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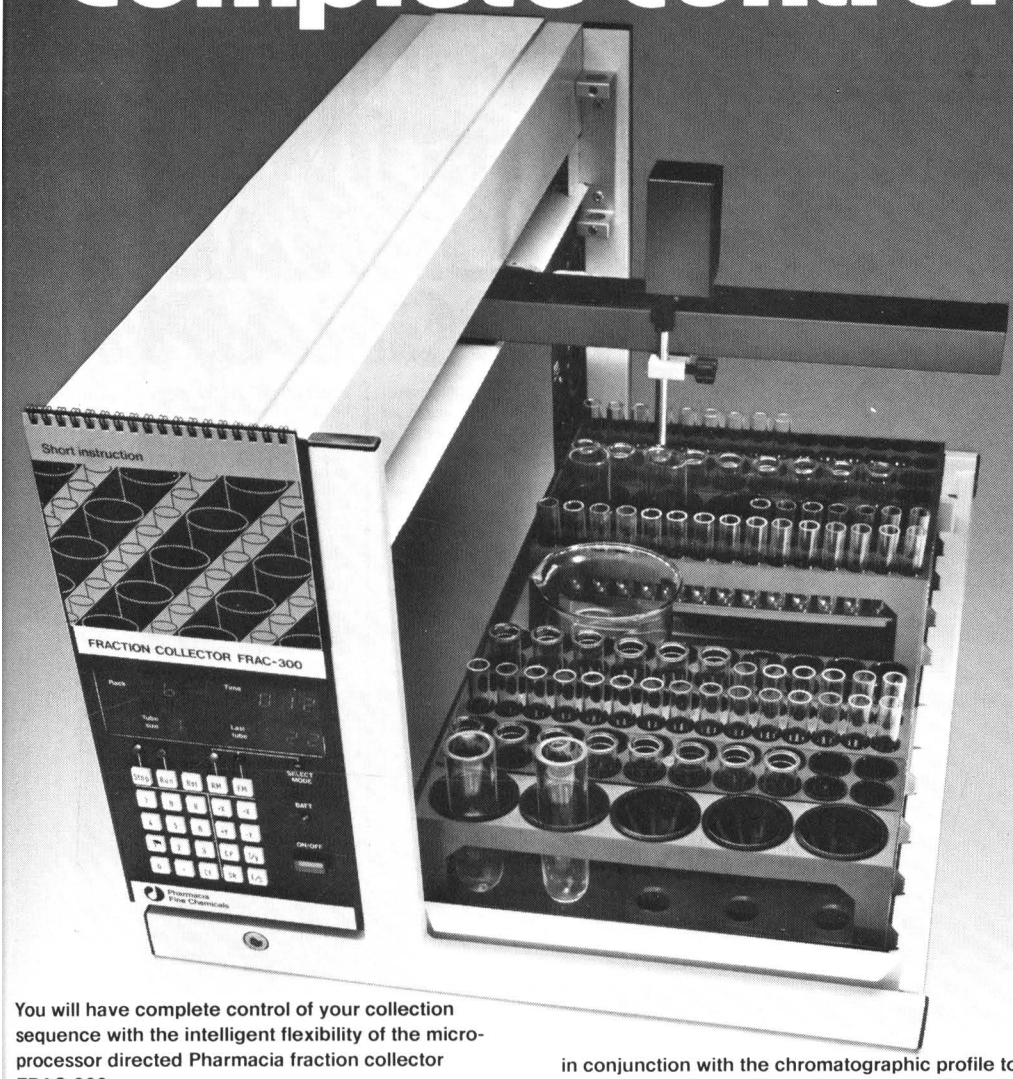

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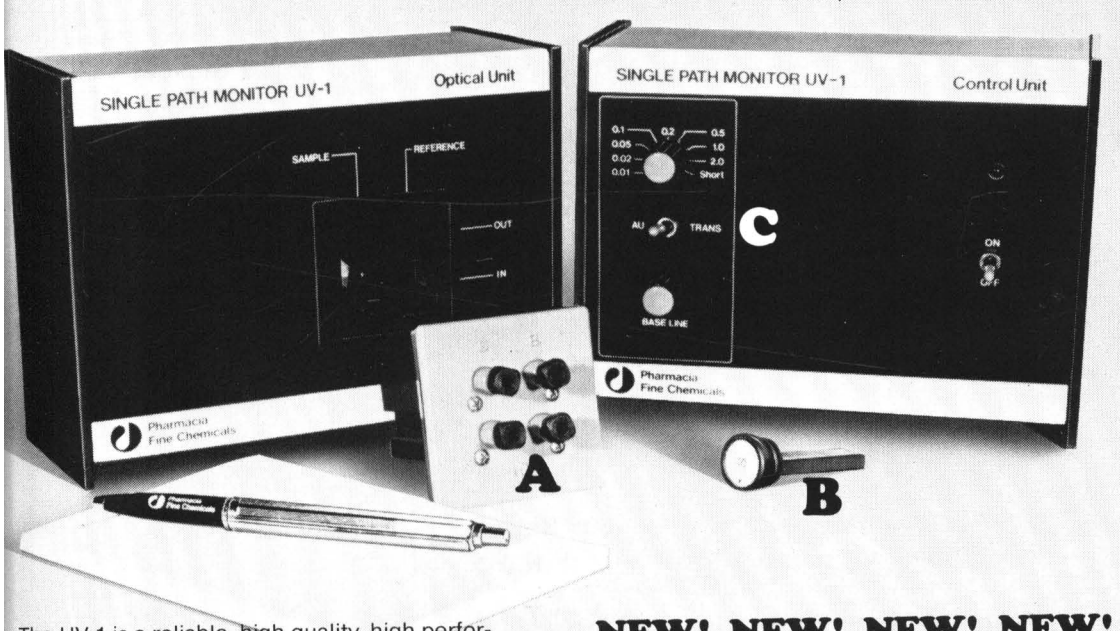

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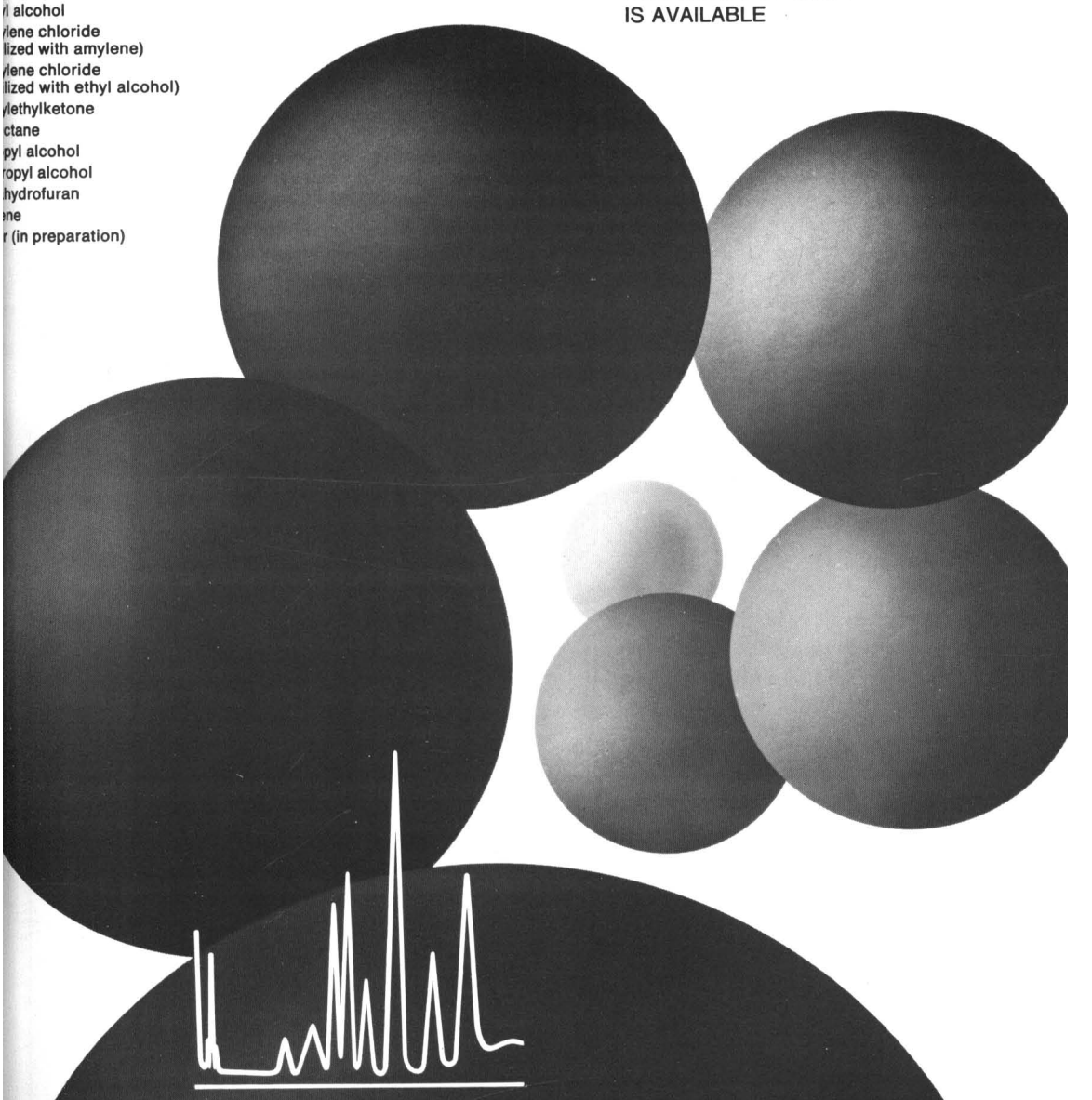

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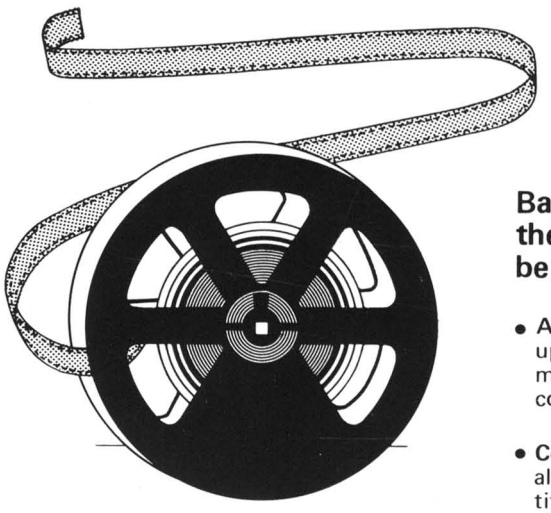

