux radicaux R et OR pour les composés tétracoordonnés du phosphore. Des recherches sont encore à faire pour compléter ce travail, notamment en ce qui concerne l'épaisseur et la nature des couches.

La combinaison de ces solvants avec ceux mis au point pour la séparation des

organophosphorés acides[1] doit offrir un grand nombre de possibilités de séparations par chromatographie bidimensionnelle.

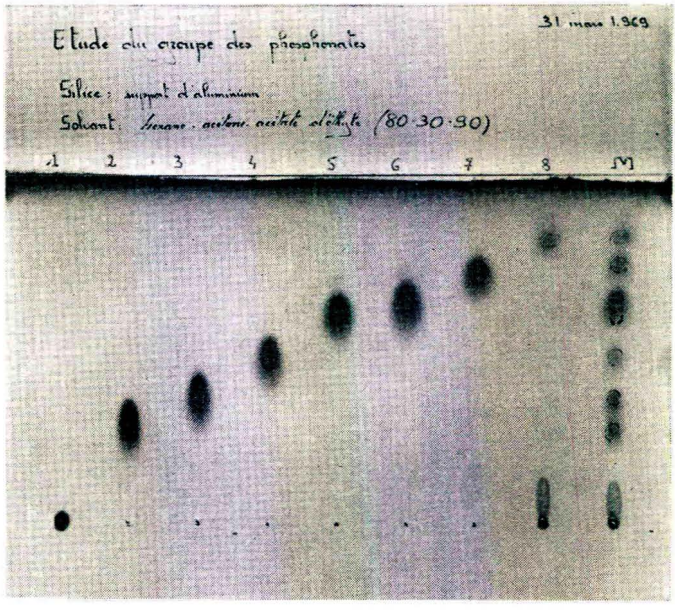


Fig. 2. Exemple de chromatogramme obtenu. 1 = $C_6H_5P(O)(OH)_2$; 2 = $C_6H_5CH_2P(O)(OCH_3)_2$; 3 = $C_6H_5P(O)(OCH_3)_2$; 4 = $C_6H_5P(O)(OC_2H_5)_2$; 5 = $C_6H_5P(O)(OC_3H_5)_2$; 6 = $C_6H_5P(O)(OC_3H_7)_2$; 7 = $C_6H_5P(O)(OC_4H_9)_2$; 8 = $C_6H_5P(O)(OC_6H_5)_2$ impur; M = mélange des 8 composés précédents.

RÉSUMÉ

Un certain nombre de solvants à base d'hexane, acétone, acétate d'éthyle et alcool butylique tertiaire ont été mis au point pour la séparation, par chromatographie sur couches minces de silice, d'organophosphorés à l'intérieur des groupes: phosphines, phosphites, phosphates, phosphonates, phosphinates et oxydes de phosphine.

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CHROM. 4402

# METABOLISM OF NATURALLY OCCURRING PROPENYLBENZENE DERIVATIVES

## I. CHROMATOGRAPHIC SEPARATION OF NINHYDRIN-POSITIVE MATERIALS OF RAT URINE

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SUMMARY

Following oral or intraperitoneal administration of myristicin, safrole, isosafrole, asarone (*trans*) or β-asarone (*cis*) to male rats, basic ninhydrin-positive substances were excreted in the urine. These materials were separated and partially characterized by thin-layer chromatography. The same animals when given a control dosage of safflower oil did not excrete these ninhydrin-positive materials.

There is an apparent requirement of a side chain double bond for the production of these products, with greater enhancement by the *trans* isomer than the *cis*. It is suggested that these urinary ninhydrin-positive materials are probably substituted phenylisopropylamines or amphetamines.

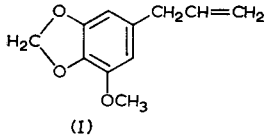
## INTRODUCTION

Substituted allyl and propenylbenzene derivatives are widely distributed in nature. The compounds have been isolated and identified in nutmeg[1], parsley[1], parsnip[2], carrots[3], bananas[4], processed tobacco[5] and many other natural oils and flavoring materials with which individuals have contact.

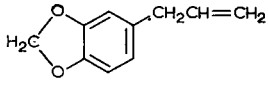
Ingestion of considerable quantities of some of these environmental agents produces significant physiological changes. In the case of nutmeg, myristicin (1-methoxy-2,3-methylenedioxy-5-allyl-benzene) is considered in part to be responsible for a narcotic effect[1]. Depending upon the drug and quantities ingested, these physiological changes[6–8] vary from (a) drop in blood pressure, (b) nausea, and (c) cyanosis to (d) heightening of ego, (e) inability to carry on intellectual processes, (f) insomnia and/or even (g) death. The mechanism by which these materials bring about these responses is unknown.

To account for the observed physiological response, it has been suggested[1] that the substituted benzene derivative may be converted biologically to amphetamines.

The present investigation was undertaken to effect the isolation, separation, and identification of the various urinary metabolites of myristicin (I), safrole (II), isosafrole (III), asarone (IV), $\beta$-asarone (V), and dihydrosafrole (VI) following their oral and intraperitoneal administration to male rats, in order to determine if exposure or ingestion of these environmental agents (and other related compounds) would constitute a potential hazard.




(I)

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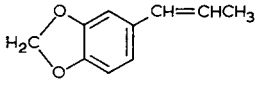


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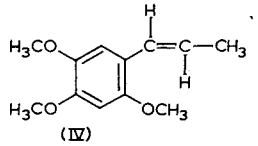


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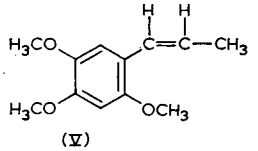
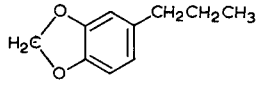



(V)




(VI)

## EXPERIMENTAL

### *Materials*

All of the compounds that were administered to the animals were of 99 % purity or greater as determined by TLC, GLC, IR spectroscopy, and NMR. Myristicin was obtained from parsley seed oil (Fritzsche) by preparative GLC on a 12 ft. $\times$ $\frac{1}{2}$ in. 10 % OV-1 column at 215° with a helium flow of 100 ml/min using the Hewlett-Packard 5750B gas chromatograph or on a 10 ft. $\times$ $\frac{3}{4}$ in. 25 % SE-30 column at 235° with a helium flow of 200 ml/min using the Nester Faust 850 Prepkromatic gas chromatograph, followed by column chromatography on silicic acid. Asarone (*trans*) and $\beta$-asarone (*cis*) were obtained from an enriched $\beta$-asarone mixture (Fritzsche) by preparative GLC, on a 12 ft. $\times$ $\frac{1}{2}$ in. 10 % OV-17 column at 250° with a helium flow of 100 ml/min using the Hewlett-Packard 5750B gas chromatograph. The safrole derivatives were commercial preparations (J. T. Baker) which were further purified by silicic acid chromatography. All organic solvents were Baker analyzed reagent grade.

### *Compound administration, urine collection and extraction*

Male Sprague Dawley rats (200–500 g) were given 0.30 ml safflower oil orally or intraperitoneally (i.p.). The animals were then placed in polycarbonate metabolic

cages* which separated the urine from the feces, and the urine was collected for the desired time (1–3 days). Twice each day, urine was removed from the cages and immediately frozen until the time of extraction.

The same animals after the control period were then given 75–300 mg/kg of the desired compound in safflower oil (1:1, w/w). The rats were returned to the metabolic cages and urine was collected for periods up to six days. Pooled urine for each day was kept frozen until extraction. The treated animals after about one week could be re-fed if desired. During the entire urine collection, the rats were housed in a supervised area with free access to food and water.

An aliquot of pooled urine for each control and treated day was extracted as described in Fig. 1. The organic solvent from each of the acidic, neutral, basic and alkaloid fractions was then removed under nitrogen at 30° using a rotary flash evaporator. The final residue was dissolved in spectro-grade chloroform (Matheson, Coleman and Bell) to a final volume so that the ratio of the volume of the final solution to that of the original urine was 1:150. The chloroform solution was then stored in teflon-capped vials in the freezer for later use.

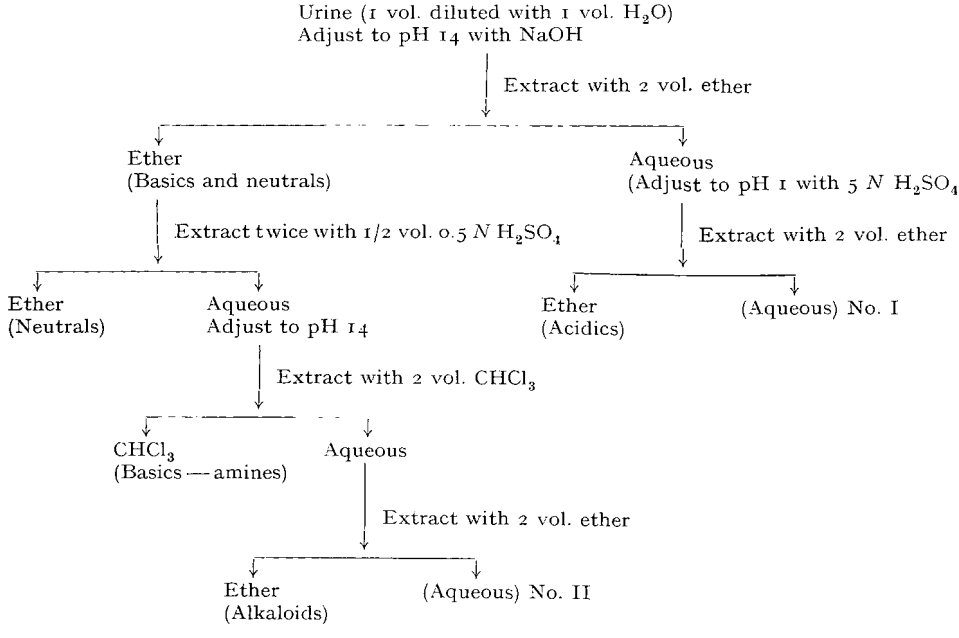



Fig. 1. Extraction of urine.

*Thin-layer chromatography*

Precoated 250-$\mu$ Silica Gel GF plates** were activated at 110° for 10 min. The plates were then scored into lanes and the appropriate amount of sample was applied. In all studies, one to four times as much control sample was applied as treated sample. The plates were then developed 140 mm in lined glass tanks using the desired solvent

* Obtained from Aloe Scientific Co., Chamblee, Ga., U.S.A.
** Obtained from Analtech, Inc., Wilmington, Del., U.S.A.

system. The solvent systems used for the neutral fractions were: (A) benzene, (B) hexane–diethyl ether–acetic acid (90:10:1)[9], and (C) hexane–diethyl ether–methanol–acetic acid (85:20:3:3)[10]. Table I represents the TLC separation obtained for the neutral administered compounds. Using the above three solvent systems, the presence of any of the administered compounds could be detected in the neutral urine fraction very easily.

TABLE I

RELATIVE $R_F$ VALUES OBTAINED ON TLC SEPARATION OF SUBSTITUTED PROPENYL BENZENE DERIVATIVES

Solvent systems: (A) Benzene.
(B) Hexane–diethyl ether–acetic acid (90:10:1).
(C) Hexane–diethyl ether–methanol–acetic acid (85:20:3:3).

| *Compound* | *Solvent system* | | |
|---|---|---|---|
| | *A* | *B* | *C* |
| Asarone | 0.24 | 0.26 | 0.58 |
| Myristicin | 1.00 | 1.00 | 1.00 |
| Safrole | 1.27 | 1.58 | 1.00 |

The basic and alkaloid fractions were examined using the following solvent systems: methanol, methanol–water–acetone–acetic acid (200:20:20:10), methanol–water–acetone–pyridine (200:20:20:10), and chloroform–methanol–acetic acid (75:25:5).

After development, the plates were air dried, examined under UV light (2537 and 3660 Å), and then exposed to iodine vapor and/or sprayed with a particular chemical reagent (for the neutral and basic urine fractions, ninhydrin[11], chromotropic acid[12,13], and 2,4-dinitrophenylhydrazine[11] were used).

## RESULTS AND DISCUSSION

### *Myristicin*

Following oral or i.p. administration of pure myristicin (75–300 mg/kg) to rats, unmetabolized myristicin is excreted in the urine during the first 17 h after administration. On 10 to 50-fold concentration of tissue extracts, there appears to be no significant accumulation of myristicin in blood, intestine, liver, heart, lung, spleen, and adipose tissue.

In addition to the unmetabolized myristicin excreted in the urine during the first 17 h., two very polar basic ninhydrin-positive materials are present in urine after oral or i.p. administration of myristicin. Semiquantitatively, these basic ninhydrin-positive materials are excreted maximally 24–48 h after administration. Three days after administration of myristicin there is a definite decrease in the excreted ninhydrin-positive materials. By the fourth or fifth day the basic ninhydrin-positive materials are absent. The urine extract of the treated animal by the fifth day is equivalent to that of the control animal, which received none of the test compound. This excretion pattern can be monitored with maximal excretion between 24–48 h and

with the absence of these basic ninhydrin-positive materials from urine by 4–5 days.

The production of these ninhydrin-positive basic materials is in response to administration of myristicin. The same animals when given only safflower oil do not excrete ninhydrin positive materials with an $R_F$ value relative to the standard 3,4-methylenedioxyphenyl-isopropylamine $\geqslant$ 1.0 in the basic urine fraction. It is very probable that these ninhydrin-positive materials are not formed by intestinal flora since the same results were obtained for oral and i.p. administration. A chromatogram of the ninhydrin-positive basic materials after i.p. injection of myristicin is shown in Fig. 2. Both of the basic materials when sprayed with ninhydrin and then heated are dark pink in color. Positive identification of these materials is presently being elucidated.

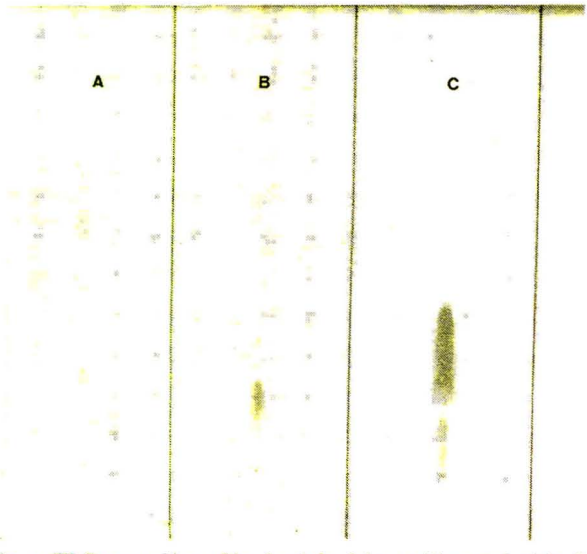



Fig. 2. TLC separation of basic ninhydrin-positive materials. Solvent system: chloroform–methanol–acetic acid (75:25:5). Basic urine fractions: (A) i.p. administered myristicin; (B) orally administered safrole; (C) orally administered asarone (*trans*).

*Safrole*

Similar experiments to those described for myristicin were also carried out with safrole. Ninhydrin-positive basic materials were also excreted in urine of rats treated with safrole. Maximal excretion of these ninhydrin-positive materials occurred during the 24–48 h period. Four ninhydrin-positive materials were separated using the methanol system. Upon spraying with ninhydrin and heating, the most polar material from safrole ($R_F$ relative to the standard amine of 1.0) was pink while the next less polar material (relative $R_F$ 1.7) was purple. The two remaining less polar ninhydrin-positive material had a relative $R_F$ of 3.9 and 4.5, respectively, in the methanol system. These two much less polar ninhydrin-positive materials (relative $R_F$ 3.9 and 4.5) may be decomposition products, as will be discussed later.

Attempts to isolate and identify the major ninhydrin-positive materials of safrole (relative $R_F$ 1.0) have been unsuccessful to date. Preparative TLC of the safrole basic fraction in the methanol system on either silica gel or kieselgur followed by

elution with methanol and rechromatography in the methanol system destroys the ninhydrin-positive material. Even during storage at 0° under nitrogen there is a decrease in ninhydrin-positive materials in a given basic fraction. With decomposition of the ninhydrin-positive material which contains a nitrogen with at least one hydrogen atom attached, a compound containing a carbonyl group is generated. This carbonyl-containing compound reacts slowly with 2,4-dinitrophenylhydrazine on thin-layer plates, absorbs in the IR region at 1720 $cm^{-1}$, has no absorption at 3600–3200 $cm^{-1}$, and is now ninhydrin-negative. The carbonyl-containing compound has been found under all conditions investigated thus far, while analogous treatment of control urine does not yield a carbonyl or ketone material.

