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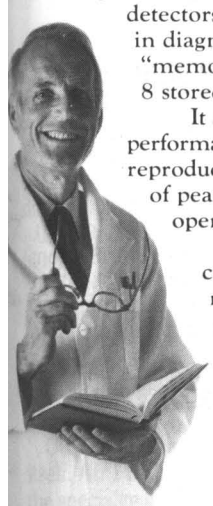

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JOURNAL OF CHROMATOGRAPHY

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INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

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FREE DISPLACEMENT ELECTROPHORESIS (ISOTACHOPHORESIS): AN ABSOLUTE DETERMINATION OF THE KOHLRAUSCH FUNCTIONS AND THEIR USE IN INTERACTION STUDIES

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(Received January 28th, 1980)

SUMMARY

The Kohlrausch regulating functions (the ω-functions) have been calculated for the moving zones in carrier-free displacement electrophoresis (isotachophoresis) experiments. We verified experimentally that the ω-functions of the different migrating zones have the same value at the steady state. From studies of the Kohlrausch functions it is easy to decide whether interaction between the constituents of a zone occurs.

INTRODUCTION

The theory of electrophoretically displaced boundaries was outlined by Kohlrausch in 1897[1]. Kohlrausch introduced the ω-function ("die beharrliche Funktion"), defined as $\omega = \Sigma c_j/m_j$, where c_j is the concentration and m_j the mobility of the ion j. Kohlrausch derived mathematically that the ω-function has the same value in two phases separated by an electrophoretically moving boundary. In spite of the great importance of this statement in theoretical treatments of all kinds of electrophoresis methods, to the best of our knowledge, no experiments to verify it have been published. The reason could be the lack of electrophoresis techniques that permit accurate, simultaneous measurements of the concentrations c_j and the mobilities m_j. The electrophoresis apparatus used has some unique features that render it very suitable for such measurements (see Discussion). For reasons also given under Discussion we chose to determine the Kohlrausch functions in displacement electrophoresis (isotachophoresis) experiments.

THEORETICAL

Only the theory of displacement electrophoresis that is of interest for our verification experiments is given here. For a more exhaustive treatment, see refs. 2 and 3.

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The electrophoretic mobility of an ion is defined as its velocity at unit field strength:

$$m = v/E \tag{1}$$

where m = mobility ($m^2/Vsec$), v = velocity (m/sec) and E = electrical field strength (V/m)*.

The field strength can be expressed as

$$E = \frac{I}{A\kappa} = \frac{i}{\kappa} \tag{2}$$

where I = current (A), A = cross-sectional area (m^2), κ = conductivity ($\Omega^{-1}m^{-1}$) and i = current density (A/m^2).

Combination of eqns. 1 and 2 provides the mobility equation:

$$m = \frac{vA\kappa}{I} \tag{3}$$

which often is used for the calculation of ion mobilities.

The determination of counter-ion mobility demands further consideration. The procedure is as follows. The current density in a solution is a function of the ion concentrations and velocities:

$$i = F\Sigma c_j z_j v_j \tag{4}$$

F = Faraday's constant $\approx$ 96 488 A·sec/equiv., c_j concentration of the ion j (mol/m^3) and z_j = charge of the ion j (equiv./mol) (z and v, as well as m in eqn. 3, are given positive signs for cations and negative signs for anions).

Consider a zone α consisting of an ion L and a counter ion R (Fig. 1). (We assume that we perform the experiments at an intermediate pH where the contribu-

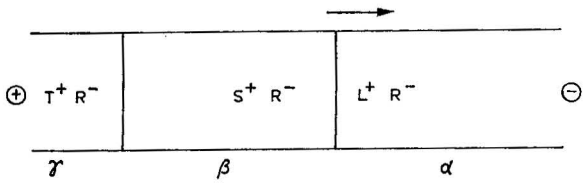

Fig. 1. Schematic representation of displacement electrophoresis at the steady-state. The leading zone α, the sample zone β and the terminating zone γ consist of the cations L^+, S^+ and T^+, respectively, and the common counter ion R^-. The arrow indicates the direction of migration of the boundaries between the zones.

* CGS or MKS units are often used in papers dealing with the fractionation and characterization of biological material. As this paper is of a physical character we have employed SI units, which are commonly used in physics.

tions from H^+ and OH^- to the Kohlrausch function are negligible.) Then, according to eqn. 4

$$i = F(c_L z_L v_L + c_R z_R v_R) \tag{5}$$

Electroneutrality demands that

$$c_L z_L + c_R z_R = 0 \tag{6}$$

Combination of eqns. 1, 5 and 6 gives

$$i = F c_L z_L E(m_L - m_R) \tag{7}$$

Introduction of eqn. 2 gives a function suitable for the calculation of counter-ion mobility:

$$m_R = m_L - \frac{\kappa}{F c_L z_L} \tag{8}$$

The ω-functions of the zones α, β and γ in Fig. 1 can be determined from the mobility values (eqns. 3 and 8), and the measured ion concentrations and their valencies:

$$\omega^\alpha = \frac{c_L z_L}{m_L} + \frac{c_R^\alpha z_R}{m_R^\alpha} \tag{9a}$$

$$\omega^\beta = \frac{c_S z_S}{m_S} + \frac{c_R^\beta z_R}{m_R^\beta} \tag{9b}$$

$$\omega^\gamma = \frac{c_T z_T}{m_T} + \frac{c_R^\gamma z_R}{m_R^\gamma} \tag{9c}$$

The ratio of the ω-functions for the two zones α and β can be calculated either directly from eqns. 9a and 9b or from the following equation, obtained by combining eqns. 9a, 9b, 3, 8 and 6:

$$\frac{\omega^\alpha}{\omega^\beta} = \frac{\kappa^\beta}{\kappa^\alpha} \cdot \frac{\dfrac{I}{c_S z_S} - FvA}{\dfrac{I}{c_L z_L} - FvA} \tag{10a}$$

A similar equation for the zones α and γ is obtained by combining eqns. 9a, 9c, 3, 8 and 6:

$$\frac{\omega^\alpha}{\omega^\gamma} = \frac{\kappa^\gamma}{\kappa^\alpha} \cdot \frac{\dfrac{I}{c_T z_T} - FvA}{\dfrac{I}{c_L z_L} - FvA} \tag{10b}$$

These equations were also used to estimate the uncertainty in the calculated values of the ω-function ratio (see Table II).

The values of the ω-functions in parentheses in Tables IIa and IIb were calculated from eqns. 9a and 9b, putting $m_R^\alpha = m_R^\beta =$ the arithmetic mean of the measured counter-ion mobilities in the zones α and β, and in Table IIc from eqns. 9a and 9c, putting $m_R^\alpha = m_R^\gamma =$ the mean value of the counter-ion mobilities in the zones α and γ.

EXPERIMENTAL

Reagents

All chemicals [potassium acetate, cobalt(II) acetate, copper(II) acetate, acetic acid and hydrochloric acid] were of pro analisi purity. Tris(hydroxymethyl)aminomethane (Tris) and 5-sulphosalicylic acid (SSA) were purified by recrystallization in 40% ethanol.

Apparatus

All experiments were carried out in the free solution electrophoresis equipment previously described in detail[4–6]. The electrophoresis chamber was a 40-cm long, slowly rotating horizontal quartz tube with an inner diameter of approximately 2.5 mm. The rotation has been shown to give efficient stabilization against convectional disturbances. A syringe pump was connected to one electrode vessel, making it possible to apply a controlled liquid flow in the electrophoresis tube. The apparatus was equipped with a photoelectric scanning device, giving the ultraviolet absorption pattern of the zones on the strip chart of a recorder. The scanning wavelength was 280 nm; the background absorption at 320 nm was automatically subtracted by means of a rotating filter.

Loading the electrophoresis tube

The left electrode vessel and half of the tube were filled with leading buffer with the aid of a syringe. A sample zone about 10 cm long (*ca.* 0.5 ml) was applied in contact with the leading buffer while the tube was rotating. The remainder of the tube and the right electrode vessel were then filled with terminating buffer.

Electrophoresis

After the electrophoresis current had been switched on, a counterflow of leading buffer was applied to keep the front boundary of the sample zone stationary in the tube, permitting complete adaptation of the sample zone concentration to the leading zone. The steady-state sample concentration was considered to be reached when the scanner trace showed that the concentration was constant throughout the zone and no further change in zone length could be detected (Fig. 2). The counterflow was turned off and the migration distance of a moving boundary was then determined by scanning the tube at accurately measured time intervals (the position P of a boundary was taken as the point of the boundary that corresponded to half the height of the scanning trace; see Fig. 2b). The migration velocity thus obtained for one boundary is equal to those of all other boundaries (as they all move with the same speed when the steady state has been attained). The runs were performed at 20 °C.

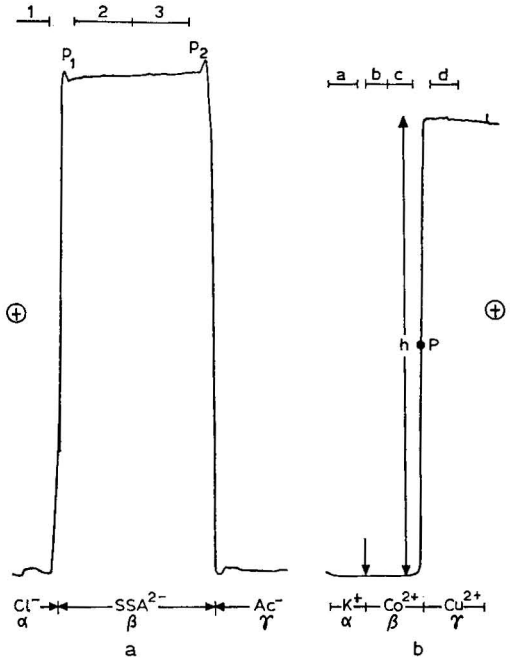

Fig. 2. Strip-chart recorder traces of scans of the electrophoresis tube at 280 nm. The ions of the zones are indicated below the curve. (a) Anionic system, Cl^-–SSA^{2-}–Ac^- with $Tris^+$ as counter ion; (b) cationic system, K^+–Co^{2+}–Cu^{2+} with Ac^- as counter ion. Recovered fractions are marked by numbers 1–3 and letters a–d.

Collection of fractions

When the front boundary of the sample zone had reached the end of the tube, the voltage was switched off and the zones were collected, starting with the terminating solution. Each zone was withdrawn with a dry syringe.

The absorbances of the collected fractions were measured with a Beckman ACTA CIII spectrophotometer at wavelengths of 297, 510 and 760 nm for SSA^{2-} (the anion of 5-sulphosalicylic acid), Co^{2+} and Cu^{2+}, respectively. Standard graphs of absorbance against concentration were constructed at the pH of the zones.

The conductivities were measured with a Radiometer CDM3 conductivity meter, equipped with a CDC314 microcell.

RESULTS

One anionic and one cationic system were investigated.

Anionic system

The leading solution consisted of hydrochloric acid and Tris in the molar ratio 1:2, ensuring maximal buffering capacity and good reproducibility. The pH obtained was 8.3 at 20 °C. Three concentrations of leading solution were analysed (see Table Ia). 5-Sulphosalicylic acid (SSA) was used as the sample (the sample applied was prepared from the acid and Tris in the molar ratio 1:4). At pH 8.3

the acid appears almost completely as SSA^{2-} since $pK_2 \approx 3$ and $pK_3 \approx 11$. The sample concentration used was close to the expected steady-state concentration. The terminating solution consisted of HAc and Tris, Tris initially being at the same concentration as in the hydrochloric acid–Tris buffer in the leading solution.

Electrophoresis was performed as described above. After 2-4 h the concentrations in the sample zone had become adapted to those of the leading zone and the concentrations in the terminating zone to those of the sample zone. Fig. 2a shows the recorder trace of a scan of the tube. The SSA zone could easily be detected by its UV absorbance. The small peaks p_1 and p_2 at the zone boundaries in Fig. 2a were probably due to light deviation caused by refractive index differences between the zones (Schlieren effects). Neither the leading chloride zone nor the terminating acetate zone showed any absorbance at 280 or 320 nm.

The migration velocity of the front boundary of the SSA zone was determined. The standard deviation of this determination was very low, as shown in Fig. 3. The slope (= migration velocity) was calculated by the method of least squares.

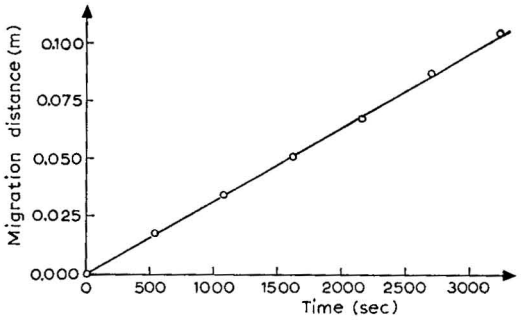

Fig. 3. Migration distance of the boundary between the Cl^- zone and the SSA^{2-} zone of the anionic system (see Fig. 2a) at different times. The migration distance of the boundary was obtained from the scanning pattern on the recorder chart.

When the electrophoresis was completed, the contents of the tube were withdrawn in fractions 1–3, as indicated in Fig. 2a. The conductivity of each fraction and the SSA concentration in fraction 2 were determined, and their measured values are listed in Table Ia. The ion mobilities and the values of the ω-function of the different zones were calculated from these values by eqns. 3, 8 ,9 and 10 (see Table IIa). For the calculation of the Kohlrausch regulating functions we used both the calculated mobilities of the counter ion and their arithmetic means. The values of the ω-functions in parentheses in Tables IIa, IIb and IIc refer to the mean values. When comparing the ω-functions in Table II it is more suitable to consider those calculated from the mean values of the mobilities because these functions according to the treatment given in refs. 1 and 7, have the same value only if the mobility of an ion is the same in both phases.

Cationic system

KAc–HAc (pH 5.40) was chosen as the leading solution and $CuAc_2$–HAc (pH 5.10) as the terminating solution. The applied sample contained $CoAc_2$ and

TABLE I

EXPERIMENTALLY DETERMINED PARAMETERS

(a) The Cl^- (α) and SSA^{2-} (β) zones (see Fig. 2a):

$^cCl^-$ (mol/m³)	κ^α $(\Omega m)^{-1}$	$^cSSA^{2-}$ (mol/m³)	κ^α $(\Omega m)^{-1}$	$I \cdot 10^3$ (A)	$v \cdot 10^5$ (m/sec)	$A \cdot 10^6$ (m²)
25.0	0.206	11.3	0.145	0.495	3.22	4.56
50.0	0.386	22.2	0.253	2.48	8.15	4.56
100	0.734	43.3	0.484	4.99	7.93	4.56

(b) The K^+ (α) and Co^{2+} (β) zones (see Fig. 2b):

$^cK^+$ (mol/m³)	κ^α $(\Omega m)^{-1}$	$^cCo^{2+}$ (mol/m³)	κ^β $(\Omega m)^{-1}$	$I \cdot 10^3$ (A)	$v \cdot 10^5$ (m/sec)	$A \cdot 10^6$ (m²)
100	0.800	37.5	0.404	2.44	2.52	6.25
200	1.55	74.7	0.689	4.90	2.70	6.25

(c) The K^+ (α) and Cu^{2+} (γ) zones (see Fig. 2b):

$^cK^+$ (mol/m³)	κ^α $(\Omega m)^{-1}$	$^cCu^{2+}$ (mol/m³)	κ^γ $(\Omega m)^{-1}$	$I \cdot 10^3$ (A)	$v \cdot 10^5$ (m/sec)	$A \cdot 10^6$ (m²)
100	0.800	46.5	0.238	2.44	2.52	6.25
200	1.55	100	0.369	4.90	2.70	6.25

HAc in concentrations approximately equal to the steady-state concentrations. Two leading ion concentrations were used, namely 100 and 200 mol/m³ (see Tables Ib and Ic). The counter-flow was applied for 2–4 h. Fig. 2b shows the scanner trace. The Cu zone could easily be detected by its UV absorbance. The Co zone showed no UV absorbance, but was easily localized by its red colour. The zone length could therefore be measured manually with a ruler. The position of the boundary between the leading K zone and the Co zone (indicated in Fig. 2b by an arrow) was established by such a measurement. The fractions that were analysed are indicated as a–d.

The migration velocity was calculated in the same way as for the anionic system; the boundary between Co and Cu was used for this calculation. The measured parameters of the cationic system are listed in Tables Ib and Ic. The related calculated mobility and ω-function values are presented in Tables IIb and IIc.

Error analysis

All possible care has taken to minimize the errors in the measurements. The uncertainty of a measured physical parameter consists of a standard deviation of the measurement and a systematic error related to experimental procedures. The latter was estimated according to the precision of the equipment. The limits of the errors of the calculated mobilities and ω-functions in Tables IIa, IIb and IIc were estimated using the equation

$$\frac{\Delta f}{f} = \sqrt{\sum_i \left(\frac{(\Delta f)i}{f}\right)^2} \tag{11}$$

TABLE II

CALCULATED PARAMETERS, INCLUDING THE ω-FUNCTIONS

(a) The Cl^- (α) and SSA^{2-} (β) zones (see Fig. 2a):

$^cCl^+$ *(mol/m³)*	*(Eqn. 3)* $m_{Cl^-} \cdot 10^8$ *(m²/V·sec)*	*(Eqn. 8)* $m_{Tris^+} \cdot 10^8$ *(m²/V·sec)*	*(Eqn. 3)* $m_{SSA^{2-}} \cdot 10^8$ *(m²/V·sec)*	*(Eqn. 8)* $m_{Tris^+} \cdot 10^8$ *(m²/V·sec)*	*(Eqn.9a)* $\omega^\alpha \cdot 10^{-9}$ *(mol·V·sec/m⁵)*	*(Eqn. 9b)* $\omega^\beta \cdot 10^{-9}$ *(mol·V·sec/m⁵)*	*(Eqn. 10a)* $\omega^\alpha/\omega^\beta$
25.0 ± 0.13	−6.10 ± 0.19	2.44 ± 0.15	−4.30 ± 0.13	2.34 ± 0.17	1.44 ± 0.06 (1.46)	1.49 ± 0.09 (1.47)	0.96 ± 0.06 (0.99)
50.0 ± 0.25	−5.78 ± 0.18	2.22 ± 0.15	−3.79 ± 0.12	2.12 ± 0.15	3.12 ± 0.14 (3.17)	3.26 ± 0.20 (3.22)	0.96 ± 0.06 (0.99)
100.0 ± 0.5	−5.32 ± 0.16	2.29 ± 0.14	−3.51 ± 0.11	2.28 ± 0.15	6.25 ± 0.25 (6.26)	6.27 ± 0.35 (6.26)	1.00 ± 0.06 (1.00)

(b) The K^+ (α) and Co^{2+} (β) zones (see Fig. 2b):

$^cK^+$ *(mol/m³)*	$m_{K^+} \cdot 10^8$ *(m²/V·sec)*	$m_{Ac^-} \cdot 10^8$ *(m²/V·sec)*	$m_{Co^{2+}} \cdot 10^8$ *(m²/V·sec)*	$m_{Ac^-} \cdot 10^8$ *(m²/V·sec)*	$\omega^\alpha \cdot 10^{-9}$ *(mol·V·sec/m⁵)*	$\omega^\beta \cdot 10^{-9}$ *(mol·V·sec/m⁵)*	$\omega^\alpha/\omega^\beta$
100 ± 2	5.16 ± 0.18	−3.13 ± 0.23	2.61 ± 0.09	−2.97 ± 0.15	5.13 ± 0.31 (5.22)	5.40 ± 0.23 (5.33)	0.95 ± 0.07 (0.98)
200 ± 4	5.34 ± 0.19	−2.69 ± 0.23	2.37 ± 0.08	−2.41 ± 0.13	11.2 ± 0.7 (11.6)	12.5 ± 0.6 (12.2)	0.89 ± 0.07 (0.95)

(c) The K^+ (α) and Cu^{2+} (γ) zones (see Fig. 2b):

$^cK^+$ *(mol/m³)*	$m_{K^+} \cdot 10^8$ *(m²/V·sec)*	$m_{Ac^-} \cdot 10^8$ *(m²/V·sec)*	$m_{Cu^{2+}} \cdot 10^8$ *(m²/V·sec)*	$m_{Ac^-} \cdot 10^8$ *(m²/V·sec)*	$\omega^\alpha \cdot 10^{-9}$ *(mol·V·sec/m⁵)*	*(Eqn. 9c)* $\omega^\gamma \cdot 10^{-9}$ *(mol·V·sec/m⁵)*	*(Eqn. 10b)* $\omega^\alpha/\omega^\gamma$
100 ± 2	5.16 ± 0.18	−3.13 ± 0.23	1.54 ± 0.05	−1.12 ± 0.07	5.13 ± 0.31 (6.66)	14.4 ± 0.8 (10.4)	0.36 ± 0.03 (0.64)
200 ± 4	5.34 ± 0.19	−2.69 ± 0.23	1.27 ± 0.04	−0.64 ± 0.05	11.2 ± 0.7 (12.0)	46.9 ± 3.2 (27.8)	0.24 ± 0.02 (0.43)

where $(\Delta f)i$ denotes the relative difference in f when the parameter p_i is replaced by $p_i + \Delta p_i; f = f(p_i); \Delta p_i$ is the standard error of p_i.

DISCUSSION

Method

The electrophoresis equipment that was used has the following characteristic features that make it suitable for the present investigation. (a) Electrophoresis is performed in a carrier-free solution; it is accordingly not necessary to compensate for disturbances caused by the presence of supporting media (for instance, in the form of gels of polyacrylamide or gradients of sucrose) and viscosity-increasing agents (for instance, methylcellulose). (b) With the photoelectric scanner one can determine accurate migration velocities and easily decide when the steady state has been attained. (c) Fractions can be withdrawn after completion of a run; the conductivity and ion concentration of any zone can therefore be measured with high accuracy. (d) The power supply is equipped with a current stabilizer; variations in the temperature of the cooling water therefore have very little influence on the migration velocities of the zones[3]. (e) Electroendosmotic flow is eliminated.

In displacement electrophoresis all adapted zones migrate with the same velocity; in addition, the number if ionic species in the zones is smaller than in zone electrophoresis or moving boundary electrophoresis. The former method therefore has the advantage that it requires fewer measurements than the other two for the calculation of the ω-functions. This is the main reason why we chose to utilize the displacement technique in this investigation. The conclusions drawn from this study are, however, relevant also to zone and moving boundary electrophoresis.

That the steady state had been attained in our experiments is evident not only from the shape of the curves in Fig. 2a and b but also from the fact that the conductivities and absorbances of fractions 2 and 3 in Fig. 2a (and b and c in Fig. 2b) differed by at most 2%. In one control experiment the SSA^{2-} zone was withdrawn in eight fractions. Accurate measurements showed that the SSA concentration in the fractions deviated less than 0.5% from the arithmetic mean.

Theory

In the deduction given by Kohlrausch[1] and Longsworth[7] a prerequisite for the ω-functions to have identical values in all zones moving in an electrical field is that the mobility of an ion has the same value in the different zones. It is therefore necessary to consider the constancy of the mobilities of the counter ions (which are the only ions in our experiments, except the negligible H^+ and OH^- ions, that appear in more than one zone). Table IIa shows that the mobility of $Tris^+$ (within experimental error) has the same value in the α and β phases. The data in Table IIb indicate that it is more uncertain whether this is true for the Ac^- ion in the K^+–Co^{2+} system. It is obvious, however, that the Ac^- ion has a different mobility in the presence of Cu^{2+} than in the presence of K^+ (Table IIc). These considerations are in agreement with the experimentally found ratio between the ω-functions: in Table IIa the ratio is close to unity; Table IIb shows possibly some deviation from unity, while a very striking deviation is observed in Table IIc. It should be stressed that the ionic concentrations c_j in the calculations of the ω-functions were set equal to the

total concentration of the ion j, which is far from correct for the Ac^- ion in the Cu^{2+} zone (Table IIc).

The above great difference between the values of the ω-functions for two moving zones is due to complex formation between Cu^{2+} and Ac^- (ref. 8). Generally, one can state that significant differences between the Kohlrausch functions, calculated with the assumption that no complex formation occurs (as we have done in this paper), are an indication of molecular interactions. A closer inspection of the Kohlrausch functions may in some instances give important information about the nature of ionic complexes, particularly if ion mobilities and ion concentrations are replaced by constituent mobilities and constituent concentrations (see ref. 7).

ACKNOWLEDGEMENT

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ROLE OF THE CHARGE NUMBER OF THE COUNTER-IONIC CONSTITUENT IN THE SEPARATION OF ANIONS BY ISOTACHOPHORESIS*

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SUMMARY

The influence of the different charge numbers of the ionic forms of the buffering counter-ionic constituents on the effective mobilities of anions at pH 6.0 was investigated. It is shown that a proper choice of the charge number of the counter-ionic constituent can be used as an effective tool in optimization of the operating conditions in the separation of anions by isotachophoresis. The successful separation of a group of anions at pH 6.0 using 1,3-bis[tris(hydroxymethyl)methylamino]propane as the buffering counter constituent in the leading electrolyte (which could not be performed at this pH when other constituents were used for this purpose) illustrates the practical possibilities of this approach. Ca^{2+} (a complex-forming cation) and doubly protonated diaminopropane (a non-complexing cation) were used as co-counterions in the leading electrolytes to show the different natures of their interactions with the same group of anions.

INTRODUCTION

There are several means by which the effective mobilities of ionic constituents can be affected in a desired way and, consequently, their isotachophoretic separations effected[1]. Of these, the pH dependences of the effective mobilities are mostly used, and in some instances complex formation is a good alternative[2,3]. When the charge numbers of the components to be separated differ, their effective mobilities exhibit

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different concentration dependences. This property can also be of practical use in isotachophoresis[1].

Supposing that no complex formation occurs, in the above concentration dependences both electrophoretic and relaxation effects[1,4–7] and ion pairing[4–7] are involved. In the terms that are used to correct conductivities or mobilities for electrophoretic and relaxation retardations, in addition to concentrations of the ions and parameters describing the properties of the solvent, ionic charges are also involved[1,4–7]. The electrophoretic and relaxation retardations are long-range effects and should be completely independent of any short-range parameters such as ionic dimensions[6]. On the other hand, ion pairing should include all short-range effects[6] (ion–ion and ion–solvent interactions).

Everaerts *et al.*[1] have shown that corrections of the calculated conductivities of the zones of some divalent cations and anions for the electrophoretic and relaxation effects were sufficient to obtain theoretically predicted dependences of the thermal step heights on isotachopherograms on the resistivities of the zones. This means that for the counter-ionic constituents used and the ionic constituents investigated ion pairing is negligible. Moreover, it is known from conductivity measurements[8] that strong 2–2 electrolytes which have separated their charge-carrying groups by an inert framework can serve as good model constituents with no ion pairing concentrations up to at least 5 m*M*.

The above facts provide the possibility of a qualitative interpretation of the results presented in this work. For greater detail, an extensive literature dealing with many aspects of the theory of electrolytic conductance is available (*e.g.*, refs. 5–7).

The aim of this work was to show how different counter-ionic constituents buffering at the same pH, while differing in the charge numbers of their ionic forms at this pH, influence the effective mobilities of anions. It will be shown that a proper choice of the charge number of the counter-constituent gives some practical possibilities for the separation of anions by isotachophoresis. At pH 6.0 doubly protonated diaminopropane[7] (a non-complexing cation) and Ca^{2+} (a complex-forming cation) used at the same concentrations in the leading electrolytes can give an insight into the nature of the interactions that are responsible for the retardations of anion in these instances. The former represents mainly retardation due to the electrophoretic and relaxation effects, while the latter introduces a combined effect of both the long-range and short-range interactions.

As previously no attention had been paid to the subject dealt with in this paper, an attempt is made here to explain some of the phenomena observed.

EXPERIMENTAL

An instrument for isotachophoresis similar to that described by Everaerts *et al.*[1] was used, provided with a conductivity detection cell designed by Stankoviansky *et al.*[9]. A fluorinated ethylene–propylene copolymer (FEP) capillary tube of I.D. 0.3 mm was used.

Chemicals were of pro analisi purity and some of them were purified by conventional methods.

Histidine (HIS), 1,3-bis[tris(hydroxymethyl)methylamino]propane (bis-tris-propane or BTP) and 2-[N-morpholino]ethanesulphonic acid (MES) were bought

from Sigma (St. Louis, MO, U.S.A.). 1,2-Dimorpholinylethane (DME) was obtained from K & K (Plainview, NY, U.S.A.) and lysine (LYS) and 1,3-diaminopropane (DAP) from Fluka (Buchs, Switzerland). The other chemicals were supplied by Lachema (Brno, Czechoslovakia).

As additives to the leading electrolytes[1], Mowiol 8-88 (Hoechst, Frankfurt, G.F.R.) or hydroxyethylcellulose (Polysciences, Warrington, PA, U.S.A.) were used at 0.05% and 0.2% concentrations, respectively.

RESULTS AND DISCUSSION

The operating systems used are listed in Table I. The concentration of the leading anion (chloride, 0.01 *M*) and the pH of the leading electrolytes (6.0) were kept constant. Counter-ionic constituents buffering at pH 6.0 and differing in the charge numbers of their ionic forms were varied.

TABLE I

OPERATING SYSTEMS

Additives to the leading electrolytes: Mowiol 8-88 or hydroxyethylcellulose. Terminating anions: 0.005 *M* MES or 0.005 *M* capronate. For the abbreviations used, see Experimental.

Parameter	*System No.*										
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
Leading anion	Cl^-	Cl^-	Cl^-	Cl^-	Cl^-	Cl^-	Cl^-	Cl^-	Cl^-	Cl^-	Cl^-
Concentration (*M*)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Counter ion	HIS	HIS	HIS	HIS	DME	BTP	DME	DME	DME	DME	HIS
Co-counter ion	—	DAP	DAP	DAP	—	—	LYS	LYS	HIS	DAP	Ca^{2+}
Concentration (*M*)	—	0.001	0.002	0.003	—	—	0.002	0.006	0.006	0.002	0.002

Some of the operating systems were used for several reasons:

(1) to explain the observed changes in the effective mobilities of anions for different buffering counter-constituents by using the results obtained in other systems;

(2) to compare retarding efficiencies of co-counter ions carrying the same or different charges;

(3) to compare the effective mobilities of anions for the co-counterions carrying the same charges while differing in the interactions involved (*e.g.*, long-range effects and complex formation);

(4) to investigate dependences of the effective mobilities of anions carrying different charges on the concentration of a co-counterion in the leading electrolyte.

It is assumed in the following discussion that only negligible differences in the pH of the zones exist when different buffering counter constituents (small differences in their p*K* values) are used at the same pH and/or the buffering capacities of the leading electrolytes containing non-buffering co-counter ions are sufficient. These assumptions are supported by several facts: at pH 6.0 the anions studied behave more or less like strong electrolytes; no phenomena known to occur when a leading electrolyte with unsufficient buffering is used[1] were observed (the anode compartment of the separation unit was filled with an equimolar solution of histidine and histidine

hydrochloride to prevent the protons formed in this compartment from entering the separation compartment); very good reproducibility of the step heights of the zones on the isotachopherograms was found for all of the operating systems.

As the constituents studied are almost fully dissociated at pH 6.0, their ionic mobilities and in part also their association equilibria (ion pairing) are affected when the counter constituents are changed.

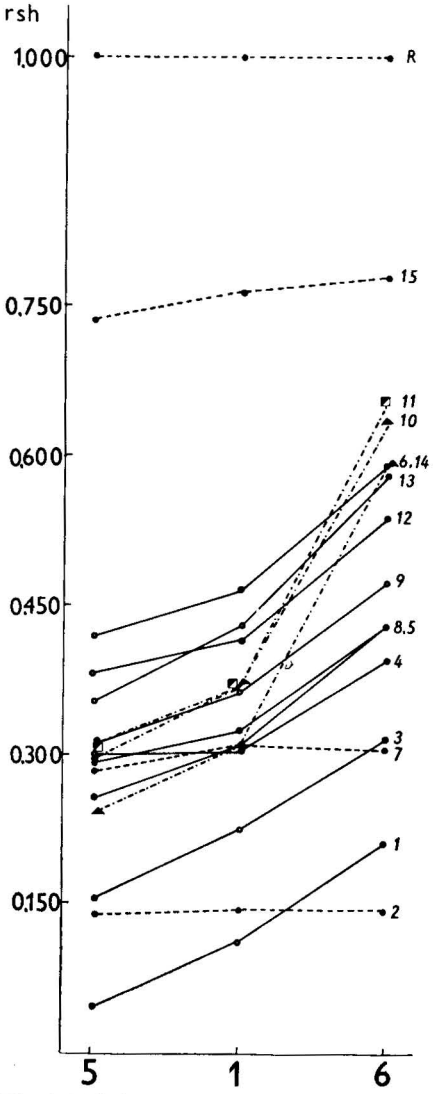

Fig. 1. Relative step heights (rsh) of the anionic constituents relative to trichloroacetate for different counter-ionic constituents buffering at pH 6.0. 1 = Oxalate; 2 = chlorate; 3 = tartronate; 4 = fumarate; 5 = malonate; 6 = *trans*-aconitate; 7 = formate; 8 = tartrate; 9 = malate; 10 = citrate; 11 = isocitrate; 12 = pyrazole-3,5-dicarboxylate; 13 = pyrazine-2,3-dicarboxylate; 14 = maleate; 15 = acetate; R = trichloroacetate. For compositions of the operating systems, see Table I. – – –, Monovalent; –.–.–, trivalent; ——, divalent.

The step heights of the anions on the isotachopherograms relative to trichloroacetate (see ref. 1, p. 307) are used for the evaluation of the results.

The relative step heights of the anions for different counterconstituents buffering at pH 6.0 are given in Fig. 1. In some instances, as can be seen from the data, different orders of some anions in the steady state can be expected on changing one counter constituent for another that differs in the charge numbers of its ionic forms. The isotachopherograms in Fig. 2 clearly illustrate this fact. A complete separation of a group of anions was achieved using a leading electrolyte containing BTP as the counter constituent. Histidine and DME did not effect this separation at the same pH.

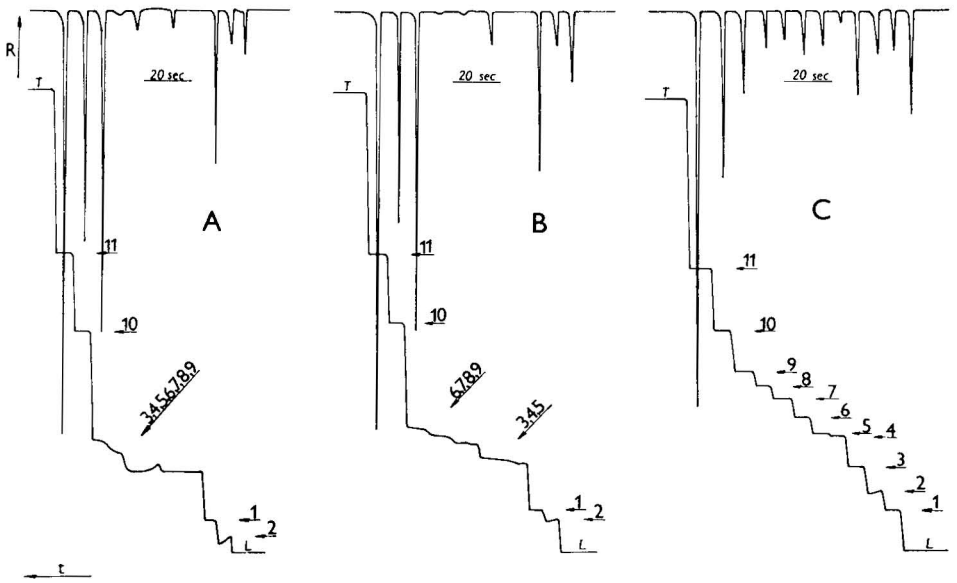

Fig. 2. Isotachopherograms for the separation of a group of anions at pH 6.0. Different counter-ionic constituent buffering at this pH were used in the leading electrolytes: A, DME (system 5); B, histidine (system 1); C, BTP (system 6). A 1-μl volume of the sample (1 = chlorate; 2 = oxalate; 3 = formate; 4 = fumarate; 5 = tartrate; 6 = malate; 7 = pyrazole-3,5-dicarboxylate; 8 = pyrazine-2,3-dicarboxylate; 9 = citrate; 10 = acetate) was injected in all instances. L = Leading anion (chloride); T = terminating anion (capronate); R = increasing resistance; t = time. The driving current was 50 μA.

The above discussion implies that the separation effect of BTP at pH 6.0 can be ascribed to the higher positive charges of its ionic forms (doubly positively charged acidic form and a singly positively charged basic form) relative to those of histidine and DME. In other words, retardations of the separands due to the electrophoretic and relaxation effects are different in the operating systems used. Among the parameters that describe these effects (*e.g.*, ref. 1, p. 36), the ionic charges of the counter constituents were changed in the operating systems, so that their influence on the effective mobilities is of major importance. The extent to which ion pairing contributes to the retardations of mono- and divalent anions seems marginal with respect to the findings of Atkinson *et al.*[8]. However, for trivalent anions and/or for counter

constituents distributed into ionic forms carrying higher positive charges than those used in this work, ion pairing could play a dominant role[10].