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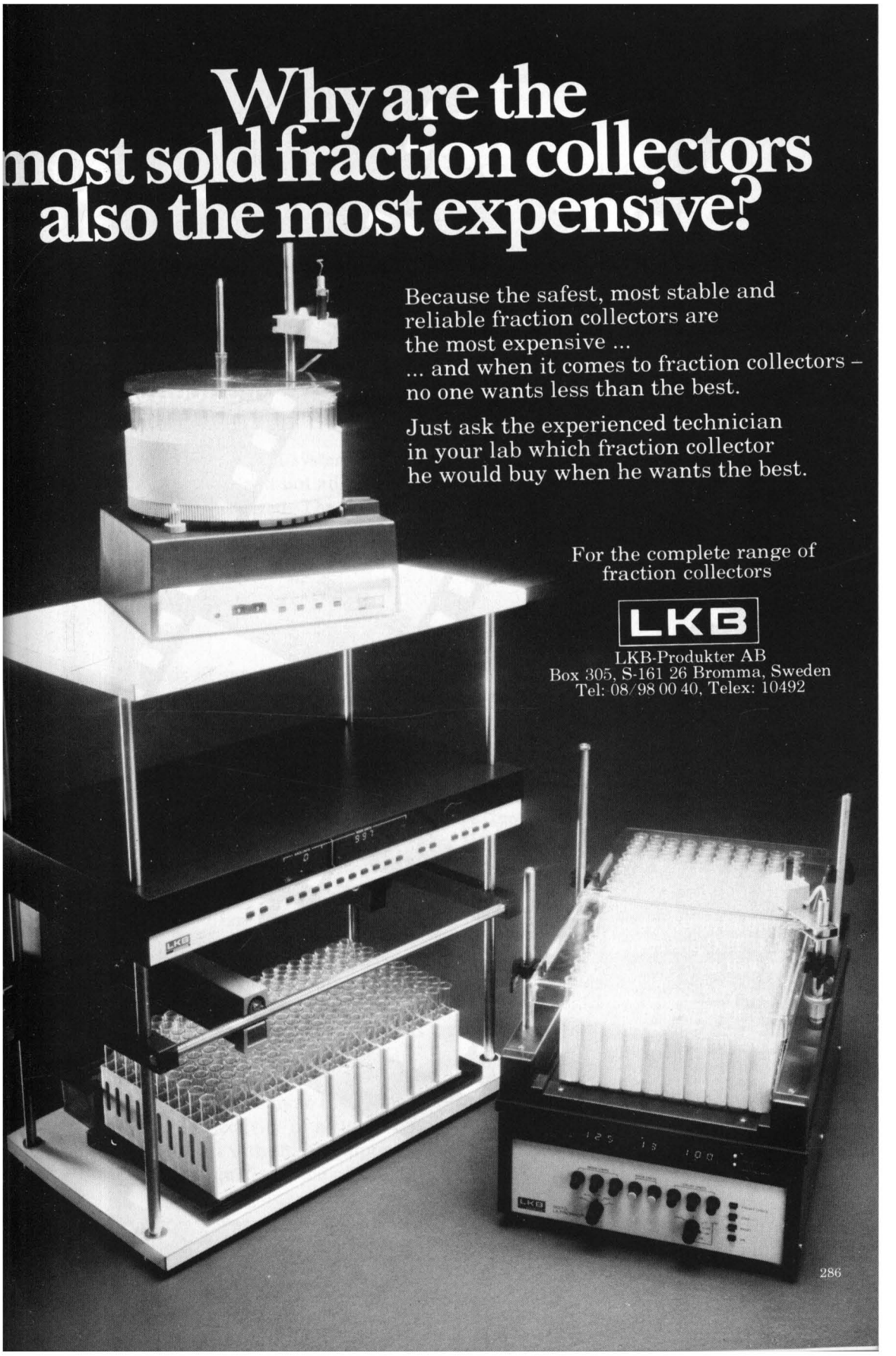
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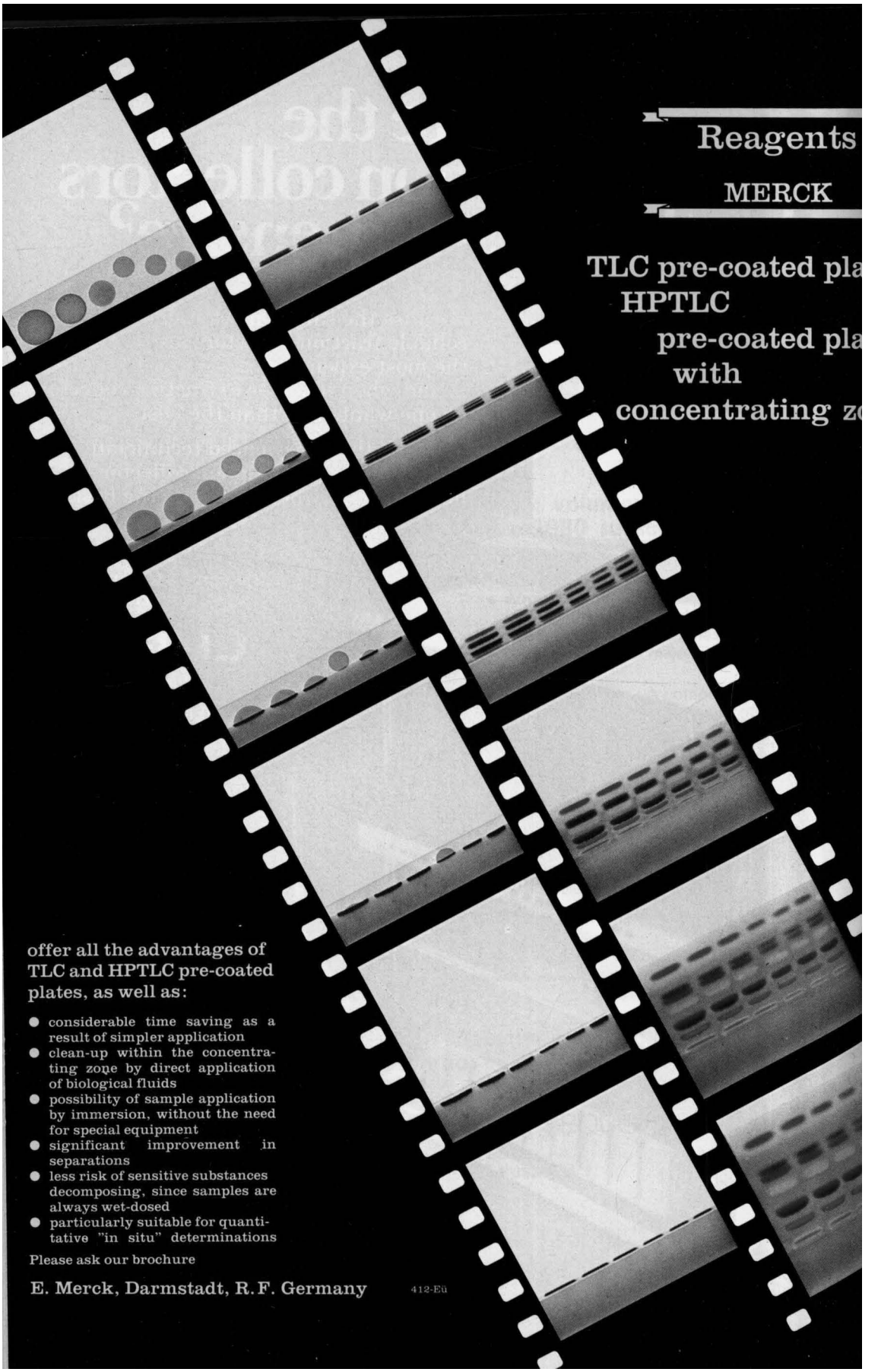

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RELATIVE RETENTION EXPRESSIONS IN CHROMATOGRAPHY

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The Perkin-Elmer Corporation, Norwalk, CT 06856 (U.S.A.)
(First received April 24th, 1980; revised manuscript received June 19th, 1980)

SUMMARY

Computers and data systems usually calculate relative retention as the ratio of two retention times and not adjusted retention times as it should be if theoretical relationships are considered. The purpose of this paper is to demonstrate that such relative retention data can directly be related to the true relative retention (separation factor) and to basic chromatographic relationships.

INTRODUCTION

The basis of separation and identification in chromatography is the relative retention called also the relative volatility or separation factor (α). It is expressed as the ratio of the distribution constants (partition coefficients) of two solutes (sample components)*:

$$\alpha = K_i/K_j \tag{1}$$

Due to the basic chromatographic relationships the ratio of the capacity factors (capacity ratios; k) or the adjusted retention times (t'_R) or volumes (V'_R) may be substituted for the distribution constants:

$$\alpha = k_i/k_j = t'_{Ri}/t'_{Rj} = V'_{Ri}/V'_{Rj} \tag{2}$$

The relative retention (separation factor) is a thermodynamic function which may be related to a number of other physico-chemical values, *e.g.*, to solute chemical potentials, the mole fractions of two solutes in the vapor and liquid phases at equilibrium or to the activity coefficients at infinite dilution and the saturation pressures of the solutes[1].

In the above equations subscripts i and j may have different meanings. In identification i usually refers to the solute of interest and j to the standard while when investigating the selectivity of a stationary phase and the separation of two solutes, i and j refer to them with the assumption that $k_i > k_j$.

As seen in eqn. 2, t'_R, the adjusted retention time is used in the calculation of

* For the meaning of the symbols see the listing at the end of the paper.

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relative retention. The reason for this is that we only consider the selective retardation of the solutes by the stationary phase and this is expressed by this value. The holdup time while contributing to the overall retention of the solutes by the column, is not related to the specific interactions between solutes and the stationary phase; the holdup time is only a function of column length and mobile phase velocity, regardless of the stationary phase and the particular solutes.

Due to the fact that the relative retention (α) is related only to the selective retardation of the solutes by the stationary phase, its value is not related to a given column or instrument; it should remain the same if the same two solutes are analyzed on any instrument and column, assuming that the same stationary phase (and, in liquid chromatography: mobile phase) is used at the same temperature.

This brief discussion makes it clear that, from the theoretical point of view, relative retention calculation must be based on the adjusted retention times.

In spite of this, however, practically all modern chromatography data systems calculate the "relative retention time" (RRT) as

$$\text{RRT} = t_{Ri}/t_{Rj} = V_{Ri}/V_{Rj} \tag{3}$$

in other words, using the retention times (volumes) and not the adjusted retention times (volumes). These values cannot be generalized from one instrument or column to another, even if prepared with the same stationary phase and operated at the same temperature. On the other hand, in a given laboratory and a given system (*i.e.*, instrument and column) such data can be used equally well for identification, by building up data collections through the analysis of standards, particularly by utilizing the speed and automation of present-day data systems. In other words, in spite of the fact that the RRT values do not conform to the chromatographic theory, they are useful in a given laboratory.

It is generally not known that the RRT values can be related to the true relative retention values. Investigation of these relationships is the subject of this paper.

In the discussion below, the symbol α will be used for the true relative retention (separation factor) and the symbol α^* for the RRT, *i.e.*, the ratio of the two retention times. The two solutes will be characterized by the subscripts 1 and 2 assuming that $t_{R2} > t_{R1}$ and that t'_{R2} (t_{R2}) is in the numerator.

RELATIONSHIP BETWEEN α AND α^*

As seen the true relative retention may be expressed as the ratio of the two adjusted retention times or capacity factors:

$$\alpha = t'_{R2}/t'_{R1} = k_2/k_1 \tag{4}$$

On the other hand, α^* is equal to the ratio of the two retention times:

$$\alpha^* = t_{R2}/t_{R1} \tag{5}$$

In order to express α^* as a function of the capacity factors, we first write the retention times as the sum of the adjusted retention times and the hold-up time, and then divide both the numerator and the denominator by the holdup time:

$$\alpha^* = \frac{t'_{R2} + t_M}{t'_{R1} + t_M} = \frac{(t'_{R2} + t_M)/t_M}{(t'_{R1} + t_M)/t_M} = \frac{k_2 + 1}{k_1 + 1} \tag{6}$$

Comparing eqn. 6 with eqn. 4 it is clear that if k_1 and $k_2 \to \infty$, then $\alpha^* \to \alpha$; on the other hand, at low values of the capacity factor, $\alpha^* \neq \alpha$, and we may add that $\alpha^* < \alpha$. This is clear from Fig. 1 which plots α^* against the capacity factor of the second peak (k_2) for four values of the true relative retention (α); for the calculation of α^*, eqn. 8 was used.