The carbonyl-containing material which was generated from the ninhydrin-positive material (relative $R_F$ 1.0) has an $R_F$ relative to the standard amine or most polar ninhydrin spot from safrole of 5.5–6.0 in the methanol system. Using 1-(3,4-methylenedioxyphenyl)-penten-3-one-1) (J. T. Baker) as a standard in the benzene and hexane–diethyl ether–methanol–acetic acid solvent systems, the carbonyl-containing material which was generated from the ninhydrin-positive material has a relative $R_F$ of 1.0 in both cases. As is indicated by the above results, this material is much less polar than the ninhydrin-positive material. These results also indicate that the nitrogen may have been lost during the breakdown process. Treatment of the carbonyl-containing material with chromotropic acid on TLC gives a positive reaction for formaldehyde. This indicates that the methylenedioxy ring may also be intact. Further identification and characterization of the ninhydrin-positive materials and also the ketone materials from safrole is in progress.

*Isosafrole*

Isosafrole was administered to rats as a *cis–trans* mixture. Pooled urine was extracted as described earlier and the presence of ninhydrin-positive materials in the basic fraction was investigated. Results very similar to those of safrole were obtained for isosafrole. Two ninhydrin-positive materials with $R_F$ relative to the standard amine of 1.0–2.0 were present. With ninhydrin one material stained pink and the other purple. Preparative TLC of the basic urine fractions from isosafrole also gave rise to a 2,4-dinitrophenylhydrazine-positive material (ninhydrin-negative). As discussed earlier, decomposition of the ninhydrin-positive urinary constituent of safrole and isosafrole produces carbonyl-containing materials which are ninhydrin-negative. These carbonyl and ninhydrin-positive materials are presently being identified and characterized.

*Dihydrosafrole*

Dihydrosafrole was administered orally (75–300 mg/kg) to male rats; pooled urine was extracted and the basic fractions were chromatographed as described earlier. As shown in Table II, there is no detectable quantity of ninhydrin-positive material with a relative $R_F \geqslant 1.0$ as for safrole and isosafrole. The only ninhydrin-positive material that is present in the treated dihydrosafrole urine is a faint purple spot at the origin using the methanol systems. The results for dihydrosafrole urine are equivalent to the control urine and suggest that the production of the ninhydrin-positive material with a relative $R_F \geqslant 1.0$ is dependent upon the presence of an allyl or propenyl double bond in the compound administered. Considering that the double

TABLE II

$R_F \times 140$ VALUES OBTAINED ON TLC SEPARATION OF NINHYDRIN-POSITIVE BASIC MATERIALS

Abbreviations: PSB = pooled safrole basics; PCB = pooled control basics; DHSB = dihydrosafrole basics; 3,4-MDPIA = 3,4-methylenedioxyphenyl-isopropylamine. The color of the ninhydrin-positive spot is given in parentheses.

| *Fraction* | *Solvent system* | |
|---|---|---|
| | *Methanol* | *Chloroform–methanol–acetic acid (75:25:5)* |
| PSB | 12–15 (purple)<br>32–38 (purple) | 58–65 (purple) |
| PCB | 0–10 (pink-purple) | 15–20 (pink-purple)<br>30 (pink |
| DHSB | 0 (purple) | 20 (purple)<br>20–25 (purple) |
| 3,4-MDPIA | 10–43 (red-purple) | 40–70 (red-purple) |

bond is aminated to produce these ninhydrin-positive materials, the propyl derivative could not give rise to ninhydrin-positive materials similar to those of safrole or isosafrole unless there was first a dehydrogenation to produce an allyl or propenyl derivative.

*Asarone and β-asarone*

Additional experiments were carried out to determine the specificity requirement for a *cis* or *trans* double bond. Asarone (*trans* isomer) or β-asarone (*cis* isomer) was administered orally or i.p. to rats as described earlier. The basic urine fractions were examined by TLC. From earlier experiments with isosafrole, a *cis–trans* mixture, there does not seem to be a great difference in the amount of ninhydrin-positive materials excreted per volume of urine for safrole as compared to the same volume of urine after isosafrole treatment.

All urine extracts were concentrated as described earlier. Equal amounts of the basic fractions from myristicin, safrole, isosafrole, asarone (*trans*) and β-asarone (*cis*)-treated urine were applied to silica gel plates and then developed in the desired solvent system.

In comparison to safrole and myristicin, the asarone (*trans*)-treated urine contained very much more of the ninhydrin-positive material. As discussed earlier for myristicin and safrole, the maximum excretion for the basic ninhydrin-positive materials occurred 24–48 h after administration. For asarone, the *trans* isomer, the majority of the ninhydrin-positive material was excreted during the first 17 h after administration. One day after administration of asarone, very little ninhydrin-positive material with a relative $R_F \geqslant 1.0$ was excreted. In contrast, treatment with β-asarone, the *cis* isomer, produced only a small amount of ninhydrin-positive material in the urine even after two or three days. As is shown in Fig. 2, asarone, the *trans* isomer, produced much more (10 to 50-fold) ninhydrin-positive material than did either the *cis* isomer, safrole, or myristicin.

Using two-dimensional TLC and varying solvent systems, at least three ninhydrin-positive components from the asarone-treated basic urine fraction were separated (Fig. 3). After spraying with ninhydrin and heating, there are two pink spots

and one purple spot. There seem to be also acidic groups present in these materials.

It can be concluded from these preliminary investigations that after treatment of rats either orally or i.p. with myristicin, safrole, isosafrole, asarone (*trans*), or β-asarone (*cis*), basic ninhydrin-positive materials are excreted in the urine. There seems to be a prerequisite of a double bond for the production of these ninhydrin-positive materials. As suggested by unpublished work of BARFKNECHT[8], amphetamine-type materials may be produced in rats after feeding of allylbenzene. More specifically, the *trans* isomer of asarone is a better precursor to the ninhydrin-positive materials than the *cis* isomer. Control rats which did not receive the desired compound do not excrete these ninhydrin-positive materials in urine. These ninhydrin-positive basic materials break down very easily to produce ninhydrin-negative carbonyl-containing compounds.

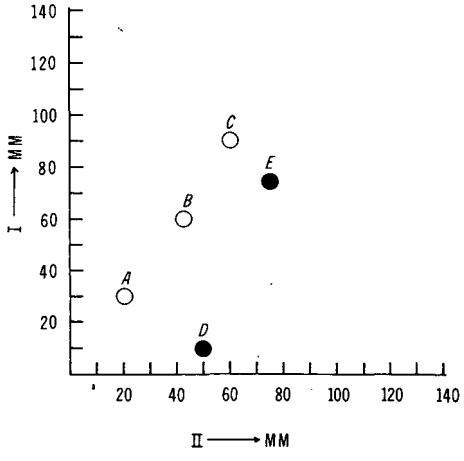



Fig. 3. Two-dimensional TLC separation of the asarone (*trans*) basic urine fraction. Solvent systems: ●, (I) (vertical), methanol–water–acetone–pyridine (200:20:20:10) 140 mm; (II) (horizontal), methanol–water–acetone–acetic acid (200:20:20:10) 140 mm. ○, (I) (vertical), methanol–water–acetone–acetic acid (200:20:20:10) 140 mm. (II) (horizontal), methanol–water–acetone–pyridine (200:20:20:10) 140 mm. Sprayed with ninhydrin. (A) $R_F$ 30↑; 20 (pink); (B) $R_F$ 60↑; 40 (purple); (C) $R_F$ 90↑; 60 (pink); (D) $R_F$ 10↑; 50 (purple); (E) $R_F$ 75↑; 75 (pink).

It is very probable that these ninhydrin-positive materials in urine are phenylisopropylamines or amphetamines which could bring about the psychotropic effect as described earlier for nutmeg and other natural products that contain these constituents. Further identification and characterization of these ninhydrin-positive materials will help to clarify the role of these components in the metabolism of the various substituted benzene derivatives.

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CHROM. 4368

# SEPARATION OF VITAMIN $K_2$ ISOPRENOLOGUES BY REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A simple thin-layer chromatographic system for the separation of the isoprenologues of vitamin $K_2$ is described. This system employs hexadecane-impregnated Kieselguhr thin-layer plates and an acetone–water solvent system. Using α-[1-$^{14}$C]-naphthol or 2-[$^{14}$C]methyl-1,4-naphthoquinone and DL-[5-$^{3}$H]mevalonate, the separation of the isoprenologues was confirmed. Each isoprenologue was recovered quantitatively and its proportion determined either by ultraviolet spectroscopy or radioactivity in the ring nucleus. *Staphylococcus aureus* synthesizes vitamin $K_2$ isoprenologues with 0, 5, 10, 15, 20, 25, 30, 35, 40, and 45 carbon atoms in the side chain. Vitamin $K_2$-0 represents about 6%; $K_2$-5 through 30, between 1 and 1.5% each; $K_2$-35, 20%; $K_2$-40, 60%; $K_2$-45, 6% of the total. *Haemophilus parainfluenzae* forms 2-demethyl vitamin $K_2$ isoprenologues with 0, 5, 10, 15, 20, 25, 30, 35, 40 and 45 carbon atoms in the side chain.

## INTRODUCTION

Vitamin $K_2$* is a naphthoquinone with isoprenoid side chains and is found in the respiratory systems of bacteria. When the formation of the membrane bound respiratory system is induced in *Staphylococcus aureus*, there is both a marked increase in the level of vitamin $K_2$ and a shift in the proportion of the major isoprenologues[1]. To examine how the synthesis of vitamin $K_2$ isoprenologues could be related to the formation of the electron transport system, a rapid method that would allow determination of the specific activities of the various isoprenologues of vitamin $K_2$ was needed. Reversed-phase thin-layer chromatography (TLC) or paper chromatographic systems in which the supporting phase was impregnated with paraffin oil, polyamide, silver nitrate or silicone oil have been described[2–4]. Complex impregnating agents often contain components that interfere with the spectral determination of the quinone. When highly purified hexadecane was used to form reversed-phase thin-layer plates, the isoprenologues could be separated. Each isoprenologue could be recovered

* Vitamin $K_2$-30 indicates 2-methyl-1,4-naphthoquinone with a polyisoprenoid side chain at position 3 containing 30 carbon atoms in the following configuration: $-(CH_2-CH=C(CH_3)-CH_2)_n$.

quantitatively and its concentration determined by the absorbance at 248 mμ without further purification. When necessary the contaminating hexadecane was readily removed.

## MATERIALS AND METHODS

### *Materials*

α-[1-$^{14}$C]naphthol and 2-[$^{14}$C]methyl-1,4-naphthoquinone were supplied by Amersham/Searle, Des Planes, Ill. Dibenzylethylenediamine-DL-[5-$^{3}$H]mevalonate was supplied by New England Nuclear Corporation, Boston, Mass. Kieselguhr G and Silica Gel G (Brinkmann Instruments, Inc., Westbury, N. Y.) and hexadecane-99 %, olefin free (Matheson, Coleman and Bell, Cincinnati, Ohio), were used to make the TLC plates. Highest purity commercial solvents were utilized without further purification. Synthetic vitamin $K_2$ isoprenologues were supplied through the generosity of O. ISLER, Hoffman-La Roche, Basle, Switzerland.

### *Growth of bacteria*

The strain, media, growth and harvesting conditions of *S. aureus* have been described[1]. The bacteria were grown for 14 h in the presence of 50 μCi each of a $^{14}$C- and a $^{3}$H-labeled substrate per 1500 ml to introduce radioactivity into the vitamin $K_2$. *Haemophilus parainfluenza* was grown, harvested and $DMK_2$ extracted as discussed previously[5].

### *Extraction of the lipids and purification of the vitamin $K_2$*

Bacteria were harvested by centrifugation and the lipids extracted with a modified BLIGH AND DYER procedure[6] as follows: The bacterial pellet was suspended in 30 ml of phosphate buffer and 75 ml of methanol and 37.5 ml of chloroform were added. The one-phase mixture was shaken in a separatory funnel and allowed to stand for at least 2 h. Then 37.5 ml of chloroform and 37.5 ml of water were added, the mixture shaken, and the phases allowed to separate. The lower phase was filtered through about 5 g of anhydrous sodium sulfate. The extract was dried in a stream of nitrogen and the residue was redissolved in a small volume of chloroform. This solution was applied as a series of spots near the bottom of a Silica Gel G thin-layer plate prepared as previously described[1]. The quinone fraction ($R_F$ 0.6) was separated from the phospholipids and polar carotenoids (origin) and non-polar carotenoids (solvent front) by ascending chromatography with a solvent of chloroform–isooctane (2:1). The quinone can be detected as a spot which quenches U.V. light (360 mμ). There was no spectral evidence for contamination of the purified quinone by carotenoids or lipid phosphate. The pure vitamin $K_2$ was recovered and eluted from the Silica Gel G as described[1].

### *Reversed-phase chromatography*

Kieselguhr G was spread to a thickness of 50 μ on glass chromatoplates and impregnated with hexadecane by ascending chromatography with 5 % hexadecane in chloroform. The plates were dried in air for a few minutes and used immediately. The naphthoquinone, after initial purification, was chromatographed by ascending chromatography in a solvent of acetone–water (95:5) (ref. 2) saturated with hexadecane. The chromatography was completed in about 45 min. The plate was allowed

to dry in air for a few minutes. The center of the plate was covered with Saran wrap (Dow Chemical Corp., Midland, Mich.) and the edges of the plate were sprayed first with 1% sodium borohydride in 50% ethanol and then with 0.2% aqueous neotetrazolium. The neotetrazolium was dissolved in 4 ml of 95% ethanol and made up to 200 ml with water. The quinones appeared as red spots without heating. If the quinones were radioactive, they were localized by autoradiography. The separated naphthoquinone isoprenologues were quantitatively recovered from the channels protected by the Saran Wrap by picking up the Kieselguhr in sealing tubes (with reduced ends and a coarse fritt, Corning 39580) with a vacuum, inverting the tube and eluting with 3 ml of chloroform followed by 3 ml of methanol and 3 ml of chloroform. Vitamin $K_2$ and hexadecane can be separated by TLC in Silica Gel G with solvents of 5% chloroform in hexane or 10% chloroform in methanol. U.V. spectra of the quinones were determined in isooctane using a Cary 15 spectrophotometer[7]. Reversed-phase chromatography of quinone isoprenologues on vaseline-impregnated paper with a solvent of dimethylformamide–water (32:1) was performed as described[8].

*Degradation of vitamin $K_2$*

Approximately 180 nmoles of purified vitamin $K_2$ labeled with $^{14}C$ from α-naphthol and $^{3}H$ from mevalonate were degraded by refluxing for 4 h in 10 ml of acetone containing 6.3 mmoles of crystalline potassium permanganate. The mixture was then cooled and filtered through Whatman No. 1 filter paper. The residue was washed with a small volume of acetone to remove unreacted permanganate and then with 150 ml of boiling water to recover the phthalic anhydride. The water extract was adjusted to pH 2.0 with hydrochloric acid and extracted three times with equal volumes of diethyl ether. The ether was washed with water and the solution was concentrated to a small volume and transferred to a silica gel thin-layer plate. The phthalic anhydride ($R_F$ 0.78) and phthalic acid ($R_F$ 0.40) derived from the ring of the naphthoquinone were separated from the degradation products of the side chain by ascending chromatography with a solvent of ethanol–water–12 *N* ammonium hydroxide (25:3:4). The phthalic acid and anhydride were detected as areas that quench U.V. light, and they were recovered from the silica gel as described[1].

*Determination of radioactivity*

Radioactivity was measured in a Packard Scintillation Spectrometer Model 2311 in a scintillation fluid of 9.25 m*M* 2,5-bis[2-(5-*tert.*-butyl-benzoxazoyl)]-thiophene (BBOT) in toluene. Radioactive samples were dried in the scintillation vials before adding the scintillation fluid. Quinones containing $^{14}C$ and $^{3}H$ were counted under conditions such that the $^{14}C$ channel = 0.017 $^{3}H$ + 0.542$^{14}C$ and the $^{3}H$ channel = 0.282 $^{3}H$ + 0.0002$^{14}C$ in the toluene scintillator. Under these conditions the efficiency of counting was 42% for $^{14}C$ and 7.6% for $^{3}H$. For autoradiography the thin-layer plates were placed on Kodak no-screen X-ray film as described[9].