Figs. 1 and 2 indicate smaller differences in the effective mobilities of the anions for systems 1 and 2. Histidine and DME were used as the buffering counter-ionic constituents. Again, the charge numbers of their ionic forms can explain the differences observed. Histidine (at pH 6.0) is distributed into a doubly positively and a singly negatively charged acidic form and a singly positively and a singly negatively charged basic form. On the other hand the acidic form of DME at this pH has only a single positive charge, while its basic form is uncharged. If it is assumed that only

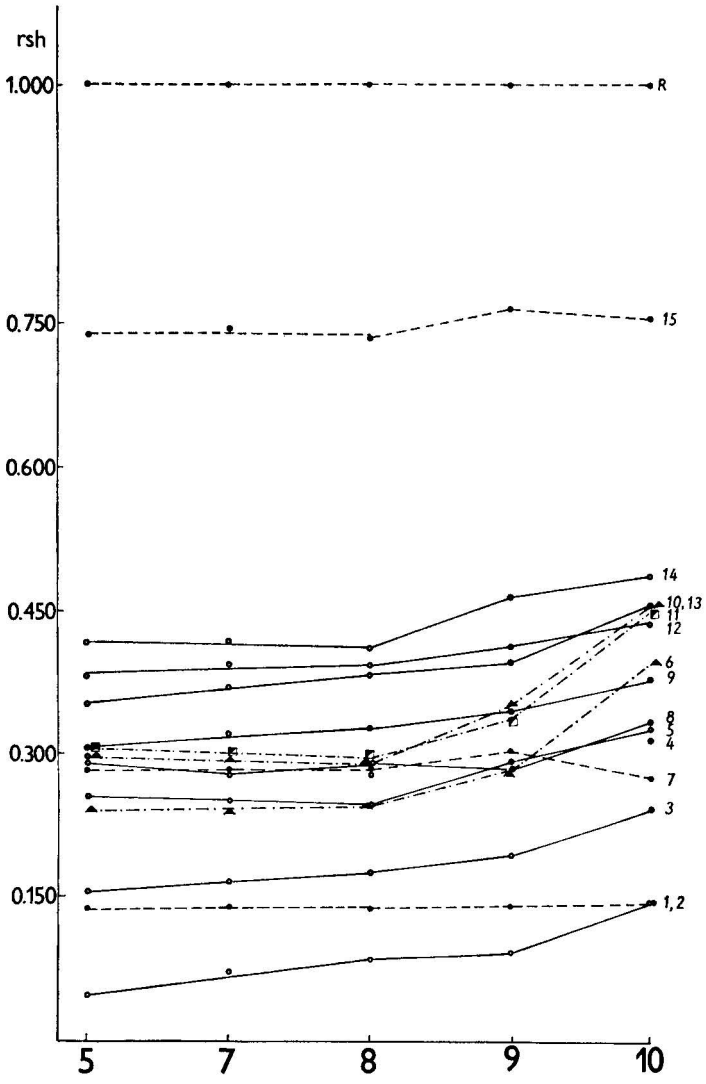

Fig. 3. Relative step heights of the anions at pH 6.0 for different co-counter ions. Information on the operating systems is given in Table I. For numbering of constituents, see Fig. 1. – – –, Monovalent; ——, divalent; –.–.–, trivalent.

the long-range effects are responsible for the different effective mobilities of the anions in these operating systems, histidine must exhibit a higher positive net charge than DME.

Further, the effective mobilities of the anions were measured in systems 7–10 to compare the effects of the same charge type of zwitterionic constituents and to relate them to the results obtained for the system with no co-counter ion (system 5) and to those when 2 m*M* DAP^{2+} was used (system 10). In these experiments histidine

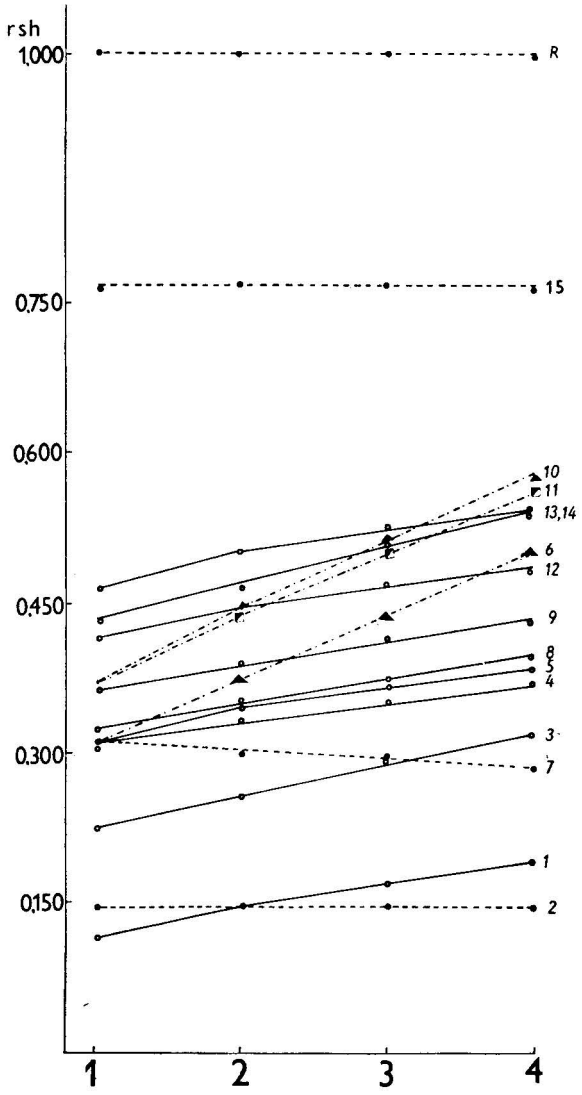

Fig. 4. Relative step heights (rsh) of the anions for different concentrations of DAP^{2+} in the leading electrolytes. Information on the operating systems is given in Table I. For numbering of constituents, see Fig. 1. – – –, Monovalent; ——, divalent; –.–.–, trivalent.

and lysine were used as the co-counter ions. The relative step heights of the anions are given in Fig. 3. For some of them differences in their effective mobilities for the leading electrolytes containing histidine and lysine at the same concentrations can be seen. In general, histidine is a more retarding constituent than lysine, which is unexpected because the opposite would be observed with respect to the above results as lysine at pH 6.0 is completely in the form of a doubly positively charged and a singly negatively charged ion, whereas histidine is only half in this form (see above). A reasonable explanation of these results could only involve higher intramolecular shielding of the opposite charges in the molecules of lysine relative to histidine, rather than a higher intermolecular association of the anions with histidine. The former also explains the smaller than expected long-range effects for lysine at pH 6.0. Nevertheless, further research is necessary in order to obtain a full explanation of these observations.

To show the influence of the concentration of a doubly positively charged co-counter ion on the effective mobilities of the anions, a series of experiments in systems 1–4 were carried out. The results (Fig. 4) show that retardations of the anions are proportional to their charges. In this way it seems possible to estimate the charge numbers of the separated components to allow their identification from a universal detector response[11].

Some anions can be separated by isotachophoresis using labile complex equilibria[2,3]. The isotachopherograms in Fig. 5 show the differences in the order of the components in the steady state when a non-complexing cation (DAP^{2+}) in the

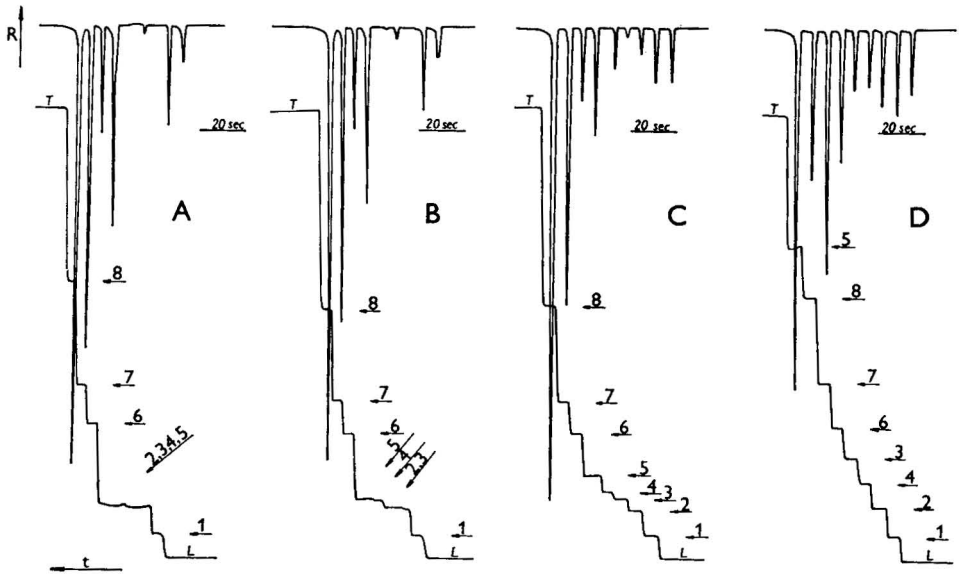

Fig. 5. Isotachopherograms for the separation of a group of anions at pH 6.0. Effects of a complex-forming cation and a non-complexing cation when used as co-counter ions in the leading electrolytes. A, DME (system 5); B, histidine (system 1); C, 2 m*M* DAP^{2+} (system 3); D, 2 m*M* Ca^{2+} (system 11). A 1-μl volume of the sample (1 = chlorate; 2 = formate; 3 = tartrate; 4 = α-ketoglutarate; 5 = citrate; 6 = acetate; 7 = lactate; 8 = capronate) was injected in all instances. L = Leading anion (chloride), T = terminating anion (MES), *R* = increasing resistance, *T* = time. The current was stabilized at 50 μA.

leading electrolyte was replaced with a complex-forming cation (Ca^{2+}) at the same concentration. This indicates the different natures of the interactions that are responsible for the separations in these instances: a more specific retardation of the complex-forming cation on the one hand and a less specific retardation due mainly to the electrophoretic and relaxation effects on the other. For this group of anions we can again see a difference in their effective mobilities when histidine and DME were used as counter constituents.

At a lower pH of the leading electrolyte (3.0–5.0) a similar behaviour of anionic constituents was found when buffering counter constituents differing in their charge numbers were used. Moreover, some phenomena that disturbed the separations of some anions were observed when counter constituents carrying higher positive charges at these pH values were used. Further research on this aspect is being carried out in order to obtain an explanation of the phenomena observed.

CONCLUSIONS

The charge numbers of the ionic forms of the buffering counter-ionic constituents play an important role in separations of anions by isotachophoresis. Therefore, the counter constituents should not only be chosen for their buffering properties at a particular pH but also the charge numbers of their ionic forms should be taken into account.

A proper choice of the buffering counter-ionic constituent can increase the number of components resolved at a given pH or at least a higher resolution rate[12] can be achieved in this way. Obviously, when an attempt is made to reproduce published results the above facts must be borne in mind, otherwise contradictory results could be obtained.

Information concerning of the charges of the separated anions at a given pH can be obtained when the results obtained in at least two operating systems (differing, for example, in the charge numbers of the ionic forms of the counter-ionic constituents used) are compared.

The nature of the separation effects depends on both the charge numbers and the structural factors of the ionic constituents involved in the ionic interactions.

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GAS CHROMATOGRAPHIC DETERMINATION OF NITRITE IN FOODS AS TRIMETHYLSILYL DERIVATIVE OF 1*H*-BENZOTRIAZOLE

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SUMMARY

1,2-Diaminobenzene reacts with nitrite in acidic solution to form 1*H*-benzotriazole, which can be extracted into ethyl acetate. After evaporation of the ethyl acetate, 1*H*-benzotriazole is determined as its trimethylsilyl derivative by gas–liquid chromatography on a column of 15% SE-30 on Chromosorb G HP at 200 °C with flame-ionization detection. The nitrite concentration is calculated from the peak height. Amounts of 0.5–10 μg of nitrite-nitrogen can be determined. For the determination of nitrite in foods, clean-up of the crude extracts by ion-exchange column chromatography allows the satisfactory elimination of interferents and permits concentrations down to 0.41 ppm to be determined. The recovery of nitrite added to foods at the 4.1 ppm level ranges from 94.6 to 98.7% and at the 8.2 ppm level it ranges from 95.2 to 98.8%. The trimethylsilyl derivative of 1*H*-benzotriazole was identified as 1-trimethylsilylbenzotriazole by combined gas chromatographic–mass spectrometric examination and nuclear magnetic resonance spectrometry.

INTRODUCTION

Sodium nitrite is widespread in nature and is also used as a food preservative. In recent years, there has been concern over the potential health danger from nitrite additives in foods because of the possibility that nitrite may react with secondary amines present in the body and form carcinogenic nitrosamines[1]. In several countries official tolerance limits have been established, and it is important that sensitive and accurate methods be available for the determination of nitrite. Such methods should also be simple and rapid and capable of determining nitrite in various types of real samples.

There are numerous methods for determining nitrite, including colour reactions and absorption measurement, UV and IR spectrophotometry, fluorimetry, polarography and gas chromatography. Many colorimetric methods[2–6] have been reported and in more recent methods sulphanilic acid is diazotized and coupled with 1-naphthylamine or N-(1-naphthyl)ethylenediamine to form a coloured azo dye[7,8]. All of these colorimetric methods are limited by the fact that occasionally turbid and

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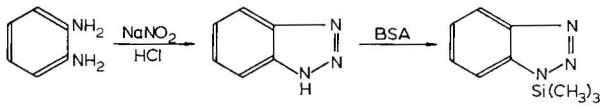
slightly coloured food extracts can affect the colour of the azo dye and, consequently, the accuracy of the nitrite determination.

Recently, the determination of nitrite by gas–liquid chromatography (GLC) has been described[9–11]. Wu and Peter[11] applied an electron-capture detector (ECD) to nitrobenzene after nitration of nitrite and benzene, and found a detection limit of about 0.04 ppm of nitrite; however, this method was not suitable for routine use because of the complex procedure and the vigorous reaction conditions required. Akiba *et al.*[9] studied the determination of nitrite as 1*H*-benzotriazole after reaction with 1,2-diaminobenzene[12] in acidic solution by GLC with a flame-ionization detector (FID) and found it difficult to obtain good accuracy and sensitivity; they recommended the use of another method.

However, we have found that the trimethylsilyl (TMS) derivative of 1*H*-benzotriazole is over 40 times more sensitive than 1*H*-benzotriazole in GLC; it can be prepared quantitatively by reaction with N,O-bis(trimethylsilyl)acetamide (BSA) in ethyl acetate. This reaction scheme is as follows:



The TMS derivative was detected quantitatively with a detection limit of 0.5 ng for nitrite-nitrogen (NO_2-N). Nitrite in foods was extracted with an alkaline solution and purified by ion-exchange column chromatography[10,13] [Dowex 1-X4 (Cl^-)]. This GLC method is simple and sensitive and offers a practical means of determining nitrite in various foods. The recovery of nitrite added to foods was satisfactory.

EXPERIMENTAL

Reagents and apparatus

All water used for preparing solutions was triply distilled and deionized. Sodium nitrite was dried at 100 °C under vacuum immediately before use. A stock nitrite solution was prepared by dissolving 0.493 g of sodium nitrite in distilled water and diluting to 1000 ml to give a concentration of 10 μg/ml of NO_2-N. 1,2-Diaminobenzene solution (0.1 %, w/v) was prepared by dissolving 0.1 g of the reagent (special high grade material, recrystallized three times from benzene before use) in 100 ml distilled water.

The ion-exchange resin Dowex 1-X4 (Cl^-) used in the clean-up stage was obtained from Muromachi Kagaku Kogyo (Tokyo, Japan). The silylating reagents used were BSA–trimethylchlorosilane (TMCS), BSA, TMCS–hexamethyldisilazane (HMDS), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and N-trimethylsilylimidazole (SIM), all obtained from Tokyo Kasei Kogyo (Tokyo, Japan). The internal standard solution for GLC was prepared by dissolving 50 μg of fluorene in 1 ml of ethyl acetate.

The column packing materials for GLC, *viz.*, Chromosorb G HP, SE-30, DC-200, OV-101, OV-17 and Triton X-305 were obtained from Nishio (Tokyo, Japan). All other reagents and solvents were of high purity and were obtained from Wako (Tokyo, Japan). For identification of the trimethylsilyl derivative of 1*H*-benzotriazole,

a Shimadzu LKB-9000 combined gas chromatograph–mass spectrometer was used; for GC, a glass tube (1.5 m × 3 mm I.D.) packed with 15% of SE-30 on Chromosorb G HP (80–100 mesh) was fitted. The flow-rate of helium was 30 ml/min, and the column temperature was 200 °C. For mass spectrometry (MS), the separator temperature was 260 °C and that of the ion source was 290 °C. The trap current was 60 μA. The electron energy was 70 eV and the accelerating potential was 3.5 keV. Nuclear magnetic resonance (NMR) spectra were measured at 60 Hz with a Varian EM-60 spectrometer.

Preparation of TMS derivative of 1H-benzotriazole

A suitably diluted solution of nitrite or the effluent from the ion-exchange column was placed in a 100-ml beaker and adjusted to pH 1.0–1.5, then 1 ml of 1,2-diaminobenzene solution was added. After reaction at 80 °C with occasional shaking for 10 min in a water-bath and cooling to room temperature, the solution was re-adjusted to pH 2.0–2.5 and transferred into a 100-ml separating funnel, then 5 g of sodium chloride and 10 ml of ethyl acetate were added. The mixture was shaken vigorously for 5 min and the ethyl acetate layer separated and dried with 2 g of anhydrous sodium sulphate. The ethyl acetate extract was placed in a 50-ml round-bottomed flask with a ground-glass stopper, and the solvent was removed by evaporation under reduced pressure at room temperature. To the dried residue was added 1 ml of internal standard solution and 50 μl of BSA, and the reaction was allowed to proceed at room temperature for 10 min (although it was usually complete after 5 min). A 3-μl volume of the final solution was injected into the gas chromatograph.

Gas–liquid chromatography

A Shimadzu GC-5AIFF gas chromatograph with an FID was used for all analyses. The column consisted of a glass tube (1.5 m × 3 mm I.D.) packed with 15% of SE-30 on Chromosorb G HP (80–100 mesh) and was conditioned at 200 °C; the detector and injector temperature were 290 °C and the flow-rates of nitrogen carrier gas, hydrogen and air were 40, 40 and 800 ml/min, respectively.

Calibration graph

A series of working-standard nitrite solutions were prepared by diluting the stock solution with water. Aliquots were placed in a beaker to give amounts of 0.5, 1.0, 3.0, 5.0, 7.5 and 10.0 μg of NO_2-N. According to the procedure described above, 10 ml of ethyl acetate extract were obtained in each instance, and then removed by evaporation. After trimethylsilylation by addition of BSA and the internal standard solution to the residue, a 3-μl aliquot of the mixture (1050 μl) was injected into the GLC column. As shown in Fig. 1, the retention time of the TMS derivative relative to that of fluorene was 0.63. The peak-height ratio of the TMS derivative to fluorene was plotted against the amount of NO_2-N analysed; a typical standard graph is shown in Fig. 2.

Extraction and clean-up procedure

To 10 g of finely ground sample in a 100-ml flask with a ground-glass stopper were added 40 ml of hot (70–80 °C) water (pH 9.0); after occasional shaking in a water-bath at 80 °C for 40 min and cooling to room temperature, the extracted solution was

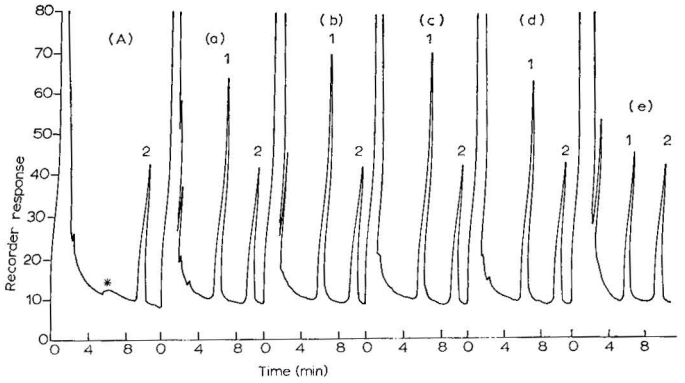


Fig. 1. Gas chromatograms of 1*H*-benzotriazole (A) and the TMS derivative (a–e). The silylating agents added to 42.5 μg of 1*H*-benzotriazole were (a) HMDS–TMCS, (b) BSA, (c) BSA–TMCS, (d) BSTFA and (e) SIM. The reagents were dissolved in 1 ml of ethyl acetate, and the sample size was 3 μl. Peaks: 1, TMS derivative of 1*H*-benzotriazole; 2, fluorene; *, 1*H*-benzotriazole.

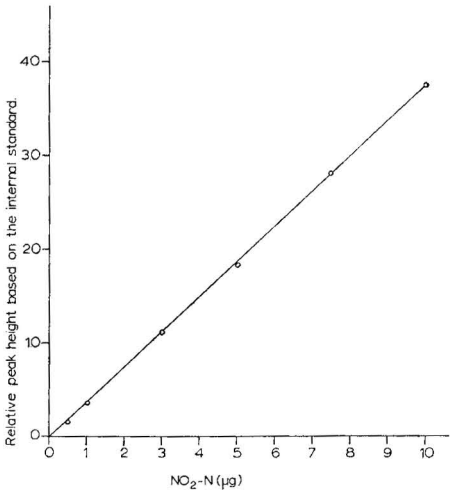


Fig. 2. Calibration graph for nitrite-nitrogen. Silylation was carried out at room temperature for 10 min. The sample size for GLC was 3 μl; the column temperature was 200 °C and the nitrogen flow-rate was 40 ml/min. The abscissa shows the nitrite-nitrogen content of the reaction mixture, and the ordinate the detector response measured as the peak height relative to the internal standard (fluorene; 50 ng per μl of reaction mixture).

filtered and diluted accurately to 100 ml with water. A 40-ml volume of the filtrate was decanted and passed through an ion-exchange column (30 × 1.0 cm I.D.) containing Dowex 1-X4 (which was regenerated with 1 *N* sodium hydroxide solution and 1 *N* hydrochloric acid before use). The column was then eluted successively with 200 ml of water, 50 ml of 0.1 % sodium chloride solution and 25 % sodium chloride solution at a rate of 1 ml/min. The elution with 25 % sodium chloride solution was continued until the effluent volume reached 25 ml. After adjusting the pH to 1.0–1.5, the eluate was reacted with 1,2-diaminobenzene, extracted with ethyl acetate and then evaporated as described above.

The dry residue was silylated and analysed by GLC as described above. The contents of nitrite in foods were determined from the peak heights relative to that of the internal standard on the gas chromatograms, and comparison with calibration graphs.

RESULTS AND DISCUSSION

Standard assay

For the GLC assay using the described procedure, there was a linear relationship between peak height and amount of NO_2-N. As shown in Fig. 2, the calibration graph was linear from 0.5 to 10 μg of NO_2-N, and the average relative standard deviations of four determinations were 0.4% for 1.0 μg, 0.5% for 5 μg and 1.0% for 10 μg of NO_2-N; the reproducibility was considered to be satisfactory.

Production of 1H-benzotriazole

The influence of pH on the reaction of 1,2-diaminobenzene with nitrite to form 1*H*-benzotriazole was studied by mixing 5.0 μg of NO_2-N and 1 ml of 1,2-diaminobenzene solution. The relative yields obtained after 15 min were 86.0% at pH 0.5, 100% at pH 1.0, 99.2% at pH 1.5, 98.6% at pH 2.0, 88.3% at pH 2.5, 83.3% at pH 3.0 and 46.5% at pH 3.5; therefore, pH 1.0 was adopted as optimal.

The course of the reaction at different temperatures is shown in Fig. 3. A constant peak height was obtained after 10 min at room temperature and 3 min at 80 °C. After 10 min at 80 °C, the amount of 1*H*-benzotriazole present slowly decreased, and therefore the reaction was further studied at high temperatures. The relative yields obtained after 20 min were 93.6% for 100 °C and 63.2% at 150 °C. The use of a long reaction time at high temperatures was not desirable, and therefore 80 °C was adopted.

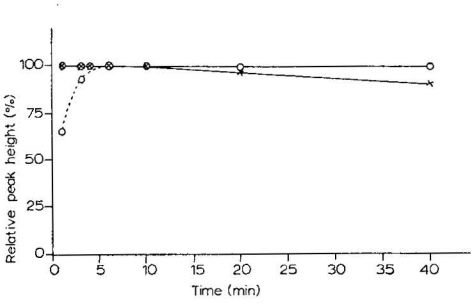


Fig. 3. Time course of production of the 1*H*-benzotriazole. To 5 μg of NO_2-N was added 1 ml of 0.1% 1,2-diaminobenzene at various temperatures, followed by silylation according to the described procedure and analysis by GLC. ○---○, Room temperature; ○—○, 50 °C; ×—×, 80 °C.

If we assume that 1 mol of 1,2-diaminobenzene reacts with 1 mol of nitrous acid, then 39 μg of 1,2-diaminobenzene is required for 16.8 μg of nitrous acid (5 μg of NO_2-N). The relative yields of 1*H*-benzotriazole for various amounts of 1,2-diaminobenzene added to 16.8 μg of nitrous acid in a total of 26 ml of solution were 96.5% for 50 μg of 1,2-diaminobenzene, 99.4% for 100 μg, 99.8% for 200 μg, 100% for 300 μg, 98.9% for 500 μg and 99.7% for 1000 μg at 80 °C with a reaction time

of 10 min. To some extent, therefore, addition of 1*H*-benzotriazole in excess gave a good result, and in practice 1 ml of a 0.1 % reagent solution was used.

Extraction

1*H*-Benzotriazole can be extracted into an organic solvent over the pH range 2–7. When the pH of the aqueous phase is higher than the pK_a (the acid dissociation constant of monoprotonated 1,2-diaminobenzene), the excess of reagent would be extracted into an organic solvent together with the 1*H*-benzotriazole. Consequently, 1*H*-benzotriazole should be extracted at a pH lower than the pK_a. When the pH of the aqueous phase was 4.0 or above, it was impossible to analyse 1*H*-benzotriazole. Therefore, the optimum pH range of the extraction adopted was 2.0–2.5. The use of various solvents as extractants was examined. When a polar solvent such as ethyl, *n*-propyl or *n*-butyl acetate was used, the extraction yield of 1*H*-benzotriazole was high, but it was lower if a non-polar solvent such as *n*-hexane was used. Thus, ethyl acetate, the most volatile of the selected polar solvents, was adopted.

Influence of evaporation of the solvent on the recovery of 1H-benzotriazole

Prior to trimethylsilylation of 1*H*-benzotriazole it was necessary to evaporate the ethyl acetate, with the risk of loss of volatile 1*H*-benzotriazole. A 10-ml volume of ethyl acetate which contained 42.5 μg of 1*H*-benzotriazole (corresponding to 5 μg NO_2-N) was evaporated under reduced pressure at room temperature. No loss of 1*H*-benzotriazole during the evaporation was observed. However, after the ethyl acetate had been removed and 1*H*-benzotriazole remained as the residue, the decrease in the amount of 1*H*-benzotriazole was significant, as shown in Table I. When the evaporation was performed at up to 40 °C, a significant decrease in 1*H*-benzotriazole was observed. The recoveries obtained after 15 min were 73.4 % at 50 °C, 23.5 % at 80 °C and 20.9 % at 100 °C. Therefore, the sample should be trimethylsilylated within 1 min after removal of the ethyl acetate at room temperature.

TABLE I

DECREASE OF 1*H*-BENZOTRIAZOLE RESIDUE AFTER EVAPORATION OF THE SOLVENT

1*H*-Benzotriazole content before evaporation was 42.5 μg (NO_2-N: 5.0 μg). The tests were carried out at room temperature.

Time after completion of evaporation (min)	*1H-Benzotriazole recovery (%)*	*Time after completion of evaporation (min)*	*1H-Benzotriazole recovery (%)*
0	100	5	100
1	100	10	97.5
2	100	15	92.7
3	100	30	90.7

Trimethylsilylation of 1H-benzotriazole

The chromatograms of the TMS derivative of 1*H*-benzotriazole are shown in Fig. 1. The retention time of the TMS derivative was 6.0 min. The optimal amount of reagent and the optimal reaction time were investigated by using BSA, and the

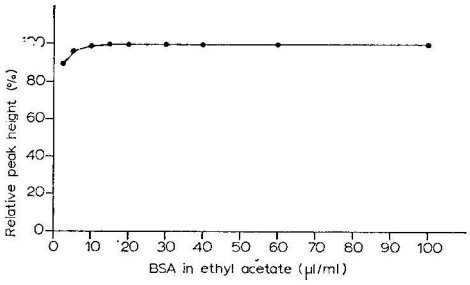


Fig. 4. Effect of amount of BSA on production of the TMS derivative of 1*H*-benzotriazole. To 42.5 μg of 1*H*-benzotriazole (corresponding to 5.0 μg NO_2-N) was added BSA in 1 ml of ethyl acetate at room temperature, and the product was analysed by GLC after 15 min.

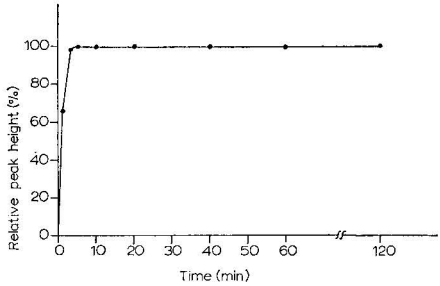


Fig. 5. Time course of TMS derivative production after addition of BSA to 1*H*-benzotriazole. To 42.5 μg of 1*H*-benzotriazole (corresponding to 5.0 μg NO_2-N) was added BSA in ethyl acetate (50 μl/ml) at room temperature, and the product was analysed by GLC.

results are shown in Figs. 4 and 5. For 42.5 μg of 1*H*-benzotriazole (corresponding to 5.0 μg NO_2-N), at least 72.5 μg of BSA in 1 ml of ethyl acetate were required. The reaction proceeded fairly rapidly and when BSA solution in ethyl acetate (50 μl/ml) was added to the solid residue of 1*H*-benzotriazole, the yield of the TMS derivative reached 100% within 10 min.

On comparing the reactivities of various trimethylsilylating reagents towards 1*H*-benzotriazole, it was observed that the reaction of BSTFA was slower that those with reagents BSA–TMCS, BSA and TMCS–HMDS and that the reaction with SIM was not complete even after 24 h. BSA–TMCS, BSA and TMCS–HMDS gave good chromatograms and these reagents are suitable for the silylation of 1*H*-benzotriazole. *n*-Hexane, ethyl acetate, cyclohexane, 4-methylpentan-2-one, dimethyl sulphoxide, tetrahydrofuran and acetonitrile were tried as reaction solvents. The most suitable were ethyl acetate and *n*-hexane and the least suitable pyridine and methanol, as shown in Table II. We chose ethyl acetate because of its good solvent properties for 1*H*-benzotriazole and fluorene.

Gas chromatographic sensitivity

Columns containing SE-30 (15%, w/w), DC-200 (15%, w/w), OV-17 (15%,

TABLE II

SOLVENT DEPENDENCE OF PRODUCTION OF THE TMS DERIVATIVE OF 1*H*-BENZOTRIAZOLE

Silylation and GLC conditions as in Fig. 2. Each reaction mixture contained 42.5 μg of 1*H*-benzotriazole (corresponding to 5 μg NO_2-N) and 50 μl of BSA.

*Solvent**	*Relative peak height (%)*	*Solvent**	*Relative peak height (%)*
Ethyl acetate	100	Dimethyl sulphoxide	21.8
n-Hexane	100	Dimethylformamide	16.4
Acetonitrile	100	Pyridine	7.3
Tetrahydrofuran	81.8	Methanol	3.6
Acetone	45.5		

* Fluorene (50 μg) was dissolved in 1 ml of each solvent.

w/w), OV-101 (15%, w/w) and Triton X-305 (15%, w/w), on Chromosorb G HP, were tested. Except with Triton X-305, the columns showed the peak for the TMS derivative of 1*H*-benzotriazole; particularly good peak characteristics and sensitivity were achieved with SE-30 under the conditions described above. A high temperature and a short column were preferable for the GLC of the TMS derivative of 1*H*-benzotriazole. At 200 °C, a 1.5-m column containing SE-30 on Chromosorb G HP gave a good gas chromatogram, the retention times of 1*H*-benzotriazole and the TMS derivative relative to that of the internal standard were 0.60 and 0.64, respectively. The ratio of the peak height for the same molar concentration of 1*H*-benzotriazole and the TMS derivative was 1:40, and therefore the peak characteristics of the TMS derivative were better than those of the parent 1*H*-benzotriazole (see Fig. 1). After trimethylsilylation, the reaction mixture should be injected into the gas chromatograph as soon as possible; at room temperature, the sample was stable for at least 24 h, but the content of the TMS derivative decreased to 94.8% in this period.

Interferences

Sodium nitrite can be extracted from foods with an alkaline solution and subsequently separated from the alkaline solution by ion-exchange chromatography. The simple and rapid extraction and clean-up procedures based on this principle permit the determination of nitrite in foods by GLC without effects from interfering substances. To investigate the effects of preservatives such as sorbic acid, benzoic acid, dehydroacetic acid, butylhydroxyanisole and butylhydroxytoluene on the determination, 42.5-μg portions of 1*H*-benzotriazole (corresponding to 5 μg NO_2-N) were added to 0.5–10.0 mg of various preservatives, and each mixture was analysed by direct silylation without clean-up procedure. As shown in Table III, when more than 5 mg of most preservatives were present (for example, 5 μg of NO_2-N and 10 mg of preservatives), the clean-up procedure described removed most of the

TABLE III

INFLUENCE OF FOOD ADDITIVES ON RECOVERY OF NITRITE-NITROGEN

Each amount of food additive was added to a mixture of 42.5 μg of 1*H*-benzotriazole (corresponding to 5 μg NO_2-N), 50 μl of BSA and 1 ml of internal standard solution. Silylation and GLC conditions as in Fig. 2.

Additive	*Amount added (mg)*			
	0.5	*1.0*	*5.0*	*10.0*
Sorbic acid	100	100	100	61.3 (98.2)**
Benzoic acid	100	100	100	50.2 (99.1)
Dehydroacetic acid	100	100	95.0	25.0 (98.5)
Butylhydroxyanisole	—* (100)	—* (100)	—* (98.7)	— (77.9)
Butylhydroxytoluene	93.0 (100)	60.0 (100)	40.0 (100)	— (82.3)

* Quantitative determination impossible.

** Values in parentheses are recoveries after clean-up.

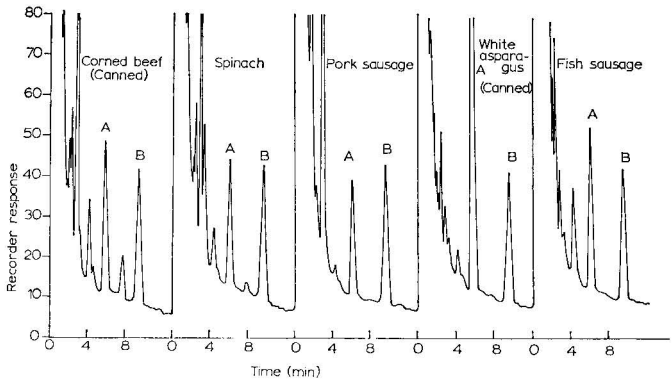


Fig. 6. Gas chromatograms of silylated extracts of various foods. Sample size, 3 μl. Peaks: A, 1-trimethylsilylbenzotriazole; B, fluorene.

amount present. As shown in Fig. 6, the silylated extracts obtained from foods gave gas chromatograms with good peak characteristics.

Application and recoveries

Nitrite added to 10-g samples of pork sausage, corned beef (canned), fish sausage, spinach and white asparagus (canned), chopped and then ground in a porcelain pestle and mortar, was determined by the proposed method. The recoveries of 4.1 and 8.2 ppm of nitrite, given in Table IV, ranged from 94.6 to 98.7% for 4.1 ppm and 95.2 to 98.8% for 8.2 ppm. The detection limit was 0.31 ppm.

TABLE IV

PERCENTAGE RECOVERIES OF NITRITE ADDED TO VARIOUS FOODS AT THE 4.1 AND 8.2 ppm LEVELS

Each result is the average of four determinations.

Sample	*Amount of nitrite-nitrogen added (μg)*	
	12.5	*25.0*
Fish sausage	98.7	98.4
Corned beef (canned)	97.5	98.1
Pork sausage	94.6	95.2
White asparagus (canned)	98.4	98.8
Spinach	96.7	97.3

Identification of the TMS derivative of 1H-benzotriazole

GC–MS. The mass spectrum of the product from the reaction of 1,2-diaminobenzene and nitrous acid was identical with the standard spectrum of 1*H*-benzotriazole, with ion peaks at *m/e* 119 (M^+), 91 (M^+-N_2) and 76 (−NH). The mass spectrum corresponding to the peak obtained by silylation and GLC separation of the 1*H*-benzotriazole are shown in Fig. 7, *viz.*, *m/e* 191 (M^+), 176 (M^+-CH_3), 118 (−Si (CH_3)), 90 ($-N_2$) and 75 (−NH). The parent peak (*m/e* 119) for 1*H*-benzotriazole and at *m/e* 191 for the TMS derivative correspond to the molecular weight of each compound. The shift of the peaks from *m/e* 191 to 118 for the TMS derivative could be ascribed to 1-de-trimethylsilylation and the subsequent shift from *m/e* 118

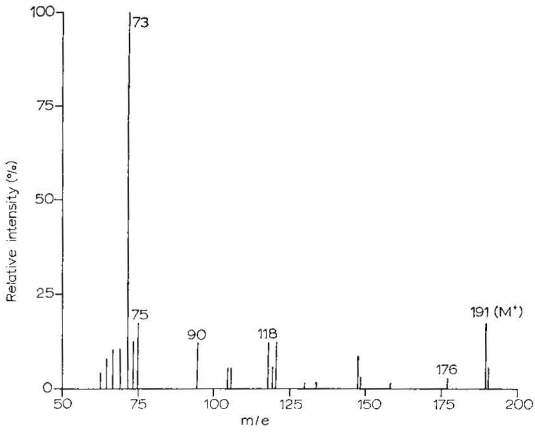


Fig. 7. Mass spectrum of 1-trimethylsilylbenzotriazole.

to 75 for the 1*H*-benzotriazole ion could be attributed to degradation of the triazole ring.

NMR spectrometry. Portions of 30 mg of 1*H*-benzotriazole dissolved in acetone were silylated with 100 μl of BSA. In the NMR spectrum of 1*H*-benzotriazole dissolved in acetone, signals appear at $\delta = 7.35$–7.93 ppm (multiplet; 4H) which is indicative of an aromatic compound, and at $\delta = 14.56$ ppm (wide singlet peak; 1H), which is indicative of the NH group. As shown in Fig. 8, in the NMR spectrum of the TMS derivative dissolved in acetone the singlet peak (H) at $\delta = 14.56$ ppm has disappeared, which suggests the loss of the NH group in the triazole ring. The difference in the chemical shifts makes it possible to distinguish 1*H*-benzotriazole and the TMS derivative.