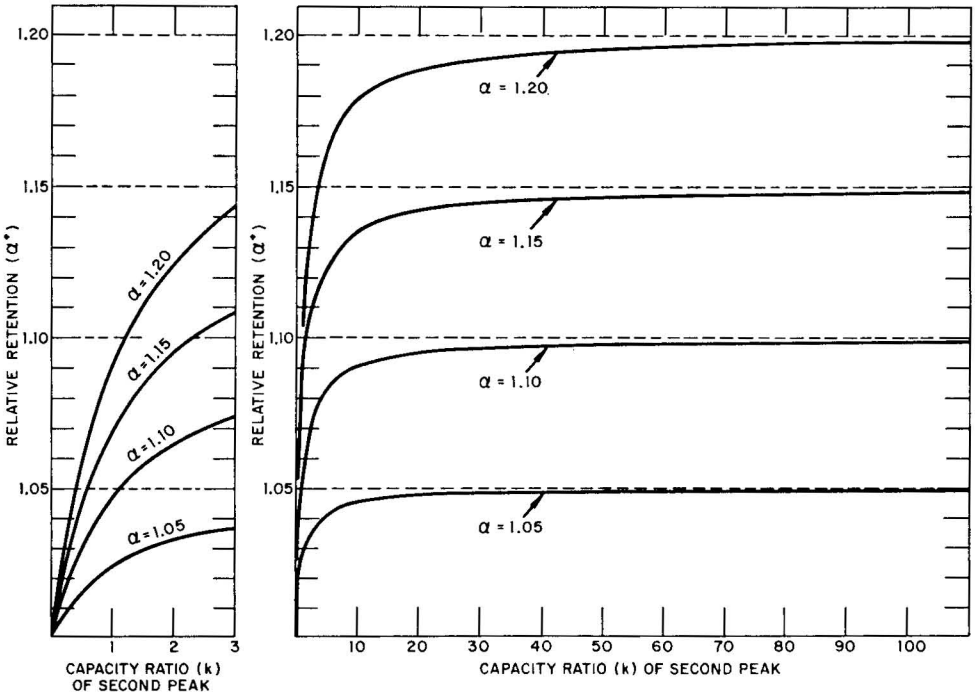

Fig. 1. Plots of α^* against the capacity factor (k) for given true relative retention (α) values.

By substituting eqn. 4 into eqn. 6, α^* can be expressed as a function of α:

$$k_1 = k_2/\alpha \tag{7}$$

$$\alpha^* = \frac{k_2 + 1}{(k_2/\alpha) + 1} = \frac{\alpha(k_2 + 1)}{k_2 + \alpha} \tag{8}$$

Similarly we can deduct that

$$\alpha^* = \frac{\alpha k_1 + 1}{k_1 + 1} \tag{9}$$

In turn, we can express α as a function of α^*:

$$\alpha = \frac{\alpha^* k_2}{(k_2 + 1) - \alpha^*} \tag{10}$$

$$\alpha = \frac{\alpha^*(k_1 + 1) - 1}{k_1} \tag{11}$$

Thus, if k_1 or k_2 are known, α^* can be calculated from α and *vice versa*.

The relationships expressed in eqns. 8–11 underline again the importance of the capacity factor (k) values representing one of the most fundamental terms in chromatography. Since for their calculation knowledge of the holdup time is necessary, it would be very important to have enough information available in each chromatographic run to permit this calculation[2].

THE FUNDAMENTAL RESOLUTION EQUATION AND α^*

The fundamental relationship of chromatography expressing peak resolution (R_s) as a function of the theoretical plate number (n), HETP (h), capacity factor (k), relative retention (α) and column length (L) is as follows[1]:

$$R_s = \frac{\sqrt{n_2}}{4}\left[\frac{\alpha - 1}{\alpha}\frac{k_2}{k_2 + 1}\right] = \frac{1}{4}\sqrt{\frac{L}{h_2}}\left[\frac{\alpha - 1}{\alpha}\frac{k_2}{k_2 + 1}\right] \tag{12}$$

or

$$n_2 = \frac{L}{h_2} = 16R_s^2\left(\frac{\alpha}{\alpha - 1}\right)^2\left(\frac{k_2 + 1}{k_2}\right)^2 \tag{13}$$

In eqns. 12 and 13 the plate number, plate height and capacity factor refer to the second peak ($k_2 > k_1$). Let us see how could α^* be used in these expressions.

From eqn. 8 we can express ($\alpha^* - 1$):

$$\alpha^* - 1 = \frac{\alpha(k_2 + 1)}{k_2 + \alpha} - 1 = \frac{(\alpha - 1)k_2}{k_2 + \alpha} \tag{14}$$

Dividing eqn 14. by eqn. 8 we get:

$$\frac{\alpha^* - 1}{\alpha^*} = \frac{(\alpha - 1)k_2}{k_2 + \alpha}\frac{k_2 + \alpha}{\alpha(k_2 + 1)} = \frac{\alpha - 1}{\alpha}\frac{k_2}{k_2 + 1} \tag{15}$$

If we compare eqn. 15 with eqn. 12 it is evident that the right-hand-side of eqn. 15 is equal to the bracketed term in eqn. 12. In other words,

$$R_s = \frac{\sqrt{n_2}}{4}\left(\frac{\alpha^* - 1}{\alpha^*}\right) = \frac{1}{4}\sqrt{\frac{L}{h_2}}\left(\frac{\alpha^* - 1}{\alpha^*}\right) \tag{16}$$

and similarly

$$n_2 = \frac{L}{h_2} = 16R_s^2\left(\frac{\alpha^*}{\alpha^* - 1}\right)^2 \tag{17}$$

In other words, using α^* we can directly obtain the number of theoretical plates needed to achieve a desired resolution (R_s) for a peak pair (or the resolution corresponding to a column with a given length and efficiency), without the need to establish the corresponding capacity factor value.

RELATIONSHIP OF THE PLATE NUMBERS

Eqn. 17 looks similar to the expression describing the number of effective plates (effective plate number, N), a term which can be traced back to Purnell*[3,4] and obtained its name from Desty[5]:

$$N_2 = \frac{L}{H_2} = 16R_s^2\left(\frac{\alpha}{\alpha - 1}\right)^2 \tag{18}$$

where H is the height equivalent to one *effective* plate (HEEP) or effective plate height. Using the respective symbols of A and A^*:

$$A = \frac{\alpha}{\alpha - 1} \text{ and } A^* = \frac{\alpha^*}{\alpha^* - 1}$$

and dividing eqn. 17 by eqn. 18 we get:

$$n/N = (A^*/A)^2 \tag{19}$$

In other words, the ratio of the two terms in the right-hand-side of the equation gives the ratio of the number of theoretical and effective plates.

NOTE

It should be noted that α^* is equivalent to the "separation factor" of Glueckauf[6]. As pointed out by Tang and Harris[7], confusing it with the real separation factor (*i.e.*, α), particularly in the interpretation of Glueckauf's widely reproduced charts predicting the number of theoretical plates needed to obtain a given purity of products for the separation of species having a given "separation factor" (see *e.g.* Keulemans[8]) led to conflicting and highly misleading conclusions.

LIST OF SYMBOLS[6]

A	symbol for $\alpha/(\alpha - 1)$
A^*	symbol for $\alpha^*/(\alpha^* - 1)$
h	height equivalent to one theoretical plate; theoretical plate height, HETP
H	height equivalent to one effective plate; effective plate height, HEEP
k	capacity factor, capacity ratio
K	distribution constant, partition coefficient
L	column length
n	number of theoretical plates, theoretical plate number
N	number of effective plates, effective plate number
R_s	peak resolution
RRT	see α^*

* In Purnell's original equations the symbols can easily be misinterpreted. Using our present-day symbols[6] his V_R is V_R', V_d is V_M, and V_R' is V_R^o ($V_R^o = jV_R$, the retention volume corrected for gas compressibility).

t_M holdup time: retention time of a solute not retained by the stationary phase
t_R retention time (measured from the instant of sample introduction)
t'_R adjusted retention time; $t'_R = t_R - t_M$
V_M holdup volume: retention volume of a solute not retained by the stationary phase
V_R retention volume (measured from the instant of sample introduction)
V'_R adjusted retention volume; $V'_R = V_R - V_M$
α relative retention, separation factor; $\alpha = t'_{Ri}/t'_{Rj} = V'_{Ri}/V'_{Rj}$
α^* "relative retention time" (RRT); $\alpha^* = t_{Ri}/t_{Rj} = V_{Ri}/V_{Rj}$

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INFLUENCES OF DETECTOR TIME CONSTANT VARIATIONS ON EFFICIENCY CALCULATIONS IN THE STANDARDISATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC COLUMNS

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(First received January 28th, 1980; revised manuscript received April 15th, 1980)

SUMMARY

This paper reports the influences of detector time constant variations on plate count calculations and peak retention times in the standardisation of high-performance liquid chromatographic columns. A recommendation is made here that column efficiencies should be quoted at zero time constant in order to remove the variability in plate count introduced by varying time constant. For detectors which do not have variable time constant controls, it is recommended that the calculation of plate numbers should be made on solutes having capacity factors in the region 5–6.

Further recommendations made are that the peak symmetry correlation ratio should be used to evaluate system performance only when calculated at zero time constant and that the detector time constant should not exceed one hundredth of the peak width for peaks used in the calculation of plate numbers.

INTRODUCTION

Peak asymmetry is an important aspect in relation to resolution and column performance in high-performance liquid chromatography (HPLC). The major contributions to peak tailing arise from voids or channelling in the column itself and also from extra-column effects chiefly due to solute diffusion in tube connectors, precolumns, cell volume of the detector and the injector system[1–3]. A further contribution which has not often been recognised is that improper time constant settings on UV detectors of the HPLC system can also cause peak asymmetry.

Two important terms, the "time constant" (τ) and the "response time" (t) are often referred to in the literature. The former term is the time required for the recorder to reach 63.2% of its final value and the manner in which the detector time constant affects peak shape and peak height has been briefly discussed by Stewart[4]. The latter term is the time required for the recorder to reach 99.7% of its final value and is equal to five times the time constant.

In a study of the use of a modified scanning spectrophotometer as a variable-wavelength detector for HPLC, Higgins[5] noted that HETP (height equivalent to a theoretical plate) values and peak symmetry were dependent on the time constant of

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the detector. We have explored these effects in detail and this paper presents a discussion of the serious errors which can result when column efficiency (expressed as the number of theoretical plates) is calculated using peaks which are distorted due to incorrect settings of time constant. In addition, the influence of detector time constant on retention times of solutes and their analytical sensitivities is also discussed.