## RESULTS

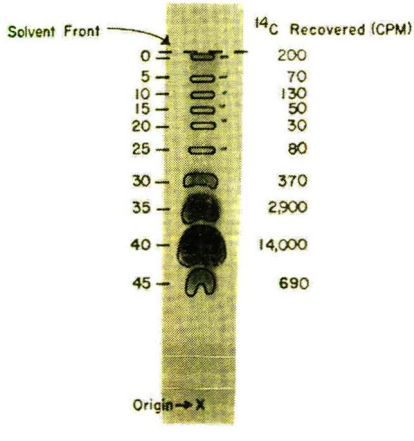
*Separation and recovery by reversed-phase TLC*

A purified vitamin $K_2$ preparation isolated from *S. aureus* grown in the presence of 2-[$^{14}C$]methyl-1,4-naphthoquinone was chromatographed on a hexadecane-impreg-

nated thin-layer plate. The plate was dried in air and placed in contact with a Kodak no-screen X-ray film for ten days. The film was developed and the silica gel corresponding to each dark spot on the autoradiogram was separated and eluted. A total of 18,500 c.p.m. $^{14}C$ was applied to the plate. The total $^{14}C$ recovered from the ten fractions was 18,000 c.p.m. for a 97 % recovery. The autoradiogram of the separation with the total $^{14}C$ recovered in each isoprenologue is illustrated in Fig. 1.

*Identification of the isoprenologues*

The chromatographic mobility of a purified vitamin $K_2$ preparation from *S. aureus* was compared with synthetic isoprenologues of vitamin $K_2$ in two chromatographic systems. The $R_M$ values $\{\log(1/(R_F)-1)\}$ (ref. 10) of isoprenologues in both the reversed-phase TLC system and a reversed-phase paper system agree with those of the synthetic standards. The $R_M$ values are linear for the isoprenologues with 15 to 50 carbon atoms (Fig. 2). *H. parainfluenzae* contains isoprenologues of 2-demethyl



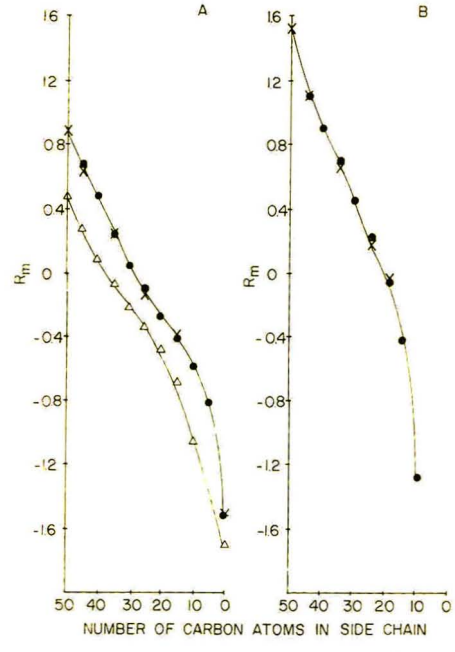




Fig. 1. Autoradiogram of vitamin $K_2$ isoprenologues. *S. aureus* grown in the presence of 2-[$^{14}C$]-methyl-1,4-naphthoquinone was harvested, the lipids extracted and the vitamin $K_2$ isolated and purified. The purified vitamin $K_2$ was spotted on a hexadecane-impregnated Kieselguhr thin-layer plate and chromatographed using acetone–water (95:5), saturated with hexadecane as solvent. The chromatogram was then placed in contact with Kodak no-screen X-ray film for ten days. The Kieselguhr corresponding to each radioactive spot was removed and the vitamin $K_2$ assayed for radioactivity as described. The total radioactivity of each isoprenologue is given on the autoradiogram, as counts per minute.

Fig. 2. $R_M$ values of vitamin $K_2$ and $DMK_2$ isoprenologues. (A) vitamin $K_2$ isoprenologues isolated from *S. aureus* (●), synthetic vitamin $K_2$ isoprenologues (×) and $DMK_2$ isolated from *H. parainfluenzae* (△) were separated on hexadecane-impregnated thin-layer plates as in Fig. 1. (B) vitamin $K_2$ isoprenologues isolated from *S. aureus* (●) and synthetic vitamin $K_2$ isoprenologues (×) were separated on vaseline-impregnated papers.

vitamin $K_2$ ($DMK_2$) (ref. 5) that can also be separated by the reversed-phase TLC system (Fig. 2).

*Labeled isoprenologues*

Mevalonic acid is preferentially incorporated into the carbon side chain of vitamin $K_2$ isoprenologues in *S. aureus*. The vitamin $K_2$ was isolated from cells grown for about 13 divisions with 25 $\mu$Ci each of $\alpha$-[1-$^{14}$C]naphthol and DL-[5-$^{3}$H]-mevalonate per 1500 ml. About 180 nmoles of quinone containing 15,000 c.p.m. $^{14}$C and 46,000 c.p.m. $^{3}$H were oxidized with permanganate and the phthalic acid and phthalic anhydride recovered from the products. Approximately 87 % of the $^{14}$C and 13 % of the $^{3}$H were recovered in the phthalic acid and phthalic anhydride. Presumably, the 13 % of the ring $^{14}$C not recovered in the phthalic derivatives could be found in levulinic acid derived partly from the ring or in other products of ring degradation. LEISTNER *et al.*[11] found that all the $^{14}$C in vitamin $K_2$ formed from $\alpha$-[1-$^{14}$C]naphthol-in *Bacillus megaterium* was recovered in the ring after permanganate oxidation. In *S. aureus* vitamin $K_2$-0, about 10–14 % of the $^{3}$H can be recovered from cells grown

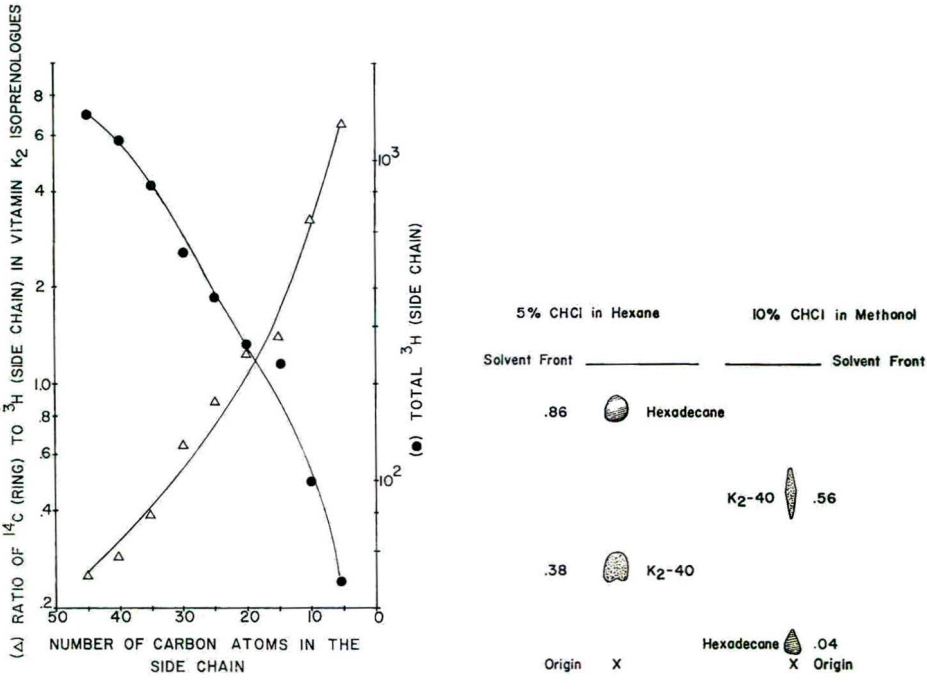



Fig. 3. Ratio of $^{14}$C to $^{3}$H in the ring and side chain of vitamin $K_2$ isoprenologues. The isoprenologues of vitamin $K_2$ labeled with $^{14}$C in the ring and $^{3}$H in the side chain were separated and assayed for radioactivity as described in Fig. 1. △ indicates the ratio of $^{14}$C in the ring to $^{3}$H in the side chain; ● indicates the total specific activity of $^{3}$H in the side chain of each isoprenologue.

Fig. 4. Separation of vitamin $K_2$-40 and hexadecane. TLC using the solvents illustrated allowed separation of the quinone from the hexadecane. The quinone was recovered quantitatively from the silica gel. The quinone was detected as a dark spot with U.V. light. The hexadecane can be seen as an oily spot on the plate after drying.

with DL-[5-$^3$H]mevalonate (unpublished data). Thus, it is reasonable to expect that 10–14 % of the $^3$H from DL-[5-$^3$H]mevalonate and all the $^{14}$C from α-[1-$^{14}$C]naphthol be found in the ring of vitamin $K_2$.

The total specific activity of $^3$H in the side chain of each isoprenologue of vitamin $K_2$ should increase with the increase in number of isoprenoid units in the side chain. The ratio of ring $^{14}$C to side chain $^3$H should decrease with increasing number of isoprenoid units in the side chain. In fact both these suppositions are true as illustrated in Fig. 3. To calculate the $^3$H in the side chain, the proportion of each isoprenologue was calculated from the $^{14}$C and the calculated proportion then used to calculate a specific activity for $^3$H and $^{14}$C in the isoprenologues. From the data on vitamin $K_2$-0, the specific activity of $^3$H in the ring could be measured directly. The $^3$H specific activity of vitamin $K_2$-0 was then used to correct the total specific activity in each of the other isoprenologues for the $^3$H in the ring. The assumption is made that all the isoprenologues have the same specific activity of $^3$H and $^{14}$C in the ring.

*Isolation of isoprenologues*

The isoprenologues can be recovered quantitatively from the hexadecane-impregnated thin-layer plates (Fig. 1). The hexadecane recovered in this operation does not interfere with the spectral determination of the isoprenologues but would interfere with mass spectral or nuclear magnetic resonance studies in which pure isoprenologue is required. The hexadecane can be readily separated from the isoprenologue by TLC with a solvent of 5 % chloroform in hexane or 10 % chloroform in methanol (Fig. 4). The isoprenologues can be recovered from the Silica Gel G with the sealing tubes, described above, eluted quantitatively and the solvents removed by evaporation *in vacuo*.

## DISCUSSION

The principal advantage of hexadecane-impregnated TLC for the separation of vitamin $K_2$ isoprenologues is that the separated isoprenologues can be recovered quantitatively and assayed by U.V. spectroscopy without further purification. The hexadecane can be easily removed by TLC yielding pure isoprenologues of vitamin $K_2$. The separation is complete, rapid and the recovery quantitative using materials that are available commercially. Previous methods[2–4] of reversed-phase chromatography using complex mixtures of hydrocarbons or silicone oils contain contaminants which interfere with the determination of U.V. spectra or do not give as good a separation. Use of highly purified tetradecane, octadecane and eicosane as the impregnating agent gave poor separation of the vitamin $K_2$ isoprenologues. Perhaps these hydrocarbons would be useful with terpenes of a different type.

The study of the isoprenologues of vitamin $K_2$ is useful in that the isoprenologue proportions vary with pathogenicity[12] or in the membrane modification concommitant with formation of the electron transport system in *S. aureus*[1]. Multiple isoprenologues of vitamin $K_2$ and $DMK_2$ have been shown in other bacteria[7,13–15]. A detailed study of the synthesis by *S. aureus* of the isoprenoid side chain of vitamin $K_2$ will be presented elsewhere.

ACKNOWLEDGEMENTS

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CHROM. 4363

# L'EMPLOI D'ORDINATEURS ÉLECTRONIQUES DANS L'ÉLABORATION DE SÉPARATIONS D'IONS INORGANIQUES PAR CHROMATOGRAPHIE SUR COLONNE

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SUMMARY

*The use of computers in the elaboration of separations of inorganic ions by column chromatography*

This article describes the use of computers in the elaboration of separations of inorganic ions by ion exchange. The use of computers as an aid in documentation (through the constitution of data and literature reference files) was investigated as well as their use in cases where decisions concerning the techniques to be employed have to be made. The optimalisation of separations was also studied.

## INTRODUCTION

Il y a une dizaine d'années les données disponibles pour élaborer des séparations d'ions inorganiques par chromatographie sur colonne consistaient en une dizaine de tableaux de constantes de distributions comme ceux de KRAUS ET NELSON[1] et de STRELOW[2] et en quelques centaines de fiches additionnelles au maximum. Depuis lors une véritable explosion dans le nombre des informations disponibles a eu lieu en partie par l'acceptation comme techniques de valeur des méthodes d'échange d'ions classiques et en partie par le développement de nouvelles méthodes, aussi bien en ce qui concerne les phases mobiles (emploi de milieux aqueux–organiques mixtes et de hautes concentrations en sels ou acides) que les phases stationaires (échangeurs inorganiques, "reversed phase", échangeurs cellulosiques, résines complexantes). Tout ceci a amené trois laboratoires, où l'on emploie fréquemment l'échange d'ions pour un grand nombre d'applications radiochimiques, à collaborer pour étudier la possibilité d'em-

ployer les ordinateurs en premier lieu comme aide dans la documentation et en deuxième lieu pour permettre de prendre des décisions sur les techniques à employer et de les optimaliser.

Nous décrirons ci-dessous les travaux réalisés ou en cours dans ces laboratoires.

Les informations peuvent être obtenues de trois façons: (1) coëfficients de distribution par équilibrage des phases mobiles et liquides (batch experiments) ou par chromatographie sur colonne; (2) pourcentages d'élution ou d'adsorption; (3) séparations décrites dans la littérature.

## (1) COËFFICIENTS DE DISTRIBUTION

A la Section d'Applications des Radioéléments (Département des Radioéléments du C.E.A.) à Grenoble, le premier objectif dont la réalisation est en cours est de constituer sur cartes mécanographiques une bibliothèque des coëfficients de distribution entre ions inorganiques et résines organiques du type Dowex 1 et Dowex 50.

On définit un *milieu* par (a) le type de résine (cationique ou anionique); (b) la nature du coëfficient de distribution ($D_V$ ou $K_d$); (c) la nature de la phase mobile (acide inorganique ou organique pur, mélange d'acides, mélanges acides–solvants organiques); (d) un code affecté à cette phase; (e) *l'échelle* des variations de concentration de la phase mobile. Celle-ci est identifiée par: un code concernant les variations de molarité, de pH ou de concentration en volume; et une référence à une table d'échelles. Il a paru utile de diversifier ces échelles pour profiter au maximum des 15 positions disponibles qui définissent par points une "courbe"; (f) la référence bibliographique.

Dans un milieu donné, l'ensemble des courbes concernant les différents ions pour lesquels on dispose de mesures de coëfficients de distribution est représenté par un paquet de cartes au format suivant: (i) reprise des codes milieu 1–4 ci-dessus; (ii) le symbole et la valence de l'ion; (iii) une suite de *valeurs des coëfficients* correspondant aux différentes positions de l'échelle; (iv) un code numérique, renvoyant à une table des ions, lequel peut être plus commode à manipuler au cours des calculs.

Les valeurs des coefficients sont relevées soit manuellement sur des tableaux publiés tels quels, soit mécanographiquement à partir des graphiques (*cf.* KRAUS ET NELSON[1]): une interpolation quadratique ou cubique est effectuée si nécessaire. Les valeurs obtenues sont perforées avec les conventions suivantes: 0 indique une absence de mesure; −1, une hydrolyse partielle; 9999 toute valeur supérieure à ce chiffre (rare); aucune décimale n'est retenue en dessus de 10, une décimale au-dessous.

Quant à l'utilisation de ces données, on peut envisager trois étapes: d'abord opérer une intercomparaison des mesures compilées, une normalisation par exemple des $K_d$ en $D_V$, ce qui peut amener une certaine expérimentation complémentaire; enfin une édition tenue à jour des résultats.

Dans la perspective des problèmes pratiques de séparations spécifiques ou par groupes, on a commencé à écrire des programmes de classements—dans un milieu donné—par ordre croissant ou décroissant de $D_V$. On peut aller plus loin en opérant une recherche systématique des sous-ensembles minimum ou maximum comprenant ou excluant l'ion ou les ions considérés, à travers l'ensemble des milieus de la bibliothèque.

Enfin, dans l'optique de la chromatographie, on envisagera des calculs d'une séquence complète de séparation en fixant, par exemple, les dimensions de colonnes et débits d'éluant de l'expérience—ou bien en tenant ces paramètres pour des variables à optimiser.

Une autre alternative a été essayée à l'Institut de Chimie Nucléaire à Gand. L'unité d'information reste la courbe. Toutefois, au lieu de la charactériser par des points fixes on la représente par une équation dont on calcule les paramètres par la méthode des moindres carrés. Comme il s'agit de séparations radiochimiques, il a semblé à l'auteur du programme que seules les séparations rapides étaient intéressantes c'est à dire celles où un élément est retenu ($D_V \geqslant 10$) et l'autre élué ($D_V \leqslant 5$).

On divise donc la zone des molarités en zones d'adsorption, d'élution et de comportement intermédiaire qu'on charactérise par un code chiffré. Pour savoir si deux éléments peuvent être séparés, il suffit de voir pour quelles molarités ces codes sont différents. Ensuite, on peut optimaliser en recherchant les molarités pour lesquelles l'élution est la plus rapide.