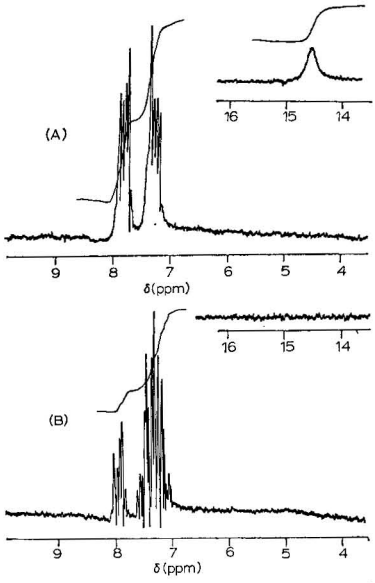


Fig. 8. NMR spectra of 1*H*-benzotriazole (A) and 1-trimethylsilylbenzotriazole (B) in acetone at 60 Hz.

From this series of experiments, it was concluded that the TMS derivative was 1-trimethylsilylbenzotriazole.

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TRIMETHYLSILYL-ESTER PFLANZLICHER SÄUREN UND IHRE ANWENDUNG IN DER GASCHROMATOGRAPHIE

DARSTELLUNG, KINETIK DER SILYLIERUNG UND EINFLÜSSE VERSCHIEDENER LÖSUNGSMITTEL AUF AUSBEUTE UND STABILITÄT DER DERIVATE

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SUMMARY

Trimethylsilyl esters of plant acids and their application in gas–liquid chromatography. Preparation, kinetics of silylation and solvent effects on quantitative reaction and stability of the derivatives.

Gas–liquid chromatographic separation of phosphate and organic acids in plant extracts as trimethylsilyl (TMS) derivatives is usually carried out in relatively high boiling solvents (pyridine, dimethylformamide) using internal standards which undergo no reaction with the silylating agent.

It is shown that acetone is advantageous to conventional solvents concerning boiling point, dissolving of the acids and the fact that extremely anhydrous conditions are not needed. Moreover, the reproducibility of results is essentially better for most compounds than with pyridine.

An internal standard forming TMS derivatives compensates for variable reaction conditions and permits the analysis of samples over an extended period of time, since its derivatives are subjected to a similar decomposition as the substances analysed. Butylmalonate met all these requirements.

The kinetics of the silylation reaction and the decomposition of the derivatives were investigated for phosphate and a lot of biologically interesting plant organic acids.

EINLEITUNG

Für die gaschromatographische (GC) Trennung pflanzlicher organischer Säuren werden vor allem Methylester und Trimethylsilyl (TMS)-Ester herangezogen. Nur in wenigen Fällen ist die Flüchtigkeit der Säuren für eine direkte GC-Analyse ausreichend.

Die Methylierung wird entweder durch Gemische aus wasserfreiem Methanol mit HCl bzw. BF_3 oder durch Diazomethan erreicht[1]. Störungen durch C=C und

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C=O Gruppen können Mehrfachpeaks zur Folge haben[2]. Diese Nebeneffekte können durch Darstellung der TMS-Ester vermieden werden, für deren Bereitung bereits eine Vielzahl Reagenzien von unterschiedlicher TMS-Donorstärke entwickelt wurden. Üblicherweise gelangen für organische Säuren Trimethylchlorosilan (TMCS), Gemische aus TMCS und Hexamethyldisilazan (HMDS)[3,9] sowie N,O-bis(TMS)-trifluoroacetamid (BSTFA) und N,O-bis(TMS)acetamid (BSA)[4,6,11] zur Anwendung. Nebenreaktionen, die zu Mehrfachderivatbildung führen, wurden nur bei Vorhandensein enolisierbarer Ketogruppen (z.B. bei α-Ketoglutarat) beobachtet, sie können durch Methoximierung der Carbonyle ausgeschlossen werden[4,8].

Die genaueste Methode zur quantitativen Bestimmung der Komponenten ist die Zugabe eines internen Standards (I.S.). Bei der Wahl der Standardsubstanz ist aber darauf Rücksicht zu nehmen, dass nicht nur Fehler durch schwankende Einspritzmengen und Detektorempfindlichkeit ausgeglichen werden, sondern dass auch Unterschiede in den Reaktionsbedingungen und der kontinuierliche Abbau der Derivate über längere Zeiträume mit erfasst werden. Letzteres ist eine essentielle Voraussetzung für den Einsatz in automatischen Probengebern. Dabei wird ja eine grössere Zahl Ansätze zur gleichen Zeit bereitet und anschliessend sequenziell vom Automaten zur Analyse herangezogen.

Als Standardsubstanzen werden in der Literatur fast ausschliesslich aromatische Verbindungen ohne funktionelle Gruppen vorgeschlagen, so etwa Diphenyl[5,16]. Sie können den vorhin erläuterten Anforderungen nicht genügen, da sie nicht silyliert werden und damit Schwankungen in den Reaktionsbedingungen und dem langsamen Abbau der TMS-Ester bei längeren Wartezeiten zwischen Bereitung und Analyse des Reaktionsgemisches nicht unterworfen sind wie die zu analysierenden Substanzen. Ausserdem muss darauf geachtet werden, dass eine vollständige Trennung der Standardsubstanz von den Komponenten der Analysenlösung erzielt wird.

Die Silylierung wird entweder lösungsmittelfrei[13], in Pyridin[8,11] oder Dimethylformamid[12] durchgeführt. Da die verwendeten Lösungsmittel aber relativ hohe Siedepunkte aufweisen (Pyridin: 116 °C, Dimethylformamid: 153 °C bei 1 bar) und Säuren nur sehr langsam lösen, wurde nach einem leichter flüchtigen und besser lösenden Solvens gesucht.

Die lösungsmittelfreie Silylierung befriedigt nicht, da die Reaktionsgeschwindigkeit durch die Auflösung der Festsubstanzen bestimmt wird und damit ziemlich gering ist[14].

Zur GC-Trennung der TMS-organischen Säuren ist eine stationäre Phase geringer Polarität ausreichend. Die erforderliche thermische Stabilität weisen Silikone auf, etwa die Präparate SE-30 und SE-52[9,11]. Wegen seiner hohen thermischen Belastbarkeit wurde hier Dexil 300 verwendet.

EXPERIMENTELLES

Die in natürlichen Proben häufig auftretenden Komponenten Glykolsäure, Oxalsäure, Malonsäure, Äpfelsäure, Citronensäure sowie anorganisches Phosphat* wurden als repräsentativ für den Flüchtigkeitsbereich der TMS-Ester pflanzlicher Säuren für die Untersuchung ausgewählt.

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* Zur Bestimmung von anorg. Phosphat als TMS-Derivat siehe Hashizume und Sasaki[15].

Als I.S. diente Butylmalonsäure (p.A., 1.6 mg/ml in Aceton p.A; Merck, Darmstadt, B.R.D.).

Die organischen Komponenten (p.A.) wurden zu je 1.6 mg/ml in Aceton (p.A.) gelöst. Phosphat lag als wässrige Orthophosphorsäure (85% H_3PO_4 p.A.; Merck) 1:250 verdünnt. Der genaue PO_4-Gehalt ist durch elektrochemische Titration leicht zu ermitteln.

In 2-ml Probefläschchen (Hewlett-Packard HP 62311-S29) wurden 0.1 ml Phosphatlösung im Vakuum bei Zimmertemperatur (20 °C) abgetrocknet, danach 0.5 ml Aceton-Lösung der organischen Säuren zugesetzt. Nach neuerlichem Entfernen des Lösungsmittels im Vakuum wurden 0.5 ml I.S.-Lösung zugesetzt, einige Sekunden leicht geschüttelt und 0.3 ml BSA (Pierce, Rockford, IL, U.S.A.) zugezetzt. Für die Untersuchungen mit anderen Lösungsmitteln wurde eine I.S.-Lösung gleicher Konzentration mit dem betreffenden Lösungsmittel an Stelle der I.S.-Lösung in Aceton eingesetzt. Die volumetrischen Operationen wurden mit Hamilton-Spritzen 725 N, 1001 LTN und Eppendorf-Pipetten 0.1 ml durchgeführt. Anschliessend wurde die Reaktionslösung in 0.07-ml Portionen auf 0.2-ml fassende Glaseinsätze aus 5 × 0.5 mm Glasrohren, passend in die HP 62311-S29 Probefläschchen aufgeteilt[8] und die Fläschchen mit PTFE-Gummi Bördelkappen verschlossen. Die Ansätze wurden sodann bei Zimmertemperatur (20 °C) bis zur Analyse aufbewahrt. So konnten zehn identische, in ihrer Zusammensetzung nicht von Pipettierungsfehlern behaftete Reaktionsansätze im automatischen Probengeber eingesetzt werden.

Die Analyse wurde anschliessend mit einem Gaschromatograph Hewlett-Packard HP 5835A mit automatischer Einspritzeinheit 7671A (Spritze Hamelton 701 RN) durchgeführt.

Säule: Dexil 300 GC (Applied Science Labs., State College, PA, U.S.A.) 3%ig auf Chromosorb W HP, 80–100 mesh (Pierce Eurochemie, Rotterdam, Niederlande); Säulendimension 6 ft. × 2 mm; on-column injection; einsäuliger Betrieb.

GC-Konditionen: Initialtemperatur 105 °C; Rate 3 °C von 0–10 min (105–135 °C), 5 °C von 10–23 min (135–200 °C); Endtemperatur 200 °C; Einspritzeinheit 220 °C; Detektor: Flammenionisationsdetektor (FID) 300 °C; Signalabschwächung 2^8; Die Abgase wurden durch Absaugen mittels Wasserstrahlvakuum unschädlich gemacht; Gesamtlaufzeit 25.0 min; Zyklusdauer (von Einspritzung zu Einspritzung) 30.0 min; Trägergas N_2 p.A. wasserfrei, Durchfluss 20 ml/min; Einspritzvolumen 1.6 μl.

ERGEBNISSE

Vergleich der Lösungsmittel

Für diese Untersuchungen wurden 3 Lösungsmittel gewählt: (a) Dichlormethan p.A. wasserfrei, Siedepunkt 41 °C bei 1 bar; (b) Aceton p.A., Wassergehalt max. 0.3%, Siedenpunkt 56 °C; (c) Pyridin p.A. wasserfrei, Siedenpunkt 116 °C.

Die Auswahl von a und b wurde nach dem Gesichtspunkt vorgenommen, möglichst niedrig siedende Lösungsmittel, die keine Nebenreaktionen mit dem TMS-Donor eingehen können, zu finden. Pyridin (c) ist das für Silylierungen gebräuchlichste Lösungsmittel, während etwa Aceton kaum verwendet wird*.

* Collier und Grimes[13] zeigen die Verwendung von Aceton als Lösungsmittel für den internen Standard, dieser wird aber erst nach der (lösungsmittelfreien) Silylierungsreaktion zugesetzt.

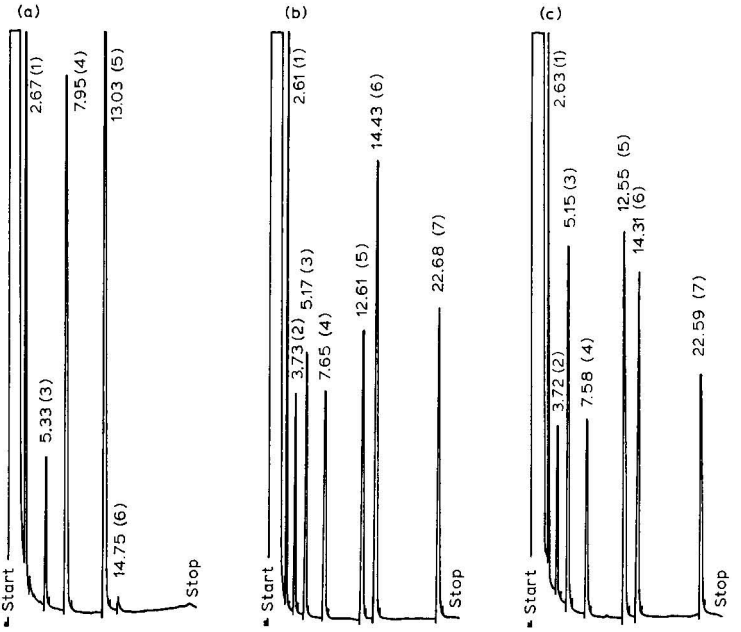

Fig. 1. Gaschromatogramme der TMS-Ester der untersuchten Substanzen in den Lösungsmitteln Aceton (a), Pyridin (b), Dichlormethan (c). Konzentrationen und Derivatisierungsprozess sind im Abschnitt Experimentelles angeführt. Die in der Mischung enthaltenen Komponenten sind: Glykolat (1), Oxalat (2), Malonat (3), Orthophosphat (4), I.S. Butylmalonat (5), Malat (6), Citrat (7). Zu jedem peak erscheint ferner die Retentionszeit in Minuten.

Fig. 1a zeigt, dass Dichlormethan einige Komponenten nicht löst und daher nicht verwendet werden kann.

Aceton und Pyridin als Solventien (Fig. 1b und c) zeigen, abgesehen von einem bei Pyridin etwas stärkeren "tailing" des Lösungsmittelpeaks, der die Bestimmung von Glykolat stört (siehe Tabelle I), im detector response und damit in der Ausbeute an

TABELLE I

VERGLEICH DER REPRODUZIERBARKEIT DER QUANTITATIVEN BESTIMMUNG MIT UNTERSCHIEDLICHEN LÖSUNGSMITTELN

n = Anzahl der Bestimmungen, $\bar{Q}$ = Mittelwert der Quotienten $area_x/area_{I.S.}$, s_Q = Standardabweichung, $s_Q(\%)$ = Standardabweichung in % des Mittelwertes.

	Lösungsmittel							
	Aceton				*Pyridin*			
	n	$\bar{Q}$	s_Q	$s_Q(\%)$	n	$\bar{Q}$	s_Q	$s_Q(\%)$
Glykolat	76	0.9946	0.0183	1.83	76	0.9587	0.1642	17.12
Oxalat	76	1.0045	0.0478	4.78	76	1.1183	0.0916	7.74
Malonat	76	0.9709	0.0226	2.33	76	0.9816	0.0154	1.57
Orthophosphat	76	1.0027	0.0145	1.45	76	0.9866	0.0308	3.12
Malat	76	1.0104	0.0133	1.31	76	1.0071	0.0064	0.64
Citrat	76	1.0324	0.0393	3.80	76	1.0167	0.0457	4.49

TMS-Ester der untersuchten Substanzen nur sehr geringfügige Differenzen. Deutliche Unterschiede treten jedoch im Fall der Reproduzierbarkeit der Ergebnisse hervor, diese ist bei Anwendung von Aceton in 3 Fällen (Glykolat, Oxalat und Citrat) wesentlich besser (siehe Tabelle II). Die Werte von Malonat, Phosphat und Malat unterscheiden sich nur geringfügig.

TABELLE II

DETECTOR-RESPONSE-VERGLEICH BEI VERWENDUNG VERSCHIEDENER LÖSUNGSMITTEL

Die Analysenbedingungen sind im Abschnitt Experimentelles angegeben. Die Analyse erfolgte 60 min nach Bereiten des Ansatzes. Es wurden die Quotienten $Q = \text{area}_x/\text{area}_{\text{I.S.}}$ und die Standardabweichungen aus jeweils 4 Bestimmungen erreichnet und auf $Q = 1.000$ bezogen. n = Zahl der Bestimmungen; s_Q = Standardabweichung, auf $Q = 1.000$ bezogen.

Substanz	*Lösungsmittel*			
	Aceton		*Pyridin*	
	n	s_Q	n	s_Q
Glykolat	4	0.0022	4	0.0147
Oxalat	4	0.0065	4	0.0453
Malonat	4	0.0041	4	0.0054
Orthophosphat	4	0.0007	4	0.0008
Malat	4	0.0017	4	0.0031
Citrat	4	0.0040	4	0.0215

Zur Untersuchung der Reproduzierbarkeit der mit diesen beiden Lösungsmitteln gewonnenen Ergebnisse bei längerer Aufbewahrung der Reaktionsansätze wurden die Quotienten $Q = \text{area}_x/\text{area}_{\text{I.S.}}$ bezogen auf $Q = 1000$ bei $t = 60$ min für Zeiten von 60 bis 600 min nach Bereiten der Ansätze in 30-min Intervallen ermittelt. Die Konzentration der Testsubstanzen und die Derivatisierungsprozedur sind im Abschnitt Experimentelles angeführt. Jeder Ansatz wurde 4× wiederholt.

Die Ergebnisse sind in Tabelle I zusammengestellt. Es zeigt sich bei Verwendung von Pyridin als Lösungsmittel deutlich schlechtere Reproduzierbarkeit der Resultate bei den Komponenten Glykolat, Oxalat, Phosphat und Citrat, bei Malonat und Malat ist sie geringfügig besser. Das schlechte Ergebnis bei Glykolat hängt mit der langsamen Eluierung von Pyridin von der Säule und dem dadurch verursachten Zusammenhängen des Lösungsmittelpeaks mit dem TMS-Glykolatpeak zusammen.

Insgesamt gesehen sind die mit Aceton gewonnenen Ergebnisse wesentlich gleichmässiger.

Kinetische Untersuchung der Silylierungsreaktion

Die Aufbauphase der TMS-Ester. Hiefür wurden die Reaktionszeiten unter 60 min betrachtet. Es wurden Analysen identischer Ansätze (Lösungsmittel Aceton) jeweils nach 10, 20, 30 und 60 min durchgeführt und jeder Ansatz 4× wiederholt. Die so erhaltenen Werte für die Peakflächen wurden auf $C = 1000$ bei $t = 60$ min bezogen und aus den 4 Parallelansätzen Mittelwert und Standardabweichung errechnet. Daraus wurde die Kurve Konzentrationsverlauf der TMS-Ester/Zeit nach der Formel

$$C = 1 - e^{-kt} \tag{1}$$

errechnet:

Substanz	*k*
Glykolat	0.321
Oxalat	0.180
Malonat	0.327
Orthophosphat	0.252
Malat	0.238
Citrat	0.158
I.S. (Butylmalonat)	0.238

Die untersuchten Substanzen sind in der Geschwindigkeit ihrer Trimethylsilylierung in homogener Lösung sehr unterschiedlich. Eine äusserst schnelle Reaktion, wie sie Donike[14] für Ephedrinchlorid angibt, konnte hier nicht festgestellt werden. So reagieren die Komponenten Oxalat und Citrat sehr langsam, was die niederen *k*-Werte zeigen. Wesentlich schneller verläuft die Reaktion bei Glykolat und Malonat, während die übrigen Komponenten und der I.S. zwischen diesen beiden Extremen liegen. Trotzdem ist eine Analyse von ausgezeichneter Genauigkeit schon knapp nach Bereiten des Reaktionsansatzes möglich, da der I.S. durch seine mittlere Reaktionsgeschwindigkeit ausgleichend wirkt.

Auch durch den Einsatz anderer, für die Analyse organischer Säuren und ähnlicher Stoffklassen vorgeschlagener TMS-Donorsubstanzen ist keine wesentliche Beschleunigung der Silylierung zu erreichen. Ausserdem erfordert die Anwendung halogenhaltiger Silylierungsmittel (BSTFA, TMCS) spezielle Vorsichtsmassnahmen zur Vermeidung von Korrosion und Gesundheitsschäden beim Bedienungspersonal.

Stabilität der Derivate über längere Zeiträume. Hiefür wurde der Bereich zwischen 60 und 600 min nach Bereiten der Ansätze betrachtet. In dieser Zeit können bei einer Zyklusdauer von 30 min 19 Proben analysiert werden (das entspricht etwa der Kapazität des verwendeten Probengebers).

Wie für den Abschnitt *Die Aufbauphase der TMS-Ester* wurden 4 identische Ansätze (Lösungsmittel Aceton) bereitet und nach einer Wartezeit von 60 min in 30-min Intervallen analysiert. Konzentrationen, Derivatisierungsprozedur und GC-Konditionen sind im Abschnitt Experimentelles erläutert. Die Werte für die Peakflächen wurden auf $C = 1000$ beit $t = 60$ min bezogen. Dieser Zeitpunkt wurde gewählt, da die leichtest zersetzlichen Derivate (TMS-Oxalat und -Malonat) hier den Kulminationspunkt ihrer Konzentration erreichen.

Zur Auswertung wurde die Regressionsgerade berechnet:

Substanz	n^*	*Gleichung der Regressionsgeraden*
Glykolat	76	$C = 0.9847 + 1.825 \cdot 10^{-5} t$
Oxalat	76	$C = 0.9801 - 3.234 \cdot 10^{-5} t$
Malonat	76	$C = 0.9886 - 5.972 \cdot 10^{-5} t$
Orthophosphat	76	$C = 0.9821 + 3.462 \cdot 10^{-5} t$
Malat	76	$C = 0.9815 + 6.645 \cdot 10^{-5} t$
Citrat	76	$C = 0.9817 + 3.827 \cdot 10^{-5} t$
I.S. (Butylmalonat)	76	$C = 0.9831 + 2.807 \cdot 10^{-5} t$

* n = Zahl der Bestimmungen.

Dabei lassen sich die untersuchten Substanzen in 3 Gruppen einteilen:

(a) Diese Gruppe bildet sehr rasch zersetzliche Derivate (Oxalat, Malonat).

(b) Hier ist die maximale Ausbeute noch nicht erreicht, die Konzentration steigt stetig an (Malat).

(c) Die Komponenten weisen nur noch einen leichten Konzentrationsanstieg auf (Phosphat, Glykolat, Citrat, I.S.).

Die Wahl des Standards wurde auch danach vorgenommen, die unterschiedliche Stabilität der Derivate auszugleichen. Butylmalonat gehört der Gruppe (c) an und erfüllt daher diese Anforderung.

Weitere Tests ergaben, dass die Stabilität der Derivate ausreicht, um auch mit über 50 h alten Reaktionsansätzen noch hinreichend genaue Analysenresultate zu erzielen.

Die Reproduzierbarkeit der Analyse mit internem Standard

Hiefür wurden die Ergebnisse der Kurzzeitversuche 10–60 min und der Langzeittestreihen 60–600 min (jeweils in Aceton als Lösungsmittel) als Quotienten $Q = \text{area}_x/\text{area}_{\text{I.S.}}$ dargestellt und auf den Wert $Q = 1000$ bei $t = 60$ min bezogen. Jede Testreihe wurde 4× wiederholt.

Mittelwerte, Standardabweichung und die Regressionsgeraden wurden für jede Komponente ermittelt und in Tabelle III zusammengestellt. Die graphische Darstellung der Regressionsgeraden zeigt Fig. 2.

TABELLE III

REPRODUZIERBARKEIT DER QUANTITATIVEN BESTIMMUNGEN BEI ANWENDUNG VON BUTYLMALONAT ALS I.S. (LÖSUNGSMITTEL ACETON)

n = Zahl der Bestimmungen; $\bar{Q}$ = Mittelwert der Quotienten $\text{area}_x/\text{area}_{\text{I.S.}}$; s_Q = Standardabweichung; $s_Q(\%)$ = Standardabweichung in % des Mittelwertes.

Substanz	n	$\bar{Q}$	s_Q	$s_Q\%$	*Gleichung der Regressionsgeraden*
Zeitbereicht t = 10–60 min					
Glykolat	16	1.0304	0.0424	4.11	$C = 1.0419 - 44.00 \cdot 10^{-5} t$
Oxalat	16	0.9714	0.0251	2.58	$C = 0.9597 + 44.53 \cdot 10^{-5} t$
Malonat	16	1.0135	0.0115	1.13	$C = 1.0258 - 46.92 \cdot 10^{-5} t$
Orthophosphat	16	1.0041	0.0096	0.96	$C = 1.0129 - 33.22 \cdot 10^{-5} t$
Malat	16	0.9968	0.0041	0.41	$C = 0.9982 - 5.65 \cdot 10^{-5} t$
Citrat	16	0.9619	0.0186	1.86	$C = 0.9545 + 28.08 \cdot 10^{-5} t$
Zeitbereich t 60–600 min					
Glykolat	76	0.9946	0.0183	1.84	$C = 0.9946 + 0.434 \cdot 10^{-5} t$
Oxalat	76	1.0045	0.0478	4.75	$C = 1.0198 - 4.631 \cdot 10^{-5} t$
Malonat	76	0.9709	0.0226	2.32	$C = 0.9984 - 8.336 \cdot 10^{-5} t$
Orthophosphat	76	1.0027	0.0145	1.44	$C = 1.0010 + 0.485 \cdot 10^{-5} t$
Malat	76	1.0103	0.0133	1.32	$C = 1.0006 + 2.951 \cdot 10^{-5} t$
Citrat	76	1.0323	0.0392	3.80	$C = 1.0280 + 1.207 \cdot 10^{-5} t$

Dabei ist zu ersehen, dass bei Berücksichtigung des Mittelwertes eine durchschnittliche Analysengenauigkeit von $\pm$ 2.21% im Zeitbereich von 10 bis 600 min nach Bereiten des Reaktionsansatzes erzielt werden kann (wobei die am stärksten schwankende Komponente, Oxalat, über eine Abweichung von $\pm$ 4.75% nicht

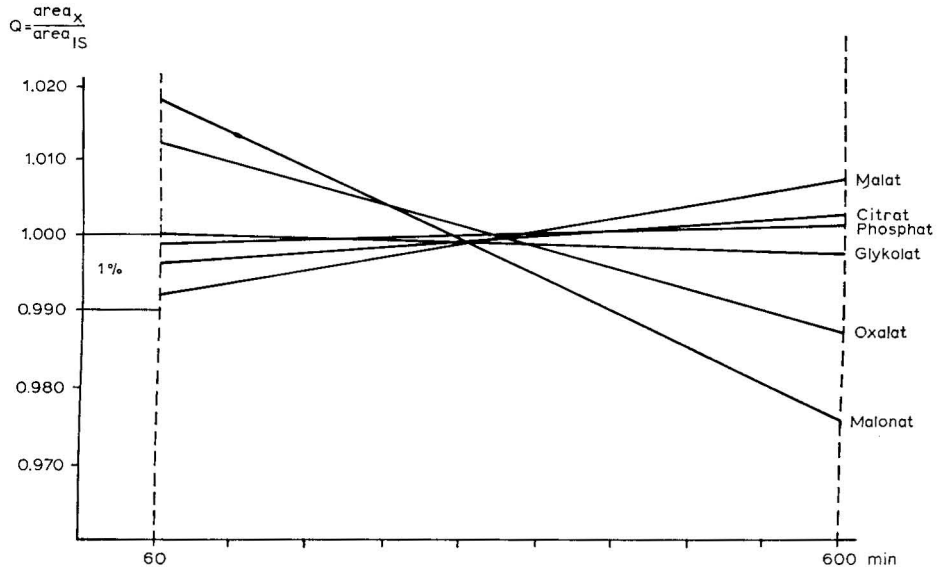

Fig. 2. Regressionsgerade der Quotienten Q = area$_x$/area$_{I.S.}$ bezogen auf die Mittelwerte$\bar{Q}$ der untersuchten Substanzen (Gleichung der Regressionsgeraden in Tabelle III).

hinausgeht). Dieser Wert kann durch Anwendung der Formel für die Regressionsgerade noch weiter verbessert werden.

DISKUSSION

Für Silylierungsreaktionen werden üblicherweise relativ hoch siedende Lösungsmittel, zumeist Pyridin[7,11] und wasserfreies Arbeiten vorgeschlagen. Auf der Suche nach einem niedriger siedenden Lösungsmittel wurde unter anderem Aceton erprobt, wobei entscheidend bessere Löslichkeit der untersuchten organischen Säuren beobachtet werden konnte. Der geringe Wassergehalt des benützten Präparates (max. 0.3%) stört die Silylierungsreaktion in keinem Fall, da der molare Überschuss an Silylierungsmittel ausreichend gross ist. Für einen Ansatz wie im Abschnitt Experimentelles beschrieben bedeutet dies: BSA (Molekulargewicht 203.46, Dichte 0.830 g/ml); Testsubstanzen (11.2 mg/ml Gesamtkonzentration, Molekulargewicht durchschnittlich 125, durchschnittlich 2.6 silylierbare Gruppen pro Mol Säure); Aceton (max. Wassergehalt 0.3% = 3 mg Wasser/ml Aceton); Gesamte Silylierungskapazität: 0.300 ml BSA = 2.46 mval TMS-Gruppen; Verbrauch durch die Testsubstanzen: 0.500 ml Lösung = 0.12 mval Säure $\triangleq$ 4.9% der Silylierungskapaiztät; Verbrauch durch den Wassergehalt: 0.500 ml Aceton $\triangleq$ 0.083 mmol Wasser $\triangleq$ 3.4% der Silylierungskapazität.

Bei den hier eingesetzten Mengenverhältnissen werden also durch den Wassergehalt des Lösungsmittels 3.4% der Silylierungskapazität aufgebraucht, eine Menge, die angesichts des 12 fachen Überschusses an TMS-Donor (in jedem Ansatz werden insgesamt 8.3% der Silylierungskapazität verbraucht) die mühevolle Prozedur der Herstellung wasserfreien Acetons nicht rechtfertigt. Ausserdem verbessert der Wassergehalt die Löslichkeit der hydrophilen Säuren.

Die Resultate der Untersuchungen zeigen, dass die Schwankungen in der Ausbeute der Derivate bei Anwendung von Aceton als Lösungsmittel geringer und damit die Analysenergebnisse weit besser sind als im Fall von Pyridin.

Weitere Vorteile gegenüber herkömmlichen Verfahren bietet die Anwendung einer silylierbaren Standardsubstanz. Sie ermöglicht eine exakte Analyse im untersuchten Zeitbereich von 10–600 min. Das Einhalten einer bestimmten Reaktionszeit (wie bei der Anwendung nicht silylierbarer Standardsubstanzen) wird dadurch vermieden.

DANK

Die vorliegende Arbeit wurde durch den Fonds zur Förderung der wissenschaftlichen Forschung im Rahmen von Projekt Nr. 3042 unterstützt. Herrn Dr. Georg A. Janauer sei für seine Anregungen und die Unterstützung beim Aufarbeiten der Daten sowie für die kritische Korrektur des Manuskriptes an dieser Stelle bestens gedankt.

ZUSAMMENFASSUNG

Die zur GC-Trennung von Phosphat und pflanzlichen organischen Säuren als TMS-Derivate bisher vorgeschlagenen Methoden arbeiten mit relativ hoch siedenden Lösungsmitteln (Pyridin, Dimethylformamid) und nicht derivatisierbaren internen Standards.

Es konnte nunmehr gezeigt werden, dass Aceton gegenüber den herkömmlichen Lösungsmitteln entscheidende Vorteile hat (niedrigerer Siedepunkt, besseres Lösungsvermögen für die untersuchten Substanzen, ohne Entfernen von Wasserspuren verwendbar) und ausserdem verglichen mit Pyridin die Reproduzierbarkeit der Analysenresultate wesentlich besser ist.

Ein silylierbarer interner Standard gleicht nicht nur unterschiedliche Reaktionsbedingungen aus, sondern ermöglicht auch die unterschiedlich lange Aufbewahrung der Reaktionsansätze vom Zeitpunkt der Bereitung bis zur Analyse, da sein TMS-Derivat ebenso wie die der untersuchten Substanzen einem, wenn auch langsamen Zerfall unterliegt. Butylmalonsäure entsprach diesen Anforderungen.

Die Kinetik der Silylierungsreaktion und des Abbaues der Derivate wurde untersucht und das unterschiedliche Verhalten biologisch interessanter pflanzlicher Säuren dokumentiert.

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PRACTICAL ASPECTS OF THE PREPARATION AND CHROMATOGRAPHY OF THE TRIMETHYLSILYL ETHERS OF ECDYSTEROIDS

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SUMMARY

Some of the difficulties encountered in the silylation of ecdysteroids are described, together with methods for avoiding them. Standard procedures are given for the preparation of trimethylsilyl ethers of ecdysteroids in biological samples and their analysis by gas chromatography with electron capture detection. This is considered to be the most efficient method for ecdysteroid determination in most arthropod tissues.

INTRODUCTION

The analysis of the steroidal insect and crustacean moulting hormones, or ecdysteroids, is currently of great interest to invertebrate physiologists and embryologists. We have reviewed the methods available and made some comparison of their advantages and disadvantages[1]. Excluding the non-specific, though sensitive, radioimmunoassay method, the two techniques of most promise are high-performance liquid chromatography (HPLC) and gas chromatography (GC). Advances in HPLC have improved that method through increased resolution but sensitivity is still limited by the detector (*cf.*, ref. 2). In our hands, the determination by GC using electron capture detection (GC–ECD) of the trimethylsilyl ethers of the ecdysteroids is preferred[3–6]. Although the method requires derivative formation, it has the advantages of great sensitivity, of selectivity, requires the least lengthy preliminary clean-up, and is least subject to losses during handling.

However, the preparation and handling of silyl ethers and the use of the electron capture detector present problems for those not experienced in their use, therefore a description of some of the problems encountered and methods for avoiding or overcoming them are important for those wishing to determine ecdysteroids in this way. Methods for ecdysteroid determination, found necessary and satisfactory in the hands of several workers, are described in this paper.

EXPERIMENTAL

Purification of solvents

Toluene for GC–ECD was purified by shaking twice with small portions of

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conc. sulphuric acid, then washing with distilled water and 5% aqueous sodium hydrogen carbonate to remove all traces of acid. The toluene was dried over anhydrous magnesium sulphate, distilled from phosphorus pentoxide and stored over molecular sieves 4A. Its purity was checked periodically by evaporating a 10-ml portion to 200 μl with a stream of nitrogen and injecting 2 μl onto the gas chromatograph fitted with a ^{63}Ni ECD. The solvent peak should be no more than 1 min in breadth.

Pyridine for silylation reactions was distilled from calcium hydride and stored over molecular sieves. Methanol and diethyl ether were dried with magnesium and sodium respectively in the conventional way.

Cleaning glassware

Glassware was cleaned by soaking in a bath of alkaline detergent overnight and then rinsing thoroughly with water and finally acetone. Reacti-vials used for the silylation reaction were cleaned by soaking in chromic acid overnight, washing with aqueous sodium bicarbonate solution and rinsing repeatedly with distilled water. They were finally rinsed with acetone and baked dry at 140 °C for at least 30 min. Material which was not removed by chromic acid was removed with a commercial powdered pumice abrasive ("Briz") and the Reacti-vials were then washed with water and soaked in chromic acid as above.

Cleaning the electron capture detector

Frequent use of the ECD eventually leads to contamination and build-up of a deposit. Cleaning must depend upon the design and makers instructions. With the Pye 104 detector, the central collecting electrode was best cleaned with metal polish and the barrel was cleaned in an ultrasonic bath for 1–2 h in hexane or toluene. The bath was monitored for radioactivity after the washing but none was ever detected.

Silica for thin-layer chromatography

Commercial silica for thin-layer chromatography (TLC) was purified to remove electron-capturing impurities. Silica gel P F_{254} (Merck, Darmstadt, G.F.R.; 1 kg) was suspended in methanol (2 l) by stirring mechanically for 2–3 h, filtered with vacuum and washed with methanol (1 l) and diethyl ether (0.5 l). The resulting cake was broken up and dried at room temperature.

The glass plates were washed with detergent, rinsed with distilled water, dilute acid and again with water. The purified silica was slurried in distilled water and plates of 0.6 mm thickness prepared in the usual way and dried and activated by heating at 100 °C for 1 h and then stored over saturated sodium chloride solution to produce uniform deactivation.

Preparation of N-trimethylsilylimidazole

All stages of reaction were carried out with as careful an exclusion of moisture as was practical. Imidazole (27.2 g, 0.4 mole) was heated under reflux for 2 h with hexamethyldisilazane (48.4 g, 0.3 mole) and conc. sulphuric acid (two drops). The product was distilled fractionally under reduced pressure to give N-trimethylsilylimidazole (TMSI, 46.8 g, 84% based on imidazole) as a colourless mobile oil, b.p. 91 °C at 12 mmHg. The product was stored by transferring to 1-ml ampoules under

nitrogen and sealing with a flame. Its activity was checked by the rate of silylation of a pure ecdysteroid (see below).

Preparation of trimethylsilyl ethers of pure ecdysteroids

Both ecdysone and 20-hydroxyecdysone (Simes, Milan, Italy) were used. A sample of ecdysteroid (0.2–1.0 mg) was weighed on a microbalance and dissolved in acetone to give (typically) 250 μg ml^{-1}. Several 40-μl aliquots of this were evaporated to dryness in Reacti-vials (Pierce and Warriner, Chester, Great Britain) with a stream of warm nitrogen. Purified pyridine (65 μl) and TMSI (35 μl) were added to each, the vials sealed with screw caps and heated at 120 °C for various periods, from 30 min to 6 h. Each tube in turn was cooled and 10 μl of solution withdrawn and diluted with ECD toluene to give 1–2 ng μl^{-1} and 1 μl of this solution was injected onto the GC. The course of reaction was monitored to find the time required for complete conversion to a single derivative. The derivative, once formed, was stable for several weeks in excess TMSI if the Reacti-vial was kept closed in a refrigerator.

Preparation of biological sample

The biological material to be examined (10–300 g as necessary) was ground in methanol (5 ml g^{-1}) with a high shear stainless-steel grinder (Unishear Mixers, Audnam, Stourbridge, Great Britain) and filtered through sintered glass. The residue was blended twice more with smaller volumes of methanol and filtered. The insoluble residue was discarded.

For smaller samples, such as insect eggs, the sample (0.5–2.0 g) was ground in a glazed mortar with methanol-washed sand and methanol (200 ml). The slurry was filtered as above and the residue extracted twice more with methanol and filtered.

The methanol extracts were reduced to dryness on a rotary evaporator with vacuum at 50 °C. The resulting residue was partitioned between light petroleum (b.p. 40–60 °C) and aqueous methanol (1:4). The light petroleum was extracted twice more with aqueous methanol before being discarded.

The aqueous methanol extracts were reduced to dryness at 50 °C in the same way, the residue partitioned between butanol and water, and the butanol phase was washed twice with water. The combined aqueous phases were washed twice with butanol. The aqueous portion contained any polar conjugates of ecdysteroids, and was evaporated to dryness at 50 °C if these were to be hydrolysed and the ecdysteroids examined, otherwise it was discarded.