EXPERIMENTAL

Instrumentation and reagents

The liquid chromatograph used consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000 solvent pump, Model U6K injector, Model 450 variable-wavelength detector and an Omniscribe Model B5217-1 recorder. The detector was fitted with a continuously adjustable variable time constant control consisting of a simple first-order resistance–capacity (RC) Butterfield filter with normal roll-off characteristics of −3dB at the cut-off frequency and −6dB per octave. Because this control was difficult to set reproducibly, it was replaced by a 10-position switch with discrete resistors instead of the continuously variable potentiometer formerly fitted. Use of this switch enabled settings of time constant in the range 0.1–1.1 sec in increments of 0.1 sec. No attempt was made to compensate observed chromatographic data for the response characteristics of the recorder, since the same recorder was used for all measurements.

Analytical grade methanol was triply distilled from all glass apparatus. Acetonitrile was purchased from Ajax, Sydney, Australia (Spectrograde) and water was distilled using Millipore Milli-Q water purification system. All other reagents were used as purchased without further purification.

Chromatographic procedure

Separation by HPLC was accomplished using a μBondapak C_{18} column (30 cm × 3.9 mm O.D., Waters Assoc.). The mobile phases used were acetonitrile–water (60:40) for the first test solution and acetonitrile–water (50:50) for the second test mixture. The mobile phase flow-rate was 2.5 ml/min producing a back pressure of 2000 p.s.i. and the detector was operated at 254 nm with a sensitivity setting of 0.1 a.u.f.s. The first test solution contained 26 mg acenaphthene in 100 ml of methanol–water (60:40), and the second test mixture contained phenol (20.3 mg), *p*-cresol (19.3 mg), 2,5-xylenol (19.9 mg), anisole (20.5 mg) and phenetole (20.5 mg) in 100 ml of methanol–water (50:50). In all runs, 15 μl of the respective test solution was injected using a 25-μl syringe. All separations were carried out at 20°C with a recorder chart speed of 2 in./min.

The peak symmetry correlation test entailed removal of the HPLC column from the system and the inlet and outlet tubes to the column were then joined by a suitable connector. The sample solution consisted of 0.1 ml of acetone in 100 ml of methanol and 10 μl of this solution were injected. The mobile phase was pure methanol with a flow-rate of 0.5 ml/min. The wavelength of the detector was fixed at 254 nm using a sensitivity of 0.1 a.u.f.s, and the recorder chart speed was 5 in./min.

RESULTS AND DISCUSSION

Effect of time constant on peak shape, height and retention

Increase in detector time constant results in a reduction in peak height (H), an increase in peak skewness and a shift in the peak maximum towards longer retention. These effects are illustrated in Fig. 1 which shows the separation of the test mixture obtained at two extreme settings of detector time constant.

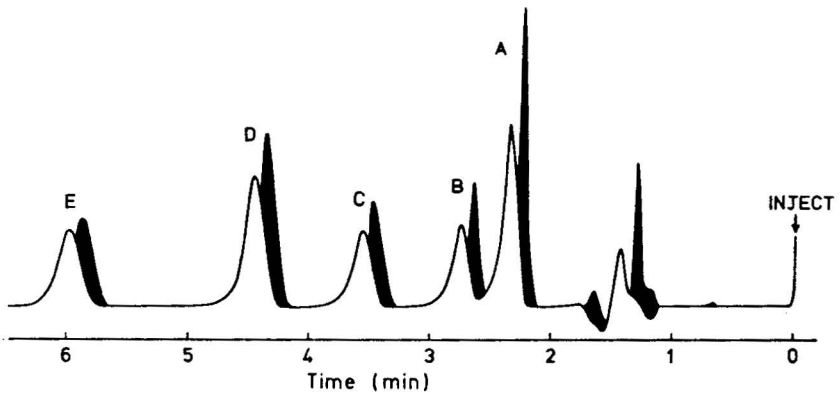

Fig. 1. Chromatogram of test mixture recorded at two different values of time constant. Dark peaks: $\tau = 0.1$; light peaks: $\tau = 1.1$. A = phenol; B = paracresol; C = 2,5-xylenol; D = anisole; E = phenetole.

The precise manner in which the above peak parameters are affected by detector time constant will be dependent on the type of filter used in the detector. In our case, the detector employed a simple Butterfield type filter and the results presented in this paper would be applicable to detectors equipped with this type of filter.

Linear relationships were observed between time constant and both peak height and capacity factor of the solute, however skewness and time constant were non-linearly related with the greatest increases in skewness being observed at the longer values of time constant, that is, those values preferred for heavy damping of baseline noise.

Effect of time constant on HETP calculation

The efficiency of the column, expressed as the number of theoretical plates (N), was calculated using the 4σ, 5σ and half-height methods with acenaphthene as solute and at various settings of detector time constant. The calculated value of N was observed to decrease linearly with increasing values of τ, in confirmation of results presented by Higgins[5]. This effect was quite pronounced. For example at $\tau = 0.1$ sec, $N = 3870$ whilst at $\tau = 1.1$ sec, $N = 2190$.

Most manufacturers of HPLC columns include in the column specifications a guaranteed minimum value of N, without specifying a corresponding value of τ. From our results, we suggest that a more logical procedure to use when reporting values of N would be to remove the time constant effect by quoting the value of N at zero time constant. To do this requires that detectors have a variable, calibrated time constant

control or an in-line RC filter (as used by Higgins) to allow at least three values of τ to be used in construction of a graph of N *vs.* τ. Extrapolation of this graph to $\tau = 0$ provides the required value of N. An alternative procedure will be suggested below for situations where modification of detector electronics is not possible.

Preliminary work had indicated that the dependence of both peak height and the number of theoretical plates on time constant was governed by the capacity factor of the peak used. The percentage decrease in H and N obtained by changing the time constant from 0.1 sec to 1.1 sec was calculated for each of the solutes in the test mixture (Fig. 1) and compared with the capacity factor of the solute (measured at zero time constant by extrapolation). It was found that the time constant effect on H and N, as reflected by the decrease in these parameters when τ is increased, is much less for the longer retained solutes. This is due to the fact that the broad peaks observed at high capacity factors are much less susceptible to changes in τ than are sharp, narrow peaks. It can be concluded from these results that calculations of N based on a solute with a capacity factor in the region of 5–6 would give a value closer to that obtained by the extrapolation method discussed earlier (*i.e.* the value of N at zero time constant) than for a faster eluted peak. Thus if no variable time constant control is present and electronics modification is not desirable, then selection of a solute with a high capacity factor for calculations of N would be a suitable compromise.

This contention is supported by the following values of N: 5470 (calculated by the method of extrapolation to zero time constant) and 5350 (calculated using phenotole which has a capacity factor of 5.85 in a mobile phase of acetonitrile–water (45:55) with a detector time constant of 0.6 sec).

Some authors and instrument manufacturers have suggested that suitable values of time constant can be selected by reference to the peak width. Stewart[4] recommends that time constant should not exceed one tenth of peak width (w) and this recommendation is based on treatment of the peak shape as a normal distribution. The handbook for the detector used in this study makes the same suggestion. In order to evaluate the suitability of this recommendation for data to be used in column efficiency calculations, we determined the value of N at various ratios of τ/w using acenaphthene as solute. The results showed that the plate count was reduced from its theoretical maximum (at zero time constant) as the ratio τ/w was increased. A reduction of 10% or less in plate count was achieved only for values of τ/w of 0.01 or less. The conclusion arising from this study is that the selection of time constant settings such that $\tau/w \leqslant 0.1$ is inappropriate when the chromatogram is to be used for plate count calculations. In this case, $\tau/w \leqslant 0.01$ should be used.

Peak symmetry correlation test

The symmetry correlation ratio is expressed as $R_S = 0.8\ (A/B)$ where A is the base width of a peak measured at 4.4% of the peak height and B is the base width between tangents. For a perfectly Gaussian peak, this ratio is equal to one. R_S is often used as an indicator of the efficiency of the chromatographic system with the column removed; high values of R_S generally reflect malfunctions such as cracked cell windows, excessive dead volume in the injector or tubing etc. This ratio was calculated at various values of τ and was found to increase linearly with τ. It follows that the peak symmetry correlation test can be validly applied only when the time constant effect is removed. To do this, the linear R_S *vs.* τ plot should be extrapolated to $\tau = 0$ and the

resulting value of R_S used as a measure of system performance. In our laboratory, a value of $R_S \leqslant 1.3$ indicated proper functioning of the chromatograph.

CONCLUSIONS

The effect of detector time constant on peak height, peak skewness, capacity factor, number of theoretical plates and the peak symmetry correlation ratio has been examined. The results have led to four recommendations for minimising the effect of time constant on the above parameters and these are:

(1) Column efficiencies (expressed as the number of theoretical plates) should be determined at zero time constant by extrapolation of the linear N *vs.* τ plot.

(2) If no variable time constant control is fitted to the detector, then calculation of N using a peak of a solute with capacity factor 5–6 ensures minimal time constant effect on the value obtained.

(3) The peak symmetry correlation ratio R_S should be determined at zero time constant by extrapolation of the linear R_S *vs.* τ plot.

(4) The commonly held idea that a value of $\tau/w \leqslant 0.1$ is optimal for general chromatography is inappropriate when a peak is to be used for efficiency calculations. In this case $\tau/w \leqslant 0.01$ should be used.

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QUANTITATIVE STRUCTURE–RETENTION RELATIONSHIPS IN CATION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

A study of quantitative structure–retention relationships has been made in the cation-exchange chromatography of primary monoamines, such as aliphatic amines, β-phenylethylamine and amino alcohols. Retention data for twelve amines on three columns in the K^+, Na^+ and Li^+ forms were analysed. The relationship between the Stokes radius of the counter ion and retention was elucidated by comparing the selectivity coefficients of amines for the three different counter ions. The structure parameters, τ, of methyl, phenyl, hydroxyl and ionized amino groups were obtained as the increments in the selectivity coefficient of the amine, $\Delta \ln K_M^{Amine}$, on replacing a hydrogen atom with these groups.

INTRODUCTION

Liquid chromatography has contributed greatly to the separation and analysis of various compounds. However, optimization of the elution conditions for new problems still require much effort and time. Many studies have been made of the relationship between retention and chromatographic conditions.

In a previous paper[1], the chromatographic behaviour of primary amines in cation-exchange chromatography was investigated. The correlation of the distribution coefficient with the composition of the mobile phase was elucidated, and the influence of the degree of dissociation of the amines on retention was expressed by an experimental equation.

This paper describes the effect of the molecular structure of amines on retention in ion-exchange chromatography. The contribution of hydrophobic interactions to the selectivity coefficient is discussed. Hydrophobic properties of amine molecules with respect to the resin matrix are measured by applying the method of quantitative structure–activity relationships (QSAR). Recently, a study of QSAR, developed by Hansch[2], has been applied to the evaluation of the contribution of substituents to chromatographic retention[3–6].

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EXPERIMENTAL

Methylamine, ethylamine, *n*-propylamine, *n*-butylamine, isobutylamine, *n*-amylamine, isoamylamine, *n*-hexylamine, ethanolamine, 2-aminobutanol, 5-aminopentanol, 6-aminohexanol and β-phenylethylamine were purchased from Nakarai Chemicals (Kyoto, Japan) or Tokyo Chemicals (Tokyo, Japan). Other chemicals were obtained from Nakarai Chemicals or Wako (Osaka, Japan).

A Hitachi Model KLA-3B amino acid analyser (Hitachi, Tokyo, Japan) was employed throughout this work. The chromatography was carried out on a 3 × 0.6 cm column of Aminex A-4 sulphonated polystyrene cation-exchange resin (Bio-Rad Labs., Richmond, CA, U.S.A.) with a flow-rate of 30 ml/h at 50°C. Potassium borate, sodium borate and lithium borate (pH 8.0) buffers were used as eluents. A 0.1-μmole amount of each amine was applied to the column and detected with the ninhydrin system at 440, 570 and 640 nm.