Cette méthode possède donc certains avantages pour l'étude d'une séparation de deux éléments. Pour plusieurs éléments et pour la constitution d'une bibliothèque, la méthode devient difficile à manier et on en a donc arrêté l'étude pour de tels problèmes.

(2) POURCENTAGES D'ADSORPTION

Depuis quelques années le laboratoire d'Analyse par Activation du C.C.R. Euratom d'Ispra s'est engagé dans l'étude de substances inorganiques comme adsorbants dans la séparation de radioéléments par chromatographie sur colonne. Un certain nombre de résultats a déjà été publié[3–7]. Ces résultats ont été classés sur cartes perforées et ont été codés de la façon suivante: (a) nom de l'échangeur inorganique; (b) nom de la phase mobile (= éluant); (c) symbole de l'élément chimique envisagé; (d) code alphanumérique du comportement observé. Les codes choisis pour représenter ces comportements sont repris au Tableau I.

TABLEAU I

CODE ALPHANUMÉRIQUE DU COMPORTEMENT D'ADSORPTION

| | |
|---|---|
| ** | Adsorption à 100% |
| * | Adsorption à plus de 95% |
| oo | Élution à 100% |
| o | Élution à plus de 95% |
| (* | Adsorption probable |
| (o | Élution probable |
| / / | Partage à ± 50% |
| (/ | Partage probable |
| = | Incertain ou non étudié |

La carte perforée contient la date de l'expérience et un code d'identification. D'autres cartes sont nécessaires à l'ordinateur et en particulier celles contenants les noms des échangeurs et les noms des éluants.

Le Tableau II donne les couples adsorbant–éluant utilisés et le nombre d'élé-

TABLEAU II

NOMBRE D'INFORMATIONS OBTENUES POUR LES COUPLES ADSORBANT–MILIEU UTILISEES

| | 1MHNO3 | 1MHCLO4 | 1MHCL | 1MH2SO4 | 7MHNO3 | 6MHCLO4 | 6MHF | 6MHCL | 14MHNO3 | 12MHCL | 0.1MHNO370C | 3MH2SO4 | 10MHCL | 1MHCLO490C | 7MHNO390C | 0.1MHNO3 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SNO2 | 59 | 47 | 57 | | 56 | | 46 | | 46 | | 41 | | | | | | 352 |
| MNO2 | 50 | 51 | | | | | 52 | | 51 | | 61 | | | | | | 265 |
| SB2O5 | 36 | 51 | | | 32 | 36 | 53 | 58 | 53 | 60 | | | | | | | 379 |
| AL2O3 | | 50 | | | 48 | | | | | | | | | 46 | 42 | | 186 |
| SIO2 | | 49 | | | 49 | | | | | | | | | | | | 98 |
| PIROLUSITE | | 50 | | | | | 50 | | 51 | | | | | | | | 151 |
| ZP | | | | 35 | 36 | 33 | | 33 | | | | | | | | | 137 |
| PTIA | | | | 34 | 41 | 38 | | 38 | | | | | | | | | 151 |
| SNP | | | | 33 | 34 | 33 | | | | | | 26 | | | | | 126 |
| CUS | | | | 33 | 34 | 34 | | 34 | | | | | | | | | 135 |
| CUCL | | | | 34 | | 36 | 36 | | | | | | | | | | 106 |
| CECOO2 | | | | 37 | | | 39 | | | | | | | | | | 76 |
| PBF2 | | 32 | | | | | 32 | | | | | | | | | | 64 |
| CDO | | | | | | | | | | | | | | | | 59 | 59 |
| CDS | | | | | | | 35 | | | | | | | | | | 35 |
| SB2O3 | 21 | | | | 21 | | 20 | | 22 | | | | | | | | 84 |
| SB2O4 | 36 | | | | 36 | 36 | 36 | | 36 | | | | 36 | | | | 216 |
| DOWEX1X8 | | | | | 32 | 32 | 33 | 33 | | | | | | | | | 130 |
| DOWEX50X8 | | | | | 32 | 33 | 33 | 32 | | | | | | | | | 130 |
| ZPSPRL | | | | | 34 | 34 | | 33 | | | | | | | | | 101 |
| SB2O5SPRL | | | | | 32 | | | 33 | 32 | | | | | | | | 97 |
| | 202 | 298 | 57 | 238 | 517 | 345 | 465 | 294 | 291 | 60 | 102 | 26 | 36 | 46 | 42 | 59 | 3076 |

ments dont le comportement a été étudié pour chaque couple. Les données (à présent plus de 3000 informations) sont exploitées essentiellement par les programmes TABLEAUX et SERACE (Séparation Radiochimique avec Calculatrice Electronique).

Le programme TABLEAUX permet en premier lieu le transfer des données sur bande magnétique. A partir de la date où le comportement d'un élément vis à vis d'un couple adsorbant–éluant à été déterminé, la bande est mise à jour soit en ajoutant de nouvelles données, soit en substituant les anciennes par de plus récentes. En deuxième lieu ce programme permet de classer les résultats:

(i) sous forme de tableaux périodiques des éléments (Fig. 1) pour chaque couple adsorbant–éluant;

(ii) sous forme de tableaux résumés permettant une meilleure visualisation du comportement observé pour chaque éluant ou pour chaque adsorbant.

Le programme SERACE est destiné à choisir une procédure pour séparer un élément d'un certain nombre d'autres. Comme données une liste d'éléments dont le premier est à séparer des autres suffit. Pour chaque élément indiqué le programme construit des sous-matrices ayant pour colonnes les éluants et pour lignes les adsorbants. Les éléments de ces matrices, qui représentent le comportement d'un élément observé pour chaque couple éluant–milieu, ont été réduits à quatre types. On considère comme séparables les éléments à codes

** oo // =

Le dernier code groupe tous les comportements qui n'appartiennent pas aux trois groupes précédents. L'ensemble de ces sous-matrices (dont un example est donné à la Fig. 2) constitue une matrice à trois dimensions qui comprend toutes les informations relatives aux élements à séparer. A partir de cette matrice, un certain nombre d'opérations logiques sont exécutées permettant d'indiquer quelle est la suite de couples adsorbant–éluant à utiliser pour obtenir la séparation désirée. SERACE a été limité à une séparation en trois étapes. Dans le cas oú le résultat est insuffisant on peut appliquer le programme à nouveau aux éléments qui ne sont pas encore séparés. Toutefois la séparation en plus de trois étapes peut être considérée comme peu économique. La sortie du programme SERACE pour une séparation du Zr d'un mélange Fe–Cr est donnée au Tableau III.

### (3) LES SÉPARATIONS DÉCRITES DANS LA LITTÉRATURE

On trouve dans la littérature un grand nombre de séparations déjà mises au point. On y emploie outre les phases stationaires et mobiles classiques, des éluants et des adsorbants pour lesquels des coëfficients de distribution n'ont pas été déterminés. En plus on cite souvent des détails importants tels que les dimensions des colonnes et les débits optimaux. L'exploitation de ces données a été étudiée à l'Institut de Chimie Nucléaire de l'Université de Gand.

Les données du programme EZRA consistent en une référence de l'article en question, éventuellement des cartes portants les remarques et les cartes avec la phase stationaire, les éléments et les éluants. Ces dernières emploient un code numérique (Fig. 3). Le code pour les éléments est le même que celui employé à Grenoble (pour permettre d'éventuels échanges).

31 catégories de phases stationaires sont prévues (6 résines polymériques, 13 échangeurs inorganiques, 10 systèmes de partition en phase inversée, 2 divers) et 71

FILTER $MnO_2$ — 100 o/o RETAINED **
OVER 95 o/o RETAINED *
MEDIUM 0.1M $HNO_3$ 70°C — 100 o/o ELUTED oo
OVER 95 o/o ELUTED o
PROBABLY RETAINED (*
PROBABLY ELUTED (o
PARTIALLY RETAINED //
PROBABLY DISTRIBUTED (/
UNCERTAIN =

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| oo LI | BE | | | | | | | | | | | B | C | N | O | oo F |
| oo NA | oo MG | | | | | | | | | | | oo AL | SI | (/ P | (/ S | oo CL |
| ** K | oo CA | ** SC | ** TI | ** V | ** CR | ** MN | ** FE | oo CO | oo NI | oo CU | oo ZN | ** GA | ** GE | ** AS | ** SE | oo BR |
| ** RB | oo SR | ** Y | ** ZR | ** NB | ** MO | oo TC | (/ RU | RH | ** PD | ** AG | oo CD | ** IN | ** SN | ** SB | ** TE | ** I |
| oo CS | oo BA | LAN | ** HF | ** TA | ** W | oo RE | ** OS | oo IR | oo PT | oo AU | ** HG | TL | PB | BI | PO | AT |
| FR | RA | ACT | | | | | | | | | | | | | | |
| LANTHANIDES | | ** LA | ** CE | PR | ** ND | ** PM | SM | ** EU | GD | ** TB | DY | HO | ER | TM | YB | LU |
| ACTINIDES | | AC | TH | ** PA | oo U | oo NP | PU | AM | CM | BK | CF | DATE = 31 10 68 | | | | |

Fig. 1. Sortie du programme TABLEAUX.

| | 1MHNO3 | 1MHCLO4 | 1MHCL | 1MH2SO4 | 7MHNO3 | 6MHCLO4 | 6MHF | 6MHCL | 14MHNO3 | 12MHCL | 0.1MHNO370C | 3MH2SO4 | 10MHCL | 1MHCLO490C | 7MHNO390C | 0.1MHNO3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SNO2 | oo | // | ** | = | oo | = | oo | = | oo | = | ** | = | = | = | = | = |
| MNO2 | // | // | = | = | = | = | oo | = | oo | = | ** | = | = | = | = | = |
| SB2O5 | ** | // | = | = | oo | // | oo | oo | oo | oo | = | = | = | = | = | = |
| AL2O3 | = | oo | = | = | oo | = | = | = | = | = | = | = | = | // | oo | = |
| SIO2 | = | // | = | = | // | = | = | = | = | = | = | = | = | = | = | = |
| PIROLUSITE | = | oo | = | = | = | = | oo | = | oo | = | = | = | = | = | = | = |
| ZP | = | = | = | ** | ** | ** | = | ** | = | = | = | = | = | = | = | = |
| PTIA | = | = | = | ** | // | ** | = | oo | = | = | = | = | = | = | = | = |
| SNP | = | = | = | // | oo | oo | = | = | = | = | = | oo | = | = | = | = |
| CUS | = | = | = | oo | oo | oo | = | oo | = | = | = | = | = | = | = | = |
| CUCL | = | = | = | oo | = | oo | oo | = | = | = | = | = | = | = | = | = |
| CECOO2 | = | = | = | oo | = | = | ** | = | = | = | = | = | = | = | = | = |
| PBF2 | = | = | = | // | = | = | // | = | = | = | = | = | = | = | = | = |
| CDO | = | = | = | = | = | = | = | = | = | = | = | = | = | = | ** | = |
| CDS | = | = | = | = | = | = | oo | = | = | = | = | = | = | = | = | = |
| SB2O3 | = | = | = | = | = | = | = | = | = | = | = | = | = | = | = | = |
| SB2O4 | // | = | = | = | oo | oo | oo | = | oo | = | = | = | oo | = | = | = |
| DOWEX1X8 | = | = | = | = | oo | oo | ** | // | = | = | = | = | = | = | = | = |
| DOWEX50X8 | = | = | = | = | oo | // | oo | oo | = | = | = | = | = | = | = | = |
| ZPSPRL | = | = | = | = | // | // | = | // | = | = | = | = | = | = | = | = |
| SB2O5SPRL | = | = | = | = | oo | = | = | oo | oo | = | = | = | = | = | = | = |

Fig. 2. Example de sous-matrice: élément Zr. Symboles: **, adsorbé; oo, elué; //, partagé; = non testé.

TABLEAU III

SORTIE DU PROGRAMME SERACE

GLI ELEMENTI ZR FE TRATTATI CR SONO I 3 SEGUENTI

| FASE 1 | | FASE 2 | | FASE 3 | |
|---|---|---|---|---|---|
| $SNO_2$ | ZR | | | | |
| 1MHCL | * * | | | | |
| ZP | ZR | | | | |
| $7MHNO_3$ | * * | | | | |
| ZP | ZR | | | | |
| $6MHCLO_4$ | * * | | | | |
| ZP | ZR | | | | |
| 6MHCL | * * | | | | |
| DOWEX1X8 | ZR | | | | |
| 6MHF | * * | | | | |
| $SNO_2$ | ZR | $SNO_2$ | ZR | | |
| 0.1$MHNO_3$70C | * * | 1MHCL | * * | | |
| ZP | ZR | ZR | ZR | | |
| 1$MH_2SO_4$ | * * | $7MHNO_3$ | * * | | |
| ZP | ZR | ZP | ZR | | |
| 1$MH_2SO_4$ | * * | $6MHCLO_4$ | * * | | |
| ZP | ZR | ZP | ZR | | |
| 1$MH_2SO_4$ | * * | 6MHCL | * * | | |
| $SNO_2$ | ZR | $SNO_2$ | ZR | ZP | ZR |
| 0.1$MHNO_3$70C | * * | $7MHNO_3$ | o o | $7MHNO_3$ | * * |
| $SNO_2$ | ZR | $SNO_2$ | ZR | DOWEX1X8 | ZR |
| 0.1$MHNO_3$70C | * * | 6MHF | o o | 6MHF | * * |
| $MNO_2$ | ZR | $MNO_2$ | ZR | DOWEX1X8 | ZR |
| 0.1$MHNO_3$70C | * * | 6MHF | o o | 6MHF | * * |
| $SB_2O_5$ | ZR | $SB_2O_5$ | ZR | ZP | ZR |
| 1$MHNO_3$ | * * | $7MHNO_3$ | o o | $7MHNO_3$ | * * |
| $SB_2O_5$ | ZR | $SB_2O_5$ | ZR | DOWEX1X8 | ZR |
| 1$MHNO_3$ | * * | 6MHF | o o | 6MHF | * * |
| $SB_2O_5$ | ZR | $SB_2O_5$ | ZR | ZP | ZR |
| 1$MHNO_3$ | * * | 6MHCL | o o | 6MHCL | * * |
| PTIA | ZR | PTIA | ZR | ZP | ZR |
| 1$MH_2SO_4$ | * * | 6MHCL | o o | 6MHCL | * * |
| PTIA | ZR | PTIA | ZR | ZP | ZR |
| $6MHCLO_4$ | * * | 6MHCL | o o | 6MHCL | * * |

catégories d'éluants (27 acides et solutions de sels minéraux, 10 mélanges d'acides, 14 mélanges de solvants inorganiques et organiques, 13 éluants complexants, 7 divers).

Les données sont classées sur les cartes de la façon suivante: (a) le nombre de cartes nécessaires pour décrire une séparation; (b) le code de la résine; (c) les codes des éléments élués avec le premier groupe et le code de l'éluant avec lesquels ces éléments sont élués; (d) les codes des éléments du deuxième groupe, le code du deuxième éluant etc.

Un example d'entrée et de sortie est donné à la Fig. 3.

Le programme EZRA peut répondre à un certain nombre de questions. On peut non seulement demander l'inventaire de toutes les séparations se trouvant en bibliothèque mais aussi toutes les séparations concernant un ou plusieurs éléments, toutes les séparations d'un élément donné d'un groupe d'autres éléments donnés, les séparations qui emploient une phase stationaire ou mobile donnée ou enfin toutes les séparations employant une phase stationaire et un éluant donnés.

```
1 6 31237 67237  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0
CABELL MILNER A C A 13 258 55
STATIONARY PHASE  AN S
HCL HF NB
HCL HF TA
10CHERNOBROV KOLONINA A A 8 4596 61
1 7 17237 31237 11201 67237  0  0  0  0  0  0  0  0  0  0  0  0
CHERNOBROV KOLONINA A A 8 4596 61
STATIONARY PHASE  AN W
HCL HF FE
HCL HF NB
HCL    TI
HCL HF TA
10DENISOVA CVETKOVA ZAV LAB 27 656 61
1 7  6201 22288  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0
DENISOVA CVETKOVA ZAV LAB 27 656 61
STATIONARY PHASE  AN W
HCL    AL
H2O    GA
10EDGE J CHROMAT 526 61
1 6 29264 54 50264  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0
EDGE J CHROMAT 526 61
STATIONARY PHASE  AN S
ETHNO3 Y
ETHNO3 LA ND
10FARIS ANAL CHEM 35 520 60
1 6 31203 67203  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0
FARIS ANAL CHEM 35 520 60
STATIONARY PHASE  AN S
HF     NB
HF     TA
10FISCHER MELOCHE ANAL CHEM 24 1100 52
1 6 32287 69201  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0
FISCHER MELOCHE ANAL CHEM 24 1100 52
STATIONARY PHASE  AN S
BASE   MD
HCL    RE
10FRESEN VAN GOGH VAN PINXTEREN PHARM WEEKBL 95 33 50
1 6104218106287  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0
FRESEN VAN GOGH VAN PINXTEREN PHARM WEEKBL 95 33 50
STATIONARY PHASE  AN S
CLORID F
BASE   SO4
10GERDES RIEMAN A C A 27 113 62
1 6 19201 20201 80201 17201 21205 44202  0  0  0  0  0  0  0  0  } INPUT
GERDES RIEMAN A C A 27 113 62
STATIONARY PHASE  AN S
HCL    NI                                                        }
HCL    CU                                                        } OUTPUT
HCL    PB                                                        }
HCL    FE                                                        }
HOX    ZN                                                        }
HNO3   SN                                                        }
10GILLIS EECKHOUT SPEECKE C A 48 9862 54
1 6 31240 67201  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0  0
GILLIS EECKHOUT SPEECKE C A 48 9862 54
STATIONARY PHASE  AN S
HCLHOX NB
HCL    TA
10GIUFFRE CAPIZZI ANN CHIM (IT) 49 1419 59
```

Fig. 3. Entrée (INPUT) et sortie (OUTPUT) du programme EZRA.