The material obtained after evaporation of the butanol was submitted to a third partition system which depended upon the sample material, either equal volumes of ethyl acetate and water, discarding the ethyl acetate, or hexane–2-propanol–water (5:15:36) discarding the hexane. In each case the less polar phase was washed twice with the aqueous phase before being discarded.

The aqueous portion was evaporated to dryness under vacuum at 50 °C. Evaporation can be hastened by addition of 1-butanol and removing a butanol–water azeotrope. The residue was transferred to a centrifuge tube with redistilled methanol (15 ml) and the volume reduced to 2 ml with a stream of warm nitrogen. This volume was transferred in 250-μl portions to a 1-ml Reacti-vial, and evaporated to dryness with warm nitrogen between additions. The residue was dried *in vacuo* at 57 °C for 1 h. The residue, which should amount to 100 mg or less and preferably spread as a

thin film on the walls of the tube, so that drying was efficient, was silylated as described earlier in pyridine (200 μl) and TMSI (100 μl). The time of heating required was about 80% of that required for the pure ecdysteroid and was found by trial and error using a biological sample to which pure ecdysteroid had been added.

Thin-layer chromatography of silyl ethers

The pyridine solutions after silylation of ecdysteroids was reduced in volume while still warm by blowing a jet of nitrogen onto the surface, and the remainder applied as a band to the origin of a TLC plate (20 × 20 cm) prepared as described above, the Reacti-vial was rinsed with toluene, which was also applied to the origin. The plate was immediately developed in ethyl acetate–toluene (3:7) until the solvent front had travelled 15 cm. The plate was removed, dried quickly with a hair dryer and the silica from R_F 0.5 to 0.9 removed and packed into a glass column (15 × 1.0 cm), the lower end of which held a glass wool plug. The silica was eluted with diethyl ether (15 ml). The ether was evaporated, taking care that water did not condense inside the tube. The residue was taken up in a known volume of purified toluene and diluted suitably for GC–ECD.

Gas chromatography columns

Columns used were 1.5 m × 4 mm coiled glass columns packed with 1.5% (w/w) OV-101 silicone phase on Chromosorb Q (100–120 mesh). The material was handled very carefully during coating and packing the column to prevent breaking of the particles and exposure of uncoated surfaces. The column was conditioned at 340 °C for 24 h before use. In repeated use, retention times slowly decreased, and resolutions deteriorated. Column life could be prolonged by replacing the first few centimetres of packing from time to time (approximately every 300 injections) and injecting 10-μl samples of "Silyl-8" (Pierce and Warrener) onto the column at 250 °C ensuring that the detector was disconnected.

Injections were made directly "on column" with an 11-cm needle which reached into the top of the column packing. Injection into a heated injector block with a shorter needle is *not* recommended.

A Pye Series 104 gas chromatograph fitted with flame ionization (FID) and ^{63}Ni ECD detectors was used. Nitrogen, freed of traces of oxygen with an "Oxy-trap" and dried by passing over molecular sieves, was used as carrier gas, flow-rate 50–60 ml min^{-1}, oven temperature 270–280 °C, detector temperature 300 °C. When the carrier gas was switched off, a purge of 15 ml min^{-1} of nitrogen was maintained through the detector.

DISCUSSION

Ecdysteroids have the disadvantages of sensitivity to acid[7] (dehydration at the 14α-OH group), to alkali[7] (the unsaturated ketone) and to heat (non-specific dehydration) and their high polarity conferred by several hydroxyl groups makes them susceptible to irreversible adsorption on activated surfaces such as alumina[8] and silica[9]. They have the advantage of possessing a strongly absorbing ultraviolet chromophore in the unsaturated ketone ($\lambda_{max.}$ 240 nm, $\varepsilon \approx 12{,}000\ l\ mol^{-1}\ cm^{-1}$). This absorption is

useful for UV detection after HPLC, but a very large number of compounds absorb in the same region.

Where a relatively "clean" material is to be examined, *e.g.*, phytoecdysteroids[10], HPLC is sufficient, but many arthropod tissues require extended "clean-up" before ecdysteroids are freed of co-eluting substances.

Ecdysteroids also possess the advantage of a strongly electron-capturing electrophore[11], which is possessed by a relatively small number of compounds. The method for determining ecdysteroids described here takes advantage of the selective electrophore and attempts to avoid exposure to acids, alkalis, heat and active adsorbents used in chromatography.

The basis of the method is to use several solvent partitions and to resort to chromatography only after conversion to a non-polar derivative. In this way a partially purified concentrate of derivatives of ecdysteroids is prepared and this is clean enough for their selective detection by an electron capture detector.

Determination of any substances in the nanogram range requires care in avoiding contamination and ensuring reproducibility. For the present work it is important to avoid contact with chlorinated solvents since organohalogen compounds are strongly electron capturing. The toluene used for GC and the silica gel for TLC need particular care and must be specially purified. Apparatus must be clean, and traces of acid introduced from chromic acid cleaning must be rigorously removed. In particular, the Reacti-vials used for the silylation reaction must be very clean and free of acid or alkali. The build-up of a silicious deposit on glassware from use of silylating reagents has, in some unknown way, a deleterious effect upon the compounds. Traces of sodium acetate have a catalytic effect upon the rate of the silylation reaction, as do imidazole and other unidentified substances in the biological sample. Since sodium acetate–acetic acid is a common buffer system, acetate can be introduced accidently into ecdysteroid samples where enzymic hydrolysis has been used. This was discovered in hydrolysing ecdysteroid conjugates with the mixture of enzymes from the snail *Helix pomatia*. When an acetate buffer was used for the enzymic step, abnormal results were found in the GC step, and the effect could be reproduced by adding sodium acetate to the silylation mixture. When phosphate buffer was used, the silylated ecdysteroids behaved normally.

The ECD is not as simple to operate as the FID. The price of its greater sensitivity is that it is more easily contaminated. Some instructions in the handling of the detector are therefore included. Detector design differs with the manufacturer and some experimentation must be made using pure ecdysteroids to find the optimum operating conditions, of current, pulse spacing, sensitivity and so on. Because of the nature of the electron-capturing process, the highest practical operating temperature of the detector gives greatest sensitivity[11].

The reactivity of TMSI has been found to vary widely and in an unpredictable way. Some material purchased by us was quite unusable because even prolonged heating in pyridine did not produce satisfactory silylation of ecdysteroids and reactivity seemed to vary widely from batch to batch. We have therefore prepared our own TMSI as described. This material is consistent in its reactivity and separate 1-ml vials sealed for a long time, can be opened and transferred to a Reacti-vial sealed with a rubber serum cap and portions drawn from it to give reproducible reaction times. If the reaction is carried out for 10 min at room temperature, ecdysone tetrakis trimeth-

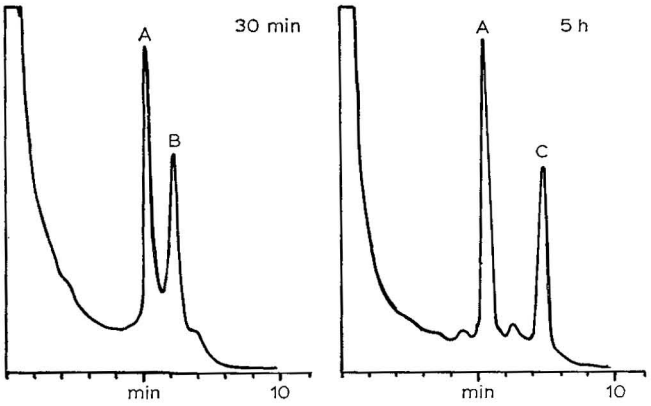

Fig. 1. Examples of gas chromatography traces of the derivatives of ecdysteroids after silylation for 30 min and 5 h. Peaks: A = tetrakis TMS ether of ecdysone; B = tetrakis TMS ether of 20-hydroxyecdysone; C = pentakis TMS ether of 20-hydroxyecdysone. Chromatographic conditions as in text.

ylsilyl ether and 20-hydroxyecdysone tetrakis trimethylsilyl ether are formed. Even when left overnight at room temperature, no further silylation occurs. If the mixture is heated to 110 °C for 5 h, then ecdysone still remains as the tetrakis ether (longer reaction begins to produce the pentakis ether) and 20-hydroxyecdysone is converted to the pentakistrimethylsilyl ether (Fig. 1). The very short reaction time has the advantage of speed of analysis. The longer reaction time with heating, has the advantage that the derivatives formed from ecdysone and 20-hydroxyecdysone are better resolved in GC (Table I).

TABLE I

GAS CHROMATOGRAPHIC RETENTION TIMES OF TMS ETHERS OF ECDYSTEROIDS ON A 1.5% OV-101 COLUMN OF 1.5 m LENGTH WITH NITROGEN CARRIER GAS AT 60 ml min^{-1}

Parent ecdysteroid	*No. of TMS groups*	*Retention time (min)* **	*Column temperature (°C)*
Ecdysone	4	1.85	280
	5	1.65	280
20-Hydroxyecdysone	4	2.25	280
	5	2.45	280
	6	1.90	280
Inokosterone*	4	2.55	280
	5	2.85	280
	6	2.20	280
2-Deoxy-20-hydroxyecdysone	4	3.9	280
	5	2.4	280
Poststerone	2	0.6 (1.3)	280 (260)
	3	0.9	260
Cyasterone		8.0	280
3-Dehydroecdysone	3	2.15	280
3-Dehydro-20-hydroxyecdysone	4	2.70	280

* Commercial inokosterone apparently consists of two C-25 epimers[13] which are not resolved under these conditions.

** Not the same column as used for the figures.

The user must decide which method, cold reaction or with heating, is preferable. If the reaction is heated, to produce the penta-ether of 20-hydroxyecdysone, then because of the variability of the TMSI reagent, the time necessary for reaction of either ecdysone or 20-hydroxyedysone is first found, using pure materials. Several 10-μg samples of compound are silylated for varying periods at any convenient temperature within the range 100–140 °C and the extent of reaction monitored by GC (Fig. 2). Using one sample and withdrawing aliquots at time intervals is not satisfactory because of the unavoidable exposure to moisture at each opening of the Reacti-vial. Once the reaction time has been decided, practice shows that a slightly shorter time is required for ecdysteroids in a biological sample.

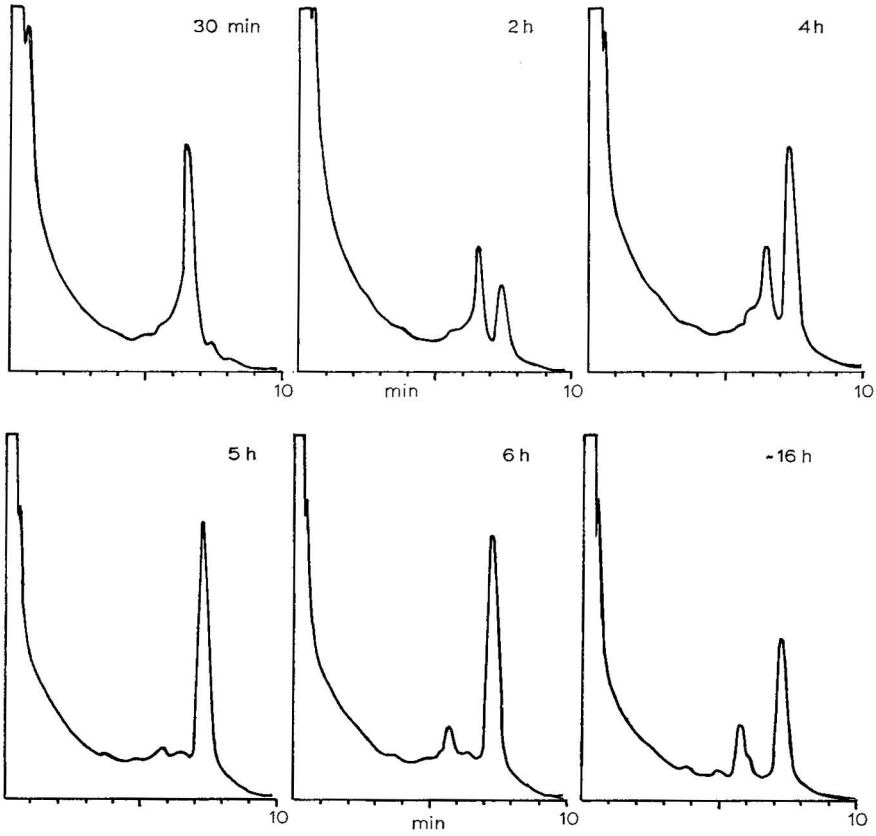

Fig. 2. Gas chromatographic trace of silylation products of 20-hydroxyecdysone after varying periods of time showing the formation of a single derivative (the tetrakis ether) after 30 min and again at 5 h (the pentakis ether). The chromatographic and silylation conditions were as described in the text.

Tailing and non-Gaussian peak shape in GC can be attributed to adsorption on the column walls or uncoated support. It can be corrected by treatment with "Silyl-8" (Fig. 3).

It is advisable to add a known quantity of pure ecdysteroid to a biological sample known to contain little or no ecdysteroid, to give a concentration within the

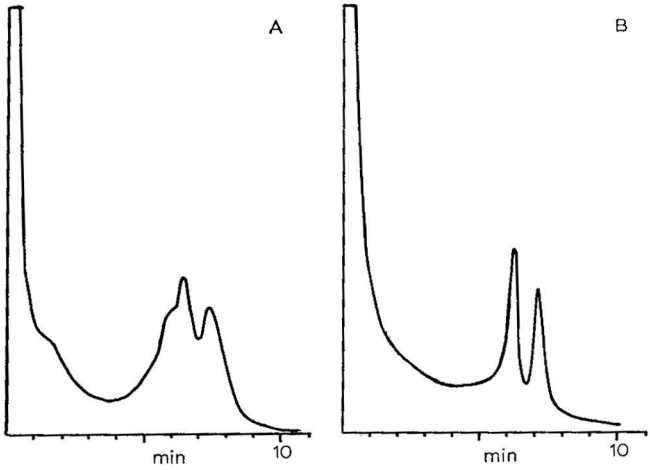

Fig. 3. Effect of condition of the column on peak shape. A sample of 20-hydroxyecdysone was silylated for 2 h to give a mixture of two silyl ethers. This mixture was injected onto the chromatography column at 285 °C, with the carrier gas flowing at 60 m min^{-1}. On a column which has been in use for some time, the result was as in A. The column was then treated with 10 μl of "Silyl-8" (with the detector disconnected) and the sample reinjected, after conditions had returned to equilibrium, to give the result in B.

range of experimental values and to carry out the extraction and derivatization procedures to test the efficiency of recovery. As the limit of detection is approached, manipulation losses increase and a correction factor may have to be found from these results and applied to experimental data. An internal standard provides a useful check on recovery and reproducibility. A substance such as cyasterone or makisterone A can be used. Cyasterone has the disadvantage of having an inconveniently long retention time and consequently a broad peak shape. Makisterone A, which is commercially available has the advantage of retention time closer to ecdysone and 20-hydroxyedcysone, but could conceivably co-elute with a natural ecdysteroid and obscure it.

Using whole adult male *Schistocerca gregaria* as ecdysteroid-free samples, we obtained recoveries for the complete isolation procedure of 95% for 10^{-4} g of added hormone, falling to 85% recovery for 10^{-8} g added hormone.

For the TLC step alone, recoveries were quantitative within the range of 10^{-4} to 10^{-8} g, but recovery fell to $\approx$ 60% when smaller quantities of silylated ecdysteroid were spread on the plate.

Removal of water and methanol from the biological sample in the Reacti-vial before silylation is also important. For large samples ($\approx$ 300 mg) a thick gum may form at the bottom of the tube. This may dry on the surface but retain solvent underneath. A smaller sample should be used if possible or else the material should be spread as a thin film on the walls of the tube before the solvent evaporates. The sample should redissolve completely in the pyridine, if necessary by warming and shaking before silylation.

If a greater proportion of TMSI is used for silylation, to overcome losses of reagent from moisture, then it is important that imidazole should not crystallize from the reaction on cooling. The crystalline imidazole can occlude the silylated ecdysteroids

and lead to completely negative results. If 100 ng of silylated ecdysteroid is co-precipitated with 1 mg of crystalline imidazole, this represents only a 0.01 % contamination of the imidazole.

Once prepared, the trimethylsilyl ethers of ecdysteroids are relatively stable, in the pyridine–TMSI mixture, for several months, and can be heated to 100 °C for several days without decomposition. Trimethylsilyl ethers are subject to hydrolysis, particularly when catalysed by acids. They are therefore of limited stability on TLC plates, and it is advisable to carry out TLC operations as quickly as possible and elute from the silica immediately after chromatography. In a few cases we have found silica gel for TLC caused hydrolysis of the ethers (Fig. 4) but the problem was solved as soon as a new batch of silica was purchased. The R_F values of some ecdysteroid silyl ethers are given in Table II.

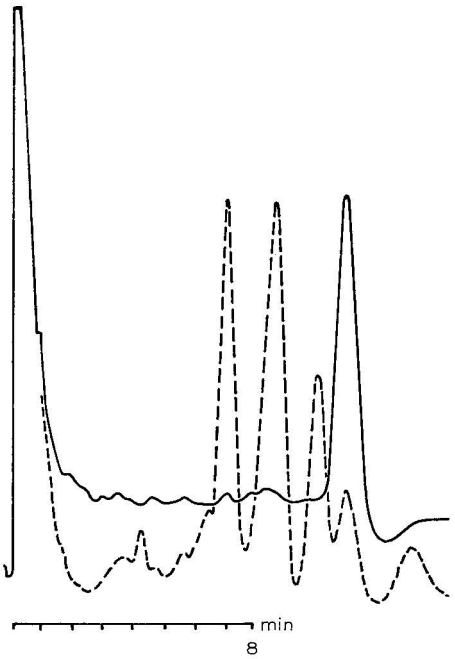

Fig. 4. Effect of thin-layer chromatography on unsatisfactory silica gel. Full line, 20-hydroxyecdysone pentakis silyl ether before thin-layer chromatography. Broken line, same product after TLC. This sample was chromatographed on a different column (longer retention times) than that used for the other figures.

Preparation of biological material

The extent of the preliminary clean-up of a biological sample before formation of the trimethylsilyl ethers and GC must be found by trial and error. In our experience, three solvent partitions is quite sufficient for samples of whole locust bodies, locust haemolymph could be determined after only two solvent partitions, but locust faeces contained much more interfering substances and samples of barnacles (*Balanus balanoides*) are still too impure after three solvent partitions for satisfactory GC. The criterion of purification is whether the large solvent peak elutes before the ecdysteroids,

TABLE II

R_F VALUES OF SOME ECDYSTEROID TMS ETHERS ON SILICA GEL (FROM REF. 1)

Parent compound	*Hydroxyl groups silylated*	*Solvent system*	
		Toluene–ethyl acetate (9:1)	*Toluene–ethyl acetate (7:3)*
2β,3β,14α-Trihydroxy-	2β,3β	0.39	—
-5β-cholest-7-en-6-one	2β,3β,14α	0.51	—
Ecdysone	2β,3β,22,25	—	0.69
	2β,3β,14α,22,25	0.58	—
20-Hydroxyecdysone	2β,3β,22,25	—	0.54
	2β,3β,20,22,25	0.22	0.67
	2β,3β,14α,20,22,25	0.69	0.75

so that maximum detector sensitivity is available or whether the ecdysteroid peaks are superimposed on a falling baseline (Fig. 5).

Partition between hexane or light petroleum (b.p. 40–60 °C) and methanol–water (4:1) is best carried out first. This removes a large quantity of non-polar lipids and avoids emulsion formation as far as is possible. Systems such as hexane–water cause formation of very stable emulsions.

The second solvent system is butanol–water, which removes unwanted polar compounds in the aqueous phase, which also contains many polar ecdysteroid conjugates[5,12].

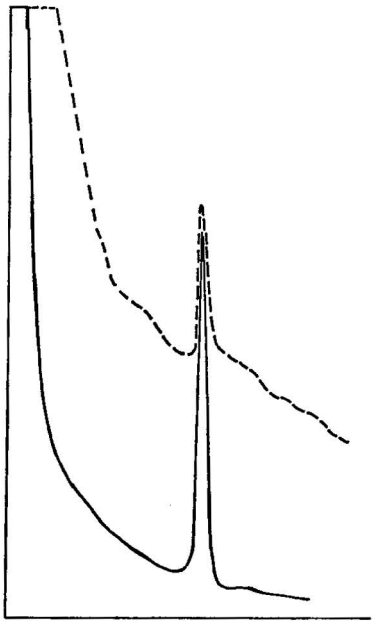

Fig. 5. Comparison of satisfactory purification (full line) and insufficient purification (broken line) for satisfactory quantification. The material was a sample of extract of the barnacle *Balanus balanoides* to which pure ecdysone had been added.

The third solvent partition, if necessary to reduce the volume of the sample or to further purify it, is found by trial and error. Ethyl acetate–water (1:1) or hexane–isopropanol–water (5:15:36) are two useful alternatives. The common ecdysteroids partition into the aqueous phase of both systems.

If further purification is necessary, chromatography on Sephadex, a non-polar reversd-phase material such as Bondapak C_{18} or a weak absorbent such as Floridin earth is helpful, but not alumina or silica.

The most frequently encountered ecdysteroids are easily resolved by the GC conditions. We have experienced no difficulty in distinguishing them by their retention times, some of which are listed in Table I. New ecdysteroids make rather greater demands on techniques and quantities of material for identification[13].

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THIN-LAYER CHROMATOGRAPHY OF CHLORINATED GUAIACOLS

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SUMMARY

The thin-layer chromatography of guaiacol and six chlorinated guaiacols has been studied on silica gel with 40 neutral and acidic solvent systems. Standard deviations and relative differences in the R_F values were used for selecting the most suitable solvents for particular separations. For group separation, dichloromethane–benzene–methanol (60:30:10) and acetone were suitable. Light petroleum (b.p. 40–60 °C)–ethyl acetate (70:30) and dichloromethane–chloroform (90:10) separated all components. Some other solvents are recommended for two-dimensional analyses.

INTRODUCTION

Thin-layer chromatography (TLC) is frequently applied for the separation of phenols. Leach and Thakore[1] used preparative TLC on silica gel for the isolation of 4,5,6-trichloroguaiacol and tetrachloroguaiacol from waste liquor from pulp bleaching. The eluents used were light petroleum (b.p. 30–60 °C)–benzene–methanol and benzene –methanol–acetic acid (50:8:4) for group separation and for the separation of the individual components, respectively. Thakore and Oehlschlager[2] separated 3,4,5-trichloroguaiacol, 4,5,6-trichloroguaiacol and tetrachloroguaiacol by TLC with chloroform–light petroleum (9:1). Chloroform[3] and different mixtures of chloroform and ethyl acetate[4] were used in separations of various chlorophenolic compounds. An advanced TLC system for the analysis of 126 different phenols has been presented[5]. In addition, alumina layers have also been applied in the TLC of a large number of *ortho*- and *para*-substituted derivatives of phenol.[6]

Chlorinated guaiacols are formed in pulp bleaching and thus occur as important environmental residues[7]. As they have been found to be extremely toxic to fish[1,8], accumulating[9] and being enriched in natural food chains[10], we have undertaken syntheses of model compounds, structural determinations and the development of analytical methods. Previous work on the TLC of chlorinated cresols[11] and catechols[12] provided a starting point for the present study.

EXPERIMENTAL

Apparatus

Pre-coated TLC plates with a silica gel G60 layer and a concentrating zone

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(10 × 20 cm, layer thickness 0.25 mm; Merck, Darmstadt, G.F.R.) were used. Each guaiacol, as a 0.5% (w/v) solution in diethyl ether, was spotted with a 10-μl Hamilton syringe, 2 μl to each spot, on a line 1.5 cm from the bottom of the plate to the concentrating zone with spot intervals of 1.2 cm. Ascending elution in a closed glass chamber (Desaga, Heidelberg, G.F.R.) was applied. Both a Desaga scale plate and a meter scale were used to measure the R_F values of the spots.

Samples

The compounds used (see Fig. 1) were guaiacol (I), 5-chloroguaiacol (II), 4,5-dichloroguaiacol (III), 4,6-dichloroguaiacol (IV), 3,5-dichloroguaiacol (V), 4,5,6-trichloroguaiacol (VI) and tetrachloroguaiacol (VII). Except for guaiacol, which was a commercial sample (Fluka, Buchs, Switzerland), the compounds were synthesized in our laboratory and their structures and purities were checked by infrared, mass, ^{1}H NMR and ^{13}C NMR spectroscopy and by glass capillary gas chromatography.

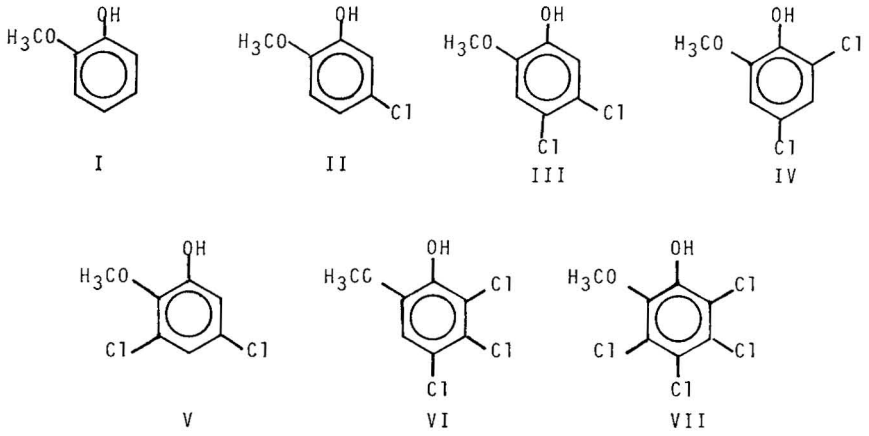

Fig. 1. Structures of guaiacol (I), 5-chloroguaiacol (II), 4,5-dichloroguaiacol (III), 4,6-dichloroguaiacol (IV), 3,5-dichloroguaiacol (V), 4,5,6-trichloroguaiacol (VI) and tetrachloroguaiacol (VII).

Solvent systems

Forty different solvent systems were examined in order to establish which gave the best spots and the most reasonable R_F values with all of the compounds studied. Owing to the use of a concentrating zone the spots were good (narrow) in all instances. The compositions (by volume) of the solvent systems were as follows:

(1) Light petroleum (b.p. 40–60 °C).
(2) Benzene.
(3) Dichloromethane.
(4) Chloroform.
(5) Diethyl ether.
(6) Ethyl acetate.
(7) Acetone
(8) *n*-Propanol.
(9) Light petroleum (b.p. 40–60 °C)–diethyl ether (70:30)
(10) Light petroleum (b.p. 40–60 °C)–ethyl acetate (70:30).
(11) Light petroleum (b.p. 40–60 °C)–acetone (80:20).
(12) Light petroleum (b.p. 40–60 °C)–*n*-propanol (90:10).

(13) Dichloromethane–chloroform (90:10).
(14) Dichloromethane–diethyl ether (95:5).
(15) Dichloromethane–ethyl acetate (95:5).
(16) Dichloromethane–acetone (95:5).
(17) Dichloromethane–*n*-propanol (95:5).
(18) Chloroform–dichloromethane (80:20).
(19) Chloroform–diethyl ether (90:10).
(20) Chloroform–ethyl acetate (95:5).
(21) Chloroform–acetone (95:5).
(22) Chloroform–*n*-propanol (95:5).
(23) Dichloromethane–chloroform–diethyl ether (85:10:5).
(24) Dichloromethane–benzene–methanol (60:30:10).
(25) Light petroleum (b.p. 40–60 °C)–dichloromethane–ethyl acetate (60:30:10).
(26) Light petroleum (b.p. 40–60 °C)–benzene–*n*-propanol (40:40:20).
(27) Benzene–acetic acid (85:15).
(28) Benzene–dichloromethane–acetic acid (60:30:10).
(29) Benzene–chloroform–acetic acid (50:40:10).
(30) Benzene–diethyl ether–acetic acid (60:40:10).
(31) Benzene–ethyl acetate–acetic acid (80:15:5).
(32) Benzene–acetone–acetic acid (80:15:5).
(33) Benzene–*n*-propanol–acetic acid (85:15:5).
(34) Light petroleum (b.p. 40–60 °C)–diethyl ether–acetic acid (80:15:5).
(35) Light petroleum (b.p. 40–40 °C)–ethyl acetate–acetic acid (80:15:5).
(36) Light petroleum (b.p. 40–60 °C)–acetone–acetic acid (80:15:5).
(37) Light petroleum (b.p. 40–60 °C)–*n*-propanol–acetic acid (80:15:5).
(38) Dichloromethane–ethyl acetate–acetic acid (80:15:5).
(39) Chloroform–ethyl acetate–acetic acid (80:15:5).
(40) Chloroform–acetone–acetic acid (80:15:5).

Chromogenic reagents

A 2% solution of 3,5-dichloro-*p*-benzoquinonechlorimine in toluene[13] and different concentrations (1–5%) of $FeCl_3 \cdot 6H_2O$ in water were tested for spot detection in order to obtain the most specific colour reaction for each compound studied.

Development of chromatograms

Development was continued until the solvent front had moved 13 cm from the boundary between the concentrating section and the silica gel section of the layer. After development the plates were dried in air at room temperature (24 ± 2 °C) for about 15 min and then sprayed with the chromogenic reagent.

RESULTS AND DISCUSSION

Colour reactions

$FeCl_3$ reagent gave light violet spots for compounds I–VII 1 h after spraying. After 2–3 days the spots changed colour to grey-green or grey-violet.

3,5-Dichloro-*p*-benzoquinonechlorimine reagent gave more specific colour reactions. The colours of the spots were compared 1 h, 24 h and 10 days after spraying.

The developing solvent influenced the colour reaction only on the basis of its acidity. On the other hand, the influence of time on the colours was substantial. The colour reactions are presented in Table I.

TABLE I

CHARACTERISTIC COLOUR REACTIONS OF GUAIACOL (I) AND CHLORINATED GUAIACOLS (II–VII) IN DIFFERENT TIMES AFTER SPRAYING TLC PLATES WITH A 2% SOLUTION OF 3,5-DICHLORO-*p*-BENZOQUINONECHLORIMINE IN TOLUENE
Amount of each compound applied: 10 μg.

Compound	*Neutral developing solvent*			*Acidic developing solvent*		
	1 h	*24 h*	*10 days*	*1 h*	*24 h*	*10 days*
I	Violet-brown	Red-brown	Brown	Orange-brown	Brown	Brown
II	Violet-blue	Red-brown	Brown	Red-orange	Brown	Brown
III	Orange-brown	Violet-blue	Red-brown	Orange-brown	Violet-brown	Violet-brown
IV	Grey-green	Red-brown	Brown	Orange-brown	Orange-brown	Orange-brown
V	Violet	Violet	Violet	Light yellow	Light violet	Violet
VI	Grey-green	Red-brown	Red-brown	Orange-brown	Violet-brown	Violet-brown
VII	Light orange	Grey-violet	Light violet	Light yellow	Light brown	Grey-violet

The colour reactions of the 3-chloro-substituted guaiacols were clearly different than those of the others. Firstly, V and VII gave much slower colour reactions and their final colours after 10 days were violet-based, whereas those of the other compounds were brownish.

R_F values

The R_F values of the spots were measured with an accuracy of better than 0.03. To achieve this, most runs had to be carried out three times and average values calculated. The results obtained with neutral (1–26) and acidic (27–40) solvent systems are given in Table II.

The standard deviations of the R_F values (s) of I–VII in each run were calculated for estimation of the separating power of each solvent system (see Table II). Large s values correspond to possible solvents for the analysis of individual components and small s values to solvents suitable for group separation.

Further evaluation of the separation in each experiment was effected by comparing the relative differences (x) of the R_F values as presented by Sattar and Paasivirta[14]:

$$x_{ij} = \frac{R_F(i) - R_F(j)}{R_F(i) + R_F(j)} \cdot 2 \qquad (1)$$

which is the same as the difference between two R_F values divided by their average. From each experiment with seven compounds (each TLC run), 21 different x values were obtained. The results for six solvent systems are presented in Table III.

The averages and sums of the x values ($\bar{x}$ and Σx) for each run were also calculated. These are measures of the relative separating powers of the solvent

TABLE II

R_F VALUES OF GUAIACOL (I) AND CHLORINATED GUAIACOLS (II–VII) ON A SILICA GEL G60 LAYER WITH DIFFERENT SOLVENT SYSTEMS

Solvent system	*Compound*							*Standard deviation of* R_F	*Development time (min)*
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*		
1	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.000	30
2	0.16	0.19	0.19	0.21	0.12	0.20	0.15	0.032	40
3	0.31	0.35	0.37	0.39	0.27	0.39	0.33	0.045	30
4	0.41	0.41	0.38	0.43	0.30	0.40	0.33	0.048	35
5	0.62	0.60	0.56	0.60	0.65	0.53	0.58	0.039	30
6	0.60	0.58	0.57	0.58	0.61	0.54	0.55	0.025	35
7	0.62	0.62	0.62	0.63	0.63	0.61	0.57	0.021	25
8	0.59	0.58	0.59	0.58	0.62	0.59	0.60	0.014	130
9	0.31	0.23	0.18	0.23	0.31	0.14	0.25	0.062	35
10	0.43	0.38	0.34	0.37	0.45	0.29	0.39	0.054	35
11	0.40	0.38	0.37	0.39	0.45	0.35	0.42	0.034	35
12	0.59	0.53	0.50	0.52	0.62	0.47	0.60	0.057	35
13	0.36	0.38	0.41	0.42	0.29	0.44	0.34	0.052	30
14	0.55	0.58	0.58	0.60	0.46	0.62	0.53	0.053	40
15	0.56	0.55	0.56	0.59	0.49	0.58	0.53	0.033	40
16	0.53	0.53	0.52	0.54	0.46	0.52	0.48	0.030	40
17	0.61	0.61	0.60	0.62	0.55	0.62	0.58	0.025	40
18	0.44	0.43	0.42	0.46	0.34	0.44	0.39	0.040	40
19	0.48	0.48	0.45	0.48	0.42	0.46	0.45	0.022	40
20	0.55	0.55	0.51	0.55	0.45	0.51	0.48	0.039	45
21	0.48	0.51	0.48	0.51	0.45	0.48	0.48	0.021	40
22	0.64	0.61	0.57	0.62	0.55	0.58	0.57	0.032	45
23	0.50	0.49	0.48	0.51	0.40	0.52	0.46	0.040	30
24	0.60	0.60	0.57	0.60	0.56	0.58	0.60	0.017	30
25	0.44	0.40	0.36	0.45	0.46	0.34	0.39	0.046	35
26	0.81	0.83	0.85	0.85	0.89	0.83	0.87	0.027	80
27	0.51	0.51	0.49	0.52	0.48	0.51	0.51	0.015	40
28	0.50	0.50	0.49	0.52	0.48	0.50	0.50	0.012	35
29	0.48	0.48	0.48	0.50	0.47	0.49	0.51	0.013	35
30	0.66	0.65	0.65	0.67	0.67	0.65	0.65	0.014	40
31	0.48	0.48	0.45	0.51	0.51	0.45	0.52	0.029	40
32	0.48	0.47	0.45	0.50	0.50	0.45	0.52	0.027	35
33	0.60	0.61	0.58	0.62	0.61	0.59	0.63	0.018	40
34	0.27	0.32	0.31	0.32	0.37	0.28	0.40	0.047	40
35	0.38	0.35	0.33	0.35	0.42	0.33	0.43	0.041	30
36	0.45	0.42	0.42	0.43	0.46	0.42	0.49	0.028	55
37	0.81	0.79	0.80	0.83	0.90	0.79	0.90	0.044	80
38	0.75	0.75	0.73	0.77	0.75	0.75	0.76	0.012	45
39	0.65	0.65	0.63	0.66	0.66	0.64	0.68	0.016	45
40	0.68	0.68	0.68	0.70	0.70	0.68	0.72	0.016	45

systems, whereas the s values give a measure of absolute separation in each experiment. All three values are useful in screening solvents for analysis or group separation purposes. More detailed information for the separation of the components is obtained from the x_{ij} matrixes (examples in Table 3) in which all x values must be other than zero for complete separation to be expected in one-dimensional elution.

The order of the R_F values of different compounds depends on their polarities

TABLE III

RELATIVE DIFFERENCES, x, BETWEEN R_F VALUES OF I–VII ON SILICA GEL G60 WITH SELECTED SOLVENT SYSTEMS

The value of each x is calculated by dividing the difference of two R_F values by their average. The averages ($\bar{x}$) and sums (Σx) of x for each run are also given.

Solvent system	x							*Average* ($\bar{x}$)	*Sum* (Σx)
	—	*II*	*III*	*IV*	*V*	*VI*	*VII*		
2	I	0.171	0.171	0.270	0.286	0.222	0.065	0.228	4.797
	II		0.000	0.100	0.452	0.051	0.235		
	III			0.100	0.452	0.051	0.235		
	IV				0.545	0.049	0.333		
	V					0.500	0.222		
	VI						0.286		
3	I	0.121	0.176	0.229	0.138	0.229	0.063	0.159	3.337
	II		0.056	0.108	0.258	0.108	0.059		
	III			0.053	0.313	0.053	0.114		
	IV				0.364	0.000	0.167		
	V					0.364	0.200		
	VI						0.167		
8	I	0.017	0.000	0.017	0.050	0.000	0.017	0.026	0.536
	II		0.017	0.000	0.067	0.017	0.034		
	III			0.017	0.050	0.000	0.017		
	IV				0.067	0.017	0.034		
	V					0.050	0.033		
	VI						0.017		
9	I	0.296	0.531	0.296	0.000	0.756	0.214	0.331	6.953
	II		0.244	0.000	0.296	0.486	0.083		
	III			0.244	0.531	0.250	0.326		
	IV				0.296	0.486	0.083		
	V					0.756	0.214		
	VI						0.564		
10	I	0.123	0.234	0.150	0.045	0.389	0.098	0.174	3.659
	II		0.111	0.027	0.169	0.269	0.026		
	III			0.085	0.278	0.159	0.137		
	IV				0.195	0.242	0.053		
	V					0.432	0.143		
	VI						0.294		
13	I	0.054	0.130	0.154	0.215	0.200	0.057	0.171	3.586
	II		0.076	0.100	0.269	0.146	0.111		
	III			0.024	0.343	0.071	0.187		
	IV				0.366	0.047	0.211		
	V					0.411	0.159		
	VI						0.256		

and the polarity of the solvent system. This gives additional structural verification of these guaiacol derivatives. For example, a change from the non-polar chloroform (4) to the polar diethyl ether (5) reverses the order of elution of compounds IV, V and VII with different polarities (see Table II).