RESULTS AND DISCUSSION

The retention of the amines was expressed as the logarithm of the selectivity coefficient, which was proportional to the free energy change associated with the distribution process. In the buffer solution of pH 8.0 most of the amine molecules are dissociated, as the dissociation constants of these amines are well below 10^{-8}. The selectivity coefficient can be expressed by the following equation:

$$\text{Ln } K_{\text{M}}^{\text{Amine}} = \ln(D_v[\text{M}^+]/[\overline{\text{M}^+}]) \tag{1}$$

where $K_{\text{M}}^{\text{Amine}}$ is the selectivity coefficient of the amine for the counter ion, M, and D_v denotes volume distribution coefficients. $[\text{M}^+]$ and $[\overline{\text{M}^+}]$ are the concentrations of univalent counter ions in the mobile phase and in the stationary phase, respectively. The $[\text{M}^+]$ value was that of the cations in the buffer salts. The $[\overline{\text{M}^+}]$ value was approximated as the concentration of the fixed ionic groups in the resin, as the amounts of the amine molecules and hydrogen ions should be negligible in the stationary phase. The concentration of the fixed ionic groups depends on the ionic form of the resin and can be calculated by correcting the weight capacity for the density and solvent content of the resin[7]. The concentration of the fixed ionic group in the K^+ form of the resin was found to be 2.73, that in the Na^+ form of the resin 2.69 and that in the Li^+ form of the resin 2.57 mequiv/ml of the resin bed. The D_v value was obtained from the retention volume, V_R, according to the usual method; $D_v = (V_R - V_0)/V_M$, where V_0 is the hold-up volume and V_M the column bed volume. The selectivity coefficients of amines were calculated by eqn. 1. Each ln $K_{\text{M}}^{\text{Amine}}$ value used here was the mean value of several determinations, using eluents with different buffer salt concentrations.

In Fig. 1 the values of ln $K_{\text{K}}^{\text{Amine}}$ and ln $K_{\text{Li}}^{\text{Amine}}$ are plotted against the value of ln $K_{\text{Na}}^{\text{Amine}}$. A linear relationship was found, with a slope of unity. The correlation equations are as follows:

$$\text{Ln } K_{\text{Li}}^{\text{Amine}} = 0.38 + \ln K_{\text{Na}}^{\text{Amine}}$$

$$\text{Ln } K_{\text{K}}^{\text{Amine}} = -0.36 + \ln K_{\text{Na}}^{\text{Amine}}$$

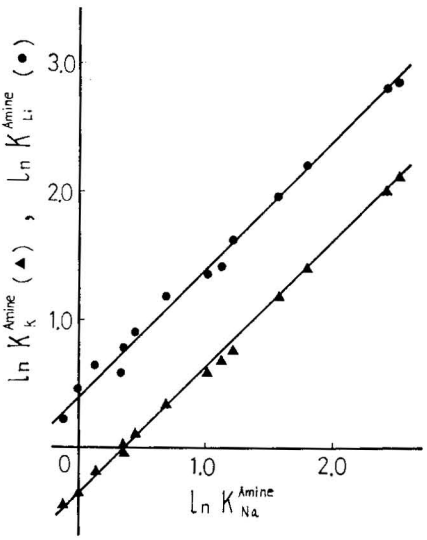

Fig. 1. Correlation between ln K_M^{Amine} values of primary monoamine measured with columns with different counter ions.

The intercepts, 0.38 and −0.36, indicate the values of ln K_{Li}^{Na} and ln K_K^{Na}, respectively. The values of the selectivity coefficients for the exchange systems Na/Li and Na/K were obtained as $K_{Li}^{Na} = 1.46$ and $K_K^{Na} = 0.70$, which compare reasonably well with values reported in the literature[8]. Additionally, ths values of K_{Li}^{Na} and K_K^{Na} obtained here could be related to the ratio of the Stokes radii (effective hydrated radii) of the ions: $r_{Li}/r_{Na} = 1.30$, $r_K/r_{Na} = 0.68$, which were calculated from Kiso's data[9] and 1.30 and 0.69 from the Landolt-Brönstein's Tabellen[10]. This shows that in ion-exchange chromatography the selectivity coefficient can be expressed as a function of the ratio of the Stokes radii of ions.

In the above discussion only ionic interactions are being considered. In fact, the selectivity coefficient of the dissociated amine depends on two different interactions; one is due to ionic interactions with the fixed ionic group of the resin and the other to hydrophobic interactions with the resin matrix. The overall selectivity coefficient can be expressed as follows:

$$\text{Ln } K_M^{Amine} = \ln(K_M^{Amine})_{ionic} + \ln(K_M^{Amine})_{hydrophobic} \quad (2)$$

The retention characteristic of each amine is dominated by the second term in eqn. 2, as the first term for compounds having the same ionized group is constant.

The effects of hydrophobic interactions on the selectivity coefficient are shown in Figs. 2 and 3. In Fig. 2 the logarithm of the selectivity coefficients of aliphatic monoamines and β-phenylethylamine for various counter ions is plotted against the carbon number of the amine, n. The values for $n = 0$ were obtained from the logarithm of the selectivity coefficients of the ammonium ion for the various counter ions. The ln K_M^{Amine} value increased with increasing carbon number. However, isobutylamine and isoamylamine were eluted faster than n-butylamine and n-amylamine, respectively.

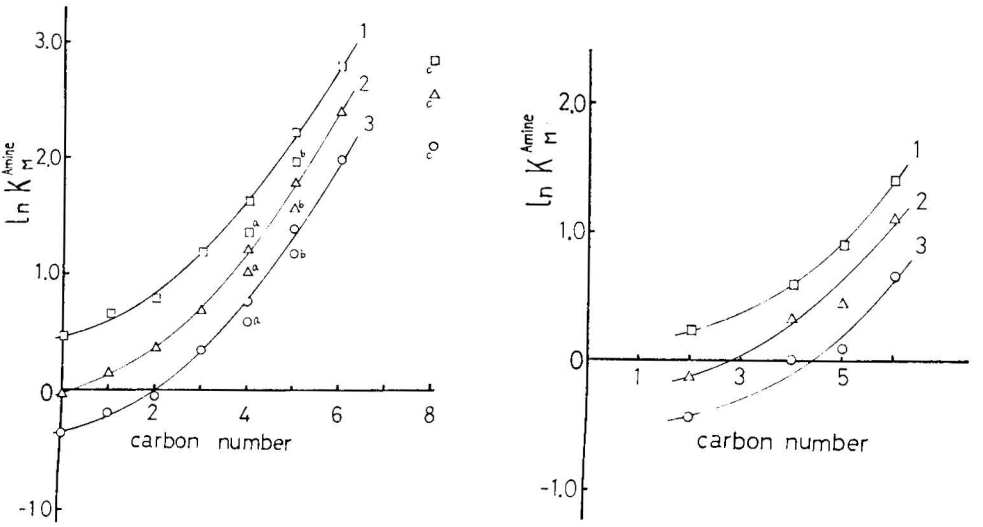

Fig. 2. Correlation between carbon number (chain length) of *n*-alkylamines and ln K_M^{Amine}. Column: 1 = K^+ form; 2 = Na^+ form; 3 = Li^+ form. (a) Isobutylamine; (b) isoamylamine; (c) β-phenylethylamine.

Fig. 3. Correlation between carbon number (chain length) of amines and ln $K_M^{Amino\ alcohol}$. Column: 1 = K^+ form; 2 = Na^+ form; 3 = Li^+ form.

The selectivity coefficient of these two amines corresponded to those of *n*-alkylamines with carbon numbers of 3.7 and 4.7, respectively. The selectivity coefficient of β phenylethylamine ($n = 8$) corresponded to that of an *n*-alkylamine with a carbon-number of 6.2.

Fig. 3 shows the plots of the logarithm of selectivity coefficients of amino alcohols against the carbon number of the main alkyl chain. Comparing the results in Fig. 3 with those in Fig. 2, it can be seen that the introduction of a hydroxyl group into the aliphatic amine molecule causes a decrease in adsorption, corresponding to a decrease in the carbon number of about 2. The results suggest that hydrophobic interactions depend on the molecular shape of the amines.

The QSAR technique developed by Hansch was applied to measurements of the hydrophobic properties of the groups constituting the amine. The increments in the ln K_M^{Amine} values on replacing a hydrogen atom by a group such as methyl, hydroxyl or phenyl were calculated from the retention data of amines. In Table I the Δ ln K_M^{Amine} values are shown as the structure parameter, τ. As retention is subjected to a linear free energy relationship, τ represents the effect of the substituted group on the free energy change of hydrophobic adsorption. A larger τ value implies that the substituted group requires a greater hydrophobic adsorption energy to interact with the resin matrix. In Table I the simple numbers before the substituents in the first column shows the position of the carbon atoms on the alkyl chain; the numbers with primes show the position of the carbon atom on the branched chain. The τ values for the same substituent obtained on the three different ionic forms of the columns did not vary considerably, and the τ values depended on the position of the substituent. The following sequence of τ values for the substituents was found: 1-CH_3 < 2-CH_3 < 3-CH_3 < 4-CH_3 < 5-CH_3 $\approx$ 6-CH_3.

TABLE I

τ VALUES

Substituent	$\Delta \ln K_M^{Amine}$	τ		
		$M = Li^+$	$M = Na^+$	$M = K^+$
1-CH_3	$\ln K_M^{Methylamine} - \ln K_M^{Ammonia}$	0.19	0.17	0.17
2-CH_3	$\ln K_M^{Ethylamine} - \ln K_M^{Methylamine}$	0.14	0.22	0.13
2′-CH_3	$\ln K_M^{Isobutylamine} - \ln K_M^{n\text{-}Propylamine}$	0.17	0.33	0.24
3-CH_3	$\ln K_M^{n\text{-}Propylamine} - \ln K_M^{Ethylamine}$	0.40	0.33	0.40
3′-CH_3	$\ln K_M^{Isoamylamine} - \ln K_M^{n\text{-}Butylamine}$	0.34	0.36	0.42
4-CH_3	$\ln K_M^{nButylamine} - \ln K_M^{n\text{-}Propylamine}$	0.44	0.53	0.42
5-CH_3	$\ln K_M^{n\text{-}Amylamine} - \ln K_M^{n\text{-}Butylamine}$	0.59	0.58	0.64
6-CH_3	$\ln K_M^{n\text{-}Hexylamine} - \ln K_M^{n\text{-}Amylamine}$	0.59	0.62	0.60
2-C_6H_6	$\ln K_M^{\beta\text{-}Phenylethylamine} - \ln K_M^{Ethylamine}$	2.07	2.16	2.17
2-OH	$\ln K_M^{Ethanolamine} - \ln K_M^{Ethylamine}$	−0.56	−0.48	−0.39
5-OH	$\ln K_M^{5\text{-}Aminopentanol} - \ln K_M^{n\text{-}Amylamine}$	−1.31	−1.35	−1.30
6-OH	$\ln K_M^{6\text{-}Aminohexanol} - \ln K_M^{Hexylamine}$	−1.39	−1.29	−1.33

Negative τ values for a substituted hydroxyl group indicate that the alcoholic hydroxyl group weakens the hydrophobic interaction to the resin matrix. The sequence of τ values for substituted hydroxyl groups was as follows: 2-OH > 5-OH ≈ 6-OH.