Pour rendre le programme plus général, les données recueillies à Ispra ont été incorporées et une annexe expérimentale au programme a été élaborée afin d'y introduire les constantes de distribution. En ce qui concerne les données d'Ispra, on a choisi un critère assez sévère. On considère notamment comme séparés les éléments à code 00 (c'est à dire à 100 % élués, réunis dans un premier groupe) et les éléments à code **, réunis dans un autre. Les autres éléments sont groupés dans un dernier groupe. A la SORTIE la ligne titre est employée pour dénoter l'échangeur et l'éluant. La ligne remarque indique comment les éléments sont classés selon leurs codes.

Ensuite suivent les deux ou trois groupes d'éléments. La Fig. 4 donne un exemple de SORTIE.

Un système similaire est expérimenté pour les constantes de distribution que le programme groupe en catégories $K_d \leqslant 5$, $5 < K_d \leqslant 10$, $10 < K_d \leqslant 20$, $20 < K_d \leqslant 50$ et $K_d > 50$, ceci pour répondre aux questions formulées ci-dessus, sauf une.

```
BEGIN FILE GUZZI
**100%RETAINED,*OVER95%RETAINED,00100%ELUTED,(*PROBABLY  RETAINED,(0PROBABLY ELUTED,//PARTIALLY RETAINED,(/PROBABLY
DISTRIBUTED,=UNCERTAIN
11AL203 7 M HNO3
324 19109 10 14 15 17 18 21 26 27 28 32 33 41 42 75 48 69 76 77
AL203 7 M HNO3
STATIONARY PHASE  ALUM
100203(/+ = 4**
HNO3   NA K  CA SC CR MN FE CO NI CU ZN GA SE RB SR Y  ZR MO TC RU PD AG CD IN CS BA CE EU YB HF RE IR PT AU HG PA CO3
I  NP
HNO3   OS
HNO3   GE NB SN SB TA BR
HNO3   AS W  PO4
11 AL203 1 M HCLO4
324 10 14 15 17 18 21 26 27 28 40 42 46 48  9 29  4 41 19110 75
 AL203 1 M HCLO4
STATIONARY PHASE  ALUM
1002 = +(3**
HCLO4  NA K  CA SC CR MN FE CO NI CU ZN GA SE RB SR Y  ZR TC RU PD AG CD IN CS BA CE EU YB HF RE PT HG BR I  CHLNP
HCLO4  TA OS IR AU PA
HCLO4  GE AS NB MO SB W  PO4
11SB205 14 M HNO3
322  8 10 14 15 17 19 20 21 22108 27 28 33 34 39 41109 48 69 75
SB205 14 M HNO3
STATIONARY PHASE  SB05
 1 00 2 0 3 (/ 4 * 5 **
HNO3   K  SC CR MN FE CO NI CU ZN GA RB SR Y  ZR TC RU3PD4CD CS EU YB RE OS PT AU HG U  RA SO4CL BR I  NP
HNO3   CA BA CE4IR
HNO3   IN TE HF W  PO4
HNO3   SB
NOTDES NA GE AS NB MO AG SN TA PA
11SB205 6 M HCLO4
222  8 35 48 28 49 52 57 64 14 15 17 18 19 20 21 42 77 73 71 27
SB205 6 M HCLO4
STATIONARY PHASE  SB05
 1 00 2 (/ 3 **
HCLO4  K  CR MN FE CO NI CU ZN RB SR RU IN CS BA CE4EU YB HF RE OS IR AU HG I
HCLO4  ZR NB
NOTDES NA SE AG CD SN SB TA W  PA
11SB205 7 M HNO3
222 48 10 15 18 76 21 77 35109 69 73 71 30202 28 52 57 64 14 42
SB205 7 M HNO3
STATIONARY PHASE  SB05
 1 00 2 (/ 3 **
HNO3   SC MN CO ZN ZR RU CS RE OS IR AU HG I
HNO3   CR SR IN SB CE4EU YB HF W
NOTDES NA K  SE RB NB AG SN TA PA
11SB205 1 00 2 0 3 (/ 4 **
322 21108 69 19 76 31202 32113202 64 26 35 46109 71 73 50 59 14
SB205 1 00 2 0 3 (/ 4 **
STATIONARY PHASE  SB05
REMARK MANKEERT
HNO3   NI ZN NB RE AU BR
HNO3   MO NP
HNO3   CR MN FE CO CU RU IN SB LA TB YB W  OS IR HG I
NOTDES NA K  SC SE RB SR ZR AG CD SN CS BA CE4EU HF TA PA
11SB205 1 M HCLO4
322107 14 18 19 20 21 22108 33 69 71 75 76 17106 29105204 15 38
SB205 1 M HCLO4
```

} INPUT

} OUTPUT

Fig. 4. Entrée et sortie du programme EZRA; phases stationaires.

Lorsqu'on demande en effet une liste de séparations possibles entre deux éléments donnés on fait intervenir aussi le concept du coëfficient de séparation $\beta$ ($\beta = K_d(1)/K_d(2)$). Les limites des catégories et les valeurs de $\beta$ acceptées comme critères de séparation, devront être soumises à l'expérimentation.

Le programme EZRA est construit de telle façon qu'on peut demander soit les séparations décrites dans la littérature soit les informations d'Ispra, soit les informations de Grenoble, soit les trois à la fois.

REMERCIEMENTS

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RÉSUMÉ

Cet article décrit l'emploi d'ordinateurs électroniques dans l'élaboration de séparations d'ions inorganiques par échange d'ions. On étudie la possibilité d'employer les ordinateurs en premier lieu comme aide dans la documentation (constitution de bibliothèques de données) et en deuxième lieu pour permettre de prendre des décisions sur les techniques à employer.

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CHROM. 4400

# CHROMATOGRAPHY AND ZONE ELECTROPHORESIS OF INORGANIC IONS DISSOLVED IN FUSED SALTS

## VI. THE ELECTROPHORETIC BEHAVIOUR OF SOME INORGANIC ANIONS IN ALKALI NITRATE AND PERCHLORATE MELTS*

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SUMMARY

The electrophoretic mobilities of some inorganic anions dissolved in molten $LiNO_3$ at 300°, (Li–K)$NO_3$ eutectic at 160°, (Na–K)$NO_3$ mixture at 250° and (Li–K)$ClO_4$ eutectic at 300° are reported and discussed.

In (Li–K)$ClO_4$ eutectic, all the anions examined, except $ClO_4^-$, move towards the cathode or stay at the point of application. This is probably due to a strong cation–anion interaction, according to the BLANDER[11] model of molten salts, occurring between the Li ions of the solvent and the dissolved anions.

From the differences between the mobilities of the chloride and bromide ions in the solvents examined, an approximate scale for the associating power of the cations of these solvents could be established.

## INTRODUCTION

The electrophoretic behaviour of inorganic anions dissolved in molten salts[1–4] has, until now, received very little attention.

Some indication concerning the type of the interaction occurring in fused salts can be deduced[5–7] from determination of the mobilities of tracer cations; hence measurements of the mobilities of tracer anions should permit a better understanding of these interactions.

The behaviour of the tracer anions was investigated by zone electrophoresis, a technique already used in tracer cation mobilities studies[1,5,8]. Various fused alkali nitrates and molten (Li–K)$ClO_4$ eutectic were used as electrolytes in order to investigate the influence of the cations and anions of the solvent on the mobilities of the tracer anions.

* This work was supported by Consiglio Nazionale delle Ricerche.

## EXPERIMENTAL

$LiNO_3$, $NaNO_3$, $KNO_3$ and $KClO_4$ were Erba RP products. $NO_2ClO_4$ was supplied by K & K Laboratories; anhydrous $LiClO_4$ was obtained according to a procedure described previously[9]. The salts were finely powdered, dried at 110° and mixed in the molar proportions $LiNO_3$–$KNO_3$ (43:57) (m.p. 132°), $NaNO_3$–$KNO_3$ (50:50) (m.p. 220°), $LiClO_4$–$KClO_4$ (76:24) (m.p. 207°) and then dried again at 110°.

The electrophoretic experiments were performed in an apparatus similar to that described in a previous paper[1]; the experimental conditions were similar to those reported in ref. 8, the cathodic and anodic reservoirs being separated from the central vessels, where the strip dipped.

To avoid explosive reactions when (Li–K)$ClO_4$ eutectic was employed as solvent, a suitable alkali nitrate mixture was used as catholite.

$Cl^-$, $Br^-$ and $ClO_4^-$ were detected radiometrically by using $^{36}Cl$, $^{82}Br$ and $^{36}ClO_4$ tracers, the distribution of their activities on the glass fiber paper being obtained by scanning each 0.5 cm of the strip.

The colorimetric detection of other inorganic anions was made by cutting the strip in several portions of 0.5 cm and dipping them in aqueous solutions of suitable reagents.

## RESULTS AND DISCUSSION

Table I shows the electrophoretic mobilities* of some inorganic anions dissolved in various fused nitrates and (Li–K)$ClO_4$ eutectic.

TABLE I

ELECTROPHORETIC MOBILITY ($cm^2/V\ sec \cdot 10^{-4}$) OF INORGANIC ANIONS IN VARIOUS FUSED SOLVENTS

| *Ion* | *(Li–K)ClO₄ T = 300°* | *LiNO₃ T = 300°* | *(Li–K)NO₃ T = 160°* | *(Na–K)NO₃ T = 250°* |
|---|---|---|---|---|
| $NO_3^-$ | 3.3 (+) | — | — | — |
| $NO_2^-$ | 2.4 (+) | — | — | — |
| $CrO_4^{2-}$ | 0.8 (+) | 0.0 | 0.3 (−) | 0.8 (−) |
| $Cr_2O_7^{2-}$ | 0.8 (+) | 0.0 | 0.3 (−) | 0.8 (−) |
| $ClO_4^-$ | 0.7 (−) | — | — | — |
| $ClO_3^-$ | — | 0.5 (−) | 0.3 (−) | 0.65 (−) |
| $BrO_3^-$ | 0.45 (+) | 0.15 (−) | 0.15 (−) | 0.3 (−) |
| $IO_3^-$ | 0.8 (+) | 0.1 (+) | 0.0 | 0.1 (−) |
| $Cl^-$ | 0.9 (+) | 0.65 (−) | 0.4 (−) | 1.0 (−) |
| $Br^-$ | 0.1 (+) | 1.1 (−) | 0.6 (−) | 0.8 (−) |
| $I^-$ | — | — | 0.7 (−) | 0.75 (−) |

(+) Movement towards cathode.
(−) Movement towards anode.

The most striking feature is that, in a (Li–K)$ClO_4$ eutectic, all the species examined, except $ClO_4^-$, travel towards the cathode, or stay at the point of application. The cationic behaviour was also confirmed for nitrate, electrophoresis experiments

* As already pointed out[8], lack of knowledge concerning the electroosmotic flow in molten media, precludes our knowing for certain whether the values represent the true mobility of the species under examination.

being carried out for a period of several hours. After 10 h of electrolysis it was noted that no traces of nitrate ion (<0.01 mg) had migrated from the cathodic vessel containing the nitrate mixture to the neighbouring vessel into which the strip dipped*.

Simple chromatographic tests on all species showing zero mobility showed that this behaviour was not due to interaction with the support.

The cationic behaviour of inorganic anions in molten perchlorates could be explained by the following two hypotheses:

(a) Strong interactions occur between the tracer anions and the cations of the melt, according to the equilibrium

$$MeO_x^{-n} + mLi \rightleftharpoons Li_mMeO_x^{+(m-n)} \text{ with } m \geqslant n \qquad x = 0,1,2\ldots$$

(b) A positively charged group is stable in this solvent, according to the Lux-Flood equilibrium

$$MeO_x^{-n} \rightleftharpoons MeO_{x-y}^{+(2y-n)} + yO^{-2} \text{ with } y \geqslant n$$

It must be noted that the oxygen ion formed in eqn. (b) can give rise to stable association, as reported by KOHLMÜLLER[10], with the lithium ions of the solvent, shifting equilibrium (b) towards the right. Thus, in both cases, the "cationic" behaviour of inorganic tracer anions is probably due to the strong associating power of $Li^+$.

Concerning the equilibrium (b), an attempt was made to introduce the species $NO_2^+$ (the Lux acid corresponding to $NO_3^-$) directly into a well-dried perchlorate melt by using nitronium perchlorate. The introduction of this salt immediately gave rise to gaseous products and the successive analysis showed no traces of nitrate or nitrite ions in the melt. It thus seems that $NO_2^+$ is not very stable in fused perchlorates and that the formation of positively charged species is probably due to equilibrium (a).

A similar equilibrium holds for tracer cations dissolved in fused salts as already discussed by LANTELME AND CHEMLA and in our previous papers in order to explain the behaviour of alkali metal ions in fused nitrates[6] and perchlorates[5], or the anionic mobilities of transition metal ions in molten (Li–K)$NO_3$[8].

The behaviour of the perchlorate ion in (Li–K)$ClO_4$ eutectic suggests that this anion is the one which interacts least with the $Li^+$ of the solvent among all the anions examined. This is in good agreement with the well-known poor complexing power of perchlorate anion in aqueous solution.

The differences between the mobilities of chloride and bromide ions, measured in the various fused solvents, are reported in Table II.

One can see that in nitrate solvents containing $Li^+$, bromide also moves faster towards the anode than chloride, while, according to the Stokes' law, the opposite should be expected. Furthermore, in this case the electrophoretic behaviour is affected by association reactions though not so evidently as in a perchlorate solvent.

The interaction of the halide anions with the cations of the solvent can be related, according to the BLANDER model[11], to the coulombic effect which is greater the smaller the distance of closest approach in the cation–anion pair.

* In this way, the use of nitrates as catholite was fully justified, since no variation in the composition of perchlorate solvent occurs.

TABLE II

DIFFERENCES BETWEEN THE ELECTROPHORETIC MOBILITY ($cm^2/V\ sec \cdot 10^{-4}$) OF CHLORIDE AND BROMIDE IONS DISSOLVED IN VARIOUS FUSED SOLVENTS

| *Solvents* | $U_{Cl^-}$ | $U_{Br^-}$ | $\Delta u$ |
|---|---|---|---|
| (Li–K)$ClO_4$ | 0.9 (+) | 0.1 (+) | +0.8 |
| $LiNO_3$ | 0.65 (−) | 1.1 (−) | +0.45 |
| (Li–K)$NO_3$ | 0.4 (−) | 0.6 (−) | +0.2 |
| (Na–K)$NO_3$ | 1.0 (−) | 0.8 (−) | −0.2 |

(+) Movement towards cathode.
(−) Movement towards anode.

When the $Li^+$ of the solvent is substituted by the less associating $Na^+$ (Table II last column) the ionic interactions are weakened and the anionic mobility of chloride becomes greater than that of bromide*.

With respect to the electrophoretic mobilities of inorganic oxyanions the situation is more complicated and comparison of the results obtained in various solvents is difficult. Data reported in Table I, seem to indicate that the interactions of these species with the cations of the solvent are due rather to polarization forces, as suggested by FÖRLAND[12], than to coulombic effect.

Unfortunately, the lack of data, such as ionic radius and polarizability of the oxyanions examined, does not allow us to attempt quantitative considerations. On the other hand we think, according to the LUMSDEN formulation[13], that both coulombic and polarization effects have to be considered to explain the interactions among the tracer anions and the cations of the solvent.