CONCLUSIONS

Solvent system 8 (*n*-propanol) gives almost identical but reasonably large R_F values (0.58–0.62) and the smallest values of s, $\bar{x}$ and Σx. The largest value of x

was only 0.067. Thus *n*-propanol could be the solvent of choice for the group separation of chlorinated guaiacols. However, the elution time is very long (130 min). Consequently, we recommend the use of dichloromethane–benzene–methanol (60:30:10) (system 24) or acetone (system 7) for the above purpose; the different separation values are almost as low and the elution times are reasoanbly short (see Table II). Small separation values were also obtained for acetic acid-containing solvents, but they cannot be used for analytical clean-up as the acid residues perturb the subsequent derivatization step in the analysis.

The solvent systems light petroleum (b.p. 40–60 °C)–ethyl acetate (70:30) (system 10) and dichloromethane–chloroform (90:10) (system 13) give x_{ij} values different from zero (see Table III) and high s, $\bar{x}$ and Σx values. Hence these solvents are recommended for the separation of the chloroguaiacols by one-dimensional TLC.

The highest overall separation power was observed for light petroleum (b.p. 40-60 °C)–diethyl ether (70:30) (system 9) (see Table III). However, two x values were zero. Hence we conclude that this solvent could be used only as the first stage in a two-dimensional TLC procedure in which the second stage is used to separate the remaining components. Such a second stage could be carried out with benzene (system 2), as from the x_{ij} matrix (Table III) the values corresponding to the zero values with solvent 9 are reasonably large.

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Note

Reversed-phase gradient high-performance liquid chromatography of procyanidins and their oxidation products in ciders and wines, optimised by Snyder's procedures

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The procyanidins of ciders and wines are based on a C-15 catechin structure examples of which are shown in Fig. 1 (ref. 1), and cover a range of molecular size from the monomeric to the heptameric. They are important to the sensory properties and browning potential of the beverage[2,3].

It has been shown previously[4] that procyanidins can be successfully separated under isocratic conditions by reversed-phase high-performance liquid chromatography (HPLC) using acidified aqueous methanol. In an attempt to improve resolution in mixtures of wide sample polarity, gradient elution was investigated using the procedures outlined by Snyder and co-workers[5,6] for optimising conditions.

The chromatographic behaviour of a solute in a mixed eluent (*e.g.* methanol–water) is described as follows[5]:

$$\log k' = \log k_w - S \cdot \varphi \tag{1}$$

where k_w = capacity ratio (k') in the weak solvent (water); φ = fraction of the strong solvent (methanol) in the eluent and S = a constant with a typical value of approximately 3.

By undertaking isocratic studies and by plotting log k' against solvent composition, the values of log k_w (intercept) and S (slope) may be determined.

The optimal conditions for gradient elution have been described as follows[5]:

$$\varphi' = b/S \cdot t_0 \tag{2}$$

where φ' = percentage increase in strong solvent per unit time (*i.e.* gradient steepness); b = a parameter with an optimal value of 0.1–0.2 (argued by Snyder on theoretical grounds and justified by experimental study) and t_0 = retention time for an unretained solute.

In using these conditions to develop separations we have noted several ways in which the chromatographic behaviour of procyanidins differs from that of smaller solute molecules.

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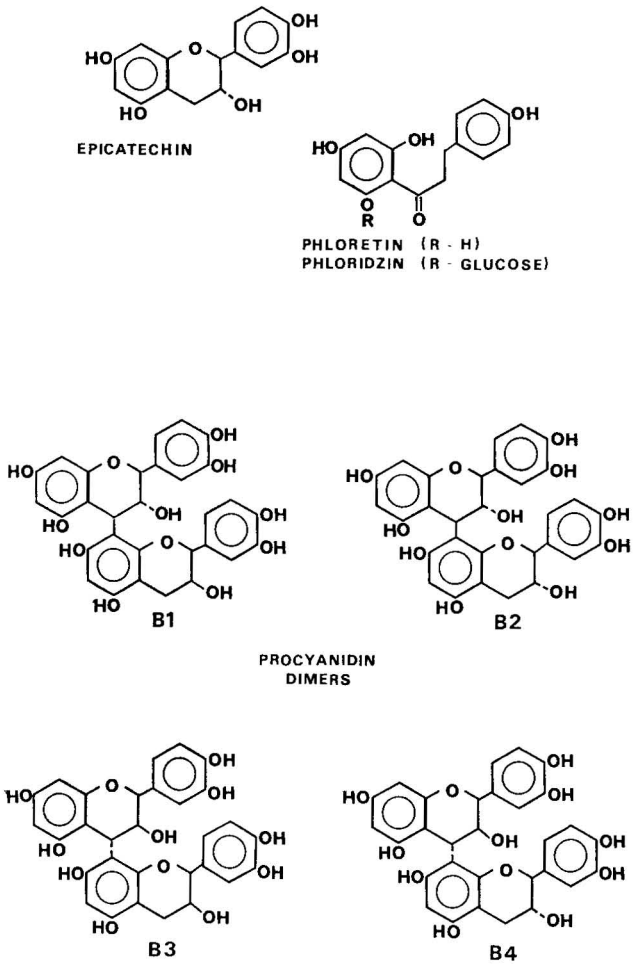

Fig. 1. Dimeric procyanidins of ciders and wines. Further polymers are built up from catechin or epicatechin in similar fashion. The phloretin glycoside, phloridzin, unique to apples, is also shown.

EXPERIMENTAL

A Spectra-Physics SP8000 machine was used, with detection on a Pye Unicam LC3 spectrophotometer at 280 nm, 0.08 a.u.f.s. Samples were generally 10 μl of 0.1–0.4% aqueous solutions of fractions derived from wines and ciders by counter-current distribution[2,3] filtered through a 0.45-μm Millipore filter before use.

Reversed-phase columns, slurry packed in the laboratory were: LiChrosorb RP-8, 10 μm (250 × 4.6 mm); Spherisorb Hexyl, 5 μm (120 × 4.6 mm) and Hypersil SAS, 5 μm (120 × 4.6 mm).

Solvent A was water, prepared through an Elga de-ioniser and charcoal column, filtered through a 0.45-μm Millipore filter before use, and acidified to pH 2.0 or 2.5 by the addition of 0.1 or 0.01% perchloric acid, respectively. Solvent B was methanol, glass distilled from KOH, filtered through a 0.45-μm Millipore filter before use.

The water was changed daily to prevent microbial growth, and the columns and system were flushed through with methanol at the end of each working day.

All separations were carried out at 45 °C. t_0 was determined by injection of 0.1% uracil on to a column eluted with 80% methanol. Other conditions are noted in the text.

RESULTS

Optimization of gradient

The initial application of eqn. 2 to separation of procyanidins, using a typical value of $S = 3$, produced very poorly resolved chromatograms as in Fig. 2. Arbitrarily chosen shallow gradients improved the resolution but led to peak broadening and reduced detection sensitivity.

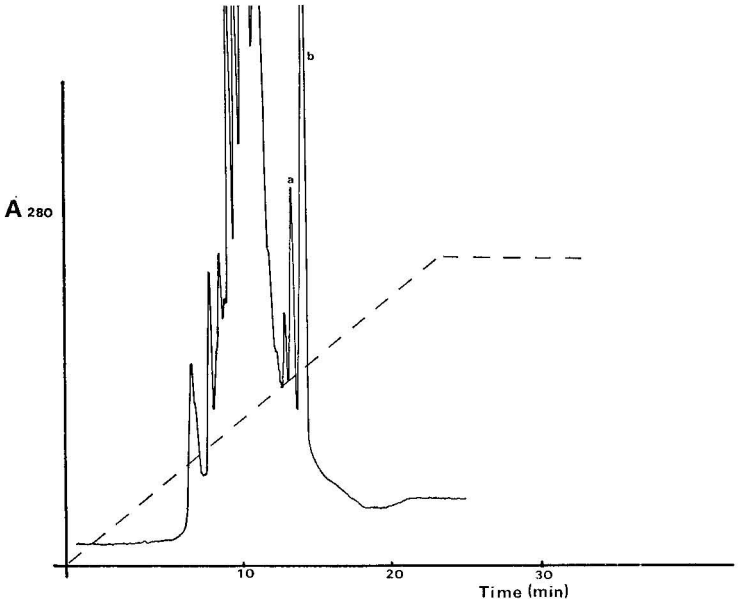

Fig. 2. Separation of a cider tannin extract ("Tremletts Bitter"). LiChrosorb RP-8. Solvent gradient (broken line) from 100% A to 100% B in 20 min. Flow-rate, 2 ml/min; t_0, 75 sec. a = Phloretin xyloglucoside, b = phloridzin.

To optimise conditions, therefore, isocratic studies of eqn. 1 were undertaken, typical results being shown in Fig. 3. These revealed that the value of S for procyanidins on LiChrosorb RP-8, for instance, takes an average value of 8 rather than the value of 3 which is usually assumed for small molecules and which is typical of the *p*-hydroxybenzoates also shown in Fig. 3. Similar plots were also obtained for Spherisorb Hexyl. The gradient for optimal resolution from eqn. 2 becomes much shallower, therefore, typical results being shown in Figs. 4 and 5. Such conditions make it possible not only to separate the major classes of procyanidins from one another, but also to resolve the four stereoisomeric dimers B1–B4.

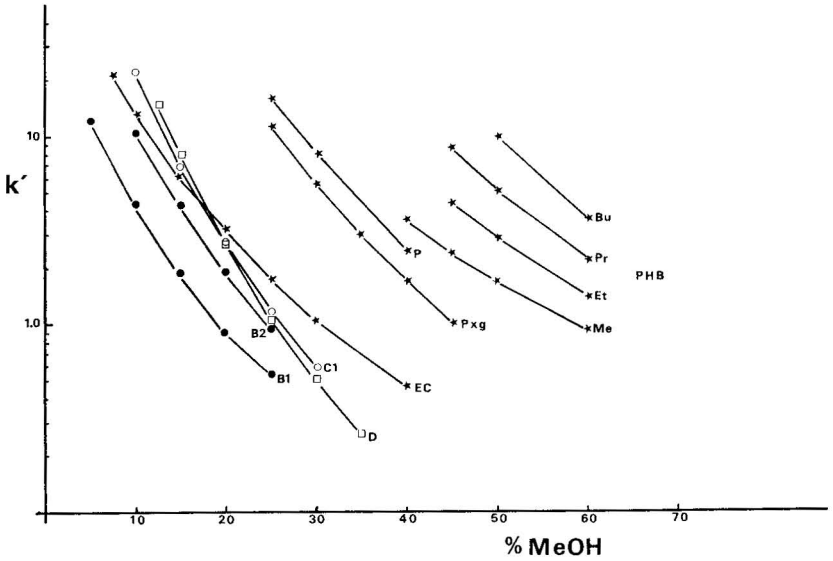

Fig. 3. Semi-log plots of k' *vs.* per cent methanol (MeOH) on LiChrosorb RP-8. Me, Et, Pr, Bu PHB = methyl, ethyl, propyl, butyl *p*-hydroxybenzoates; P = phloridzin; Pxg = phloretin xyloglucoside; EC = epicatechin; B1, B2 = procyanidin dimers; C1 = procyanidin trimer; D = procyanidin tetramer.

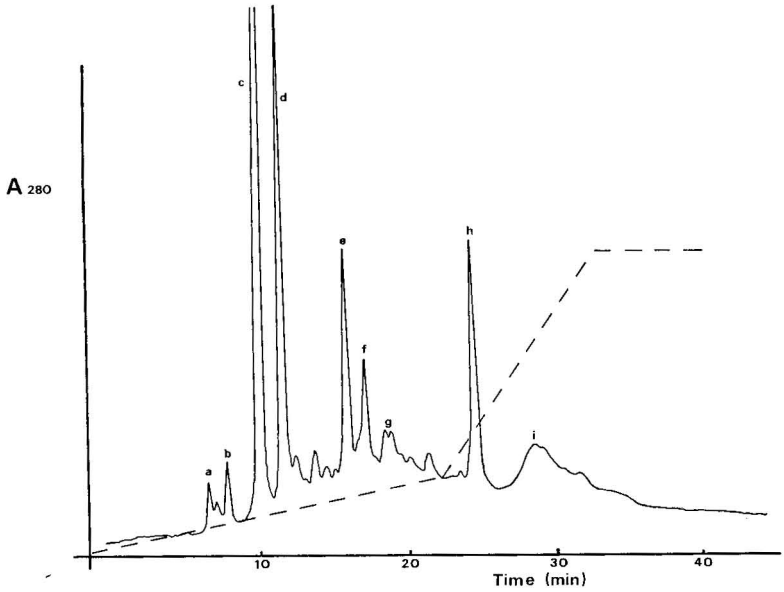

Fig. 4. Separation of a cider tannin extract ("Dabinett"). Spherisorb Hexyl. Solvent gradient (broken line) from 2% B to 25% B in 23 min, 25% B to 98% B in 10 min. Flow-rate, 1.5 ml/min; t_0, 47 sec. a = Procyanidin B3; b = procyanidin B1; c = epicatechin; d = procyanidin B2; e = procyanidin trimer C1; f = procyanidin tetramer(s); g = procyanidin pentamer(s); h = phloridzin; i = oxidised/polymeric procyanidins.

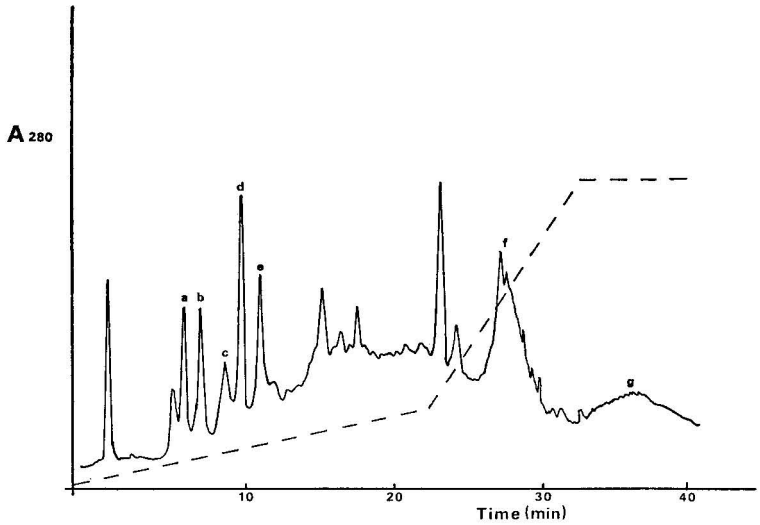

Fig. 5. Separation of a white wine tannin extract ("Müller-Thurgau"). Spherisorb Hexyl. Conditions as for Fig. 4. a = Procyanidin B3; b = procyanidin B1; c = procyanidin B4; d = epicatechin and catechin; e = procyanidin B2; f = oxidised/polymeric procyanidins; g = solvent impurities.

Relationship between S and molecular weight

Fig. 3 also shows that the value of S increases with the procyanidin molecular weight, and it was of interest to examine this relationship. However, the plots in Fig. 3 are slightly concave and therefore it is difficult to know which particular value of S should be used to characterise any particular solute. Although attempts have been made to replace eqn. 1 by a quadratic form to allow for this, it seemed that a simpler solution was presented by re-arranging Snyder's general gradient elution expression[6] into the following form:

$$S = \frac{\log(1 + 2.3\, k_0\, \varphi'\, t_0\, S)}{\varphi'/(t_g - t_0 - t_d)} \tag{3}$$

where $k_0 = k'$ for a given solute in the starting composition of the gradient; t_g = retention time of the solute in the gradient run and t_d = delay time between gradient generator and column head.

Although no simple algebraic solution of this expression is possible, a programmable pocket calculator (Texas TI-51-III) was able to provide a solution using an iterative approximation routine.

From a single gradient run at an approximately optimal value of φ', instantaneous values of S could therefore be determined for a range of procyanidins from eqn. 3. When plotted against molecular weight on a semi-logarithmic scale, as in Fig. 6, a straight-line relationship was obtained. The intercept, for the hypothetical limiting case of a procyanidin with zero molecular weight, gave a value of $S = 3.3$ which corresponds very well with the values usually adopted for small molecules.

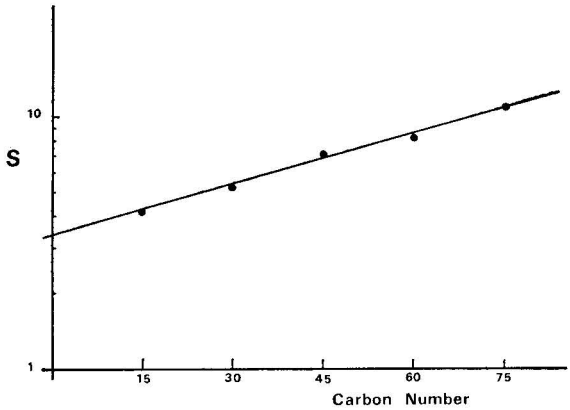

Fig. 6. Semi-log plot of S *vs.* molecular weight (carbon number) for procyanidins. 15 = Epicatechin; 30 = Procyanidin dimers, etc.

Behaviour of oxidised procyanidins

The chromatograms in Figs. 4 and 5 show a broad band which elutes after the sharp change in gradient steepness. Work with procyanidin samples from counter-current distribution which were progressively browner and more oxidised suggested that this band was associated with oxidation of procyanidins. It was further established that this band did not appear under isocratic conditions nor when operated with a continuous linear gradient. The explanation appears to be that the oxidation of procyanidins leads to an increase in ill-defined polymeric material, as has long been known[7]. Such polymeric materials do not elute with defined k' values but tend instead to be spread out over the whole area of the chromatogram. As polymeric materials, however, their S values are very high and so a rapid increase in solvent strength causes a marked depression in their k' values. Hence they are eluted as a broad band near the "new" solvent front.

Confirmation of this effect was provided by running a sample of pure epicatechin, which displayed no oxidised band, whereas an identical sample which had been allowed to brown in solution for several weeks showed a strong oxidised band after the change in gradient steepness.

DISCUSSION

It is obvious that reversed-phase gradient elution chromatography can be a powerful tool for the analysis of complex procyanidin mixtures, but the optimum conditions can only be determined with reference to studies of isocratic behaviour. Plots such as Fig. 3 also show the isocratic conditions under which certain separations are possible or impossible, and predict the reversal of elution order which may be observed when solvent strength is changed. Thus the isocratic elution order of procyanidins on Hypersil SAS in 20% methanol (see for instance, ref. 4), was in decreasing order of molecular weight, whereas by gradient elution starting at lower concentrations of methanol the order was generally reversed, as in Figs. 4 and 5. Incidentally, it was not possible to pursue detailed work on Hypersil SAS since this

particular packing is unstable below pH 3, whilst at higher pH values the procyanidins tail badly due to their slightly acidic nature. LiChrosorb RP-8 and Spherisorb Hexyl seem stable down to pH 2, however, and tailing is well supressed under such conditions.

The elution of polymeric or oxidised procyanidins as a defined band following a sharp change in gradient steepness may have considerable practical importance, since it now becomes possible to use this effect in studies of the oxidation and polymerisation of procyanidins in ciders and wines, work which has hitherto been hampered by a lack of suitable chromatographic techniques. The relationship between S and procyanidin molecular weight may also have practical significance, since it is difficult to obtain reliable molecular weight estimations for procyanidins, and chromatographic data derived from eqns. 1 and 3 may therefore be useful in supplementing other measurements on samples where molecular weight is not known.

It is expected that a correlation should be shown between the elution order for procyanidins by reversed-phase chromatography and the elution order by countercurrent distribution between ethyl acetate and water. At first sight no such correlation is apparent but, by extrapolating the plots in Fig. 3 to high concentrations of methanol where adsorptive effects are minimised, the relative chromatographic values of k' (hydrocarbon–aqueous methanol) become similar to those previously determined for the partition coefficient K (ethyl acetate–water)[8], where the smaller procyanidins have the greater partition coefficients into the hydrocarbon phase.

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Note

Program for processing amino acid data with a programmable pocket calculator

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The quantitative evaluation of a chromatogram obtained from an automatic amino acid analyzer is a time-consuming repetitive operation comprising integration of the area of each peak, after comparison with an appropriate standard. These operations can be accomplished by desk-top calculators[1] and electronic integrators[2,3]. Several authors have described the use of these devices in this field and how to reduce analysis costs[4], how to simplify the software even in the case of analyses of complex mixtures such as those of physiological fluids[5], how to minimize the effects of noisy outputs and variable retention times[6], how to detect the critical points of the chromatogram and how to prevent fluctuations in the base line[7]. However, the price of these devices (which represents 15–25% of the total cost of the apparatus, even for the simplest models) and the need for some knowledge of computer language put them out of reach of most laboratories.

Such repetitive routine calculations can be performed with the new programmable pocket calculators, whose prices are much lower than those of the instruments mentioned. Of course, in this case the calculator cannot be interfaced with the amino acid analyzer. Thus some parameters of the chromatogram peaks, such as height and width, must be measured manually, but once this is done the time required to process data is drastically shortened and the possibility of error is greatly diminished compared to full manual evaluation.

Buchanan[8] reported a program for processing amino acid data with a Hewlett-Packard HP25 calculator; however, this machine has a limited number of program steps and memories, and it can process the information for only one amino acid at a time. For each peak, 1.5–2.0 min are required to evaluate the amount in nmoles, *i.e.*, the procedure must be repeated eighteen times for protein hydrolysates, making the full elaboration of data tedious and time-consuming.

The program described in this paper is written for a Texas Instruments TI59 calculator which offers a larger number of steps and memories. It enables calculation of the whole amino acid composition just by entering in separate steps the data calculated manually (total heights, baselines and widths), which are then processed automatically. The program is divided in two sections: one for calculating correction factors from a calibration run with a standard mixture (this section requires 83 steps and 58 memory registers); and another to evaluate the amino acid composition of the sample (this section requires 150 steps and 59 memory registers).

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In the procedure proposed, the amino acid content is expressed as a percentage of the total recovered amino acids and as mg per g N (N = nitrogen). However, the program can easily be modified according to specific needs and the results can be expressed as desired (*e.g.*, residues per mole of protein, g per 100 g of protein, mg per g N, residues percent, g per 16 g N). Partial results can be displayed in any step of the program in order to record them on data sheets. The program described is designed for the evaluation of the amino acid composition of fully hydrolyzed samples (*i.e.*, eighteen amino acids), but can be modified to process more amino acids, as is required in the case of physiological fluid analysis.

The calculator needs only a few seconds to process all the data; of course, more time is required to manually enter the parameters of each peak. However, the whole procedure takes less than 6 min.

In our program the amount of each amino acid is expressed either as a percentage of the total recovered amino acids or as mg amino acid per g N, in which case the nitrogen content is determined by direct analysis of the sample. This method of expressing data is particularly useful in the analysis of food proteins. Moreover, by relating the determined data to the nitrogen content separately assayed, instead of to the total recovered amino acids, it is possible to correct the results for losses during the preparation and hydrolysis of the sample.

Table I shows the sequence in which the data for eighteen amino acids are processed. It also lists the memory addresses for total heights, net heights, baselines, widths, mg $\times$ 10^2 of each amino acid, expansion scale and optical pathway, nitrogen

TABLE I

MEMORY ADDRESSES OF AMINO ACID DATA

Expansion scale factor and optical pathway*: 19. g N/ml* (only if 1 ml is the injected volume): 59. Total mg $\times$ 10^2: 39. Counting memories: 00, 20 and 40.

$C = (\text{Height}_{\text{standard a.a.}} \times W_{\text{standard a.a.}})/(\text{Nanomoles}_{\text{standard a.a.}} \times \text{MW}) \times 10{,}000.$

Amino acid	*Total height*, Net height*	*Baseline*, Width**	*Correction factor, C*	*Amount (mg $\times$ 10^2)*
His	01	21	41	01
Lys	02	22	42	02
Arg	03	23	43	03
Asp	04	24	44	04
Thr	05	25	45	05
Ser	06	26	46	06
Glu	07	27	47	07
Pro	08	28	48	08
Gly	09	29	49	09
Ala	10	30	50	10
Cys	11	31	51	11
Val	12	32	52	12
Met	13	33	53	13
Ile	14	34	54	14
Leu	15	35	55	15
Tyr	16	36	56	16
Phe	17	37	57	17
Trp	18	38	58	18

* These data must be entered by the operator.

content of the sample and percentages of total recovered amino acids. Notice that when one amino acid is absent, the values 0 for total height, baseline and width and 1 for correction factor must be entered.

A schematic diagram of the program is shown in Fig. 1. Further details are given in Table II.

Program steps	Data Entry	Total heights and base lines
000-039	Calculation of net heights and net half heights	Net half heights displayed, net heights stored
	Data Entry	Widths, correction factors, selected scale and optical pathway, g N/ml
040-079	Calculation of the amount of each a.a.	Result stored
080-099	Calculation of the amount of recovered amino acids	Result stored
100-140	Calculation of amino acid % and mg amino acid/gN	Final results displayed

Fig. 1. Block diagram of the program.

With the suggested procedure the calculator first computes and displays net half-heights, which indicate where to evaluate the widths of the peaks and, after these data have been measured and entered, it calculates and displays the percent of each amino acid to two decimal places and the values of mg per g N approximated to an integer. However, other partial results can be displayed if the instruction "2nd Pause", which interrupts the program for 0.5 sec, is inserted after the sequence of instructions which define them.

Correction factors for each amino acid are calculated by modifying the main program after step 039, as shown in Table III. In this case the molecular weights of the amino acids must be entered in memories 41–58. Once calculated, correction factors are automatically stored in the same memories.

The performance of the main program is checked by two different types of tests. A preliminary run can be done: if, for all total heights, the digit 2 is entered and the digit 1 for baselines, widths, correction factors, expansion scale and g N, the program should display, in turn, 5.56 % and 1. Alternatively, in routine operation, the number 18 displayed at the end of each cycle indicates the correct completion of a program section. After each section, the key-stroke run/stop must be pressed in order to move the program forward.

Once correction factors are calculated and stored, eighteen peaks in a chromatogram are processed in less than 6 min, including entering the data, but excluding the manual evaluation of baselines, total heights and widths which depend on the skill of the operator.

The program steps can be recorded on magnetic cards for quick reuse. In

TABLE II

PROGRAM FOR THE CALCULATION OF AMINO ACID COMPOSITION

Program steps	*Key*	*Comments*
000	0	Values for counting memories are set.
001	STO	
002	00	
003	2	
004	0	
005	STO	
006	20	
007	2nd Lbl	The first loop is labeled. 18 is put in t register.
008	A	
009	1	
010	8	Counting program.
011	x⇄t	
012	1	
013	SUM	
014	00	
015	SUM	
016	20	
017	RCL 2nd IND	Total heights recalled; base lines subtracted.
018	00	
019	-	
020	RCL 2nd IND	
021	00	
022	=	
023	STO 2nd IND	Net heights are stored and divided by 2; base lines summed.
024	00	
025	:	
026	2	
027	+	
028	RCL 2nd IND	
028	20	
030	=	
031	2nd Pause	Net half heights diplayed
032	2nd Pause	The digit in memory 00 is compared with t. If the value is $\angle$ 18 the cycle is repeated, if = 18, is stopped.
033	RCL	
034	00	
035	INV	
036	2nd x=t	
037	A	
038	R/S	
039	0	
040	STO	Values for counting memories are reset.
041	00	
042	2	
043	0	
044	STO	
045	20	
046	4	
047	0	
048	STO	
049	40	
050	2nd LBl	The second loop is labeled.
051	B	
052	1	Counting program.
053	SUM	
054	00	
055	SUM	
056	20	
057	SUM	
058	40	

TABLE II (*continued*)

Program steps	*Key*	*Comments*
059	RCL 2nd IND	Net heights recalled, multiplied by widths, divided by correction factors, multiplied by expansion scale factor.
060	00	
061	*	
062	RCL 2nd IND	
063	20	
064	:	
065	RCL 2nd IND	
066	40	
067	*	
068	RCL	
069	19	
070	=	
071	STO 2nd IND	mg * 10^2 stored. The digit in memory 00 is compared with t. If the value is < 18 the cycle is repeated, if = 18 is stopped. 0 is set in the counting memory. Memory 39 is cleared.
072	00	
073	RCL	
074	00	
075	INV	
076	2nd x=t	
077	B	
078	R/S	
079	0	
080	STO	
081	00	
082	STO	
083	39	
084	2nd LBl	The third loop is labeled.
085	C	
086	1	Counting program.
087	SUM	
088	00	
089	RCL 2nd IND	mg * 10^{+2} recalled, summed and stored.
090	00	
091	SUM	
092	39	The digit in memory 00 is compared with t. If the value is < 18 the cycle is repeated, if = 18, is stopped.
093	RCL	
094	00	
095	INV	
096	2nd x=t	
097	C	
098	R/S	
099	0	0 is set in the counting memory.
100	STO	
101	00	
102	2nd Lbl	The fourth loop is labeled.
103	D	
104	1	Counting program.
105	SUM	
106	00	
107	RCL 2nd IND	mg * 10^{+2} recalled, divided by total mg * 10^2, multiplied by 100.
018	00	
109	:	
110	RCL	
111	39	
112	*	
113	I	
114	0	
115	0	
116	=	
117	2nd Fix	Aminoacid percentage to 2 decimal places displayed.
118	2	
119	2nd Pause	
120	2nd Pause	
121	2nd Pause	
122	2nd Pause	

(Continued on p. 74)

TABLE II *(continued)*

Program steps	*Key*	*Comments*
123	RCL 2nd IND	mg * 10^{+2} recalled
124	00	and divided by gN/ml.
125	:	
126	RCL	
127	59	
128	=	
129	2nd Fix	Results to integer
130	0	displayed.
131	2nd Pause	
132	2nd Pause	
133	2nd Pause	
134	2nd Pause	
135	RCL	The digit in memory
136	00	00 is compared with
137	INV	t. If the value is
138	2nd x=t	$\lessdot$ 18 the cycle is re-
139	D	peated, if = 18 is
140	R/S	stopped.

TABLE III

PROGRAM FOR THE CALCULATION OF CORRECTION FACTORS

Program steps	*Key*	*Comments*
000-038	The same as in the main program	
039	0	Value for counting
040	STO	program are set.
041	00	
042	2	
043	0	
044	STO	
045	20	
046	4	
047	0	
048	STO	
049	40	
050	2nd Lbl	The second loop is
051	B	labeled.
052	I	Counting program.
053	SUM	
054	00	
055	SUM	
056	20	
057	SUM	
058	40	
059	RCL 2nd IND	Net heights recalled,
060	00	multiplied by widths,
061	*	multiplied by 10000 and
062	RCL 2nd IND	divided by nanomoles
063	20	of each aa/ml of cali-
064	*	bration mixture (=40),
065	4	divided by molecular
066	0	weights, multiplied by
067	:	expansion scale factor.
068	RCL 2nd IND	
069	40	
070	*	
071	RCL	
072	19	
073	=	
074	STO 2nd IND	Correction factors to
075	40	2 decimal places sto-
076	2nd Fix	red.
077	2	The digit in memory 00
078	RCL	is compared with t. If
079	00	the value is $\lessdot$ 18, the
080	INV	cycle is repeated, if
081	2nd x=t	= 18, is stopped.
082	B	
083	R/S	

addition, if the printing device PC100 is available, the program itself and partial or final results can be printed. Thus, by avoiding any break in the program to write down the data, the time needed to process data is further reduced.

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Note

Effect of some organic buffers on the estimation of aspartic acid and resolution in amino acid analysis*

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(First received December 12th, 1979; revised manuscript received January 23rd, 1980)

The use of lithium buffers in ion-exchange column chromatography has improved the analysis of "physiological" samples containing non-protein amino acids (for example, see refs. 1 and 2). During a study of the amino acids of pea-leaf chloroplasts[3], we became aware of difficulties in the estimation of aspartic acid and also its separation from BIA, an amino compound present in peas[4]. The problem was found to be caused by the presence of some organic buffers (described by Good *et al.*[5]) used in the preparation of the chloroplasts. It is apparent that several organic buffers, including tricine, bicine, HEPES and EPPS, interfere with the resolution and estimation of aspartic acid and neighbouring compounds.

EXPERIMENTAL

A Beckman Model 119BL automatic analyser was used with a single column (240 × 9 mm) of W-2 resin. The first buffer contained lithium (citrate) at a concentration of 0.2 *N*, pH 2.83. The starting temperature was 40 °C, with a rise (to 66 °C) beginning at 44 min; this early temperature rise allowed the satisfactory resolution of asparagine, glutamic acid, glutamine and homoserine, although resolution of a few other physiological amino acids (not present in our plant samples) was impaired.

Samples for analysis were prepared from buffer solutions and amino acid standards or leaf extracts, and the pH was checked with a meter. The volume loaded was 0.5 ml.

Physiological amino acid standards were obtained from Hamilton. Amino acids were extracted from pea leaves (*Pisum sativum*) by grinding in water and immediately adding 5-sulfosalicylic acid (50 mg/ml) to precipitate proteins. After centrifugation, the solution was filtered through a Millipore cellulose mixed-ester membrane (type VM, pore size 0.05 μm). Pea-leaf extracts contained the ninhydrin-positive compound BIA, which eluted *ca.* 3 min after aspartic acid.

* Abbreviations used: BIA = β-(isoxazolin-5-on-2-yl)alanine; EPPS = N-2-hydroxyethylpiperazine-N′-3-propane sulphonic acid; HEPES = N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; MES = 2-(N-morpholino)ethanesulphonic acid; bicine = N,N-bis(2-hydroxyethyl)-lycine; tricine = N-tris(hydroxymethyl)methylglycine.

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RESULTS AND DISCUSSION

The effect of a number of organic buffers on resolution of the amino acids emerging in the early part of analysis was investigated. Amino acid samples used (50–100 nmol per amino acid) were aspartic acid alone, physiological standard mixture or pea-leaf extract. Samples were loaded at a range of pH values from 2.1 to 2.5 (2.2 is the recommended value), with the addition of up to 50 μmol of buffer. Some effects on physiological standards are shown in Fig. 1, and a more detailed survey of the effects on aspartic acid is shown in Table I.

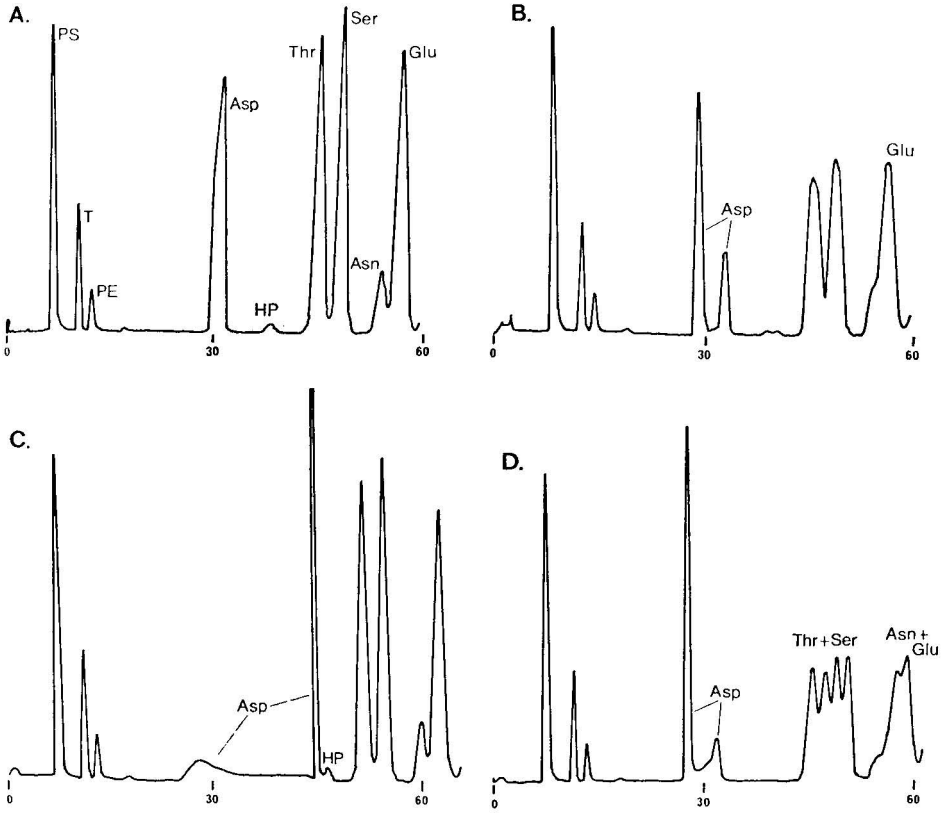

Fig. 1. Effect of organic buffers on elution of amino acids in a physiological calibration standard. The trace represents absorbance of the ninhydrin-reacted eluate, measured at 570 nm. The standard contained 50 nmol of aspartic acid. Time scale in minutes. PS = phosphoserine; T = taurine; PE = phosphoethanolamine; HP = hydroxyproline. A, standard alone; B, standard plus HEPES, (30 μmol) loaded at pH 2.2; C, standard plus tricine (25 μmol) loaded at pH 2.2; D, standard plus HEPES, (30 μmol) loaded at pH 2.5.

HEPES

In the presence of this buffer, aspartic acid emerged as two separate peaks, with an elevated baseline plateau of variable height between the peaks. In plant samples, the aspartic acid region contained three peaks, due to the presence of BIA. At pH 2.5, this effect began to appear with the addition of 12–15 μmol of

TABLE I

BEHAVIOUR OF ASPARTIC ACID IN AMINO ACID ANALYSIS, INFLUENCED BY THE PRESENCE OF ORGANIC BUFFERS IN THE SAMPLE

Aspartic acid (100 nmol) was loaded, with the organic buffer, in a sample volume of 0.5 ml, and a lithium-based analytical system was used. The figures in parenthesis represent proportions of the total aspartic acid recovered in double peaks, each peak expressed as a percentage of the total area (which includes any plateau).