In this method the structure parameter of the ionized amino group was not available. However, an approximate value for the ionized amino group could be estimated as follows. As discussed above, the contribution of ionic interactions to the selectivity coefficient can be expressed as the ratio of the hydrated radii of the ionized groups. For the exchange between the amine and counter ion, Na^+, the relationship was as follows:

$$\mathrm{Ln}\,(K_{Na}^{Amine})_{ionic} = \ln r_{Na}/r_A$$

where, r_{Na} is the hydrated radius of the sodium ion and r_A that of the ionized amino group. The hydrated radius of the ammonium ion was used as an approximate value of r_A. The $\ln r_{Na}/r_A$ was 0.38 from the values of the hydrated radii in the literature[9,10]. On the other hand, the overall selectivity coefficient, $\ln K_{Na}^{Amine(n=0)}$, was −0.04, as shown in Fig. 2. From eqn. 2, the $\ln [K_{Na}^{Amine(n=0)}]_{hydrophobic}$ value of −0.42 was obtained as the remainder on subtracting the $\ln r_{Na}/r_A$ value from the $\ln K_{Na}^{Amine(n=0)}$ value. It was estimated that the value of −0.42 was the approximate structure parameter of the ionized amino group.

In ion-exchange chromatography, ionized compounds are drawn towards the ion-exchange resin through ionic interactions between the ionized group in the molecule and the fixed ion of the resin, and this makes the hydrophobic groups in the molecule adsorb on the resin matrix. The logarithm of the selectivity coefficient could be expressed as the sum of the logarithms of the contributions of both ionic and hydrophobic interactions. The contribution of ionic interactions to the selectivity coefficient was related to the ratio of the hydrated radii of ionized amino groups and counter ions in this work. If ionized amino groups were present in the molecule the contribution of hydrophobic interactions was represented as the structure parameter,

τ, which was calculated for each substituent. The τ values showed the hydrophobic property of the methyl substituent and hydrophilic properties of hydroxyl and ionized amino substituents with respect to the resin matrix. Methyl substitution in different positions on the *n*-alkyl chain caused differences in the τ values. The τ value for the hydroxyl group was also affected by the its position in the *n*-alkyl amino alcohols. The sequences of τ-value observed for methyl and hydroxyl substituents suggest on effect of the ionized amino group on the interaction between the substituents and the resin matrix.

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METAL CHELATE AFFINITY CHROMATOGRAPHY

I. INFLUENCE OF VARIOUS PARAMETERS ON THE RETENTION OF NUCLEOTIDES AND RELATED COMPOUNDS

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SUMMARY

The influence of various parameters, such as pH, ionic strength and temperature, on the retention of different nucleotides and related compounds on copper chelate gels has been investigated in order to understand the respective roles played by the different solute constituents (*i.e.*, heterocyclic bases, sugars and phosphate groups) in the interaction and to define optimal conditions for subsequent application to the fractionation of oligo- and polynucleotides.

INTRODUCTION

The investigation of the mode of action of biological macromolecules (proteins, nucleic acids, polysaccharides, etc.) has been hampered for a long time by the lack of convenient, rapid and high-yield techniques for the isolation of these substances in a pure form. Although affinity chromatography and related methods have afforded considerable improvements during the last decade, the search for purification techniques based on new principles still remains a very challenging motivation.

The observation that amino acids and nucleotides are able to form specific complexes with metallic cations and, more generally, with electron-acceptor ligands, prompted the development of two new fractionation procedures: electron donor–acceptor (or charge-transfer) chromatography and metal chelate affinity chromatography (ligand exchange chromatography).

The latter technique has been mainly studied for protein and amino acid fractionation purposes[1–6] and has so far received only limited attention in the nucleotide field[4,7,8]. However, during the last 25 years, much work has been devoted to the study of interactions between metallic cations, especially those belonging to the first transition period, and ribo- and deoxyribonucleic acids and their constituents (bases, nucleosides,

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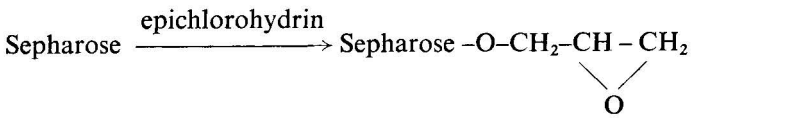
nucleotides). It turns out that all of the physico-chemical techniques employed (spectrophotometry[9–12], nuclear magnetic resonance spectroscopy[13–16] and polarography[17]) provide evidence that these compounds interact strongly with metals to form complexes involving the phosphate group and/or the heterocyclic bases, depending on the experimental conditions. Further, these interactions are specific, as striking differences occur between the purines and the pyrimidines and even among different compounds of the same class (adenine and guanine derivatives, for instance).

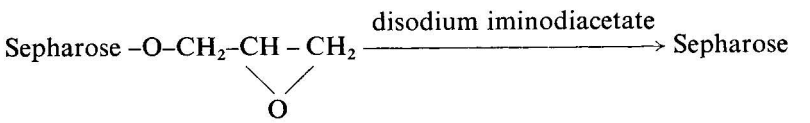
These observations led us to investigate whether these specific interactions might be used for nucleic acid fractionation on the basis of base composition by chromatography on metal chelate adsorbents, and to this end we have studied the influence of different parameters on the adsorption behaviour of nucleotides on copper chelate gels.

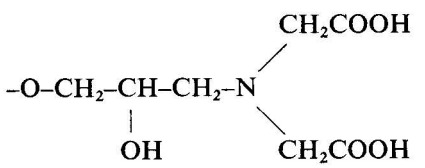
EXPERIMENTAL

The gel used for the metal chelate affinity chromatography experiments was prepared according to the following scheme:



(1)





(2)

The epoxy-activated Sepharose 6B (125 g), prepared from Sepharose 6B (Pharmacia, Uppsala, Sweden) according to the published procedure[18], was suspended in 100 ml of 2 *M* sodium carbonate solution and the mixture was shaken gently for 24 h in a water-bath (60–65°C) after the addition of 20 g of sodium iminodiacetate (Fluka, Buchs, Switzerland). After cooling the reaction vessel, the gel was carefully washed on a glass filter funnel successively with water, 0.1 *M* sodium carbonate solution, 0.01 *M* sodium acetate solution and finally water. The biscarboxymethylamino-Sepharose 6B thus obtained was kept in a cold room after addition of sodium azide (4 parts per 10,000) to prevent bacterial growth. The amount of iminodiacetic acid bound, as determined by nitrogen analysis, was 1017 μmole per gram of dry gel.

Chromatographic procedures

The biscarboxymethylamino-Sepharose 6B was packed into columns (13–15 cm × 1 cm I.D., $V_T \approx$ 10–12 ml) and washed with water prior to loading with 20 m*M* copper(II) sulphate solution (Merck, Darmstadt, G.F.R.). After saturation had been reached, excess of copper was removed by washing with 2 bed volumes of water. At this stage, the amount of Cu^{2+} bound was 899 μmole per gram of dry gel. The copper chelate adsorbent was then washed with the desired buffer until all loosely bound Cu^{2+} had been eluted.

The nucleotides and their derivatives (Sigma, St. Louis, MO, U.S.A.) were dissolved in water to a concentration of 2.5 m*M*, except for the poorly soluble adenine and guanine, which were dissolved to saturation by careful heating and were injected in 400-μl samples.

The retention of solutes is expressed in terms of reduced elution volumes (V_E/V_T), V_E being the elution volume of the maximum of the peak obtained either by recording (Altex UV minotor, RDK recorder) at 280 nm (cell 0.25 cm) or by measuring the absorbance at 260 nm (Beckman 25 spectrophotometer) of the effluent fractions collected.

In all chromatographic experiments the flow-rate was maintained at 11.7 ml/h by a peristaltic pump (Pharmacia).

RESULTS

As a preliminary control, the retention of different nucleotide derivatives was tested on Sepharose 6B and biscarboxymethylamino-Sepharose 6B in Tris–hydrochloric acid and citrate–phosphate buffers (0.05 *M*, pH 7.0, 1 *M* sodium chloride). In all instances the reduced elution volumes obtained were in the range 1.2–1.4. Hydrophobic interactions with the gel, which are likely to occur at these high salt concentrations, may account for the slight retardation observed.

Influence of buffer composition

The retention of all of the solutes tested was strongly affected by the composition of the eluting buffers, as shown in Table I.

With buffers containing substituted amino groups, the adsorption of solutes increased as the p*K* of the base involved in the buffer was decreased. This may be interpreted in terms of competition between the nucleotides and the buffer components. In fact, the metal chelate gel interacts with electron-donor solutes and therefore tends to become increasingly "saturated" during equilibration as the nucleophilic character of the buffer constituents is increased: NH_4Cl, p*K* 9.25 > Tris, p*K* 8.08 > N-ethylmorpholine, p*K* 7.65. The interaction of copper with the nucleotides is therefore less favoured as the Cu^{2+} ion becomes increasingly engaged with the electron-donor components of the buffer.

According to this interpretation, one would expect greater retardation when the elution is performed with low p*K* buffers. However, with the chosen citrate–phosphate buffer (citric acid, higher p$K = 6.39$), the opposite result was obtained, probably because citrate is a well known chelating agent for metallic cations, and is therefore able to prevent the metal from any further interaction with the solutes tested.

TABLE I

INFLUENCE OF BUFFER COMPOSITION (pH 7.0)

Compound	*Citrate–phosphate, 0.05 M/NaCl, 1 M*	*Tris–HCl, 0.05 M/NaCl, 1 M*	*Tris–HCl, 0.05 M/NH_4Cl, 1 M*	*Ethylmorpholine–acetic acid, 0.1 M/NaCl, 1 M*
Adenine	>20	>20	—	—
Adenosine	2.30	6.10	2.50	8.55
AMP	1.70	5.75	1.95	9.65
Guanine	—	>20	—	—
Guanosine	1.85	3.95	2.15	4.50
GMP	1.50	4.10	1.95	5.75
Thymine	1.35	1.35	1.35	1.40
Uracil	—	1.10	—	—
Cytosine	—	1.40	—	—

Dependence of the retention on the ionic strength and the nature of electrolytes added to the eluting buffer

The retention of nucleotides and derivatives on copper chelate gels is not based on simple electrostatic interactions, as the V_E/V_T values are actually enhanced by higher salt concentrations and the presence of chaotropic ions, as shown in Tables II–IV.