Probably the anion under investigation will be greatly affected by both, depending upon its own particular shape. The results obtained seem useful in order to have a scale for the interacting power of $Li^+$ with inorganic anions.

As reported previously[5], a relationship between this quantity and the molar concentration of $Li^+$ in the melt cannot be deduced easily when the anion of the solvent is changed.

Since the electrophoretic behaviour of the tracer anions depends upon the relative interaction of the ions with the cations of the solvent, it would seem possible to correlate the values of the differences between the mobilities of chloride and bromide** dissolved in various melts (Table II) to the amount of "free" Li ion. The data obtained suggest that the "activity" of $Li^+$ is higher in a (Li–K)$ClO_4$ eutectic than in pure $LiNO_3$, in good agreement with data obtained for the solubility of ammonia in these media[14]. Thus, a first approximate*** scale of the interacting power of the cations of the melt can be deduced from the mobility differences (which are not affected by the eventual electroosmotic flow).

This scale could be useful in order to have, among different fused salts, a first comparison criterion, which, till now, has never been stated unambiguously.

* For a more detailed discussion on the dependence of the electrophoretic mobilities of a given tracer ion on the cations and the anions present in the melt see ref. 5.

** These anions were selected since they should exhibit less complex interactions with the cations of the solvent than the other species examined.

*** Variations in viscosity and temperature should be also be taken into account, but in our case they cannot cause a reversal of the sign of the mobility differences.

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## Notes

CHROM. 4398

### Dependence of film thickness on column radius and coating rate in preparation of capillary columns for gas chromatography

Mainly because of its simplicity, the dynamic coating procedure[1] for the preparation of capillary columns for gas chromatography has been widely used, and preferred to the static version originally proposed by GOLAY[2]. KAISER[3] studied the dependence of the average thickness of the stationary phase layer, $d_F$, on the operating conditions during dynamic coating. He reported the following relation between $d_F$, the % v/v concentration of the phase in hexane solution, $c$, the velocity of the coating plug, $u$, and the inner radius of the capillary, $r$:

$$d_F = \frac{c}{100} \frac{1}{2r} (0.265\ u + 0.25) \qquad (1)$$

The wetting of capillaries was, however, studied by FAIRBROTHER AND STUBBS[4] in connection with electro-endosmotic measurements as early as 1935, and their conclusions have been more recently confirmed by other authors[5,6]. In contrast to eqn. (1), the FAIRBROTHER–STUBBS equation, adjusted to our case, relates $d_F$ *directly* to the column radius and to the *square root* of the coating rate:

$$d_F = \frac{c}{100} \frac{r}{2} \sqrt{u \frac{\eta}{\sigma}} \qquad (2)$$

where $\eta$ and $\sigma$ are the viscosity and surface tension of the coating solution, respectively.

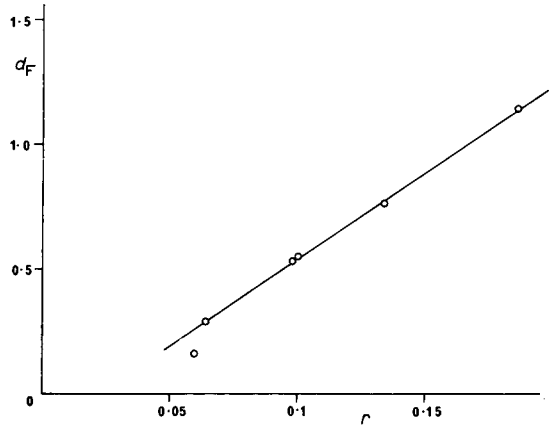


Fig. 1. Graph of film thickness, $d_F$ ($\mu$), against column radius, $r$ (mm), for vapour-phase silanised Pyrex glass capillary columns coated with a 10% v/v solution of SF-96 in toluene at 5 mm/sec.

We have been recently concerned with the reproducible preparation of highly efficient glass capillary columns for the analysis of tobacco smoke[7]. In order to establish a reliable technique for the preparation of columns of controlled efficiency and capacity, some factors affecting their coating were investigated in work to be described elsewhere[8]. A study of the dependence of $d_F$ on $r$ and $u$ (Figs. 1 and 2) showed that eqn. (2) is indeed applicable to the coating of capillary columns.

The FAIRBROTHER–STUBBS equation also has the advantage of being dimensionally correct. Moreover, the dependence of $d_F$ on the viscosity and surface tension of the coating solution is presumably responsible for the so-called "solvent-polarity effect" suggested by KAISER[3].

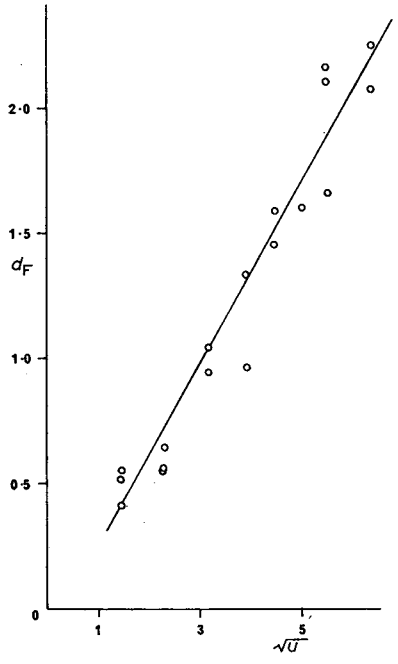


Fig. 2. Graph of film thickness, $d_F$ ($\mu$), against square root of coating velocity $u$ (mm/sec) for 0.2 mm I.D. vapour-phase silanised Pyrex glass capillary columns coated with a 10% v/v solution of SF-96 in toluene.

TAYLOR has shown[5] that the FAIRBROTHER–STUBBS equation is valid only if the product $u\eta/\sigma < 0.09$. In fact, this holds for most solutions of stationary liquids used for the coating of capillary columns, but for more viscous solutions (*e.g.*, of SE-30 or OV-1 silicone rubbers) some correction factor may be necessary.

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## A simple device for descending preparative thin-layer chromatography

Several devices for descending and ascending preparative thin-layer chromatography have been described recently[1–12]. Those which are designed for preparative chromatography using "normal" (200 × 200 mm) glass plates collect fractions by methods which either expose the substances to the atmosphere to some extent (involving scraping off the adsorbent layer), or do not completely separate them (involving washing out the fractions from the bottom of the chamber). Where the adsorbent containing the desired compound is removed and extracted with a suitable solvent, the extract usually contains fine particles of the adsorbent which interfere with spectroscopy. The complete removal of these particles by filtering or centrifuging is difficult and, further, the solvent will also be contaminated with small amounts of organic compounds present in most commercial adsorbents[13,14].

The apparatus shown in Fig. 1 has been constructed to avoid these disadvantages. It uses materials commonly available in most chemical laboratories and requires only simple skills for its construction. The apparatus has been designed for "normal" thin-layer glass plates (200 × 200 mm) but slight changes in design will permit the use of other sizes and types of plate.

The eluent transfer roll A, which rests on an open stainless steel frame E, and the adsorbent layer B attached to the stainless steel plate C, are composed of gypsum and glass powder (mixed 1:2 w/w), but other materials such as gypsum and finely ground sand or gypsum and Celite have also been shown to be suitable. The eluent roll was cast using a split teflon tube as a mould and has a glass rod for its axis. The stainless steel plate is bent in two and hangs over a glass rod J. Its front surface holds the adsorbent layer B and the plate was sandblasted to improve the adhesion of this layer. The front surface is cut in triangular shape to reduce the surface area of adsorbent and to provide a lowest point for fraction collection. The adsorbent layer can be trimmed back to reduce its surface still further.

Fractions are collected by tiny glass capillaries brought into contact with the lowest point of the adsorbent layer B. The length and diameter of the glass capillaries are rather critical. For organic solvents a minimum length of 70 mm and a maximum

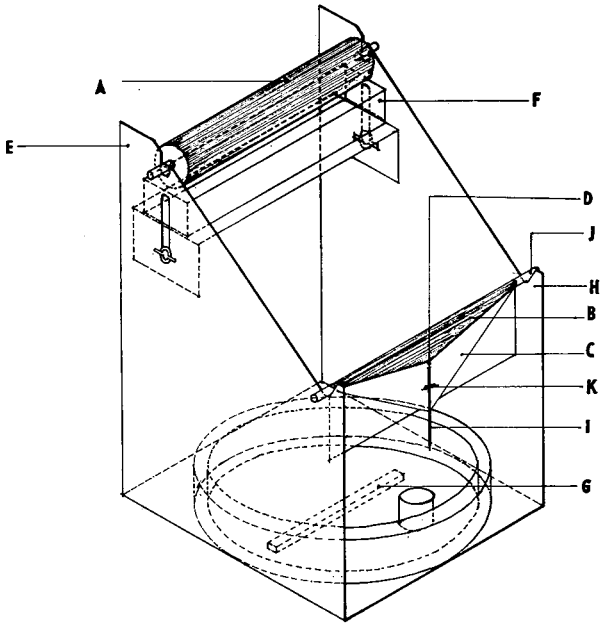


Fig. 1. A sketch of the device without thin-layer plate. For explanation of letters, see text.

inner diameter of 0.15 mm are needed. To protect these tiny capillaries, three to five of them are inserted in a melting point tube I, held in position by the weak stainless steel spring K. Trough F is made of glass but any other resistant material could be used. If the sorbent layer on the glass plate is thick and as a consequence a richer eluent supply is desired, a cotton flannel loop around the roll A dipping into the eluent trough can be used. The resistance of the material to the solvents used should, of course, be checked.

Fractions are collected in a series of small beakers placed in a glass dish floating on mercury inside a slightly larger dish. We found a large culture dish or moist chamber, 140 mm diameter and 40 mm deep, suitable for this purpose. The fraction collector is turned by an external magnet acting on the plastic covered bar magnet G placed in the inside dish. If the fraction collector is very light, it should be possible to use eluent instead of mercury to support the inner dish, but as far as we could ascertain, the mercury vapour did not interfere with the developing procedure at room temperature.

The coated glass plate is placed face down, resting on roll A and on the coated stainless steel plate C and held in position by the lugs H. The angle of rest of the glass plate does not appear to be critical. We were unable to confirm the statement of REISERT AND SCHUMACHER[10] that a steep slope in itself causes irregular development.

To view the spots we irradiated the adsorbent layer with a mercury lamp covered with a Wood's glass screen. Most commercial fluorescing adsorbents are prepared for irradiation with short wavelength UV light (254 m$\mu$), but we found such light less suitable than longer wavelengths for most cases since it cannot penetrate the glass walls of the developing chamber, it destroys some sensitive substances and it is ab-

sorbed by many solvents, etc. We therefore prepared plates containing a material (about 1 % w/w) which fluoresces at 365 mμ, such as Riedel de Haen Leuchtstoffe Grun N, Vanino's pigment and salts of 3,5-dihydroxypyrene-8,10-disulphonic acid[15]. In the few cases where the spots to be visualized did not absorb 365 mμ UV-light, we used 254 mμ sensitized plates, but in these cases the choice of eluents is restricted (must be non-absorbers for the actual wavelength) and special arrangements must be made to allow the light to enter the chamber.

No difficulty should be experienced in collecting fractions provided that the outlet capillaries are of suitable length and diameter. To avoid the eluent creeping between the capillaries and the stainless steel plate, the capillaries were inserted in a melting point tube about 10 mm shorter than the tiny capillaries in such a way that the melting point tube did not touch the eluent-carrying coated layer.

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CHROM. 4396

## The separation and detection of several mycotoxins by thin-layer chromatography

The study of mycotoxicoses[1] especially aflatoxicosis[2,3] emphasized the existence of fungal metabolites harmful to higher organisms. This evidence and the ability of various ubiquitous fungi, *e.g. Aspergillus flavus*[3] and *Penicillium islandicum*[4] to elaborate potent carcinogens prompted theories on a possible relationship between the consumption of mycotoxins and diseases of unknown etiology, *e.g.* the high incidence of hepatocarcinogenicity in Africa[5]. It is therefore essential that rapid and sensitive analytical methods be developed for the detection of these hazardous compounds in

agricultural commodities and consumer products. These methods can be used to study the factors which would influence the growth of the toxigenic fungi in nature and their production of mycotoxins. It is of importance to note that the presence only of toxigenic fungi on a specific product does not necessarily indicate the presence of any mycotoxin.

Several excellent methods have been reported for the screening and quantitative estimation of the aflatoxins (see *e.g.* Ref. 6). Recentley EPPLEY[7] introduced a method for the screening for zearalenone, aflatoxin and ochratoxin. This paper describes a new developing system for the simultaneous separation and detection of eleven different mycotoxins. The water soluble and lipid material can usually be removed from an extract of mouldy material by liquid–liquid partition. For mixtures containing acidic mycotoxins, *e.g.* cyclopiazonic acid, secalonic acid D, and ochratoxins A and B extensive purification is achieved by removal of the neutral material by standard procedure.

*Experimental*

Silica Gel G(Merck) was slurried with 0.4 *N* aqueous oxalic acid in a (1:2) ratio. The separation was achieved on 20 × 20 cm plates using an 0.25 mm layer of the above-mentioned slurry. The plates were air dried, activated at 100° for 40 min and kept at room temperature. The solvent combination used was chloroform–methylisobutylketone (4:1). The plate was spotted with each of the mycotoxins in a solution of chloroform–methanol (1:1) and allowed to develop *ca.* 14 cm from the spotting line in a tank saturated with the solvent vapour. The plate was removed from the chamber and dried at room temperature. The spots were detected by exposure to long wavelength (366 mμ) UV illumination and spraying with colour reagents.

The spray reagents used were: (a) Concentrated sulphuric acid. After spraying the plate was heated at *ca.* 110° for 10 min. (b) One per cent ethanolic ferric chloride.

TABLE I

THE SEPARATION OF MYCOTOXINS ON 0.25 mm LAYER OF SILICA GEL G IMPREGNATED WITH OXALIC ACID

| *Mycotoxin* | *Reference* | *Fungus* | $R_F$ *value* (× 100) | *Fluorescence* | *Colour reagents* | |
|---|---|---|---|---|---|---|
| | | | | | $H_2SO_4$ | $FeCl_3$ |
| Aspertoxin | 8 | *A. flavus* | 12 | light yellow | green-yellow | — |
| Ochratoxin B | 9 | *A. ochraceus* | 20 | blue | — | red-brown |
| Secalonic acid D | 10 | *P. oxalicum* | 23 | dark | light brown | light brown |
| 8α-(3-methylbutyryloxy)-4β,15-diacetoxyscirp-9-en-3α-ol | 11 | *F. tricinctum* | 28 | — | lead grey | — |
| Aflatoxin $G_1$ | 12 | *A. flavus* | 30 | green | green-grey | — |
| Aflatoxin $B_1$ | 12 | *A. flavus* | 40 | blue | green-grey | — |
| 6β-Hydroxyrosenonolactone | 13 | *T. roseum* | 44 | — | orange-red | — |
| Ochratoxin A | 9 | *A. ochraceus* | 48 | green | — | red-brown |
| Cyclopiazonic acid | 14 | *P. cyclopium* | 65 | dark | red-brown | red-brown |
| Zearalenone | 15 | *F. graminearum* | 72 | faint blue | light yellow | red-brown |
| Sterigmatocystin | 16 | *A. nidulans*<br>*A. versicolor*<br>*Bipolaris sp.* | 85 | orange | green-grey | green |

*Results and discussion*

The colours of the various mycotoxins under UV light and after spraying with the colour reagents are recorded in Table I. Also included in Table I are the fungal sources and the $R_F$ ($\times$ 100) values. The reported $R_F$ value for each mycotoxin is the average of ten independant determinations. It is apparent from the $R_F$ values that the mycotoxins are well resolved in this system.

A suitable chromogenic reagent for these mycotoxins is a solution of one per cent ceric sulphate in 6 *N* sulphuric acid. Some compounds give a characteristic colour with a specific reagent, *e.g.* cyclopiazonic acid gives a violet colour on spraying with Ehrlich reagent. Cyclopiazonic acid also turns violet-red on prolonged standing on the silica gel plates impregnated with oxalic acid.

If oxalic acid is omitted from the silica gel slurry, the mobility of the neutral metabolites are virtually unaffected, whereas the acidic compounds *e.g.* cyclopiazonic acid, secalonic acid D, and ochratoxins A and B do not move. This can be employed as a confirmation. Absolute confirmatory tests, *e.g.* by direct comparison with a standard reference sample, by physico-chemical methods or bio-assay[17] are essential for the final proof for the presence of a suspected mycotoxin in foodstuffs.

Gifts of samples of mycotoxins from several workers in the field of mycotoxin chemistry, are kindly acknowledged.