Buffer	*Amount added (μmol)*	*Loading pH*	*Time of elution of aspartic acid peak(s) (min)*	*Notes*
None	—	2.2	32	
HEPES	50	2.15	27 (49%) 33 (51%)	
	25	2.15	29 (48%) 33 (52%)	
	50	2.3	26 (33%) 34 (57%)	Plateau between peaks
	50	2.5	24 (78%) 34 (11%)	Plateau (later compounds: grossly distorted)
	25	2.5	29 (65%) 33 (17%)	Plateau (later compounds: peaks doubled)
	10	2.5	33	
EPPS	50	2.5	27 (63%) 32 (29%)	Later compounds: peaks doubled
Tricine	50	2.2	17 (24%) 45 (54%)	Plateau
	50	2.5	17 (44%) 45 (41%)	Plateau
	25	2.2	24 (20%) 42 (64%)	Plateau
	10	2.2	32 (14%) 40 (74%)	Plateau
	5	2.2	33 (10%) 39 (76%)	Plateau
Bicine	25	2.2	21 (39%) 45 (36%)	Plateau

buffer; at pH 2.15, the threshold was slightly higher. Several peaks immediately following aspartic acid were also affected, becoming first broadened (Fig. 1B), then doubled and progressively more distorted (Fig. 1D), with increasing loading pH and amount of HEPES added. Later peaks (glycine, alanine and those following) and the compounds emerging before aspartic acid (phosphoserine, taurine, phosphoethanolamine) were unaffected.

EPPS

This buffer is a homologue of HEPES, and produced very similar distortions.

Tricine

Severe effects were observed with quite low levels (5 μmol) of tricine, at a range of values of loading pH. Again, aspartic acid emerged as two peaks with an interconnecting plateau, the distance between the peaks varying with the amount of buffer added. The second peak was considerably delayed and caused late elution and some compression of later peaks (Fig. 1C). In plant samples, the compound BIA was not resolved and was completely merged with the second aspartic acid peak.

Bicine

This buffer had an effect similar to that of tricine.

Tris and MES

These buffers had little effect at levels up to 50 μmol, although at the higher concentrations a small leading fore-peak to aspartic acid was sometimes present.

Effect on analysis in sodium buffers

From a limited series of experiments, it is clear that HEPES, EPPS, tricine and bicine also influence the resolution of aspartic acid in a sodium-based analytical system. The effects are similar to those described above, although the appearance of the effects requires several fold higher levels of the organic buffers, compared with the lithium-based system.

CONCLUSIONS

Organic buffers are sometimes present in samples used for amino acid analysis, for example in preparations of purified organelles, or reaction mixtures from enzyme studies. As shown here, buffers of this type can cause serious problems in the resolution of aspartic acid and some other compounds, producing difficulties with interpretation of the chromatographic results. Accurate determination of aspartic acid content may be prevented when part of the compound emerges as a plateau region not recorded by an integrator. With HEPES and EPPS, the effect is intensified as the pH of the sample rises slightly above the recommended loading pH. Inaccurate adjustment of pH may occur with very small sample volumes, and "loading buffers" have in fact very little buffering capacity.

At first, the peak doubling noted for aspartic acid seemed to be so remarkable that the purity of the sample was suspected, but the same effect was consistently seen with a range of samples, including the aspartic acid peak in calibration standard mixtures and in plant extracts. Other workers have reported that the aspartic acid peak may undergo some distortion as the loading pH is varied[6,7], but the effects were quite small compared to the distortions described here. The nature of the buffer–amino acid interaction is not clear; possibly a buffer–aspartic acid complex is formed. Regardless of the explanation, it is clear that caution must be used when buffers such as HEPES, EPPS, tricine and bicine are present in samples that are to undergo amino acid analysis; minimum acceptable concentrations of the buffers should be used, the effect on known standards should be observed, and the pH of samples should be lowered to *ca.* 2.1.

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Note

Separation of steroid glucuronides by reversed-phase liquid column chromatography

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During pregnancy, estrogens conjugated with glucuronic acid, particularly estriol conjugates, dominate the steroids present in urine. A decrease in the excretion level of estriol indicates a possible malfunction of the placenta. These metabolites are measured routinely by a method involving extraction and derivatization by the Kober reaction followed by spectrophotometric measurements[1].

Huber *et al.*[2] and Dolphin and Pergande[3,4] have used high-performance liquid chromatography (HPLC) for the isolation of estrogen conjugates in urine after hydrolysis and extraction of the liberated aglucone. A drawback of methods involving hydrolysis is that the identity of the individual conjugates is destroyed. In order to avoid this, Van der Wal and Huber[5] used XAD-2 extraction, followed by isolation on an anion-exchange column.

The present paper describes studies of reversed-phase column chromatography of glucuronides of estrone, estradiol and estriol and a method for the isolation of estriol 16α-glucuronide from untreated pregnancy urine.

EXPERIMENTAL

Apparatus

The pump was an Altex Model 100 solvent delivery system and the detector was a Waters Model 440 with an 12.5-μl cell and wavelength of 280 nm. The columns were made of 316 stainless steel with a polished surface, equipped with Swagelok connectors and stainless-steel frits (2 μm). The column dimensions were 150 $\times$ 4.5 mm. A high-pressure injection port was used (Rheodyne, 5000 p.s.i.) with a 20-μl loop. Solvent reservoir and column were thermostated by a water-bath (HETO Type 02 PT 923 TC; Birkeröd, Denmark). The pH was measured with an Orion Research Model 801 A/digital meter, equipped with an Ingold Type 401 combined electrode. The gas chromatography–mass spectrometry (GC–MS) system was an LKB Model 2091 with electron impact (EI) ionisation. The GC column was 1.5 m of 3% SE-30.

Chemicals and reagents

1-Pentanol was of ACS reagent quality (Fisher Scientific, Pittsburgh, PA, U.S.A.). The steroid glucuronides were obtained from Sigma (St. Louis, MO, U.S.A.) and used without further purification. All other substances used were of analytical

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grade. The chromatographic support was LiChrosorb RP-8 with a mean particle diameter of 5 μm (E. Merck, Darmstadt, G.F.R.). Buffers had an ionic strength of 0.1.

Column preparation

LiChrosorb RP-8 was packed by a balanced-density slurry technique[6], suspended in carbon tetrachloride–tetrabromoethane–dioxane (1:1:1). After packing, the column was washed with hexane and acetone. The support was coated by passing the mobile phase through it until a test sample gave a constant capacity factor.

Chromatographic technique

The mobile phase reservoir was kept in a water-bath at 25 $\pm$ 0.1 °C. The column was kept in a water jacket with circulating water of the same temperature. Tubing and the injection port were insulated to avoid temperature changes. The mobile phase was prepared by saturating the phosphate buffer with 1-pentanol in a separating funnel. When a lower content of 1-pentanol was used, the saturated solution was diluted with phosphate buffer to the appropriate 1-pentanol concentration. The volume of the mobile phase was determined by injection of potassium dichromate. The samples were dissolved in the mobile phase.

Isolation and GC–MS identification of estriol 16α-glucuronide

The untreated pregnancy urine was injected directly on the reversed-phase column, and a fraction containing the peak with a capacity factor equal to that of estriol 16α-glucuronide was collected. Fractions from eight injections were pooled, acidified with hydrochloric acid to a final concentration of 2 *M* and heated for 90 min at 100 °C. The solution was extracted twice with an equal volume of water-saturated ethyl acetate. The combined extracts were evaporated to dryness.

The sample was dissolved in 20 μl pyridine, and 100 μl acetic anhydride were added. The reaction mixture was then kept in an ultrasonic bath for 5 min, heated at 80 °C for 30 min, evaporated to dryness and the residue dissolved in ethyl acetate. A 2-μl volume of the solution was injected in the GC–MS apparatus. The gas chromatograph was operated at a column temperature of 220 °C and an injector temperature of 240 °C. Helium at a flow-rate of 30 ml/min was used as carrier gas. The ion source of the mass spectrometer was operated at 260 °C with an electron energy of 70 eV.

RESULTS AND DISCUSSION

Regulation of the retention

The steroid glucuronides are weak acids with $pK_a \approx 3.5$[7]. They can be separated in reversed-phase systems with LiChrosorb RP-8 as solid phase and phosphate buffer pH 6.5, containing 1.25–2.5% of 1-pentanol as mobile phase. The capacity ratio can be regulated within rather wide limits by changes of the 1-pentanol concentration in the mobile phase, as demonstrated in Fig. 1.

In the chromatographic system used, 1-pentanol is adsorbed on the hydrophobic solid phase. If the adsorbed 1-pentanol constitutes a liquid stationary phase, the capacity ratio of the glucuronides, k'_X, is given by:

$$k'_X = (V_s/V_m) \times D = (D \times V_t/V_m) - D \quad (1)$$

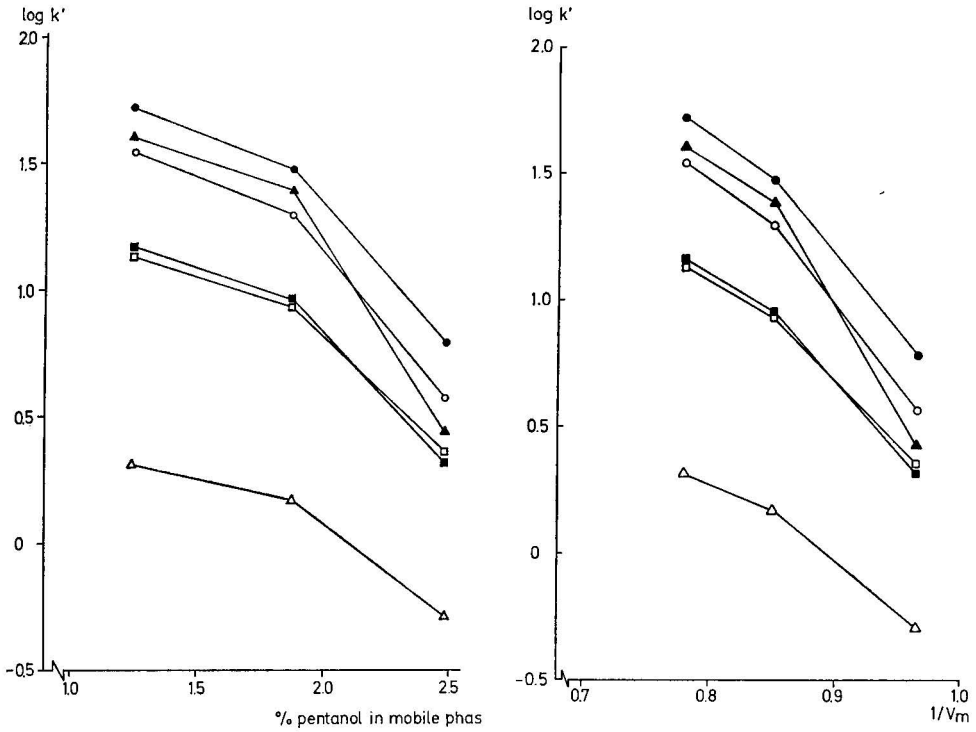

Fig. 1. Regulation of the capacity factor by addition of 1-pentanol. Mobile phase: phosphate buffer pH 6.5 + 1-pentanol. Flow-rate: 2.2 mm/sec. Support: LiChrosorb RP-8 (5 μm). Samples: ● estradiol 17β-glucuronide; ▲, estrone 3β-glucuronide; ○, estradiol 3β-glucuronide; ■, estriol 16α-glucuronide; □, estriol 17β-glucuronide; △, estriol 3β-glucuronide.

where D is the distribution ratio between stationary and mobile liquid phase, V_s and V_m are the volumes of the stationary and the mobile liquid phases, respectively, and V_t the sum of V_s and V_m. With increasing content of pentanol in the mobile phase, V_s increases[8] and V_m decreases. If eqn. 1 is valid and D is constant, the capacity factor should increase with increasing $1/V_m$. Fig. 1 shows, however, that there is a strong decrease of k' with increasing $1/V_m$. This indicates that the retention is mainly due to adsorption, which is in accordance with observations made for hydrophobic acids, amines and steroid glucuronides[8–10]. Increase of the pentanol content in the mobile phase also gives rise to changes in the separation selectivity, and there are even changes in retention order in some cases.

Separation efficiency

The separation efficiency is good in the systems containing 1.25 and 1.9% of pentanol, the reduced plate height at a flow-rate of 2.2 mm/sec being 5–9 for all compounds, except estriol 3β-glucuronide, where a reduced plate height of about 15 was obtained. Increase of the pentanol content to 2.5% strongly reduces the separation efficiency. This is illustrated in Fig. 2, which shows a drastic increase of the asymmetry of the peaks when the pentanol content is increased from 1.9 to 2.5%.

The sepation efficiency is fairly independent of the capacity ratio at $k' > 8$,

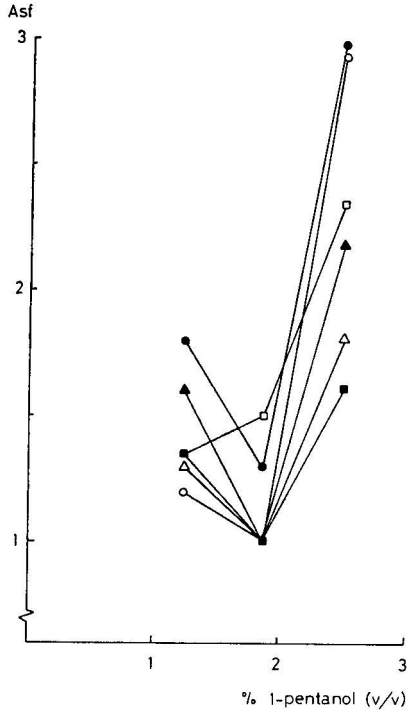

Fig. 2. Asymmetry factors obtained at different concentrations of 1-pentanol. For chromatographic data and samples see Fig. 1. Asf = back part of W_b/front part of W_b. Front and back part of W_b are calculated at the base of the peak, by drawing two tangents of the peak and a perpendicular from the formed vortex.

as demonstrated in Fig. 3. However, a considerable increase in the reduced plate height is observed when the capacity factor approaches unity, which may indicate influence of mass transfer in the stationary phase.

Chromatographic isolation of estriol conjugate from human urine

The chromatographic system could be used for isolation of estriol 16α-glucuronide from untreated human urine. A chromatogram obtained after injection of 20 μl of urine is shown in Fig. 4. Mobile phases containing 1.25% and 1.9% of pentanol gave similar chromatograms, but the retention of the glucuronide was somewhat higher at the lower 1-pentanol content.

The chromatographic systems showed good stability, capacity factors and separation efficiency remaining almost constant after injection of 50 urinary samples.

Identification the metabolite

The identity of the compound in peak 1 (Fig. 4) was established by GC–MS, as described under Experimental. The procedure involved hydrolysis with hydrochloric acid, extraction of the hydrolysis product with ethyl acetate and acetylation with acetic anhydride before injection into the GC–MS system. A solution of estriol in pyridine, treated with acetic acid anhydride in the same manner, was used as reference. The two mass spectra coincided. This indicates that the aglucone portion of the isolated compound is estriol.

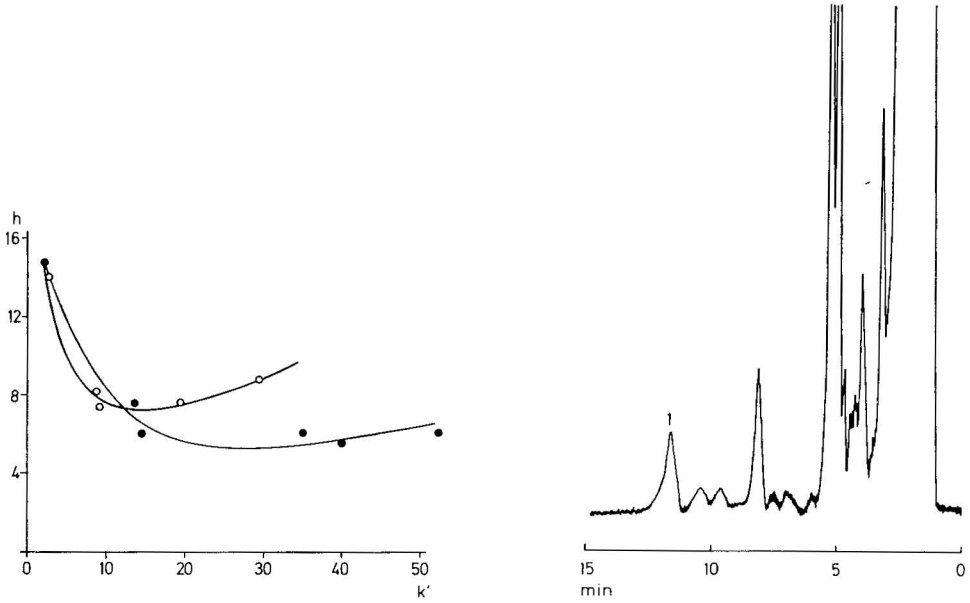

Fig. 3. Relation between the capacity factor and the separation efficiency. For chromatographic data and samples see Fig. 1.
Concentration of 1-pentanol (% v/v): ●, 1.25; ○, 1.9.

Fig. 4. Isolation of conjugated estriol from urine. For chromatographic data see Fig. 1, a.u.f.s. = 0.005.
Concentration of 1-pentanol in the mobile phase; 1.9%.
Peak 1 = estriol-16-glucuronide.

Pregnancy urine spiked with estriol 17α-glucuronide gave rise to a double peak. The addition of estriol 16β-glucuronide gave only one peak with a larger area than that obtained with unspiked urine. This indicates that the isolated metabolite is estriol conjugated at C-16 with glucuronic acid.

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Note

Simple and rapid separation of certain prostaglandins by reversed-phase high-performance liquid chromatography

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Prostaglandins (PGs) are diversely oxygenated eicosanoic acids with various physiological, pathological and pharmacological significance based on their biochemical responses. The simultaneous and quantitative determination of PGs in biological fluids is an important problem. Various methods have been developed for the determination of micro-amounts of these active but deteriorating compounds in biological samples[1]. Thin-layer chromatography[2], fluorimetry[3], gas–liquid chromatography (GC)[4,5], mass spectrometry (MS)[6] and gas chromatography–mass spectrometry (GC–MS)[7–14] have been used, and biochemical methods such as radioimmunoassay (RIA)[15] and enzyme assay[4,16], with a few bioassays by human platelet aggregation[9] and a smooth muscle response[17], are also useful. Of the biological methods, RIA has been most frequently applied in clinical investigations in recent years, but it is time consuming and has some limitations. One of the problems is the specificity and affinity of the antibody used in the RIA, as it is extremely difficult to obtain the antibody with little cross-reactivity and relatively high affinity. In addition, procedures for avoiding interferences from substances other than PGs have to be incorporated in the assay, which may also lead to a loss or alteration of PGs.

GC–MS has been shown to be the most reliable technique for the quantification of PGs. However, there are difficulties in accurate quantitative analyses, *e.g.*, complex pre-treatments and derivatization may be necessary, accompanied by changes in the PGs, in electron impact MS combined with GC[7–13] and also in ammonia chemical ionization MS combined with GC[14].

A rapid and efficient method for the isolation and purification of PGs is often required in various biological and clinical studies, and also a highly accurate analysis of many closely related PGs may be necessary. High-performance liquid chromatography (HPLC) has been increasingly applied to give separations of a series of PGs in high yields for this purpose. Fitzpatrick[18] utilized HPLC for the separation and analysis of PGs by using their *p*-nitrophenacyl esters. The preparation of the appropriate derivatives should throw light on the problem of the poorer sensitivity in the

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detection of these compounds using a fixed wavelength of 254 nm, with the drawbacks of the long time required and the decrease in the precision of the analysis caused by the derivatization. HPLC methods using silicic acid columns[19,20] and conventional reversed-phase chromatography[4,5] have not proved adequate for PG analysis because of the poor resolution and the long retention times, with unsatisfactory sample recovery.

Recently, the usefulness of an HPLC method using a reversed-phase column in combination with ordinary adsorption chromatography has been reported for the isolation of several PGs from a biological matrix[21]. However, this method required the inconvenient use of liquid scintillation spectrometry, and retention times were excessively long (about 60 min).

We describe here a simple, rapid and convenient method for the separation of prostaglandins such as 6K-$PGF_{1\alpha}$, $PFE_{2\alpha}$, PGE_2, PGE_1, PGA_2 (or PGB_2) and PGA_1 (or PGB_1) using reversed-phase HPLC.

EXPERIMENTAL

Apparatus

HPLC was carried out using an ATTO Corp. (Tokyo, Japan) solvent delivery system (Model HSLC-013-4) and a syringe-loaded loop injection valve (Model 7120) with an internal volume of 100 μl (Rheodyne, CA, U.S.A.). A column (25 cm $\times$ 4.6 mm I.D.) packed with Nucleosil 5 C_{18} (Merck, Darmstadt, G.F.R.) was used for reversed-phase chromatography. An ATTO-LDC Spectromonitor III was used to measure the absorbance at 208 nm. Chromatograms were recorded on a Rikaken R-21 recorder with a 10-mV span set at a chart speed of 2.0 cm/min. The solvent system used for elution was water–acetonitrile–tetrahydrofuran (70:30:2).

Reagents and materials

Acetonitrile and tetrahydrofuran used for the chromatography were purchased from Kanto Chemical (Tokyo, Japan). Water used for the chromatography was prepared by glass distillation and filtration with a TM-2 membrane filter (0.45 μm) (Tokyo Roshi, Tokyo, Japan). Standard samples of PGs were kindly supplied by Ono Pharmaceutical Co. (Osaka, Japan),to whom our thanks are due. All other chemicals used were special-grade materials.

Procedure

A solution of each sample of prostaglandins 6K-$PG_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, PGE_1, PGA_2, PGA_1, PGB_2 and PGB_1 as free acids in water–acetonitrile–tetrahydrofuran (70:30:2) was injected and eluted witht he same solvent system as above. The flow-rate was 1 ml/min under a pressure of 80 kg/cm^2 and the collected fractions were monitored with a UV detector at 208 nm (0.1–0.005 a.u.f.s.). Each compound separated was identified by GC–MS.

RESULTS AND DISCUSSION

The chromatographic peaks for 6K-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2 and PGE_1 occur at 2 min, 3 min 30 sec, 4 min and 4 min 40 sec, respectively, under the conditions

described above. A peak for a mixture of PGA_2 and PGB_2 and a peak for a mixture of PGA_1 and PGB_1 appear at 7 min 20 sec and 9 min 30 sec, respectively (Fig. 1). The retention times of PGA_2, PGB_2, PGA_1 and PGB_1 could be different from each other when injected separately. However, good resolutions of PGA_2 and PGB_2 and of PGA_1 and PGB_1 were not achieved with a mixture of all four. The identification of each PG separated was confirmed by GC–MS as usual[11,12]. The UV monitoring wavelength of 208 nm provided the best compromise between maximum sensitivity for each PG, efficiency of the detector and interferences from the solvents. PGs at levels of a few nanograms were detected by using this wavelength at 0.005 a.u.f.s.

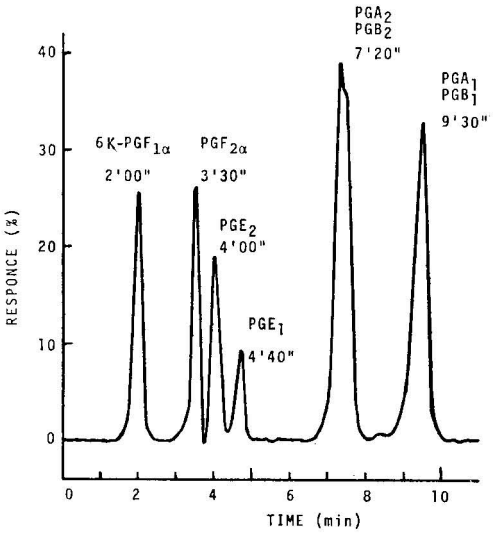

Fig. 1. Chromatogram produced by HPLC of prostaglandins (350 ng of each compound injected) as free acids on a reversed-phase column. Eluent, water–acetonitrile–tetrahydrofuran (70:30:2); pressure, 80 kg/cm^2; flow-rate, 1 ml/min; column, Nucleosil 5 C_{18} (25 cm × 4.6 mm I.D.); detection, UV, 208 nm (0.05 a.u.f.s.).

The proposed HPLC method thus seems useful for the separation of a large number of prostaglandins, including thromboxane B_2 (equivalent to thromboxane A_2) and leukotrienes, under neutral and mild conditions, in place of an alkaline and inadequate method[22]. A further study on the separation of PGA_2, PGB_2, PGA_1 and PGB_1 is in progress.

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Note

High-performance liquid chromatographic determination of major mycotoxins produced by *Alternaria* molds

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(Received February 4th, 1980)

Molds of the genus *Alternaria* are widely distributed in the environment. The genus is indigenous to soil, and many species are plant pathogens that damage crops in the field or cause postharvest decay. Because the *Alternaria* grow well at low temperatures, they are associated with extensive spoilage of fruits and vegetables held under refrigeration.

Dibenzo-α-pyrones including alternariol (AOH), alternariol monomethyl ether (AME), and altenuene (ALT)[1,2], plus tenuazonic acid (TeA), a derivative of tetramic acid (Fig. 1), have been reported as major *Alternaria* metabolites and mycotoxins[3–7]. These compounds are of current interest because of their toxicity and because they have been isolated from a number of food and feed materials contaminated with various species of *Alternaria*. Adequate methods are needed to assess the amounts of these mycotoxins in fruits and vegetables, which frequently are infested with *Alternaria* but are uncharacterized as substrates for the production of mycotoxins by this genus. Seitz and Mohr[8] reported separation of ALT, AOH and AME with gradient elution of a normal-phase, high-performance liquid chromatography (HPLC) column with a solvent system of isooctane and tetrahydrofuran. The disadvantages of gradient elution and the use of tetrahydrofuran plus our need for a rapid method for determining ALT, AOH, AME and TeA in fruits and vegetables prompted us to seek alternate HPLC parameters.

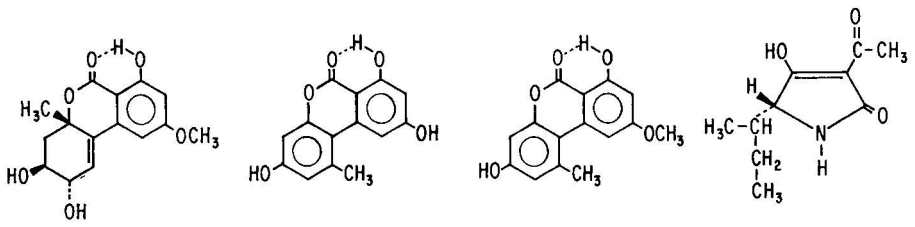


Fig. 1. Structures of four major mycotoxin metabolites produced by species of the genus *Alternaria*.

EXPERIMENTAL

Samples of AOH and AME were obtained from D. J. Harvan (National Institutes of Health, Research Triangle Park, N.C., U.S.A.). ALT was obtained from L. M. Seitz (U.S. Grain Marketing Research Laboratory, Manhattan, KS, U.S.A.). Additional quantities were prepared by fermentation of autoclaved rice with *Alternaria* strains obtained from the Northern Regional Research Center, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Peoria, Il., U.S.A. (No. 5255; donated by C. E. Main as M-1) and the American Type Culture Collection, Rockville, MD, U.S.A. (No. 34457; donated by Rosemary Burroughs as RL8442-2).

AOH, AME and ALT were isolated and purified by column and thin-layer chromatography (TLC) and recrystallization[9]. The identities of AOH, AME and ALT were confirmed by mixed melting point, R_F on TLC plates (two solvent systems) and mass spectrometry[10]. TeA was isolated by extracting a concentrated chloroform–ethanol (4:1, v/v) extract of fermented rice with aqueous 5% $NaHCO_3$, acidifying the aqueous phase to pH 2.0 and extracting it with chloroform. The identity and purity of TeA were confirmed by TLC and mass spectrometry.

Standard solutions containing 0.1 μg of ALT, AOH, AME and TeA per μl of methanol were prepared and used to evaluate HPLC separation parameters. The HPLC system was assembled from Waters Assoc.* components (solvent delivery system, Model 6000 A; injection system, Model U6K; and variable wavelength UV detector, Model 450) and a column that was 30 cm $\times$ 3.9 mm I.D. and packed with 10 μm, reversed-phase, μBondapak C_{18} (monomolecular layer of organosilane bonded to porous silica particles). The system was operated isocratically with two experimental binary solvent systems consisting of methanol–water and acetone–water. The proportions of each organic solvent and water were varied over a range of 9:1 to 6:4 (v/v), respectively, to determine optimum proportions for separation of the mycotoxins. Detector wavelengths were 324 nm for AOH, ALT and AME and 278 nm for TeA. Since acetone absorbs strongly at 278 nm, the acetone–water solvent system was not useful for separations involving TeA.

Chloroform extracts of fruits and vegetables that were infected with *Alternaria* were prepared by homogenizing 200 g of tissue in a blender, adjusting the pH of the homogenate to 2.0 and extracting with two 500 ml portions of chloroform as described by Stinson *et al.*[11]. After drying over anhydrous Na_2SO_4 and concentrating to 25 or 50 ml under a nitrogen stream, extracts were ready for HPLC analysis.

RESULTS AND DISCUSSION

With both the methanol–water and the acetone–water solvent systems, baseline separations of authentic ALT, AOH and AME were obtained with resolution greater than 1.5. As expected for these three compounds, the capacity ratio (k') (based on the unretained solvent peak) for each compound increased as the amount of water, the more polar solvent in each binary system, was increased (Table I).

* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

TABLE I

VALUES OF k' FOR ALTENUENE (ALT), ALTERNARIOL (AOH), ALTERNARIOL MONOMETHYL ETHER (AME) AND TENUAZONIC ACID (TeA) ON A REVERSED-PHASE, μBONDAPAK C_{18} COLUMN WITH TWO BINARY SOLVENT SYSTEMS CONTAINING VARIED PORTIONS OF ORGANIC SOLVENT AND WATER

$k' = (R_{t_1} - R_{t_0})/R_{t_0}$; R_{t_0} = retention time of solvent front; R_{t_1} = retention time of compound.

Solvent system	*k' Values*			
	ALT	*AOH*	*AME*	*TeA*
Methanol–water				
90:10	0.09	0.18	0.92	3.7
80:20	0.26	0.60	1.46	2.5
65:35	0.66	1.72	5.34	1.4
Acetone–water				
65:35	0.30	0.59	1.38	
60:40	0.54	1.28	2.82	

In analytical practice, the methanol–water system was not satisfactory for the analysis of chloroform extracts of *Alternaria* infected tomatoes, apples, and blueberries because an interfering substance eluted concurrently with ALT; attempts to resolve the problem by adjusting flow-rate and the ratio of methanol–water in the solvent system were unsuccessful. Acetone–water (65:35, v/v) at a flow-rate of 0.4 ml/min was entirely satisfactory for separating and quantifying ALT, AOH and AME in such extracts. In addition to extracts being spiked with authentic compounds, the identities of ALT, AOH and AME separated from extracts were substantiated further in the HPLC eluate collected in fractions corresponding to the retention volumes of the authentic compounds, then concentrated, and spotted on TLC plates; TLC R_F

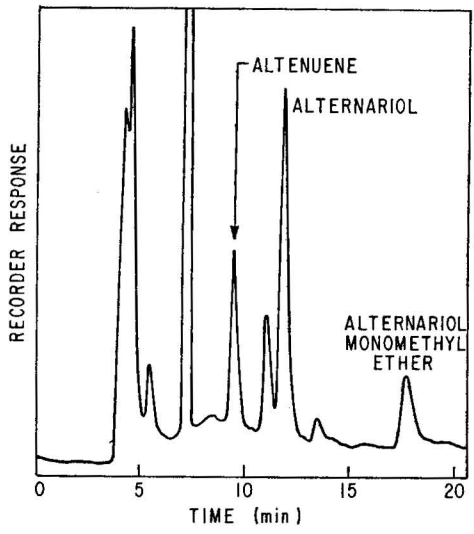


Fig. 2. HPLC separation of a chloroform extract of tomato tissue infected with *Alternaria*. HPLC parameters were: reversed-phase, μBondapak C_{18} column, 30 cm × 3.9 mm I.D.; acetone–water solvent (65:35, v/v), 0.4 ml/min; detection at 324 nm; sensitivity at 0.02 a.u.f.s.

values agreed with those of authentic compounds and no extraneous compounds were found. An example of a chromatogram obtained with a chloroform extract of tomato tissue infected with *Alternaria* is presented in Fig. 2. Standard curves were developed with known amounts of authentic ALT, AOH and AME (Fig. 3), and recorder response was linear throughout the range of weights used to establish the curve with the stated operating conditions.

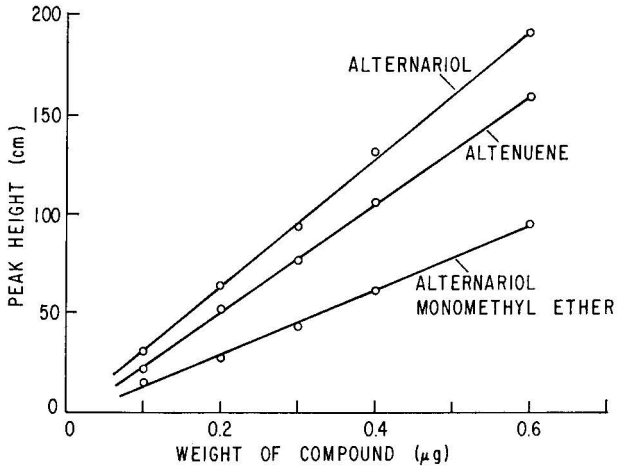
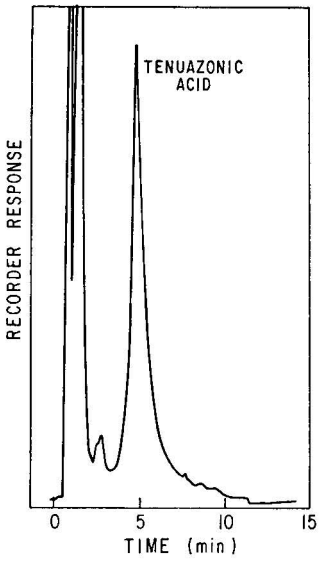


Fig. 3. Standard curves for weights of altenuene, alternariol and alternariol monomethyl ether *vs.* HPLC peak heights (measured peak height × attenuation factor).

The separation and quantitation of TeA was possible with the same μBondapak C_{18} column, but a different solvent system, methanol–water, was necessary. Other solvent systems, acetonitrile–water, ethanol–water and chloroform–methanol, were unsatisfactory. Methanol–water acidified with 0.1–0.2% acetic acid gave sharper peaks for TeA, but reproducible results were not obtained. With methanol–water, the k' value for TeA decreased with increasing proportions of the more polar solvent, water (Table I), which suggests that TeA is subject to normal-phase chromatography on this column. The optimum proportions of methanol–water were 9:1 (v/v), with a flow-rate of 2.0 ml/min for separation and quantitation of TeA in chloroform extracts of fruits and vegetables; a chromatogram obtained under these conditions with an extract of tomato tissue infected with *Alternaria* is shown in Fig. 4. No substances other than TeA were found when this fraction of the eluate was collected from twelve HPLC separations of extracts, concentrated and spotted on TLC plates. Recorder response was linear for the range of weights of authentic TeA used to establish a standard curve (Fig. 5).

Calculated as peak height (cm) divided by weight of compound (μg), relative recorder responses for ALT, AOH, AME and TeA were 264, 320, 154 and 52, respectively, under the stated conditions for analyzing extracts.

The precision of the HPLC method was determined with a chloroform extract of tomato tissue that had been inoculated with spores of a wild strain of *Alternaria* and incubated for 20 days at 20 °C. A series of 10 or 15 determinations for ALT,



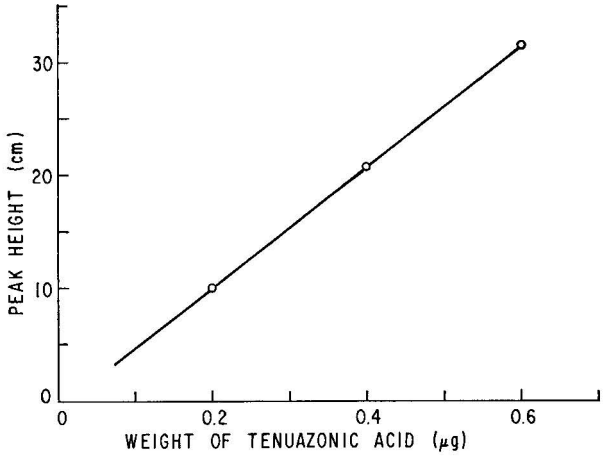


Fig. 4. HPLC separation of a chloroform extract of tomato tissue infected with *Alternaria*. HPLC parameters were: reversed-phase, μBondapak C_{18} column, 30 cm × 3.9 mm I.D.; methanol–water solvent (9:1, v/v), 2.0 ml/min; detection at 278 nm; sensitivity at 0.01 a.u.f.s.

Fig. 5. Standard curve for weight of tenuazonic acid *vs.* HPLC peak height (measured peak height × attenuation factor).

AOH, AME and TeA was made, and the standard deviation of the values obtained for the concentration of each compound was calculated (Table II).

As described herein, a reversed-phase, μBondapak C_{18} HPLC column eluted with acetone–water (65:35, v/v), for ALT, AOH and AME determinations or methanol–water (9:1, v/v), for TeA determinations now is used routinely in this laboratory to assess the contamination of a number of fruits and vegetables with the major mycotoxins produced by species of the genus *Alternaria*. The method requires only an isocratic HPLC system and readily available, relatively non-toxic solvents and has proven applicable to crude extracts of the plant materials we have analyzed thus far. These HPLC parameters should be useful for the determination of ALT, AOH, AME and TeA in extracts of other commodities, provided that interfering substances are not present in the extracts.