TABLE II

INFLUENCE OF THE IONIC STRENGTH

Compound	*Tris–HCl, 0.01 M, pH 7.0*	*Tris–HCl, 0.05 M, pH 7.0 + × mole/l NaCl*				
		0 M	*0.1 M*	*0.2 M*	*0.5 M*	*1 M*
AMP	2.35	3.0	4.90	5.65	5.5	5.75
Adenosine	7.80	5.15	6.55	6.20	5.75	6.10
GMP	2.05	2.75	4.10	4.20	3.5	4.1
Guanosine	4.6	3.65	4.20	4.0	3.6	3.9
Thymidine	—	1.3	1.2	1.15	1.15	1.2

TABLE III

INFLUENCE OF THE NATURE OF CATIONS (1 *M*) INCLUDED IN THE BUFFER (TRIS–HCl 0.05 *M*, pH 7.0)

Compound	*LiCl*	*NaCl*	*KCl*
AMP	4.4	5.75	8.5
Adenosine	5.1	6.1	6.6
GMP	3.5	4.1	5.7
Guanosine	3.9	3.9	4.1
Thymidine	1.4	1.4	1.4

The adsorption increases with the ionic strength up to a plateau at 0.1–0.2 *M* sodium chloride. Further, the retention seems to be directly related to the size of the ions involved in the eluting buffer (Li < Na < K and F < Cl < Br). KSCN, the

TABLE IV

INFLUENCE OF THE NATURE OF ANIONS (1 *M*) INCLUDED IN THE BUFFER (TRIS–HCl 0.05 *M*, pH 7.0)

Compound	*KF*	*KCl*	*KBr*	*KSCN*
AMP	4.7	8.15	8.7	1.4
Adenosine	4.15	6.1	6.6	1.8
GMP	4.95	5.25	5.3	1.25
Guanosine	3.75	3.7	3.85	1.6
Thymidine	1.3	1.3	1.3	1.3

most bulky, plays a different role, owing to its ability to react with the Cu^{2+} ions in solution.

The effect of electrolytes on the retention can be interpreted as a modification of the affinity of Cu^{2+} for its solvation water molecules; the weakening of the forces between Cu^{2+} and water induced by salt consequently facilitates the adsorption of nucleotides.

The results obtained in Tris–hydrochloric acid of low molarity (0.01 instead of 0.05 *M*) deserve special attention (Table II). From the effect of ionic strength discussed above, one would expect a slight decrease in the retention in this buffer. On the other hand, when the molarity of the buffer is lowered, the capacity of the electron-donor groups of the Tris to saturate the copper is decreased (*cf.*, Table I). More binding sites are available for the solutes, which can thus be retained more efficiently. Further, with molecules bearing a phosphate group (AMP, GMP), the low ionic strength may allow the phosphate to participate in the interaction, as pointed out by different workers[10,13–17,19–21].

The fact that nucleosides are effectively more retained supports the hypothesis according to which the lesser saturation of Cu^{2+} by the buffer components plays a major role in the retention. On the other hand, the decreased retention of nucleotides suggests that the repulsive electrostatic interactions with the matrix have, in this instance, an even more important effect.

Contribution of hydrophobic interactions in the retention process

Hydrophobic interactions with the matrix and the spacer arm are involved in the adsorption phenomenon. This is demonstrated by the fact that additives that weaken hydrophobic and other water structure-dependent interactions (*e.g.*, ethylene glycol) cause non-neglectable decreases in the V_E/V_T values (Table V).

TABLE V

INFLUENCE OF ETHYLENE GLYCOL ON RETENTION

Buffer: Tris–HCl, 0.05 *M*, pH 7.0.

Compound	*Ethylene glycol concentration (%)*	
	0	*50*
AMP	3.0	2.40
Adenosine	5.15	3.35
GMP	2.75	2.40
Guanosine	3.65	2.80

However, even in 50% ethylene glycol the retention still remains very high. This point, and the fact that the adsorption does not increase with increasing temperature (Table VI), lead to the conclusion that water structure-dependent interactions are not the main factor in the overall retention process.

TABLE VI

INFLUENCE OF TEMPERATURE ON THE RETENTION

Buffer: Tris–HCl, 0.05 *M*, pH 7.0/NaCl, 1 *M*.

Compound	*Temperature (°C)*	
	4	22
AMP	6.10	5.75
Adenosine	8.15	6.10
GMP	4.40	4.10
Guanosine	5.40	3.95

pH dependence

The pH obviously plays a major and very complex role, as it affects not only the nucleophilic character of the buffer components and the electron-donor properties of the nucleotides, but also the metal chelate structure and stability.

The data in Table VII give an example of the tremendous change in retention provoked by a variation of only one pH unit. Also, in citrate–phosphate buffer, 0.05 *M*, 1 *M* sodium chloride, pH 6.0 (results not shown), all of the solutes are readily eluted, including guanine and adenine ($V_E/V_T > 20$ in citrate–phosphate buffer, pH 7.0; *cf.*, Table I).

TABLE VII

DEPENDENCE OF THE INTERACTION ON pH

Buffer: Tris–HCl, 0.05 *M*/NaCl, 1 *M*.

Compound	*pH*	
	7.0	*8.0*
AMP	5.75	1.35
Adenosine	6.10	1.85
GMP	4.10	1.45
Guanosine	3.95	1.80

"*Cooperativity*" *effect*

The enhanced adsorption observed on passing from the mono- to the dinucleotides (Table VIII) must be due to multi-point attachment.

At pH 8.0, under conditions where guanine and adenine nucleotides are almost unretarded (*cf.*, Table VII), the presence of two purine rings in the same molecule allows differentiation between adenine and guanine derivatives, although not enough to permit a clear separation on a column of reasonable dimensions.

At pH 7.0, there is a tremendous effect of cooperativity on purine dinucleotides. On the other hand, the presence of a pyrimidine ring in the dinucleotide molecule

TABLE VIII

"COOPERATIVE" EFFECT ON RETENTION

Buffer: Tris–HCl, 0.05 *M*/NaCl, 1 *M*.

pH 7.0				*pH 8.0*			
GMP	4.10	GpG	16	GMP	1.45	GpG	3.9
AMP	5.75	ApA	35	AMP	1.35	ApA	3.6
		GpU	3.55				

does not significantly affect the retention, which remains similar to that of the corresponding purine mononucleotide.

The practical application of these observations was demonstrated in a fractionation experiment in which an artificial mixture consisting of GpU, GpG and ApA was applied on a short copper chelate gel column (length 2.7 cm, V_T 2.1 ml). The elution profile obtained reflects the complete resolution of the three compounds (Fig. 1).

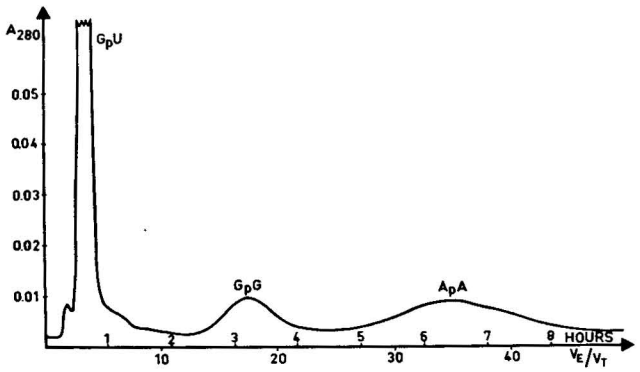


Fig. 1. Elution profile of a mixture consisting of GpU (*ca.* 0.15 mg), GpG (*ca.* 0.1 mg) and ApA (*ca.* 0.2 mg) chromatographed on copper chelate biscarboxymethylamino-Sepharose 6B. Column: 2.7 × 1 cm I.D., V_T 2.1 ml. Eluting buffer: Tris–HCl, 0.05 *M*, pH 7.0/NaCl, 1 *M*. Flow-rate: 11.7 ml/h. Profile obtained on recording at 280 nm (cell 0.25 cm).

DISCUSSION

The retention of nucleotides and derivatives on copper chelate gels appears to be complex. Electron donor–acceptor, hydrophobic and presumably also electrostatic interactions are all involved in the overall adsorption process. According to the data obtained, the retentions increase in the order purines $\gg$ pyrimidines, with adenine $>$ guanine and cytosine $\approx$ thymidine $\approx$ uracil, and generally bases $\gg$ nucleosides $\geqslant$ nucleotides.

These observations are in good agreement with results reported in the literature for species free in solution, except that guanine nucleotides and nucleosides are generally claimed to bind copper more efficiently than adenine derivatives[22]. Although both phosphate groups and heterocyclic bases have been proved to be involved in

the binding to copper when free in solution[9,13,19,22–24], our data with immobilized Cu^{2+} suggest that the adsorption proceeds mainly via interactions with the heterocyclic bases. Under our conditions a major role of phosphate group in the interaction with the metal ion seems unlikely. In fact, it is either ruled out by high salt conditions or cancelled owing to the repulsion by the negatively charged groups borne by the gel.

The fact that the bases are far more strongly adsorbed than the corresponding nucleosides and nucleotides emphasizes the negative role played by the sugar and phosphate moieties. As the formation of the glycosidic linkage to ribose or esterification at the 5′-ribose position seems to cause no major charge redistribution in the adenine ring[25], it appears therefore that steric hindrance and modification of the organization of water molecules around the solutes must be the main explanations for the effect observed.

Recently, Chow and Grushka[26] have shown that nucleosides and nucleotides can be separated by high-performance liquid chromatography on columns of silica with cobalt-complexed ligands. Together with our findings, their study indicates the potential applications of metal chelate adsorbents for nucleotide and oligonucleotide fractionation. As the main interaction involved in the adsorption process concerns the heterocyclic bases of these compounds, one can reasonably expect an extension of this system to other small heterocyclic molecules such as vitamins, hormones and drugs.

From the very high retention observed with dinucleotides one must expect considerable adsorption of solutes capable of multi-point attachment to the metal chelate gels. In connection with protein and nucleic acid purification, this may present difficulties during pH gradient elution, owing to the simultaneous leakage of metal ions[2,3,6]. The study of pre-washing conditions, allowing a subsequent enhanced stability of the chelate over a wide pH range, which is at present in progress, will complement the present work and provide the basic information required for the purification of nucleic acids by metal chelate affinity chromatography.

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STABILITÄT VON IONENAUSTAUSCHERN GEGEN SALPETERSÄURE UND BESTRAHLUNG

II. ÄNDERUNG DER SORPTIONSEIGENSCHAFTEN VON DOWEX A-1, CHELEX 100 UND WOF MC 50 NACH BEHANDLUNG MIT SALPETERSÄURE

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(Eingegangen am 4. Dezember 1979; geänderte Fassung eingegangen am 6. Juni 1980)

SUMMARY

Stability of ion exchangers against treatment with nitric acid and radiation. II. Change in sorption properties of Dowex A-1, Chelex 100 and Wof MC 50 after treatment with nitric acid

The change in the sorption properties of chelating ion exchangers with amino acetic acid and amino diacetic acid groups of the type Wof MC 50, Chelex 100 and Dowex A-1 after treatment with nitric acid is examined.

The nitric acid concentration is varied in the range 1–10.5 *N*; the reaction time varied from 0.5 to 25 h. The reaction temperature was 68°C. By destruction of the amino diacetic acid groups the sorption properties are changed.

The sorption of cations (Cs^+–0.019 *N* NH_4Cl) decreases, goes through a minimum and increases again when the COOH capacity is increased. The sorption of anions (TcO_4^-–1 *N* HNO_3) and anionic complexes ($PdCl_4^{2-}$–1 *N* HCl) decreases in correlation with the N-content.

EINLEITUNG

In einer früheren Arbeit haben wir den Mechanismus der Reaktion von chelatbildenden Ionenaustauschern mit Aminoessigsäure- und Aminodiessigsäuregruppen mit HNO_3 am Beispiel der kommerziellen Produkte Wof MC 50, Chelex 100 und Dowex A-1 untersucht[1]. Entsprechend dem gefundenen Mechanismus wird dabei die Aminodiessigsäuregruppe mit tertiärem N-Atom durch eine Nitrosierungsreaktion abgespalten, während die Aminoessigsäuregruppe mit sekundärem N-Atom in ein Nitrosamin übergeht. Anstelle der abgespaltenen Aminodiessigsäuregruppe entsteht im Harz über eine intermediäre CHO-Gruppe als neue funktionelle Gruppe eine COOH-Gruppe. Dieser Reaktionsverlauf entspricht der auch von Smith und Loeppky[2] diskutierten nitrosierenden Spaltung tertiärer Amine bei Einwirkung nitrosierender Agenzien.