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CHROM. 4424

## Anomalous behaviour of radioactive folic acid on thin-layer chromatography

The use of thin-layer chromatography to determine the purity and identity of trace amounts of organic compounds is well established. We now report an example in which the chromatographic behaviour changed with concentration of the substance under investigation.

Folic acid (pteroyl-L-monoglutamic acid) may be readily detected by TLC on cellulose powder in amounts greater than 5 $\mu$g by its appearance as a dark absorbent spot when viewed in 254 m$\mu$ light. With butanol–acetic acid–water (4:1:5, upper phase pH 2.6) as developing solvent, folic acid has an $R_F$ value of 0.0 and this conveniently distinguishes it from its fluorescent decomposition products which move away from the origin.

When [2-$^{14}$C]folic acid (0.45 $\mu$g/$\mu$l; specific activity 50.3 mCi/mmole; Radiochemical Centre, Amersham) was assayed for purity in this system a major spot, as determined by autoradiography, of an $R_F$ value of 0.42 was obtained with nothing at the origin. This led to the initial conclusion that the sample of folic acid had extensively decomposed although this radioactive material was not identical with the anticipated decomposition products. When TLC of the radioactive compound (0.45 $\mu$g/$\mu$l) was carried out in 0.1 *M* phosphate buffer (pH 7.0) and in propanol–1% aq. ammonia (2:1) the major radioactive spot had the same chromatographic behaviour as cold folic acid (5 $\mu$g/$\mu$l). When folic acid was added to the radioactive folic acid so as to produce a wide range of concentrations and TLC was carried out in butanol–acetic acid–water (4:1:5, upper phase) the $R_F$ values of the major radioactive species varied as shown in Tables I and II.

TABLE I

$R_F$ VALUES OF MIXTURES OF RADIOACTIVE AND NONRADIOACTIVE FOLIC ACID
[2-$^{14}$C]folic acid 0.45 $\mu$g/$\mu$l.

| *Nonradioactive folic acid added* | $R_F$ |
|---|---|
| 0.0 | 0.42 |
| 8 $\mu$g/2 $\mu$l | 0.00 |
| 20 $\mu$g/3 $\mu$l | 0.00 |
| 40 $\mu$g/5 $\mu$l | 0.00 |

TABLE II

$R_F$ VALUES OF MIXTURES OF RADIOACTIVE AND NONRADIOACTIVE FOLIC ACID
Mixture of 0.5 $\mu$g [2-$^{14}$C]folic acid and 10–15 $\mu$g nonradioactive folic acid.

| *Volume of solution applied* ($\mu$l) | $R_F$ |
|---|---|
| 2 | 0.0 |
| 10 | 0.0 |
| 50 | 0.50 |

These data show that folic acid in butanol–acetic acid–water has an $R_F$ value of 0.0 when applied in concentrated solutions (more than 1 $\mu g/\mu l$) and an $R_F$ value of 0.42 when applied in dilute solution (0.5 $\mu g/\mu l$).

As these differences in chromatographic behaviour could be attributed to chelation of the folic acid with metals, chromatograms were run in butanol–acetic acid–water saturated with ethylene diamine tetracetic acid and butanol–acetic acid–water containing a crystal of sodium sulphide[1]. Again dilute solutions of folic acid had an $R_F$ value of 0.49 and concentrated solutions an $R_F$ value of 0.00.

With 3% aqueous acetic acid (adjusted to pH 3.4 with NaOH) dilute solutions (0.2–0.3 $\mu g/\mu l$) of folic acid gave an $R_F$ value of 0.20–0.30 and concentrated solutions (5–7.5 $\mu g/\mu l$) an $R_F$ value of 0.00; the behaviour was not altered by the addition of ethylene diaminetetracetic acid.

With 3% aqueous ammonium chloride at pH 4.0 dilute solutions of folic acid (0.45 $\mu g/\mu l$) gave two spots ($R_F$ 0.04 and $R_F$ 0.27) coalescing into one $R_F$ (0.04) at higher concentrations (7.5 $\mu g/\mu l$) but at pH 5.5 only one spot ($R_F$ 0.30–0.40) was obtained at all concentrations.

The absence of any effect of adding sodium sulphide or ethylene diamine-tetra-acetic acid establishes that this anomalous effect is not due to chelation of the folic acid. As the variation in $R_F$ values with concentration is found only in acidic solvent systems and disappears when the pH of the 3% aqueous ammonium chloride system is changed from 4.0 to 5.5 this behaviour is due to the non-ionised acid. The variation in chromatographic behaviour with concentration of folic acid in the acidic solvents used is caused by the association of the acid, the species present at low concentrations and having the higher $R_F$ value being a monomer and the species present at higher concentrations and having the lower $R_F$ value being the associated form. The association of the folic acid molecules could be caused by intermolecular hydrogen bonding between the non-ionised carboxyl groups but this seems unlikely as the hydrogen bonds of non-ionised carboxyl groups in aqueous solutions are made preferentially with the solvent molecules and not with each other[2]. There is strong intermolecular interaction between the pteridine rings of folic acid as evidenced by its infusibility and insolubility in all solvents but aqueous alkaline solutions[1]. This intermolecular interaction would be a reasonable explanation for the association of folic acid in higher concentrations in acidic solvent systems. A similar association in aqueous solutions has been established for purines where it has been shown that purines associate with the molecular planes parallel to each other held by interactions between the $\pi$-electrons of each ring[3,4].

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CHROM. 4394

## The separation of parasympatholytics by thin-layer chromatography*

Clinical pharmacological investigations of parasympatholytics have previously been made and their presence and quantity have been determined by bioassay in different biological fluids (urine, saliva, bile, serum) of patients[1–4]. This method was found to be very sensitive but not specific for different parasympatholytic drugs.

In order to study the drug cross-tolerance developed in patients treated with parasympatholytics, we needed a method suitable for the identification and separation of several parasympatholytics simultaneously. A simple thin-layer chromatographic method for the separation and identification of various parasympatholytics simultaneously is presented in this paper.

The following solutions were used as developing solvents: chloroform–ethanol (5:5) and chloroform–ethanol (7:5). Potassium iodoplatinate reagent (3 ml of 10% $H_2PtCl_6$ solution mixed with 97 ml water, added to 100 ml of 6% aqueous KI) was used as the chromogenic reagent[5]. Silica Gel G (Merck; 25 g in 50 ml of 1.0 *N* NaOH solution) and Aluminium Oxide G (Merck; 25 g in 50 ml of water) were used as coating materials. The slurry was spread over glass plates (20 × 20 cm) to a thickness of 250 $\mu$ with a Desaga equipment. The coated plates were air dried at room temperature. The plates were activated before use by heating them at 110° for 1 h.

After activation the plates were allowed to cool on a large glass plate for 5 min, spots (usually 5 to 10 $\mu$l) of the parasympatholytic solutions under investigation were applied, and the treated plates were put into a tank equilibrated with the solvent to be used for chromatography. The atmosphere in the tank was kept saturated with the developing solvent vapour by lining the tank walls with strips of filter-paper which dip into the solvent.

Development was by the ascending method for 15–16 cm. The plates were dried at room temperature for 30 min and sprayed with the chromogenic reagent.

The following parasympatholytics were examined: atropine, Novatropine (methylhomatropinum bromatum), isopropamide (2,2-diphenyl-4-diisopropyl-amino-methyliodide) and Gastrixone (8-methyl-tropiniumbromide-xanthene-9-carboxylate). These were all obtained from the United Drug and Foodstuffs Factory, Budapest. Atropine is a tertiary amine, whereas Novatropine, isopropamide and Gastrixone are quaternary ones.

The results are presented in Table I. The spots obtained after treatment with the chromogenic reagent were of different shades of violet. The sensitivity of the method is 1 to 30 $\mu$g.

According to our results the Aluminium Oxide G was found to be suitable for the separation of each parasympatholytic drug tested when both chloroform–ethanol solvents were used, while the Silica Gel G thin-layer was found to be suitable only for the separation of atropine, isopropamide and Gastrixone. Densitometry following the chromatography was found to give a quantitative evaluation of the parasympatholytics.

Other solvent solutions[5–9] (benzene–methanol; benzene–ethanol; ethanol–am-

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TABLE I

$R_F$ VALUES × 100 FOR PARASYMPATHOLYTICS

Developed in chloroform–ethanol (5:5), chloroform–ethanol (7:3) and sprayed with potassium iodoplatinate.

| *Parasympatholytics* | *Solvent solutions* | |
|---|---|---|
| | *Chloroform–ethanol (5:5)* | *Chloroform–ethanol (7:3)* |
| *Silica Gel G impregnated with 1.0 N NaOH* | | |
| Atropine | 53 | 63 |
| Novatropine | 00 | 00 |
| Isopropamide | 48 | 30 |
| Gastrixone | 16 | 07 |
| *Aluminium Oxide G* | | |
| Atropine | 77 | 87 |
| Novatropine | 12 | 05 |
| Isopropamide | 70 | 70 |
| Gastrixone | 36 | 16 |

monia; methanol–acetone–triethylamine; chloroform–diethylamine; cyclohexane–chloroform–diethylamine; cyclohexane–diethylamine; benzene–ethyl acetate–diethylamine; dimethylformamide–diethylamine–ethanol–ethyl acetate used in different ratios) and thin-layers[9] (Silica Gel G with water and impregnated with 0.05 *N*, 0.10 *N*, 0.25 *N*, or 0.50 *N* NaOH, and Silica Gel G mixed with Aluminium Oxide G) did not give a satisfactory simultaneous separation of the parasympatholytics mentioned above.

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CHROM. 4395

## Twin-film technique for de-salting and chromatographing amino acids in two dimensions

Multiple-film (usually twin-film) laminar chromatography has two main fields of application. First, for isolating compounds which are heavily contaminated with substances having no interest for the analyst; for example, for separating unwanted iodides from organic iodo-compounds[1], or for isolating traces of pesticides from foodstuffs[2,3]. Secondly, for resolving mixtures of compounds, the components of which have widely different polarities[4]. Recently, ion-exchange resin–cellulose twin films have been used for examining urinary amino acids[5], but the procedure seems unnecessarily complicated. In addition to the labour of grinding and sieving the resin, the process requires five elutions for one-dimensional chromatography. We describe below the much simpler, two-dimensional method which has been in use in this laboratory for the past two years.

Mixtures of amino acids are easily resolved in the absence of other electrolytes, but if salts are present, the quality of the resolution depends on the salt–amino acid ratio. At low ratios (up to 4:1, molar) the amino acids form trails but their identification presents no real difficulty. At higher ratios (*e.g.*, 45:1) identification of the amino acids is impossible (Fig. 1). Natural polypeptides and the amino acids derived

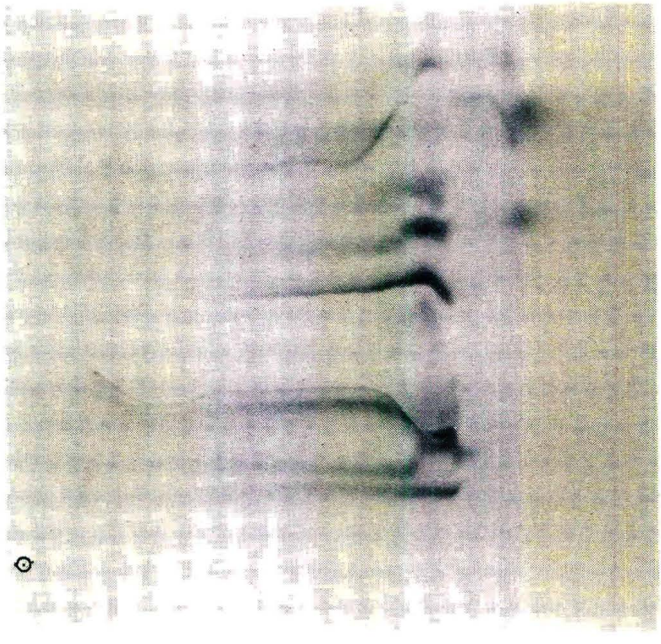

Fig. 1. Two-dimensional chromatogram of a mixture of potassium chloride and 22 amino acids (87:1, molar) on cellulose.

from them are usually accompanied by salts (chlorides). If the material has been fractionated, then buffer salts (*e.g.*, phosphates and citrates) or density gradient generators (*e.g.*, caesium sulphate or chloride) are present in relatively large amounts. Clearly, these salts must be removed before the amino acids can be chromatographed satisfactorily. On an ion-exchange resin–cellulose twin film, the amino acids may be separated from one another on the cellulose portion after the salts have been removed on the resin band. In this way the need for a separate de-salting process is eliminated.

Samples of standard mixtures containing up to twenty four amino acids were mixed with potassium chloride or sodium citrate or sodium dihydrogen phosphate; the ratio is the total weight of salt: total weight of the amino acids, where the weight may be expressed in grams or in molar terms. Chromatoplates (20 × 20 cm) carrying a sulphonated ion-exchange resin (in the $H^+$ form) on a 35 mm wide band along one edge, the rest of the plate being coated with cellulose, were used. It was very important to ensure that there were no flaws in the junction between the two substrates.

Samples were spotted on to the resin band near one corner of the plate, at a distance 5–10 mm from the film junction. If the sample was placed too far from the film junction, some of the amino acid sample, especially arginine, was not transferred to the cellulose. Conversely, if the sample was placed too near the junction, amino acids and salts were carried on to the cellulose and subsequent chromatography was unsatisfactory. The chromatogram was then developed, at 40°, with water, from the resin band to the cellulose, the front being allowed to travel to the top of the film. This elution carried cysteic acid and the salt anions to the top of the cellulose and left the amino acids and the salt cations on the resin.

After the chromatogram had been dried, the second elution, at 40°, was carried out in the same direction as the first, using a basic eluent. After the chromatogram had again been dried, a line was cut along the junction of the substrates to isolate the resin band. A second line was cut in the cellulose at the opposite edge of the plate, to isolate the band of dirt that occurs in the solvent front. Subsequently, the third elution, at right angles to the first, was carried out, at 40°, in an acidic solvent. Finally, the chromatogram was dried, sprayed with a solution of ninhydrin and stored in the dark for several hours.

Fig. 2 shows the result for a potassium chloride–amino acid ratio of 87:1 (molar). Equally good results were obtained when the salt was sodium citrate (23:1, molar) or sodium dihydrogen phosphate (50:1, molar). Samples having a higher ratio may be processed if they are placed further from the film junction, but there is a progressive loss of arginine from the pattern, and of resolution between cystine and lysine. For a caesium sulphate–amino acid ratio exceeding 200:1 (w/w) the pattern of the non-polar amino acids was fair but those amino acids which are more polar than glycine were not easily distinguishable[6].

The basic eluent used here, a modification of one described earlier[7], is less susceptible to the formation of a purple band near the acidic front.

*Experimental*

*Preparation of chromatoplates.* Shandon apparatus was used for coating batches of six plates (20 × 20 cm); the spreader-gap was set at 250 $\mu$. The spreader was divided into two compartments by a rectangular cork wedge (5 mm at the thick end) wrapped in polythene. The wedge fitted snugly into the spreader and was placed about 35 mm

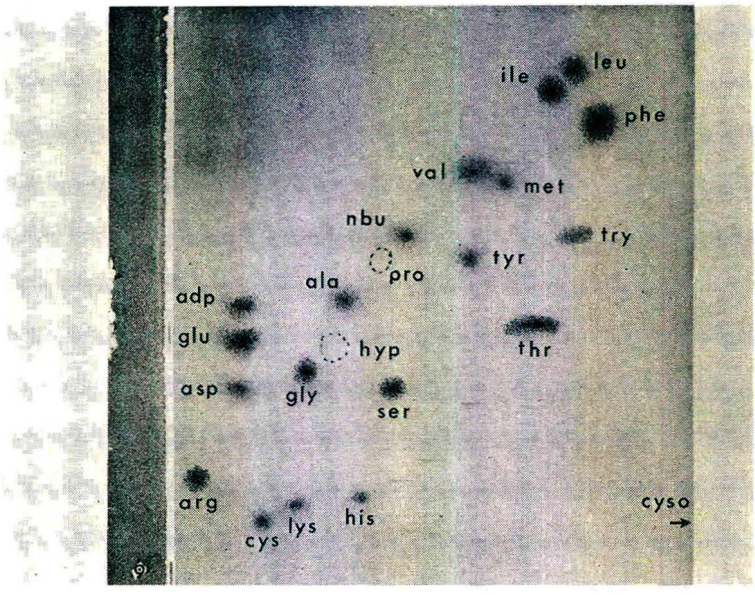


Fig. 2. Two-dimensional chromatogram of a mixture of potassium chloride and 22 amino acids (87:1, molar) on a twin-film of Amberlite CG 120–cellulose.

from one of its ends, with the thin end of the wedge just making contact with the underlying glass plate.