TABLE II

STANDARD DEVIATIONS OBTAINED FROM REPLICATE HPLC ANALYSES OF A CHLOROFORM EXTRACT OF TOMATO TISSUE INFECTED WITH *ALTERNARIA*

Compound	*n*	*Average concentration found (μg/μl)*	*S.D.*	*Relative S.D. (%)*
Altenuene	10	0.34	0.014	4.1
Alternariol	10	3.05	0.059	1.9
Alternariol monomethyl ether	10	1.01	0.048	4.7
Tenuazonic acid	15	1.32	0.071	5.4

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Note

Affinity chromatography of rat liver lactate dehydrogenase on the Remazol derivative of bead cellulose

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(Received January 2nd, 1980)

Much attention has been paid to anthraquinone–triazine derivatives, which have been used as general affinants in the affinity chromatography of enzymes[1,2] and other proteins[3]. The most frequently used carriers of these affinants are polysaccharides such as agarose and dextran. However, some of their hydrodynamic properties and the economy of their use are not always advantageous.

Cibacron Blue F3G-A has been used most frequently as an anthraquinone–triazine affinant. So far, experiments using powdered cellulose as a carrier of Cibacron Blue for the affinity chromatography of enzymes and serum proteins have not offered encouraging results[2–4]. The properties of macroporous bead cellulose, however, differ from those of cellulose. Its superior hydrodynamic properties, regular geometric shape and high porosity[5] on the one hand and its low price on the other have made it attractive for chromatographic procedures.

Previous experiments with Cibacron Blue have shown that the functional part of this large molecule, resembling the structure of nucleotides, has an anthraquinone arrangement[6]. The affinity of some enzymes, mainly NAD(P)-dependent dehydrogenases of animal origin, *e.g.*, lactate, malate and glyceraldehyde-3-phosphate dehydrogenases, towards this arrangement of the molecule is related to the presence of a "dinucleotide fold" super-secondary structure in these enzymes[1,2,6]. Our results have also indicated that some simpler anthraquinone arrangements, even without the triazine part, in the form of water-insoluble derivatives show higher affinities towards the given enzymes than the Cibacron Blue derivatives of these polysaccharides[7]. This paper deals with the application of one of these compounds, Remazol Brillant Blue R, as a derivative of bead cellulose for the affinity chromatography of lactate dehydrogenase from rat liver extracts.

EXPERIMENTAL

Preparation of the Remazol Brillant Blue derivative of bead cellulose

The Remazol Brillant Blue (RBB) derivative of bead cellulose (20–320 μm) was prepared by suspending 5 g of bead cellulose (0.61 g of dry cellulose) in 10 ml of 0.25 *M* sodium hydroxide solution followed by reaction, under continuous stirring, with 0.1 g of the dye EE AB 505 Remazol Brillant Blue R, C.I. Reactive Blue 19 (Farbwerke Hoechst, Frankfurt/Main, G.F.R.) at 25 °C for 1 h. The product was

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washed thoroughly with distilled water until the washings were colourless, then distilled water containing 0.02 % of sodium azide was added and the slurry was stored at 0–4 °C. The amount of dye bound to the dry cellulose was determined to be 65 μmole/g from the visible absorption of the solvent phase after the coupling reaction and using a molar absorptivity of 5930 l/mole·cm for RBB in water at 590 nm.

Affinity chromatography of lactate dehydrogenase from rat liver homogenate

In the preparation of 20 % ethanol–10 m*M* Tris buffer extract from rat livers[8], a 20 m*M* Tris–hydrochloric acid buffer of pH 7 containing 2 m*M* of EDTA was used instead of 0.5 *M* sodium chloride solution. Livers were obtained from male white rats (Wistar). Two procedures were applied in the purification of crude lactate dehydrogenase (LDH) by affinity chromatography on the RBB derivative of bead cellulose.

Procedure 1. A 10-ml volume of the RBB derivative of bead cellulose was washed with 25 ml of a 0.1 % solution of bovine serum albumin, 100 ml of 10 m*M* Tris–hydrochloric acid buffer (pH 7.5) containing 1 m*M* of EDTA and 2 m*M* of 2-mercaptoethanol (solution A), 100 ml of 1 *M* sodium chloride solution and finally with 200 ml of solution A. To a column thus prepared (10 ml; 20 × 0.9 cm), a portion of rat liver extract (25 ml) containing 2-mercaptoethanol at a final concentration of 2 m*M* and with the pH adjusted to 7.5 was applied. The column was then washed with 230 ml of solution A. The bound LDH was eluted with 120 ml of solution A containing 1 m*M* of reduced nicotinamide adenine dinucleotide (NADH) (solution C).

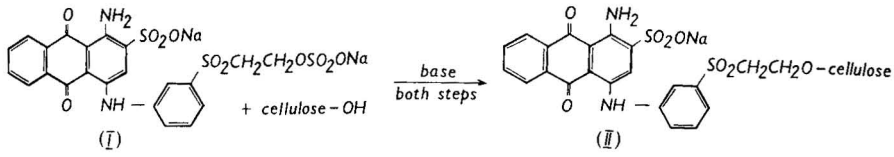
Procedure 2. This was the same as procedure 1 except that after washing with solution A elution was carried out with 100 ml of solution A containing 1 m*M* of nicotinamide adenine dinucleotide (NAD) (solution B) and finally with 160 ml of solution C.

For repeated applications, the column was finally washed, in both instances, with 60 ml of 1 *M* sodium chloride solution.

Fractions of 10 ml were collected at a flow-rate of 12 ml/h and suitable aliquots from each fraction were assayed for LDH activity and protein concentration. The catalytic activity of LDH was determined spectrophotometrically[9] and the protein concentration with bovine serum albumin as the standard[10]. Aliquots from the effluent with the highest specific activity were examined by electrophoresis on polyacrylamide gel. Slab gel electrophoresis was performed on SDS polyacrylamide gel (10 %, w/w)[11] (80 V, 30 mA) for 3.5 h at 25 °C, without addition of thiol. A 10–20-μg amount of sample was applied. Standard proteins, bovine pancreas ribonuclease and chymotrypsin, hen ovalbumin and bovine serum albumin were supplied by Calbiochem (San Diego, CA, U.S.A.).

RESULTS AND DISCUSSION

Etherification of the cellulose hydroxyl groups with Remazol Brillant Blue R (I) proceeds in two steps[12] (summarized in Scheme 1) and, in contrast with Cibacron Blue F3G-A[1–4], under mild reaction conditions. Alkali metal hydroxides promote the reaction. The vinylsulphonyl group formed in the first step etherifies the polysaccharide in the second step and the product is formed with a high degree of conversion. The RBB derivative of the bead cellulose (II) had a high stability.



(I) (II)

Scheme 1.

Of the several anthraquinone–triazine derivatives of bead cellulose, including Cibacron Blue F3G-A, the RBB derivative has been shown to be the best for chromatographic purposes [7].The RBB derivative of the bead cellulose used was found to have a binding capacity of 1.5 mg/ml of rabbit muscle LDH (Biochemica Boehringer, Mannheim, G.F.R.) or 0.6 mg/ml of bovine serum albumin (Sevac, Prague, Czechoslovakia).

In previous experiments it was shown[1,2,6,7] that NADH was the best eluent of LDH from the antraquinone–triazine derivatives of polysaccharides. The immediate elution of LDH from the RBB derivative of bead cellulose was also achieved when a 1 m*M* solution of NADH in equilibrium buffer (solution C) was used. The purification factor of LDH from the rat liver extracts may be increased by prior elution with 1 m*M* NAD (solution B), but part of the LDH is also released. Successive elution with 1 m*M* NADH, on other hand, resulted in an almost 25-fold enrichment of LDH (Table I).

The differences between elution in two steps (procedure 2) and a single elution with 1 m*M* NADH (procedure 1) are also apparent from Fig. 1. Procedure 2 gives a purer fraction of LDH. The LDH monomer, having a molecular weight of 35,000, is within the range of simple polypeptides of chymotrypsin (molecular weight 22,600) and ovalbumin (molecular weight 45,000).

The purification factor of LDH achieved by affinity chromatography of rat liver extract by the procedure 2 is higher than that achieved in four steps of the traditional seven-step procedure[8]. In addition to the practical advantage of time saving,

TABLE I

SUMMARY OF THE PURIFICATION PROCEDURES

Step No.	*Step*	*Volume (ml)*	*Protein (mg)*	*Total activity (U)*	*Specific activity (U/mg)*	*Purification factor*	*Yield (%)*
1	Procedure 1: ethanol–Tris buffer extract	25	310	473	1.5	1.0	100
2	RBB–bead cellulose, affinity elution, NADH eluate	120	18	431	24.0	15.7	91
1	Procedure 2: ethanol–Tris buffer extract	25	319	452	1.4	1.0	100
2	RBB–bead cellulose, affinity elution, NADH eluate after NAD elution	160	8	266	33.3	23.4	59

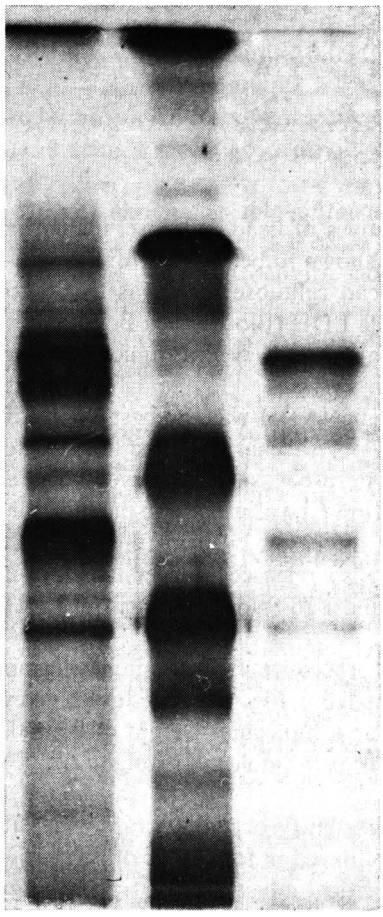


Fig. 1. Slab polyacrylamide gel electrophoresis of samples obtained from the affinity chromatography of extract of rat liver. Left, aliquot from fraction 24 (procedure 1); centre, mixture of standard proteins in the direction of electrophoresis (chymotrypsin, ovalbumin and bovine serum albumin); right, aliquot from fraction 34 (procedure 2).

the higher yields achieved are of importance. Thus, with procedure 1 a 91% yield of LDH was obtained, compared with 58% with the four-step procedure[8]. It is also of importance that the RBB derivative of bead cellulose can be used several times after previous elution with 1 *M* sodium chloride solution.

A similarly successful isolation of LDH from rabbit muscle was achieved[7]. Finally, it may be suggested that the proper selection of anthraquinone–triazine affinants linked to bead cellulose could enable us to use these products for the affinity chromatography of some other enzymes.

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Note

Detection of aminocarb and its major metabolites by thin-layer chromatography

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Aminocarb (Matacil®), 4-dimethylamino-*m*-tolyl N-methylcarbamate, a broad spectrum, non-systemic insecticide, has been used extensively for controlling the spruce budworm (*Choristoneura fumiferana* Clem.) in eastern Canada since 1976[1,2]. Very little is known about the metabolism of this pesticide in the environment. To study the fate of this chemical in the ecosystem, a sensitive method for the detection of aminocarb and its major metabolites is necessary. Strother[3] has used two-dimensional thin-layer chromatography (TLC) to isolate and identify the methylamino, amino and hydroxymethyl analogues from the *in vitro* metabolism of aminocarb by liver homogenates from humans and rats. Balba and Saha[4] obtained similar results with one-dimensional TLC using diethyl ether as the developing solvent. In this paper we describe a simple TLC technique using either (a) hexane–acetone (1:1, v/v) or (b) diethyl ether–hexane–ethanol (77:20:3, v/v) as the developing solvent for the separation of aminocarb and its major metabolites on silica gel G or silica gel F_{254}, along with two visualization techniques.

EXPERIMENTAL

Chemicals for chromatography

Analytical grade (>99%) aminocarb (4-dimethylamino-*m*-tolyl N-methylcarbamate), MA (4-methylamino-*m*-tolyl N-methylcarbamate), AM (4-amino-*m*-tolyl N-methylcarbamate), MFA (4-methylformamido-*m*-tolyl N-methylcarbamate) and FA (4-formamido-*m*-tolyl N-methylcarbamate) supplied by Chemagro (Mississauga, Canada) were used in this study.

Solvent system and development of the plates

Two types of thin-layer plates were used: (1) silica gel F_{254} (0.5 mm thick) precoated plate (20 cm × 20 cm) and (2) glass plate (20 cm × 20 cm) coated with silica gel G (0.5 mm thick). All thin-layer plates were heated in the oven at 110 °C for 1 h before use. Aminocarb and its metabolites (5 μg each in 100 μl acetone) were spotted on the plate, 1.5 cm above the lower edge, and dried under a gentle stream of nitrogen. The spot size was maintained at about 0.75 cm in diameter. The spotted plates were developed in a glass tank saturated with the developing solvent. The two

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solvent systems tested were: (1) hexane–acetone (1:1, v/v, pesticide-grade) and (2) diethyl ether–hexane–ethanol (77:20:3, v/v, pesticide-grade). The developed plates were removed from the tank when the solvent front was 15 cm from the origins. They were air dried, then sprayed with chromogenic reagents for visualization.

Spot visualization

Two spot visualization techniques were used. (1) Ninhydrin spray: the air dried plates were sprayed with sodium hydroxide solution (10% aq.) in the fume hood, heated in the oven at 60 °C for 3–5 min, then sprayed with ninhydrin (2% in ethanol), followed by heating in the oven at 60 °C for 30 min. Aminocarb and its metabolites appeared as pink spots. (2) Cholinesterase inhibition: cholinesterase was prepared from fresh pig liver[5]. The air dried plates were sprayed gradually and evenly with the pig liver homogenate until thoroughly wet. The plates were allowed to dry at room temperature for 30 min, then sprayed with the freshly prepared substrate spray in the same manner as the pig liver homogenate. The substrate spray consisted of a 20-ml mixture of two solutions as follows: solution A was prepared by dissolving 20 mg of 5-bromoindoxyl acetate in 5 ml absolute ethanol, ferrocyanide solution (0.416 g of potassium ferricyanide and 0.52 g potassium ferrocyanide in 25 ml distilled water) with 13 ml of 0.05 *M* Tris buffer. Aminocarb and its metabolites appeared as white spots on blue background 30 min after spraying.

Application in metabolite separation and identification

To evaluate the applicability of the above-described TLC method in metabolic study, an experiment to isolate and identify aminocarb and its metabolites was conducted. Rainbow trout, *Salmo gairdneri* Richardson, was exposed to 15.0 ppm of aminocarb in the aquarium at 10 °C for 144 h. At the end of exposure, aminocarb and its metabolites were extracted from fish tissues (whole fish) with ethyl acetate and analyzed by gas–liquid chromatography–alkali flame-ionization detection (GLC–AFID)[6]. The identities of aminocarb and its metabolites were also confirmed by TLC as described in this paper.

RESULTS AND DISCUSSION

The two developing solvent systems used in this study gave good separation of aminocarb and its metabolites, MA, AM, MFA, FA on both silica gel G and F_{254} (Table I). MFA and FA did not resolve completely on silica gel F_{254} using hexane–acetone (1:1, v/v) as the developing solvent. The R_F values for MFA and FA were 0.46 and 0.42, respectively. Silica gel G and F_{254} showed similar separation characteristics. In general, better resolution was obtained with hexane–acetone (1:1, v/v) as the developing solvent.

Ninhydrin is a common chromogenic reagent for the detection of amino acids, amines and amino sugars. The color reaction with the ninhydrin spray observed in this study was due to the amines formed from the alkaline hydrolysis of N-methylcarbamate esters. Consequently, this technique was specific for nitrogen. On the other hand, the cholinesterase inhibition technique was specific for the detection of cholinesterase inhibitors such as aminocarb and its metabolites that retained the carbamate moiety. Thus, a combination of both techniques will offer a high degree of specificity for detecting aminocarb and its major metabolites.

TABLE I

R_F VALUES OF AMINOCARB AND ITS MAJOR METABOLITES RESOLVED WITH TWO DIFFERENT SOLVENT SYSTEMS ON SILICA GEL G AND SILICA GEL F_{254}

Compound	*R_F values*			
	Silica gel G		*Silica gel F_{254}*	
	Hexane–acetone (1:1, v/v)	*Diethyl ether–hexane–ethanol (77:20:3, v/v)*	*Hexane–acetone (1:1, v/v)*	*Diethyl ether–hexane–ethanol (77:20:3, v/v)*
Aminocarb	0.81	0.70	0.74	0.67
MA	0.70	0.57	0.66	0.53
AM	0.53	0.40	0.52	0.37
MFA	0.45	0.30	0.46	0.28
FA	0.39	0.22	0.42	0.20

Aminocarb and its metabolites, namely MA and AM were detected in rainbow trout exposed to aminocarb for 144 h by GLC–AFID. The column (183 cm × 2 mm I.D.) used in this investigation was 1.0% OV-17 + 1.0% OV-210 on Ultra-Bond 20 M, 80–100 mesh. The column temperature was 180 °C isothermal. A typical chromatogram is given in Fig. 1. The minor peaks appeared in the chromatogram were

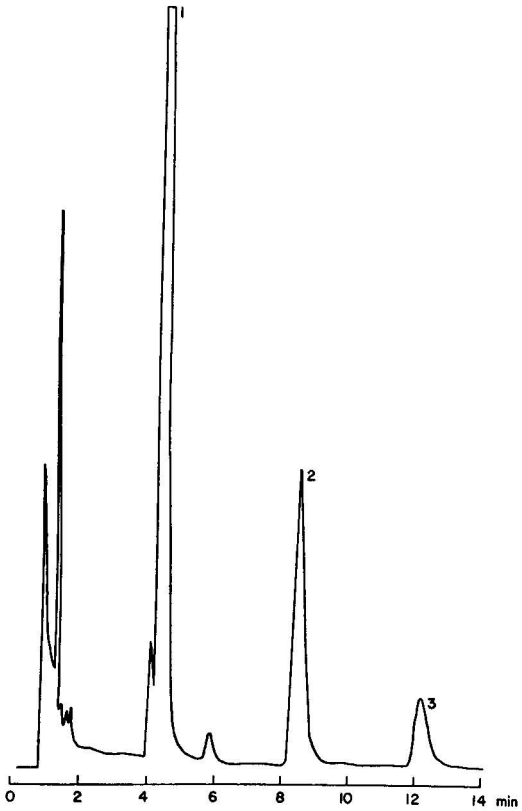

Fig. 1. Gas chromatogram of rainbow trout exposed to 15 ppm aminocarb for 144 h. Peaks: 1 = aminocarb; 2 = AM; 3 = MA.

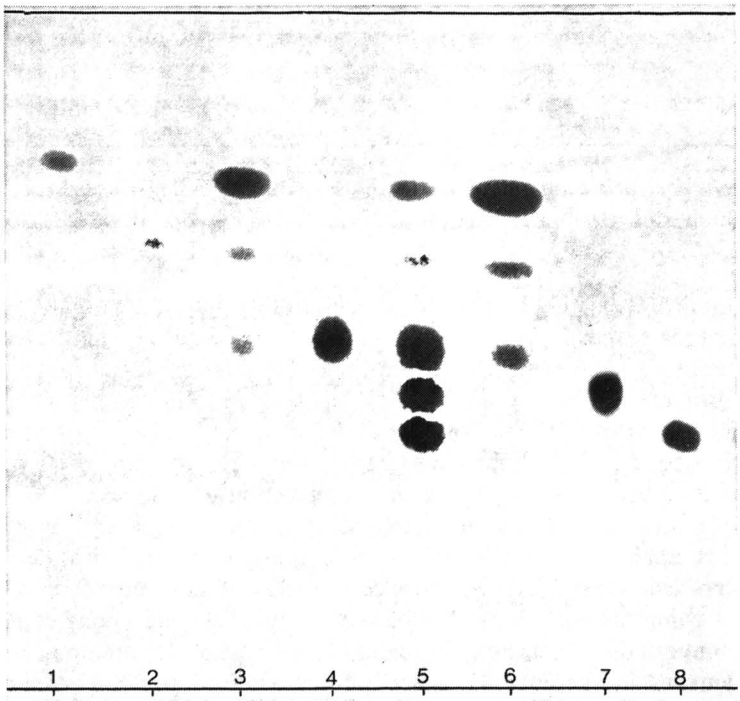

Fig. 2. Thin-layer chromatogram of rainbow trout exposed to 15 ppm aminocarb for 144 h. Spots 1, 2, 4, 7 and 8: 5 μg each of aminocarb, MA, AM, MFA and FA, respectively; spot 5: a mixture of all five; spots 3 and 6: rainbow trout tissue extracts.

also observed in fish from the control group indicating that they were naturally occurring compounds present in fish tissues. The identities of aminocarb and its 4-methylamino and 4-amino analogues were confirmed by TLC using either of the visualization techniques described in this paper. A typical thin-layer chromatogram on silica gel G using hexane–acetone (1:1, v/v) as the developing solvent and ninhydrin as the chromogenic agent is given in Fig. 2. Similar results were also obtained with cholinesterase inhibition.

In summary, the TLC technique described in this paper gives good separation of aminocarb and its metabolites and is very sensitive. Therefore, it will be a useful tool for the detection and confirmation of aminocarb and its major metabolites in environmental samples.

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Book Review

Handbook of analytical derivatization reactions, by D. R. Knapp, Wiley-Interscience, New York, Chichester, Brisbane, Toronto, 1979, XIX + 741 pp., price £ 21.50, ISBN 0-471-03469-X

The author claims that existing books and reviews on this subject are organized according to derivative or reagent type and that the practioner is also in need of a reference work that considers the sample first and looks for possibilities of how to solve his particular problem. Following this line, he has therefore limited his introduction to a very brief treatment of the philosophy of derivatization in gas chromatography, mass spectrometry and liquid chromatography. Only pre-column reactions are mentioned, without considering ion pairing or complexation techniques.

Derivative types and reagents are summarized in about 6 pages and about 10 pages are devoted to apparatus and the experimental approach in analytical derivatization on the micro-scale. This is followed by a compilation of data and references for various groups of compounds such as (1) hydroxyl, sulphydryl and epoxy compounds; (2) amino compounds; (3) carboxylic acids; (4) fatty acids; (5) amino acids and peptides; (6) aldehydes and ketones; (7) other N-functional groups; (8) phospho and sulpho compounds; (9) optical isomers; (10) fatty lipids; (11) steroids; (12) prostaglandins; (13) carbohydrates; (14) nucleotides; and (15) drugs.

For all these, possible reactions, including structures and comments for the reaction conditions and typical procedures, are presented. A more critical comparison of the various procedures could be helpful but might be difficult to realize with the vast amount of information given. References are included with each reaction, because often not enough details are available to reproduce a reaction technique without resorting to the original literature. In this sense the book only partly fulfills the function of a laboratory bench source.

As a reference source for quickly finding useful information in this field it is unsurpassed by any other publication seen so far. A comprehensive system of indexes permits easy access to this information. This includes also a derivative and reagent index and a list of suppliers world-wide. The literature is covered up to 1977 and is about as complete as it can be with such a complex subject.

The errors do not exceed the usual tolerance level in this type of reference source. Although the author states in the preface "Encyclopaedic- and compendium-type *complications* of reagents and methodology have proven to be extremely useful in the area of chemistry", it is to be assumed that he means "*compilations*" and that he intends to reduce the complications by introducing his book.

It can be concluded that this book will serve a useful purpose and should be valuable to anyone concerned with derivatization techniques in the fields of gas chromatography, mass spectrometry and liquid chromatography and to the many analytical chemists who have to deal with complex real samples.

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See 1910.

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See also 1958.

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17c. Amine derivatives and amides (excluding peptides)

See 1951.

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1922 Coutts, R.T., Jones, G.R. and Liu, S.-F.: Quantitative gas chromatography/mass spectrometry of trace amounts of glutamic acid in water samples. *J. Chromatogr. Sci.*, 17 (1979) 551-554.
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See also 1960.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

23c. Indole derivatives

See 1889, 1914, 1919.

23d. Pyridine derivatives

See 1889, 1966.

23e. Other N-heterocyclic compounds

See 1799.

24. ORGANIC SULPHUR COMPOUNDS

1930 Golovnya, R.V., Garbuzov, V.G. and Aerov, A.F.: (Gas chromatographic characteristics of sulfur-containing compounds. 5. Thiophene, furan and benzene derivatives). *Izv. Akad. Nauk SSSR, Ser. Khim.*, No. 11 (1978) 2543-2547.
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See 1807.

29f. Other types of pesticides and various agrochemicals

See 1978.

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See also 1967.

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See also 1919.

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See also 1929, 1959.

32f. Clinico-chemical applications and profiling body fluids

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33. INORGANIC COMPOUNDS

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See also 1982.

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1972 Mitchell, D.N. and Le Roy, D.J.: Theoretical and experimental behaviour of a thermal conductivity detector for the determination of the ortho/para hydrogen composition of gas mixtures in the temperature-jump region. *Can. J. Chem.*, 56 (1978) 1817-1726.

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See also 1884.

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35. SOME TECHNICAL PRODUCTS AND COMPLEX MIXTURES

35c. Various technical products

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See also 1890.

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See also 1816.

37. ENVIRONMENTAL ANALYSIS

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37d. Soil pollution

See 1976.

Liquid Column Chromatography

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4. SPECIAL TECHNIQUES

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See 2019, 2035.

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4d. Affinity chromatography

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20. ENZYMES

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20h. Isomerases

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Thin-Layer Chromatography

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37c. Water pollution

See 2864, 2958.

37d. Soil pollution

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Electrophoretic Techniques

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4c. Isoelectric focusing

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18. AMINO ACIDS AND PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS

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3174 Pham van Minh and Kadas, L.: (Polyacrylamide gel electrophoresis of milk preparations). *Elelmiszervizsgalati Kozl.*, 24 (1978) 73-77; *C.A.*, 91 (1979) 89709n.

19i. Proteins of neoplastic tissue

3175 Nakaya, K., Ushiwata, A., Nakajo, S. and Nakamura, Y.: Proteins exposed on the outer surface of the plasma membranes of AH-66 hepatoma ascites cells. *J. Biochem.*, 85 (1979) 183-189 - polyacrylamide gel.
3176 Percy, M.E., Chang, L., Demoliou, C. and Baumal, R.: The kinetics of *in vitro* reoxidation and reduction of the inter heavy-light chain disulfide bond in an unusual murine immunoglobulin G myeloma protein lacking inter-heavy chain disulfide bonds. *Can. J. Biochem.*, 57 (1979) 279-285 - SDS-polyacrylamide gel.

19j. Specific binding proteins

3177 Lapresle, C. and Wal, J.-M.: The binding of penicillin to albumin molecules in bisalbuminemia induced by penicillin therapy. *Biochim. Biophys. Acta*, 586 (1979) 106-111 - polyacrylamide-agarose gel.
3178 Sweet, G.D., Somers, J.M. and Kay, W.W.: Purification and properties of a citrate-binding transport component, the C protein of *Salmonella typhimurium*. *Can. J. Biochem.*, 57 (1979) 710-715 - isoelectric focusing, SDS-polyacrylamide gel.
3179 Williams, P.F. and Turtle, J.R.: Purification of the insulin receptor from human placental membranes. *Biochim. Biophys. Acta*, 579 (1979) 367-374 - SDS-polyacrylamide gel.

19k. Urinary proteins

3180 Barthelemy-Clavey, V., Yapo, E.A., Vanhoutte, G., Hayem, A. and Mizon, J.: Purification et caracterisation des inhibiteurs de proteases de l'urine humaine. *Biochim. Biophys. Acta*, 580 (1979) 154-165 - SDS-polyacrylamide gel, immunoelectrophoresis, isoelectric focusing.

19l. Other proteins

3181 Dahl, D. and Bignami, A.: Astroglial and axonal proteins in isolated brain filaments. I. Isolation of the glial fibrillary acidic protein and of an immunologically active cyanogen bromide peptide from brain filament preparations of bovine white matter. *Biochim. Biophys. Acta*, 578 (1979) 305-316 - SDS-polyacrylamide gel.

3182 Hayashi, H. and Koiwai, O.: (Use of two-dimensional gel electrophoresis for the identification of a gene product). *Tampakushitsu Kakusan Koso, Bessatsu*, (1978) 197-203; *C.A.*, 91 (1979) 52032k.

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20. ENZYMES

3184 Obrosov, A.N. and Ananeva, K.A.: (Some aspects of the electrophoresis of enzyme preparations). *Vop. Kurortol., Fizioter. Lech. Fiz. Kul't.*, (1979) 13-16; *C.A.*, 91 (1979) 70510u.

20a. Oxidoreductases

3185 Chang, H.-L., Holten, D. and Karin, R.: Distribution of the multiple molecular forms of glucose-6-phosphate dehydrogenase in different physiological states. *Can. J. Biochem.*, 57 (1979) 396-401 - polyacrylamide gel.

3186 Dickie, P. and Weiner, J.H.: Purification and characterization of membrane-bound fumarate reductase from anaerobically grown *Escherichia coli*. *Can. J. Biochem.*, 57 (1979) 813-821 - SDS-polyacrylamide gel.

3187 French, J.S. and Coon, M.J.: Properties of NADPH-cytochrome *P*-450 reductase purified from rabbit liver microsomes. *Arch. Biochem. Biophys.*, 195 (1979) 565-577 - SDS-polyacrylamide gel.

3188 Gustke, H.H. and Neuhoff, V.: Improved micro-scale separation of glucose-6-phosphate dehydrogenase variants. *Hoppe-Seyler's Z. Physiol. Chem.*, 360 (1979) 605-608; *C.A.*, 91 (1979) 34666r.

3189 Hansen, H.S.: Purification and characterization of a 15-ketoprostaglandin Δ^{13}-reductase from bovine lung. *Biochim. Biophys. Acta*, 574 (1979) 136-145 - isoelectric focusing, polyacrylamide gel, SDS-polyacrylamide gel.

3190 Kao, W.W.-Y. and Berg, R.A.: Cell density-dependent increase in prolyl hydroxylase activity in cultured L-929 cells requires *de novo* protein synthesis. *Biochim. Biophys. Acta*, 586 (1979) 528-536 - SDS-polyacrylamide gel.

3191 Kornbluth, R., Tracy, P.S. and Fondy, T.P.: Isoenzymic forms of NAD-linked glycerol-3-phosphate dehydrogenase from rabbit brain. *Biochim. Biophys. Acta*, 568 (1979) 273-286 - immunoelectrophoresis, SDS-polyacrylamide gel, isoelectric focusing (column and flat plate).

3192 Lopez-Barea, J. and Lee, C-Y.: Mouse-liver glutathione reductase. Purification, kinetics and regulation. *Eur. J. Biochem.*, 98 (1979) 487-499 - polyacrylamide gel.

3193 Nakai, C., Kagamiyama, H., Saeki, Y. and Nozaki, M.: Nonidentical subunits of pyrocatechase from *Pseudomonas arvilla* C-1. *Arch. Biochem. Biophys.*, 195 (1979) 12-22 - SDS-polyacrylamide gel.

3194 Schedel, M. and Trüper, H.G.: Purification of *Thiobacillus denitrificans* siroheme sulfite reductase and investigation of some molecular and catalytic properties. *Biochim. Biophys. Acta*, 568 (1979) 454-467 - SDS-polyacrylamide gel.

3195 Sim, E. and Sim, R.B.: Hydrodynamic parameters of the detergent-solubilized hydrogenase from *Paracoccus denitrificans*. *Eur. J. Biochem.*, 97 (1979) 119-126 - SDS-polyacrylamide gel.

3196 Toft, B.S. and Hansen, H.S.: Metabolism of prostaglandin E_1 and of glutathione conjugate of prostaglandin A_1 (GSH-prostaglandin A_1) by prostaglandin 9-keto-reductase from rabbit kidney. *Biochim. Biophys. Acta*, 574 (1979) 33-38 - isoelectric focusing.

3197 Weiss, H. and Kolb, H.J.: Isolation of mitochondrial succinate: ubiquinone reductase, cytochrome *c* reductase and cytochrome *c* oxidase from *Neurospora crassa* using nonionic detergent. *Eur. J. Biochem.*, 99 (1979) 139-149 - SDS-polyacrylamide gel.

20b. Transferases (excluding E.C. 2.7.-.-)

3198 Banerjee, R.K.: Characterization of an adenosine triphosphatase from myeloblasts infected with the avian myeloblastosis virus. *Eur. J. Biochem.*, 97 (1979) 59-64 - SDS-polyacrylamide gel.

3199 Billheimer, J.T., Shen, M.Y., Carnevale, H.N., Horton, H.R. and Jones, E.E.: Isolation and characterization of acetylornithine δ-transaminase of wild-type *Escherichia coli* W. Comparison with arginine-inducible acetylornithine δ-transaminase. *Arch. Biochem. Biophys.*, 195 (1979) 401-413 - polyacrylamide gel.

3200 Cano, A. and Pestana, A.: Purification and properties of a histone acetyltransferase from *Artemia salina*, highly efficient with H1 histone. *Eur. J. Biochem.*, 97 (1979) 65-72 - polyacrylamide gel.

3201 Inoue, M., Horiuchi, S. and Morino, Y.: Affinity labeling of rat kidney γ-glutamyl transpeptidase by 6-diazo-5-oxo-D-norleucine. *Eur. J. Biochem.*, 99 (1979) 169-177 - SDS-polyacrylamide gel.

3202 Kreuzaler, F., Ragg, H., Heller, W., Tesch, R., Witt, I., Hammer, D. and Hahlbrock, K.: Flavanone synthase from *Petroselinum hortense*. Molecular weight, subunit composition, size of messenger RNA and absence of pantetheinyl residue. *Eur. J. Biochem.*, 99 (1979) 89-96 - SDS-polyacrylamide gel.

3203 Ohashi, A. and Kikuchi, G.: Purification and some properties of two forms of δ-aminolevulinate synthase from rat liver cytosol. *J. Biochem.*, 85 (1979) 239-247 - polyacrylamide gel.

3204 Rubenstein, P.A. and Ivarie, R.D.: Isolation of two different molecular weight polypeptides copurifying with rat liver tyrosine aminotransferase. *Arch. Biochem. Biophys.*, 194 (1979) 299-311 - SDS-polyacrylamide gel.

20c. Transferases transferring phosphorus containing groups (E.C. 2.7.-.-)

3205 Berglund, L. and Humble, E.: Kinetic properties of pig pyruvate kinases type A from kidney and type M from muscle. *Arch. Biochem. Biophys.*, 195 (1979) 347-361 - polyacrylamide gel.

3206 Illg, D. and Pette, D.: Turnover rates of hexokinase I, phosphofructokinase, pyruvate kinase and creatine kinase in slow-twitch soleus muscle and heart of the rabbit. *Eur. J. Biochem.*, 97 (1979) 267-273 - SDS-polyacrylamide gel, immunoelectrophoresis.

3207 Kooistra, T., Duursma, A.M., Bijsterbosch, M.K., Bouma, J.M.W. and Gruber, M.: Endocytosis and breakdown of ribonuclease oligomers by sinusoidal rat liver cells *in vivo*. II. Effect of charge. *Biochim. Biophys. Acta*, 587 (1979) 299-311 - isoelectric focusing.

3208 Spassky, A., Busby, S.J.W., Danchin, A. and Buc, H.: On the binding of tRNA to *Escherichia coli* RNA polymerase. *Eur. J. Biochem.*, 99 (1979) 187-201 - polyacrylamide gel.

20d. Hydrolases, acting on ester bonds (E.C. 3.1.-.-)

3209 Axenfors, B., Andersson, I. and Augustinsson, K.-B.: Isolation and characterization of a butyryl-esterase from human erythrocytes. *Biochim. Biophys. Acta*, 570 (1979) 74-87 - polyacrylamide gel, isoelectric focusing.

3210 Delbrück, A. and Henkel, E.: A rare genetically determinated variant of pseudocholinesterase in two german families with plasma enzyme activity. *Eur. J. Biochem.*, 99 (1979) 65-69 - polyacrylamide gel.

3211 Dicou, E.L. and Brachet, Ph.: Multiple forms of an extracellular cyclic-AMP phosphodiesterase from *Dictyostelium discoideum*. *Biochim. Biophys. Acta*, 578 (1979) 232-242 - isoelectric focusing, polyacrylamide gel.

3212 Imamura, S. and Horiuti, Y.: Purification of *Streptomyces chromofuscus* phospholipase D by hydrophobic affinity chromatography on palmitoyl cellulose. *J. Biochem.*, 85 (1979) 79-95 - isoelectric focusing, SDS-polyacrylamide gel.

20e. Hydrolases, acting on glycosyl compounds (E.C. 3.2.-.-)

3213 Kanemitsu, F., Yamada, S., Kawanishi, I. and Mizushima, J.: (Analysis of fluid amylase isoenzymes by electrophoresis with cellulose acetate membrane. I. Study of analysis conditions). *Eisei Kensa*, 27 (1978) 1311-1315; *C.A.*, 91 (1979) 86207m.

3214 Kasukabe, T., Honma, Y. and Hozumi, M.: Characterization of lysozyme synthesized by differentiated mouse myeloid leukemia cells. *Biochim. Biophys. Acta*, 586 (1979) 615-623 - SDS-polyacrylamide gel.
3215 O'Neil, D.C., Bartholomew, W.R. and Rattazzi, M.C.: Antigenic homology of feline and human β-hexosaminidase. *Biochim. Biophys. Acta*, 580 (1979) 1-9 - cellulose acetate, immunoelectrophoresis (agarose gel).
3216 Tanimura, T., Kitamura, K., Fukuda, T. and Kikuchi, T.: Purification and partial characterization of three forms of α-glucosidase from the fruit fly *Drosophila melanogaster*. *J. Biochem.*, 85 (1979) 123-130 - polyacrylamide gel.
3217 Turner, B.M.: Purification and characterization of α-L-fucosidase from human placenta. pH-dependent changes in molecular size. *Biochim. Biophys. Acta*, 578 (1979) 325-336 - isoelectric focusing, SDS-polyacrylamide gel.