*Cellulose slurry*. Cellulose (MN 300; 15 g) and water (90 ml) were mixed for 40 sec in a fast mechanical homogeniser.

*Ion-exchange resin slurry*. Amberlite resin CG 120 (200 mesh; 3 g) in the $H^+$ form, water (7 ml) and a portion of the homogenised cellulose slurry (3 ml) were stirred by hand. By using a small proportion of cellulose as a binding agent for the resin, the need for grinding and sieving the resin was eliminated.

Each slurry was poured into the appropriate compartment of the spreader, and after the plates had been coated in the usual way, the films were allowed to dry at room temperature for 16 h.

*Sample*. A solution of 22 amino acids (1.25 $\mu$moles/ml of each) in water–propanol (9:1) was mixed with an equal volume of aqueous potassium chloride (2.4 *M*). In the resulting solution the weight ratio is 45:1 and the molar ratio is 87:1. Similar solutions containing 24 amino acids (0.625 $\mu$mol/ml of each) and either sodium dihydrogen phosphate (0.75 *M*) or sodium citrate (0.35 *M*) were also prepared. Samples (8 × 1 $\mu$l) of these solutions were placed near one corner of the resin band, at a distance of 5–10 mm from the film-junction.

*Development of the chromatogram*. Three successive elutions were performed in unlined tanks, all elutions being carried out at 40°.

First elution: with water, from the resin band to the cellulose, the aqueous front being allowed to travel to the top of the chromatoplate (160 mm; time required, 65 min). Subsequently, the chromatogram was dried at 40° for 2 h.

Second elution: with cyclohexanol–acetone–water–1-dimethylaminopropanol-2–diethylamine (10:5:5:1:1) in the same direction as the first elution, for 160 mm

(time required, 200 min). After the chromatogram had been dried (2 h at 40°) two parallel lines were cut in the substrate; one along the film-junction to isolate the resin band, and the other about 10 mm from the opposite edge of the cellulose to isolate the band of dirt in the front.

Third elution: with *tert.*-butanol–acetic acid–water (5:1:1) in a direction at right angles to the first elution, for 160 mm (time required, 210 min). After the substrate had been dried, it was sprayed with a solution of ninhydrin (0.2 % in butanol–acetic acid (4:1)) and the plate was stored in the dark for some hours to allow full development of the colour.

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CHROM. 4389

## Gel-electrofocusing in combination with immunodiffusion

With gel-electrofocusing[1–5] being used increasingly in protein chemistry as a new purity criterion for isolated proteins, the question of identification of the different zones focused becomes of great importance.

This communication describes a procedure where isoelectric separation of the proteins in polyacrylamide gel is used and the proteins are then identified by a method based on immunodiffusion with specific antisera[6–8]. Electrofocusing was carried out in tubes according to WRIGLY[4] using a conventional disc-electrophoresis apparatus, and on polyacrylamide plates (18 × 8 cm) using an apparatus described by AWDEH *et al.*[5].

Stock solutions for electrofocusing gels were prepared as follows: Acrylamide solution: 30 g acrylamide; 0.8 g N,N′-methylenebisacrylamide; water to 100 ml. Catalyst solution: 1.0 ml N,N,N′,N′-tetramethyl ethylene diamine; 14 mg riboflavine; water to 100 ml.

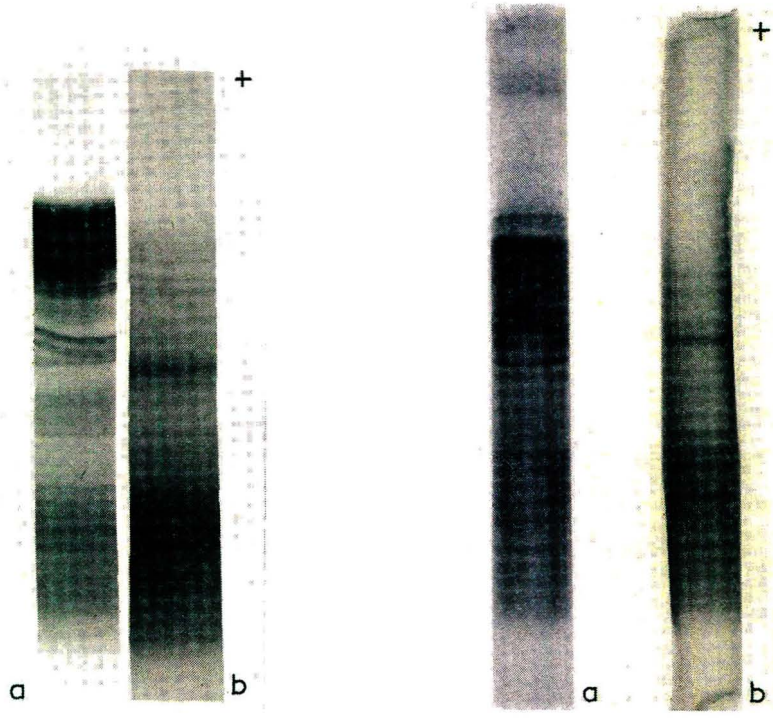


Fig. 1. Isoelectrofocusing and immunodiffusion of normal human serum on polyacrylamide gel plates. (a) isoelectric separation; (b) identical separation followed by incubation with absorbed anti-IgG serum and staining after elution of non-precipitated proteins.

Fig. 2. Isoelectrofocusing and immunodiffusion of normal human serum on polyacrylamide gel tubes. (a) isoelectric separation; (b) identical separation followed by incubation with absorbed anti-IgG serum and staining after elution of non-precipitated proteins.

Plates of polyacrylamide gel (18 × 8 cm) were prepared by polymerizing 4.4 ml normal human serum (diluted 1:50), 17.6 ml water and 11 ml gel mixture between two glass plates, 3 mm apart, by photoactivation for 1 h at 4°. (The gel mixture consisted of 2.4 ml catalyst solution, 0.9 ml ampholyte solution (40 %), pH 3–10, 9 ml acrylamide solution). The starting current was about 200 V and 10 mA. As soon as the pH gradient of carrier ampholytes was established, the current was increased to 300 V and kept constant for 36 h. The gel was then cut in two parts (length ways). One part was immediately immersed in 5 % trichloroacetic acid (TCA) and washed for 12 h to remove the ampholytes, and then stained with Coomassie Brilliant Blue R (ref. 9, Fig. 1a). A 2 mm thick layer of agar was placed on top of the other part of the gel. This agar had been obtained by mixing 50 ml of a 2 % agar solution (veronal buffer, ionic strength 0.025; pH 8.6) with 5 ml of an anti-IgG serum absorbed by using our own specific immunoadsorbent[10] (both solutions being heated to 50° before mixing); the absorbed antiserum reacts with the k and l light chains and the Fc portion of the gamma heavy chain, when tested by immunoelectrophoresis. The gel plus agar was then left at room temperature for 48 h in order that immunodiffusion

could take place. The polyacrylamide gel was then freed from the agar layer and washed for 3 days in physiological saline and a further day with 5 % TCA, before staining with Coomassie Brilliant Blue R (Fig. 1b); the TCA pretreatment improves the staining.

In the electrofocusing experiments, 0.5 ml gel mixture, 0.1 ml ampholyte (40 %) pH 3–10, and 0.9 ml of normal human serum (diluted 1:50) were polymerized by photoactivation for 1 h at 4° in 1.5 ml glass tubes (6 mm diam.). (Gel mixture: 3.0 ml acrylamide solution, 0.8 ml catalyst solution.) A current was then passed through the gel column for 3 h, starting with 1.5 mA for each gel. Twelve tubes were run simultaneously in each experiment. After focusing, the gels were removed from the tubes and treated as follows: two were immersed in 5 % TCA, washed for 12 h and stained with Coomassie Brilliant Blue R (Fig. 2a); the other ten gels were divided into pairs and immunodiffusion was carried out. Each pair was treated with 2 ml of a different specific antiserum in an 8 mm diameter tube and incubated for 24 h. The five antisera used were as follows: anti-IgG, anti-IgG/$\gamma$, anti-IgA, anti-IgM and anti-human serum albumin (HSA). Anti-IgG serum was the same as described above, the other antisera against immunoglobulins reacted only with the corresponding heavy chains, *i.e.* $\gamma$, $\alpha$ and $\mu$ respectively. After subsequent washing for 3 days in physiological saline and another day in 5 % TCA, they were stained with Coomassie Brilliant Blue R.

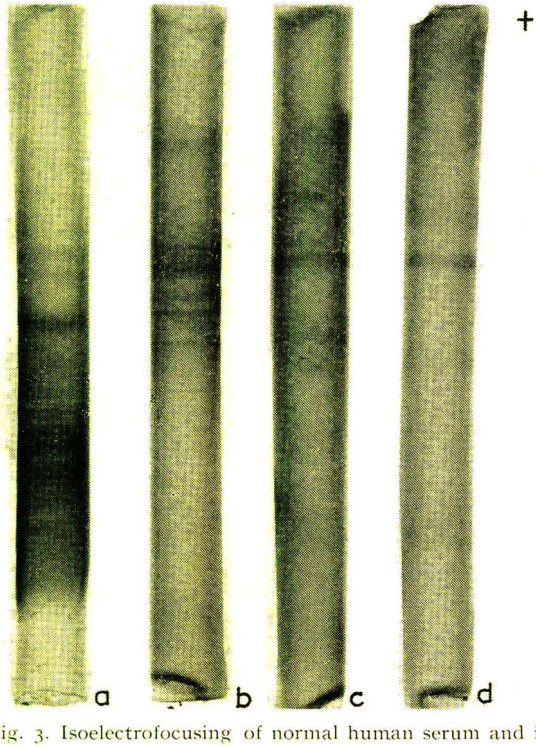


Fig. 3. Isoelectrofocusing of normal human serum and immunodiffusion with different antisera on polyacrylamide gel tubes. (a) incubation with anti-IgG/$\gamma$ serum; (b) incubation with anti-IgA serum; (c) incubation with anti-IgM serum; (d) incubation with anti-human serum albumin.

Immunoelectrofocusing patterns of IgG in normal human serum on the plates, developed with anti-IgG antibodies are shown in Fig. 1b in comparison to all serum proteins detected by TCA precipitation in Fig. 1a. Comparable results were obtained when the same system was applied to polyacrylamide tubes (Fig. 2a, b). However, it must be borne in mind that in both these cases not only the IgG but also other immunoglobulins are detected by the antiserum used which also possesses antibodies against common light chain moieties.

Precipitation bands corresponding exclusively to IgG, IgA and IgM immunoglobulins, respectively, were developed with specific anti-heavy chain sera (Fig. 3a, b, c); when these three patterns are overlapped in a logical sequence the same picture is obtained as that shown in Fig. 2b. A distinct heterogeneity was found not only in all three main immunoglobulins, but it can be shown that HSA can be separated into at least four fractions under same conditions (Fig. 3d).

The heterogeneity of human IgG immunoglobulins has also been studied using the electrofocusing method[11]; in this case the IgG was separated from the serum and electrofocused after further purification. The detection of IgG micro fractions in whole normal serum, without isolation, has been made possible by the above method, and avoids the possible alteration of the IgG molecules during the treatment. The above findings have demonstrated the microheterogeneity of all the proteins studied. The occurrence of numerous IgG immunoprecipitation bands is of particular interest. It is expected that this method will permit further identification of separated protein fractions. The above method and some results will be published in detail elsewhere.

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# News

## Meetings

SIXTH INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY

The Sixth International Symposium on Advances in Chromatography will be held at the Hotel Fontainebleu in Miami Beach, Florida, June 2–5, 1970. The format of the meeting will be similar to the previous symposia which were held in Houston, New York, and Las Vegas. The subject matter will include gas chromatography, liquid chromatography, and supercritical fluid chromatography. Participation in the symposium will be on the basis of invited papers as well as contributions from the United States and abroad. Those who wish to present papers are requested to submit an abstract of about 300 words by February 2, 1970. Complete manuscripts of accepted papers will be required by March 2, 1970.

Abstracts of papers and inquiries concerning the conference should be directed to: Professor A. ZLATKIS, Department of Chemistry, University of Houston, Houston, Texas 77004, U.S.A.

## Manufacturers' Literature

A bulletin describing a whole range of new products is available from Phase Separations Ltd. They include high pressure/accuracy gas tight syringes, Diatom W, a packing dryer for column packing preparation, a triangulator for direct area and retention time measurement, a perfluorocarbon impregnated diatomaceous support Gas-Pack F, column pack for pressure packing coiled columns, Teflon faced silicone septa, the Pierce Chemical Company steroids silylation kit, primary standards for air polutution studies, and Durapak column packing material from Waters Associates.

Also received from Phase Separations was Newsletter No. 2. Among the various items described are alumina packed glass capillary columns approximately 40 ft. in length. An example chromatogram of the analysis of commercial butane is given to show some of the potentialites of these columns.

For further information apply to the publisher under reference No. Chrom. N-247.

New catalog information is now available from the Industrial Instruments Division of the Barber-Colman Company on their solid state indicating controllers. Bulletin 1252 DB 6-2 describes the instruments available for those applications involving the control of SCR's for proportional current output, while 1252 DB 7-2 discusses the instruments which have a solid state switching output for "On–Off" control applications. Both classes of instruments make use of an eight inch scale, have solid state potentiometric measuring circuits and have temperature indication which is independent of the control circuit. Other features include proportional and automatic reset action, tamper-proof adjustments as well as thermocouple break protection.

For further information apply to the publisher under reference No. Chrom. N-251.

Recently received from Packard Instrument International S.A. (Zürich, Switzerland) is a pamphlet describing the newly introduced 7300/7400 series *gas chromatographs* which are intended primarily for research.

For further information apply to the publisher under reference No. Chrom. N-257.

The LKB instrument journal *Science Tools* contains a very detailed report on the technique of immunoelectrofocussing by N. CATSIMPOOLAS, and zone convection electrofocussing which we understand is a new technique for fractionating ampholytes in free solution by E. VALMET.

For further information apply to the publisher under reference No. Chrom. N-255.

The Jena Review 1969 No. 2 contains an article on the universal *separation chamber for zone electrophoresis* by KARL-FRIEDRICH TLACH. It is claimed that because of the new electrode system and general improvements in design better reproducible separations can be expected.

For further information apply to the publisher under reference No. Chrom. N-254.

*Pierce GC Accessories List*

Pierce Chemical Company (Rockford, Ill.) offers a new list of aids for the gas chromatographer. Included are several significantly improved accessories: liquid microsyringes, low bleed GC septa, high performance liquid phases, substrates and column packings and more than 80 trimethylsilylated compounds for use as GC standards.

For further information apply to the publisher under reference No. Chrom. N-262.

## New Materials

A new technique on thin-layer chromatography has been developed by May and Baker Ltd. In many cases of analysis it has been necessary to subject the sample to a preliminary (clean-up) procedure on a chromatographic column so that unwanted materials which would overload the plate are removed. This, however, is a time-consuming step, and several attempts have been made to modify the TLC procedure to allow complete separation on one plate. The modification announced by May and Baker consists of a shallow groove ground a short distance from one end of a standard thin-layer plate. This grooved plate, can be coated in the usual manner, but the adsorbent at the groove is about eight times thicker in the groove than on the remainder of the plate. Thus the groove permits a preliminary cleaning up procedure and the movement of the constituents to a new origin by dipping the plate into a polar solvent and eluting past the grooved portion of the plate.

These plates are marketed under the name *Chromalay* grooved plates.

For further information apply to the publisher under reference No. Chrom. N-243.

## New Apparatus

The *Coulson electrolytic conductivity detector* (CCD) offered by Tracor, Inc., can now measure sunnanogram quanities of nitrogen even in up to 40000 times excesses of other compounds. For example the volatile derivatives of amino acids are prepared by new procedures and separated by gas chromatography. Each is then measured without sample clean-up. Quantitative response is directly proportional to nitrogen present. Since clean-up procedures are no longer necessary, a complete analysis is claimed to take only 32 min. The technique is based on the MT220 gas chromatograph with the CCD system.

For further information apply to the publisher under reference No. Chrom. N-248.

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35. SOME TECHNICAL PRODUCTS AND COMPLEX MIXTURES

*35c. Complex mixtures and non-identified compounds*

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## Errata

*J. Chromatog.*, 42 (1969) 457–463.

Page 462, Table IV, the retention time of 3-$CH_2CH_3$-pyridine should be 1.01 and the ratio of retention times of 3-$COCH_3$/3-$CH_2CH_3$ should be 2.1.

*J. Chromatog.*, 43 (1969) 322–331.

Pages 328, 329, legends to Figures 3, 4, 5 and 7, $P^1$ casein should read pI casein.