20f. Other hydrolases

3218 Berezin, V.A., Reva, A.D., Shmatchenko, N.A. and Korobov, V.I.: (Isolation and purification of bovine and porcine cerebral cathepsin D). *Biokhimiya*, 44 (1979) 1030-1035 - polyacrylamide gel.
3219 Brockbank, W.J. and Lynn, K.R.: Purification and preliminary characterization of two asclepains from the latex of *Asclepias syriaca* L. (milkweed). *Biochim. Biophys. Acta*, 578 (1979) 13-22 - polyacrylamide gel.
3220 Goryukhina, O.A., Goncharova, V.P., Stepanova, I.S. and Reztsova, V.V.: (The proteinase activity of nuclei from various tissues towards endogenous histones). *Biokhimiya*, 44 (1979) 504-513 - polyacrylamide gel.
3221 Ito, T., Devaux, C., Gautray, J.P., Menard, J. and Corvol, P.: Physicochemical properties of non-activated and activated renin from human amniotic fluid. *Biochim. Biophys. Acta*, 569 (1979) 202-210 - isoelectric focusing, agarose-acrylamide gel.
3222 Pekkel, V.A. and Kipkel, A.Z.: (Purification and some physico-chemical properties of myocardial adenylate deaminase). *Biokhimiya*, 44 (1979) 1663-1672 - polyacrylamide gel.
3223 Takuma, T. and Kumegawa, M.: Regulation of trypsin-like esteroprotease synthesis by 5-dihydrotestosterone and triiodothyronine in mouse submandibular gland. *Biochim. Biophys. Acta*, 564 (1979) 335-341 - isoelectric focusing, polyacrylamide gel.
3224 Turner, G., Imam, G. and Küntzel, H.: Mitochondrial ATPase complex of *Aspergillus nidulans* and the dicyclohexylcarbodiimide-binding protein. *Eur. J. Biochem.*, 97 (1979) 565-571 - SDS-polyacrylamide gel.

20g. Lyases

3225 Gupta, S. and Acton, G.J.: Purification to homogeneity and some properties of L-phenylalanine ammonia-lyase of irradiated mustard (*Sinapis alba* L.) cotyledons. *Biochim. Biophys. Acta*, 570 (1979) 187-197 - isoelectric focusing, polyacrylamide gel (preparative).
3226 Mittal, C.K., Braughler, J.M., Ichihara, K. and Murad, F.: Synthesis of adenosine 3',5'-monophosphate by guanylate cyclase, a new pathway for its formation. *Biochim. Biophys. Acta*, 585 (1979) 333-342 - polyacrylamide gel.

20h. Isomerases

3227 Weeden, N.F. and Gottlieb, L.D.: Distinguishing allozymes and isozymes of phosphoglucoisomerases by electrophoretic comparisons of pollen and somatic tissues. *Biochem. Genet.*, 17 (1979) 287-296; *C.A.*, 91 (1979) 87480g.

20i. Ligases

3228 Baltzinger, M., Fasiolo, F. and Remy, P.: Yeast phenylalanyl-tRNA synthetase. Affinity and photoaffinity labelling of the stereospecific binding sites. *Eur. J. Biochem.*, 97 (1979) 481-494 - SDS-polyacrylamide gel.
3229 Graf, H.: Optimization of conditions for the *in vitro* formation of hybrid DNA molecules by DNA ligase. *Biochim. Biophys. Acta*, 564 (1979) 225-234 - agarose gel.

21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

3230 Lee, P.W.K. and Colter, J.S.: Studies of two temperature - sensitive mutants of Mengo virus. *Can. J. Biochem.*, 57 (1979) 902-913 - polyacrylamide-agarose gel.

3231 Lopez, J.M., Marks, C.L. and Freese, E.: The decrease of guanine nucleotides initiates sporulation of *Bacillus subtilis*. *Biochim. Biophys. Acta*, 587 (1979) 238-252 - Polygram CEL PEI (polyethyleneimine cellulose thin-layer plate).

21b. Nucleic acids, RNA

3232 Castle, T., Kreamer, W., Liu, D.S.H. and Richardson, A.: Characterization of RNA synthesis by isolated hepatocytes in suspension. *Arch. Biochem. Biophys.*, 195 (1979) 423-437 - polyacrylamide gel.

3233 Chan, S.-K. and Ball, J.K.: Investigation of the conditions of agarose gel electrophoresis for separation of viral RNAs. *Anal. Lett.*, 12 (1979) 543-554; *C.A.*, 91 (1979) 35123y.

3234 Gray, M.W.: The ribosomal RNA of the trypanosomatid protozoan *Crithidia fasciculata* physical characteristics and methylated sequences. *Can. J. Biochem.*, 57 (1979) 914-926 - polyacrylamide gel.

3225 Hofer, E., Alonso, A., Krieg, L., Schätzle, U. and Sekeris, C.E.: Purification of albumin mRNA from rat liver. Content of albumin-specific sequences in cytoplasmic and nuclear RNA. *Eur. J. Biochem.*, 97 (1979) 455-462 - agarose gel.

3236 Itoh, N., Nose, K. and Okamoto, H.: Purification and characterization of proinsulin mRNA from rat B-cell tumor. *Eur. J. Biochem.*, 97 (1979) 1-9 - SDS-polyacrylamide gel.

3237 Nichols, J.L.: "Cap" structures in maize poly(A)-containing RNA. *Biochim. Biophys. Acta*, 563 (1979) 490-495 - paper.

21c. Nucleic acids, DNA

3238 Loucks, E., Chaconas, G., Blakesley, R.W., Wells, R.D. and Van de Sande, J.H.: Antibiotic induced electrophoretic mobility shifts of DNA restriction fragments. *Nucleic Acids Res.*, 6 (1979) 1869-1879; *C.A.*, 91 (1979) 35122x.

3239 Paponov, V.D., Gromov, P.S., Sokolov, N.A., Spitkovsky, D.M. and Tseitlin, P.I.: The nature of interactions responsible for the differences in the affinities of histones for DNA. *Biochem. Biophys. Res. Commun.*, 82 (1978) 674-679 - polyacrylamide gel.

3240 Widmer, H.J., Jaggi, R.B., Weber, R. and Ryffel, G.U.: Enrichment and characterization of the DNA coding for vitellogenin in *Xenopus laevis*. *Eur. J. Biochem.*, 99 (1979) 23-29 - agarose gel.

See also 3035.

25. ORGANIC PHOSPHORUS COMPOUNDS

3241 Makan, N.R.: Phosphoprotein phosphatase activity at the outer surface of intact normal and transformed 3T3 fibroblasts. *Biochim. Biophys. Acta*, 585 (1979) 360-373 - paper.

27. VITAMINS AND VARIOUS GROWTH REGULATORS (NON-PEPTIDIC)

3242 Lindemans, J., van Kapel, J. and Abels, J.: Purification of human transcobalamin II-cyanocobalamin by affinity chromatography using thermolabile immobilization of cyanocobalamin. *Biochim. Biophys. Acta*, 579 (1979) 40-51 - SDS-polyacrylamide gel, isoelectric focusing.

3243 Panijpan, B. and Detkriangkrsikun, P.: High voltage paper electrophoresis as an alternative method for thiamin determination in the presence of substances capable of interfering with thiochrome formation. *Amer. J. Clin. Nutr.*, 32 (1979) 723-725; *C.A.*, 91 (1979) 35112u.

30. SYNTHETIC AND NATURAL DYES

30a. Synthetic dyes

3244 Banerjee, T.S., Mazumder, D., Halder, R.C. and Roy, B.R.: Detection of food colors by gel electrophoresis. *J. Food Sci. Technol.*, 16 (1979) 34-35; *C.A.*, 91 (1979) 37577y.

3245 Lavallee, D.K. and Daugherty, N.A.: The electrophoresis of indicators. An analogy to isoenzyme separation. *J. Chem. Educ.*, 56 (1979) 353-354; *C.A.*, 91 (1979) 55513d.

30b. Chloroplast and other natural pigments

3246 Nashima, K., Mitsudo, M. and Kito, Y.: Molecular weight and structural studies on cephalopod rhodopsin. *Biochim. Biophys. Acta*, 579 (1979) 155-168 - SDS-polyacrylamide gel.

31. PLASTICS AND THEIR INTERMEDIATES

See 3035.

32. PHARMACEUTICAL AND BIOMEDICAL APPLICATIONS

32f. Clinico-chemical applications and profiling body fluids

See 3219.

33. INORGANIC COUMPOUNDS

33b. Anions

3247 Booij, H.L. and Beekes, H.: Studies on the metabolism of polyphosphates in yeast cells. I. Polyacrylamide gel electrophoresis of polyphosphates. *Rec. Trav. Chim. Pays-Bas*, 98 (1979) 320-323; *C.A.*, 91 (1979) 71166y.

34. RADIOACTIVE AND OTHER ISOTOPE COMPOUNDS

See 3040.

35. SOME TECHNICAL PRODUCTS AND COMPLEX MIXTURES

35a. Surfactants

See 3035.

35d. Complex mixtures and non-identified compounds

3248 Steiner, W.W.M. and Joslyn, D.J.: Electrophoretic techniques for the genetic study of mosquitoes. *Mosq. News*, 39 (1979) 35-54; *C.A.*, 91 (1979) 16011u.

36. CELLS AND CELLULAR PARTICLES

3249 Barkas, B.V., Kornev, A.N., Mesyanzhinov, V.V., Poglazov, B.F., Turkin, A.I. and Khromov, A.S.: (Use of polyacrylamide gel electrophoresis for the preparation of oriented virus particle preparations). *Dokl. Akad. Nauk SSSR*, 245 (1979) 736-739; *C.A.*, 91 (1979) 35110s.

3250 Platsoucas, C.D., Good, R.A. and Gupta, S.: Separation of human I lymphocyte subpopulations ($T\mu$, T_γ) by density gradient electrophoresis. *Proc. Nat. Acad. Sci. U.S.*, 76 (1979) 1972-1976; *C.A.*, 91 (1979) 37272v.

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journal of chromatography news section

MEETING

13TH INTERNATIONAL SYMPOSIUM ON CHROMATOGRAPHY

The 13th International Symposium on Chromatography will be held June 30–July 4, 1980, at the Palais des Festivals et des Congres, La Croisette, B.P. 262, 06406 Cannes, France. Tel. (93) 68.12.34. Previous announcements have been published in Vols. 172 and 189, No. 1. All information can be obtained from Professor G. Guiochon, Ecole Polytechnique, Laboratoire de Chimie Analytique Physique, 91128 Palaiseau Cedex, France. Tel. 941.82.00.

The scientific program is given below.

MONDAY, JUNE 30

Plenary Session 1 *(main auditorium); Chairman: P. Tuey*

9.00 Opening address, G. Guiochon, Chairman of the Symposium

9.05 Presentation of the A.J.P. Martin Award, by the chairman of the Chromatography Discussion Group

9.15 P. Teisseire (Roure Bertrand Dupont S.A., Grasse, France), Les méthodes physico-chimiques dans l'analyse des produits naturels odorants (an outline of the lecture in English language with all slide captions will be available at the entrance of the auditorium)

10.00 Coffee break

10.30 I. Halasz (University of Saarland, Saarbrücken, G.F.R.), Semi preparative separations of complex mixtures

11.15 L.R. Snyder (Technicon Instr., Tarrytown, N.Y., U.S.A.), A new route to large column plate numbers with reasonable per-sample separation time
J. Kirkland (Du Pont de Nemours, Wilmington, Del., U.S.A.), Programmed sedimentation field flow fractionation (SFFF), a new method
M. Martin and J. Hes (Ecole Polytechnique, Palaiseau, France), Thermal field flow fractionation – laser light scattering: a powerful tandem for polymer analysis
A.V. Kiselev and D.P. Poshkus (Chem. Depart. Lomonosov State, University of Moscow and Institute of Chem. and Chem. Technol., Academy of Sciences of the Lithuanian SSR, Vilnius, U.S.S.R.), Method of determination of structural parameters of molecules from chromatographic data – Chromatoscopy

12.35 Lunch break

14.00 Poster sessions

15.00 Discussion sessions

Themes: A. Preparative chromatography
Chairman: I. Halasz
Discussion starter: K. Hupe
B. Thin layer chromatography (no discussion session)
C. Biochemical analysis
Chairman: E.C. Horning
Discussion starter: B. Maume

TUESDAY, JULY 1

Plenary Session 2 *(main auditorium); Chairman R. Rosset*

8.30 B.L. Karger, Y. Taputris, W. Lindner and J.N. Lepage (Northeastern University, Boston, Mass., U.S.A.), Ligand exchange in high performance liquid chromatography

9.15 E. Bayer (University of Tübingen, Tübingen, G.F.R.), Chiral phases as tools in biochemistry and pharmacology

10.00 Coffee break

Session 2.A: High efficiency columns *(main auditorium); Chairman: L. Ettre*

10.30 V. Pretorius, J. Rijks and J.D. Davidtz (Institute for chromatography, Univ. of Pretoria, South Africa, and Eindhoven Univ. of Technology, The Netherlands), New procedures for preparing glassy support phases for gas liquid chromatography

10.50 J.F.K. Huber, E. Kenndler and W. Nyiry (Institute of Analytical Chemistry, Univ. of Vienna, Austria) and M. Oreans (Siemens AG, Karlsruhe, G.F.R.), Quantitative analysis by multi-column gas chromatography

11.10 G. Schomburg and H. Husmann (Max-Planck-Institut, Mulheim, G.F.R.), Properties of untreated and treated glass capillary surfaces and their contribution to solute-stationary phase interaction and film fixation of the stationary liquid

11.30 M. Galli and F. Munari (Carlo Erba Strumentazione, Milan, Italy) and S. Trestianu (VEL, Leuven, Belgium), Benefits of the on-column injection system for the quantitative high resolution gas chromatographic analysis of complex mixtures

11.50 C.A. Cramers and P.A. Leclercq (Laboratory of Instrumental Analysis, Eindhoven, The Netherlands), Increased speed of analysis in directly coupled GC/MS

12.10 L. Blomberg, K. Markides and T. Wannman (Department of Analytical Chemistry, Univ. of Stockholm, Stockholm, Sweden), Glass capillary columns for gas chromatography coated with non-extractable films of cyanosilicone gums

12.30 Lunch break

Session 2.B.1: Chemical analysis *(floor 4); Chairman: P. Teisseire*

10.30 W.L. Hollaway and J.C. Bennett (Department of Med. and Microbiol., Univ. of Alabama, Birmingham, Ala., U.S.A.), Utilization of HPLC for the separation of amino acids, peptides and proteins

10.50 R. Liardon, S. Ledermann and U. Ott (Research Department, Nestlé Products, La Tour de Peils, Switzerland), Determination of D-amino acid by deuterium labelling and GC/MS selective ion monitoring

11.10 M.T.W. Hearn (MRCNZ Immunopathol. Res. Unit, Univ. of Otago Med. School, Dunedin, New Zealand), Solvophobic considerations for the separation of unprotected peptides on chemically bonded hydrocarbonaceous stationary phases

11.30 C.W. Moss (Center for Disease Control, Public Health Service, Atlanta, Ga., U.S.A.), Gas–liquid chromatography as tool in bacteriology

Session 2.B.2: Optical isomers *(floor 4); Chairman: E. Bayer*

11.50 G. Gubitz and W. Jellenz (Institut für Pharm. Chemie, University of Gratzl, Austria), Separation of optical isomers by ligand exchange chromatography using chemically bonded chiral phases

12.10 E. Grushka, R. Leshem and C. Gilon (Hebrew University, Jerusalem, Israel), The reversed-phase separation of amino acid enantiomers

12.30 Lunch break

14.00 Poster sessions: Drug analysis and manufacturer posters

15.30 Discussion sessions: Drug analysis and field flow fractionation

Themes: D. Drug analysis
Chairman: L.R. Snyder
Discussion starter: M. Uihlein

E. Manufacturer posters (no discussion session)
Chairman: P. Galais

F. Field flow fractionation
Chairman: J. Kirkland
Discussion starter: J.C. Giddings

WEDNESDAY, JULY 2

Plenary Session 3 *(main auditorium); Chairman: G. Schomburg*

8.30 P.D. Goldan and S.C. Sehsenseld (Aeronomy Laboratory, NOAA environmental research Laboratory, Boulder, Colo.), M. Satouchi (Shiga prefectural junior college, Hikone, Shiga, Japan), N.P. Phillips, M.A. Wizner and R.E. Sievers (University of Colorado, Boulder, Colo., U.S.A.), Applications of selective electron capture sensitization (SECS)

9.00 F.W. McLafferty (Cornell University, Ithaca, N.Y., U.S.A.), Mass spectrometry of high molecular weight compounds

9.45 Coffee break

Session 3.A.1: LC/MS detectors *(main auditorium); Chairman: F.W. McLafferty*

10.10 P. Arpino, J. Szafranek and G. Guiochon (Ecole Polytechnique, Palaiseau, France), P. Krien and G. Devant (Nermag, Rueil-Malmaison, France), Conditions for nebulizing liquids into a chemical ionization mass spectrometer for on-line liquid chromatography–mass spectrometry

10.30 D.E. Games, P. Hirter, W. Kuhnz, E. Lewis, K.R.N. Rao, N.C.A. Weerasinghe and S.A. Westwood (Department of Chemistry, University College, Cardiff, Great Britain), Studies of combined LC/MS with a moving belt interface

10.50 P. Vouros (Institute of Chemical Analysis, Northeastern University, Boston, Mass., U.S.A.), Ion pair reversed phase liquid chromatography–mass spectrometry

Session 3.A.2: LC detectors *(main auditorium); Chairman: C. Guillemin*

11.10 A.H.L.T. Scholten, U.A.Th. Brinkman and R.W. Frei (The Free University, Department of Analytical Chemistry, Amsterdam, The Netherlands), Photochemical reaction detectors in liquid chromatography, design, band-broadening and applications

11.30 H. Hatano, S. Rokushika, K. Makino, A. Moriya and N. Suzuki (Department of Chemistry, Kyoto University, Kyoto, Japan), Radical chromatography of stable free radicals and spin-trapped unstable radicals of amino acids, peptides and nucleotides

11.50 P.R. Brown (Department of Chemistry, University of Rhode Island, R.I., U.S.A.), A.M. Krstulovic (Department of Chemistry, Manhattan College, N.Y., U.S.A.) and R.A. Hartwick (Department of Chemistry, University of Edinburgh, Edinburgh, Great Britain), Spectroscopic and chemical characterization of peaks in biological matrices analyzed by high performance liquid chromatography

12.10 R.S. Deelder, H.A.J. Linssen, J.G. Boen and A.J.B. Beeren (DSM Research, Geleen, The Netherlands), A potentiometric membrane cell as a detector for liquid chromatography

12.30 Lunch break

Session 3.B.1: High efficiency columns *(floor 4); Chairman: J.F.K. Huber*

10.10 L. Buydens and D.L. Massart (Pharmaceutical Institute, Vrije Universiteit, Brussels, Belgium), The use of topological and/or structure – activity parameters in the prediction of gas chromatographic retention data

10.30 H.T. Badings, J.J.G. van der Pol and J.G. Wassink (Netherlands Institute for Dairy Research, Ede, The Netherlands), Preparation of wall-coated open tubular columns after surface roughening by means of amorphous silica. II. A study of factors affecting the quality of the prepared columns in gas chromatographic analyses

10.50 G. Alexander (Hungarian Academy of Sciences, Laboratory for Inorganic Chemistry, Budapest, Hungary), Surface characteristics of variously treated glasses and their role in capillary gas chromatography

11.10 S.P. Cram (Varian, Walnut Creek, Calif., U.S.A.), Mechanisms of sample introduction in high resolution gas chromatography

Session 3.B.2: Geochemical analysis *(floor 4); Chairman: N. McTaggart*

11.30 J.M. Schmitter, P. Arpino and G. Guiochon (Ecole Polytechnique, Palaiseau, France), Petroleum acids and nitrogen bases; methods of separation and full identification

11.50 S.K. Hajibrahim (Department of Chemistry, University of Riyad, Saudi Arabia), Development of HPLC for fractionation and fingerprinting of petroporphyrins

12.10 D.A. Ferguson and A.P. O'Brien (The British Petroleum Company), Characterization of "deasphaltened" petroleum residues by gel permeation chromatography

12.30 Lunch break

14.00 Poster sessions
15.30 Discussion sessions
Themes: G. Environmental analysis
Chairman: C. Guillemin
Discussion starter: R. Sievers
H. Detectors for liquid chromatography
Chairman: J.F.K. Huber
Discussion starter: R. Frei

THURSDAY, JULY 3

Plenary Session 4 *(main auditorium); Chairman: J. Tranchant*

8.30 R.P.W. Scott (Hoffmann LaRoche, Nutley, N.J., U.S.A.), Design, properties and applications of microbore columns for liquid chromatography

Session 4.A: Retention LC *(main auditorium); Chairman: B.L. Karger*

9.00 Cs. Horvath, W. Melander and A. Nahum (Department of Engineering and Applied Science, Yale University, New Haven, Conn., U.S.A.), Role and characteristics of the stationary phase in reversed phase chromatography

9.30 K. Unger, P. Roumeliotis and H. Muller (Institut für Anorganische Chemie und Analytische Chemie, Mainz, G.F.R.), Porous graphitized carbon packing as support in reverse phase ion pair chromatography

10.00 Coffee break

10.30 J.H. Knox and J. Jurand (Wolfson Liquid Chrom. Unit, Department of Chemistry, University of Edinburgh, Great Britain), Zwitterion-pair chromatography of nucleotides and other dipolar species

11.00 D.E. Martire and R.E. Boehm (Department of Chemistry, Georgetown University, Washington, D.C., U.S.A.), A unified theory of retention and selectivity in liquid chromatography

11.30 H. Engelhardt and P. Roth (Angewandte Physikalische Chemie, University of Saarland, Saarbrücken, G.F.R.), Pressure stable high capacity ion exchangers for HPLC

11.50 M.C. Hennion, C. Picard, C. Combellas, M. Caude and R. Rosset (Lab. Chimie analytique des Processus industriels, ESPCI, Paris, France), Some simple relations concerning mobile and stationary phases in normal and reversed phase chromatography

12.10 S.O. Jansson, I. Andersson and B.A. Persson (Analytical Chemistry and Biochemistry, Mölndal, Sweden), Solute–solvent interactions in reversed phase ion pair liquid chromatography of amines with pentanol and N,N-dimethyloctylamine as organic modifiers

12.30 Lunch break

Session 4.B.1: Environmental analysis *(floor 4); Chairman: R. Sievers*

9.20 D.W. Grant and R.B. Meiris (British Carbonization Research Ass., Chesterfield, Great Britain), Studies of the application of selective chromatographic and spectrofluorimetric techniques in the separation, characterization and analysis of polycyclic aromatic hydrocarbons

9.40 G. Bertoni, F. Bruner and A. Liberti (Laboratorio Inquinamento Atmosferico, Rome, Italy), Some critical parameters in collection, recovery and GC analysis of organic pollutants in ambient air with light adsorbents

10.00 Coffee break

10.30 D.A.M. Mackay and M.M. Hussein (Life Savers, New York, U.S.A.), Large bore coated columns in analysis for trace organics in water

10.50 F. Berthou, Y. Goumerlun, Y. Dreano and M.P. Friocourt (Laboratoire de Chromatographie, Biochimie, Faculté de Médecine, Brest, France), Application of gas chromatography on glass capillary columns in the analysis of hydrocarbons pollutants from Amoco-Cadiz oil spill

11.10 C. Vidal-Madjar (Ecole Polytechnique, Palaiseau, France), Quantitative analysis of chlorofluorocarbons in the atmosphere. Absolute calibration of an ECD

Session 4.B.1: Preparative scale chromatography *(floor 4); Chairman: I. Halasz*

11.30 G.B. Cox (Du Pont (UK), Hitchin, Great Britain), Column loading in isocratic and gradient mode high performance reversed phase preparative liquid chromatography

11.50 K.P. Hupe and H.H. Lauer (Hewlett-Packard GmbH, G.F.R.), Optimization of preparative liquid chromatography

12.00 Lunch break

14.00 Poster sessions

15.30 Discussion sessions

Themes: I. High efficiency columns for gas chromatography
Chairman: L.S. Ettre
Discussion starter: G. Schomburg

J. Retention mechanisms in liquid chromatography
Chairman: Cs. Horvath
Discussion starter: B.L. Karger

FRIDAY, JULY 4

Plenary Session 5 *(main auditorium); Chairman: L. Rohrschneider*

8.30 C.L. Guillemin (Rhone Poulenc, Aubervilliers, France), Daydream on process and laboratory gas chromatography

9.15 D. Deans (ICI, Billingham, Great Britain), The use of heart cutting in gas chromatography

9.45 Dr. Kelker (Farbwerke Hoechst, Frankfurt, G.F.R.), Liquid crystals in chromatography – a critical review

10.15 Coffee break

Session 5.A.1: GC detectors *(main auditorium); Chairman: H. Poppe*

10.45 MM. Coquand and Charron (G.D.G., La Plaine St.-Denis, France), Un détecteur électro-chimique pour le dosage chromatographique en exploitation des composés sulfurés présents dans les gaz naturels

11.05 R. Annino and J. Leone (Chemistry Department, Canisius College, Buffalo, N.Y., U.S.A.), High speed analysis of trace quantities of fixed gases using a correlation mode of thermal conductivity detection

11.25 K.W.M. Siu and W.A. Aue (Life Sciences Centre, Halifax, Canada), An electron capture detector in which capture and recombination can be separated

11.45 C.P.M.G. A'Campo, S.M. Lemkovitz, P. Verbrugge and P.J. van den Berg (Afd. Chem. Technol. van de Technische Hogeschool, Delft, The Netherlands), Gas chromatographic determination of water: a source of systematic error, introduced by interactions of polar compounds on porous polymer columns

Session 5.A.2: Thin layer chromatography *(main auditorium); Chairman: H. Poppe*

12.05 A.M. Siouffi (Laboratoire de Chimie Appliquée, Faculté des Sciences, Marseille III, France), Optimization in thin-layer chromatography: some practical considerations

12.30 Closing address

12.40 Lunch break

Session 5.B.1: LC detectors *(floor 4); Chairman: R. Frei*

10.45 C.E. Werkhoven-Goewie, W.M.A. Niessen, U.A.Th. Brinkman and R.W. Frei (Department of Analytical Chemistry, Free University of Amsterdam, The Netherlands), A LC reaction detector for organosulphur compounds based on a ligand-exchange reaction

11.05 B. Coq, C. Cretier and J.L. Rocca (Laboratoire de Chimie Analytique III, Université Claude Bernard, Lyon 1, France), Some factors affecting the detection in liquid chromatography sensibility level and quantitative analysis

11.25 S. Folestad and B. Josefsson (Department of Analytical Chemistry, University of Göteborg, Sweden), Chlorine selective detector for liquid chromatography

Session 5.B.2: Analysis of food, flavor and fragranges *(floor 4); Chairman: R. Frei*

11.45 E.W. Hammond (Analytical Section, Unilever Research, Bedford, Great Britain), Quantitative chromatographic analysis of lipids in foods

12.05 M. Godefroot, P. Sandra and M. Verzele (Lab. of Organic Chemistry, Ghent, Belgium), A new method for quantitative essential oil analysis

12.25 Lunch break

14.30 Poster sessions

16.00 Discussion sessions

Themes: K. Analysis of food flavor and fragrances
Chairman: L.A. Beaver
Discussion starter: R. Prevot

L. Geochemistry
Chairman: D.W. Grant
Discussion starter: P. Arpino

CALENDAR OF FORTHCOMING MEETINGS

June 10–13, 1980
Ghent, Belgium

3rd International Symposium on Quantitative Mass Spectrometry in Life Sciences
Contact: Professor A.P. De Leenheer, Laboratoria voor Medische Biochemie en Klinische Analyse, de Pintelaan 135, B-9000 Ghent, Belgium.

June 16–18, 1980
Milan, Italy

7th International Symposium on Mass Spectrometry in Biochemistry, Medicine and Environmental Research
Contact: Dr. A. Frigerio, Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy.

June 18–20, 1980
Brigham Young U, Provo, Utah, U.S.A.

2nd Symposium on Environmental Analytical Chemistry
Contact: Delbert J. Eatough, 271 FB, Thermochemical Institute, Brigham Young U, Provo, Utah 84602, U.S.A.

June 23–27, 1980
Birmingham, Great Britain

Eurochem 80
Contact: Andrew Dedman, Clapp & Polick Europe Ltd., 232 Acton Lane, London W4 5DL, Great Britain.

June 26–29, 1980
Strasbourg, France

International Symposium: Affinity Chromatography and Molecular Interaction
Contact: Dr. J.M. Egly, Faculté de Médicine, Institut de Chimie Biologique, 11 rue Humann, 67085 Strasbourg Cédex, France.

June 30–July 4, 1980
Cannes, France

13th International Symposium on Chromatography
Contact: GAMS, 88 Boulevard Malesherbes, 75008 Paris, France. (Further detai published in Vol. 172, Vol. 189, No. 1, and Vol. 194, No. 1).

July 7–11, 1980
Brussels, Belgium

2nd International Congress on Toxicology
Contact: Secretariat, SdR Assosiated, 16 Avenue des Abeilles, B–1050 Brussels, Belgium.

July 20–26, 1980
Lancaster, Great Britain

SAC 80
Contact: The Secretary, Analytical Division, The Chemical Society, Burlington House, London W1V OBN, Great Britain. (Further details published in Vol. 192 No. 1)

Aug. 24–29, 1980 San Francisco, Calif., U.S.A.	**ACS 180th National Conference – 2nd Chemical Congress of the North Amer. Continent** Contact: A.T. Winstead, 1155 16th Street, N.W. Washington, D.C. 20036, U.S.A.
Aug. 25–30, 1980 Graz, Austria	**8th International Microchemical Symposium** Contact: Prof. Dr. A. Holasek, Institut für Medizinische Biochemie, Universität Graz, Harrachgasse 21, A-8010 Graz, Austria. Tel. (0 316) 32 5 32 or 76 5 91. (Further details published in Vol. 173, No. 1)
Sep. 2–5, 1980 Prague, Czechoslovakia	**VII European Symposium on Connective Tissue Research** Contact: Dr. Z. Deyl, Physiological Institute Czechoslovak Academy of Sciences, 142 20 Budejovická 1083, Prague 4, Czechoslovakia.
Sep. 6–12, 1980 Liège, Belgium	**International Solvent Extraction Conference 1980 (ISEC '80)** Contact: Conference Secretariat ISEC '80, Department of Chemistry, University of Liège, Sart Tilman, B–4000 Liège, Belgium.
Sep. 7–12, 1980 Florence, Italy	**IUPAC International Symposium on Macromolecules (Structural Order in Polymers)** Contact: Macro IUPAC 80, Fondazione Giovanni Lorenzini, Via Monte Napoleone 23, 20121 Milan, Italy.
Sep. 9–11, 1980 Eindhoven, The Netherlands	**2nd International Symposium on Isotachophoresis** Contact: ITP 80, Afd. Instrumentele Analyse, Technische Hogeschool Eindhoven, Postbus 513, 5600 MB Eindhoven, The Netherlands.
Sep. 15–18, 1980 Liverpool, Great Britain	**Basic High-Performance Liquid Chromatography** Contact: Dr. P.A. Sewell, Department of Chemistry and Biochemistry, Liverpool Polytechnic, Byrom Street, Liverpool L3 3AF, Great Britain.
Sep. 16–19, 1980 Bratislava, Czechoslovakia	**6th International Symposium on Advances and Application of Chromatography in Industry** Contact: Dr. Ján Remeň, Analytical Section ČS VTS, pri n.p. Slovnaft, 82300 Bratislava, Czechoslovakia.
Sep. 17–18, 1980 Amsterdam, The Netherlands	**New Techniques in Analytical Chemistry** Contact: Robert S. First, Inc., 707 Westchester Avenue, White Plains, N.Y. 1060[illegible] U.S.A.
Sep. 22–26, 1980 Paris, France	**European Conference on Chemical Pathways in the Environment** Contact: Dr. C. Troyanowsky, Société de Chimie physique, 10, rue Vauquelin, F-75005 Paris, France. Tel. 707-54-48.
Sep. 28–Oct. 3, 1980 Philadelphia, Pa., U.S.A.	**7th Annual Meeting of Federation of Analytical Chemistry and Spectroscopy Societies (FACSS)** Contact: Mrs. J.G. Graselli, c/o Standard Oil Co., 4440 Warrensville Road, Cleveland, Ohio 44128, U.S.A.
Sep. 29–Oct. 3, 1980 York, Great Britain	**Modern Radiochemical Practice** Contact: The Secretary, Analytical Division, Chemical Society, Burlington House, London W1V 0BN, Great Britain.
Oct. 6–9, 1980 Houston, Texas, U.S.A.	**EXPOCHEM '80** Contact: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, Texas 77004, U.S.A. Tel. (713) 749-2623.

Oct. 6–9, 1980 Houston, Texas, U.S.A.	**Chromatography '80 – 15th International Symposium on Advances in Chromatography** Contact: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, Texas 77004, U.S.A. Tel. (713) 749 2623. (Further details published in Vol. 189, No. 1)
Oct. 19–23, 1980 Washington, D.C., U.S.A.	**Annual Meeting of Assoc. of Official Analytical Chemists** Contact: K.M. Fominaya, Box 540, Benjamin Franklin Station, Washington, D.C. 20044, U.S.A.
Nov. 11–15, 1980 Milan, Italy	**1st African and Mediterranean Congress of Clinical Chemistry** Contact: Secretariat, 1st African and Mediterranean Congres of Clinical Chemistry, Via Keplero 10, 20124 Milan, Italy.
Nov. 19–21, 1980 New York, N.Y., U.S.A.	**19th Eastern Analytical Symposium** Contact: Norman Gardner, Exposition Manager, 73 Ethel Street, Metuchen, N.J. 08840, U.S.A. Tel. (201) 548 7377.
Dec. 16–17, 1980 Brighton, Great Britain	**Chromatography, Equilibria and Kinetics** Contact: Mrs. Y.A. Fish, The Chemical Society, Burlington House, London W1V 0BN, Great Britain. Tel. 01-7349971.
Apr. 13–16, 1981 Cardiff, Wales, United Kingdom	**International Symposium on Electroanalysis in Clinical Environmental and Pharmaceutical Chemistry** Contact: Short Courses Section (Electroanalysis Symposium), UWIST, Cardiff CF1 3NU, Wales, United Kingdom.
May 11–15, 1981 Avignon, France	**5th International Symposium on Column Liquid Chromatography** Contact: Professor G. Guiochon, Ecole, Polytechnique, Laboratoire de Chimie Analytique Physique, Route de Saclay, 91128 Palaiseau, France.
June 22–26, 1981 Nijmegen, The Netherlands	**4th International Symposium on Affinity Chromatography and Related Techniques** Contact: Secretariat, 4th Int. Symp. on Affinity Chromatography and Related Techniques, Department of Organic Chemistry, Faculty of Sciences, Katholieke Universiteit, Toernooiveld, 6525 ED Nijmegen, The Netherlands.
Aug. 23–28, 1981 Espoo, Finland	**Euroanalysis IV – Triennal Conference of the Federation of European Chemical Societies** Contact: Professor L. Niinistoe, Department of Chemistry, Helsinki University of Technology, SF–02150 Espoo 15, Finland.
Aug. 30–Sep. 5, 1981 Vienna, Austria	**XI International Congress of Clinical Chemistry – IV European Congress of Clinical Chemistry** Contact: Congress Secretariat, Interconvention, P.O. Box 35, A–1095 Vienna, Austria. Tel. (0222) 42 13 52.
Sep. 1–4, 1981 Siofok, Hungary	**3rd Danube Symposium on Chromatography** Contact: Hungarian Chemical Society, H–1368 Budapest, P.O.B. 240, Hungary. Tel: Budapest 427–343. (Further details published in Vol. 189, No. 2)

PUBLICATION SCHEDULE FOR 1980

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1979	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	185 186	187/1 187/2 188/1	188/2 189/1 189/2	189/3 190/1	190/2 191 192/1	192/2 193/1 193/2 193/3	194/1 194/2 194/3	195/1 195/2 195/3	196/1 196/2 196/3	The publication schedule for further issues will be published later.			
Chromatographic Reviews			184/1	184/2					184/3				
Biomedical Applications		181/1	181/2	181/ 3-4	182/1	182/2	182/ 3-4	183/1	183/2				

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 193, No. 3, pp. 529–532. A free reprint can be obtained by application to the publisher)

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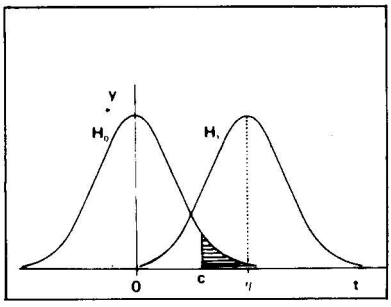
Statistical Treatment of Experimental Data

by J. R. GREEN, *Lecturer in Computational and Statistical Science, University of Liverpool, and* D. MARGERISON, *Senior Lecturer in Inorganic, Physical and Industrial Chemistry, University of Liverpool.*

Physical Sciences Data, Vol. 2



First published in 1977 and now reprinted with some minor revisions, this book is intended for researchers wishing to analyse experimental data using statistical methods. Statistical concepts and methods which may be employed, are explained, and the ideas and reasoning behind statistical methodology clarified. Formal results are illustrated by many numerical worked examples mainly taken from the laboratory. Concepts, practical methodology, and worked examples are integrated in the text.

Consideration is given in this work to a large number of practical topics which are often omitted from standard texts. These include: obtaining an approximate confidence interval for a function of some unknown parameters; testing for outliers, stabilization of heterogeneous variances, and significant differences between means; estimation of parameters after performing tests; deciding what numbers of significant figures to quote for sample means and variances; straight-line and polynomial regression, through the origin or not, using weighted points, and testing the homogeneity of a set of such lines or curves.

The many examples provided throughout the text will serve as models for the various problems encountered by the readers when employing statistical methods to treat experimental data.

In addition to research workers in universities and industry, the book will be of use for first-year students of statistics, and will be especially suitable as the basis of a graduate course in experimental sciences.

CONTENTS: Chapters: 1. Introduction. 2. Probability. 3. Random Variables and Sampling Distributions. 4. Some Important Probability Distributions. 5. Estimation. 6. Confidence Intervals. 7. Hypothesis Testing. 8. Tests on Means. 9. Tests on Variances. 10. Goodness of Fit Tests. 11. Correlation. 12. The Straight Line Through the Origin or Through Some Other Fixed Point. 13. The Polynomial Through the Origin or Through Some Other Fixed Point. 14. The General Straight Line. 15. The General Polynomial. 16. A Brief Look at Multiple Regression. Appendices: 1. Drawing a Random Sample Using a Table of Random Numbers. 2. Orthogonal Polynomials in x. References. Index.

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