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Für den praktischen Einsatz solcher Austauscher in salpetersauren Lösungen höherer Konzentration, z.B. zur Abtrennung einzelner Spaltprodukte aus Abfallösungen der Wiederaufbereitung von Kernbrennstoffen, ist es demzufolge von Interesse, welchen Einfluss die Änderungen der COOH-Kapazität, des N-Gehaltes und des Charakters der funktionellen Gruppe auf die Sorptionseigenschaften haben.

Das Ziel der vorliegenden Untersuchungen bestand deshalb vor allem darin, das Sorptionsverhalten von mit HNO_3 behandelten Chelonaustauschern zu charakterisieren. Zur Bestimmung der Kationenaustausch-, Anionenaustausch- und chelatbildenden Eigenschaften wurden die Systeme Cs^+–0.019 *N* NH_4Cl, $PdCl_4^{2-}$–1 *N* HCl, TcO_4^-–1 *N* HNO_3 und Pd(II)–3 *N* HNO_3 ausgewählt, die verwendeten Ionenaustauscher waren Dowex A-1, Chelex 100 und Wof MC 50.

EXPERIMENTELLES

Bestimmung von Verteilungskoeffizienten

Zur Charakterisierung der Sorptionseigenschaften diente der Verteilungskoeffizient. Er wurde nach der batch-Methode unter Verwendung von Radiotracern zur Markierung der interessierenden Elemente bestimmt. Als radioaktive Tracer wurden ^{99m}Tc, ^{103}Pd und ^{134}Cs eingesetzt, die Elementkonzentrationen betrugen 0.1 mg Pd/ml und 0.1 mg Cs/ml, das ^{99m}Tc wurde trägerfrei eingesetzt. Zur Bestimmung des Verteilungskoeffizienten wurden 50–100 mg des lufttrockenen Harzes mit 5–6 ml einer Lösung des interessierenden Elementes bis zur Gleichgewichtseinstellung 16–24 h geschüttelt. Anschliessend wurde das Harz durch Filtration abgetrennt und ein Aliquot der Lösung gegen einen Aktivitätsstandard radiometrisch gemessen. Das Messsystem bestand aus einem Kernstrahlungsmessgerät 20026, einem Ergebnisdrucker 23144 und einer Bohrloch-Szintillationsmesssonde VA-S-968 vom VEB Messelektronik Dresden.

Herstellung von Lösungen

Zur Herstellung einer $PdCl_2$-Lösung von 0.1 mg Pd/ml in 1 *N* HCl wurden 41.7 mg $PdCl_2$ mit der notwendigen Menge ^{103}Pd-Tracer versetzt, das Gemisch in 5–10 ml 1 *N* HCl gelöst und vorsichtig zur Trockne eingeengt. Anschliessend wurde der Rückstand in 250 ml 1 *N* HCl aufgenommen. Im Fall der $Pd(NO_3)_2$-Lösung wurden der ^{103}Pd-Tracer und 41.7 mg $PdCl_2$ in wenig 1 *N* HNO_3 aufgenommen und anschliessend in der Hitze durch Zugabe eines geringen Überschusses 0.5 *N* NaOH $Pd(OH)_2$ gefällt. Der Niederschlag wurde auf einem Glasfiltertiegel gesammelt, mit heissem Wasser neutral und chloridfrei gewaschen und in heisser 3 *N* HNO_3 gelöst. Nach Abkühlen wurde mit 3 *N* HNO_3 auf das Endvolumen von 250 ml aufgefüllt.

Zur Herstellung einer mit ^{99m}Tc-markierten TcO_4^--Lösung wurde ein entsprechendes Aliquot des Eluates eines $^{99}Mo/^{99m}Tc$-Generators mit konzentrierter HNO_3 nachoxidiert, die erhaltene Lösung vorsichtig zur Trockne eingeengt und in 100 ml 1 *N* HNO_3 aufgenommen.

Eine mit ^{134}Cs markierte Cs-Lösung mit einem Cs-Gehalt von 0.1 mg Cs/ml in 0.019 *N* NH_4Cl wurde durch Mischen von 31.7 mg CsCl mit der entsprechenden Menge ^{134}Cs-Tracerlösung, Einengen zur Trockne und Aufnahme in 250 ml 0.019 *N* NH_4Cl-Lösung erhalten.

ERGEBNISSE UND DISKUSSION

Die Sorptionseigenschaften von Chelonaustauschern, wie Wof MC 50, Dowex A-1 und Chelex 100 sind sehr stark von der H^+-Konzentration der Lösung und vom Anteil der verschiedenen funktionellen Gruppen an der Gesamtkapazität abhängig. In sauren Lösungen bei pH < 1 liegen die Aminodiessigsäure- und die Aminoessigsäuregruppen, die den Hauptteil der funktionellen Gruppen kommerzieller Austauscher dieses Typs ausmachen, in hydroacider Form vor. Aus diesem Grund haben die genannten Harze in diesem Bereich Anionenaustauscheigenschaften. Bei pH-Werten von 2–6 liegt die Aminodiessigsäuregruppe in der neutralen Säureform vor, während bei pH 7 bzw. pH 12–13 die Neutralisation der ersten bzw. zweiten COOH-Gruppe abgeschlossen ist. Im Fall der Aminoessigsäuregruppe liegt bei pH 2–5 die neutrale Säureform, bei pH 6 die Zwitterionenform und bei pH $\geqslant 11$ die Salzform vor[3].

Im Rahmen der vorliegenden Arbeit wurden die Sorptionseigenschaften folgender Harze untersucht:

(a) Je einer Versuchsserie von Wof MC 50, die bei 68°C und Reaktionszeiten von 0.5 bis 24 h mit HNO_3 einer Konzentration von 3, 6.7 und 10.5 *N* behandelt wurde;

(b) je einer Versuchsreihe von Dowex A-1, Chelex 100 und 3 weiterer Chargen Wof MC 50, die bei 68°C und Reaktionszeiten von 0.5 bis 16 h mit 10.5 *N* HNO_3 behandelt wurde.

Die Werte für die Änderung der COOH-Kapazität und den N-Gehalt der untersuchten Harze wurden früher bereits publiziert[1]. In Tabelle I sind die entsprechenden Werte für eine Wof MC 50-Probe als Beispiel zusammengestellt.

Sorption von Cs^+ aus 0.019 N NH_4Cl

In den Figuren 1 und 2 sind die Ergebnisse dargestellt. Man erkennt, dass die *K*j-Werte der Cs-Sorption sehr schnell abnehmen und dann erneut ansteigen. Die Werte für die Langzeitproben liegen deutlich über denen für die Originalharze. Der Verlauf der Cs-Sorptionskurve ist analog der der COOH-Kapazität. Eine gewisse Ausnahme bildet lediglich Chelex 100, bei dem nur ein flaches Minimum durchlaufen wird, das aber auch in diesem Fall mit dem Minimum der COOH-Kapazität übereinstimmt. Wie früher bereits diskutiert, besteht die höhere kinetische Stabilität dieses Harzes offenbar in seinem geringeren Gehalt an reduzierenden Gruppen, wodurch der Abbau der funktionellen Gruppen verlangsamt wird.

Zur Beurteilung einer möglichen Selektivitätsänderung durch den Umbau der funktionellen Gruppe kann man die scheinbare Gleichgewichtskonstante der Austauschreaktion K' verwenden, für die im Fall der Sorption von Mikromengen Cs ($RCs \ll RNH_4$) und bei Annahme eines konstanten Aktivitätskoeffizientenverhältnisses der beteiligten Ionen gilt

$$K' = K_d \cdot [NH_4^-] \cdot [COOH]^{-1}$$

In Tabelle II sind die gemessenen K'-Werte zusammengefasst. Es ergibt sich, dass K' bis zum Erreichen des Minimums der COOH-Kapazität etwas abnimmt und mit der Neubildung der COOH-Gruppen deutlich ansteigt. Daraus folgt, dass die neu gebil-

TABELLE I

ÄNDERUNG DER COOH-KAPAZITÄT UND DES N-GEHALTES VON WOF MC 50 BEI EINWIRKUNG VON HNO_3 UNTERSCHIEDLICHER KONZENTRATION BEI 68°C

C_{HNO_3} (N)	Reaktionszeit (h)	COOH-Kapazität (mäquiv./g)	N-Gehalt (mäquiv./g)
—	—	4.42	3.36
3.2	4	4.33	3.36
	8	4.18	3.36
	16	4.00	3.21
	25	2.91	2.93
6.7	1	4.37	3.36
	2	4.07	3.21
	3	3.67	3.14
	4	2.34	2.71
	4.8	2.09	2.43
	6	2.14	2.36
	7	2.29	2.21
	8	2.51	2.36
	15.8	3.58	2.07
	25	4.07	1.93
	48	4.42	1.93
10.5	0.5	3.80	3.14
	0.75	3.52	3.07
	1	1.95	2.57
	1.5	1.93	2.36
	2	3.50	2.29
	3	3.00	2.21
	4	3.50	2.07
	6	3.98	2.07
	16	4.74	2.00
	25	4.80	2.07

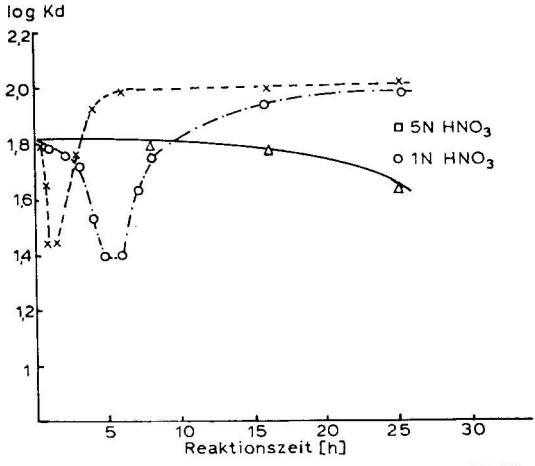

Fig. 1. Sorption von Cs^+-Ionen aus 0.019 *N* NH_4Cl an mit HNO_3 bei 68°C behandelten Wof MC 50-Proben. △, 3.2 *N* HNO_3; ○, 6.7 *N* HNO_3; ×, 10.5 *N* HNO_3.

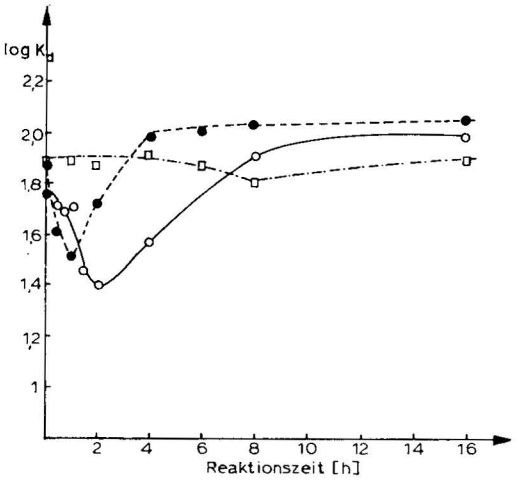

Fig. 2. Sorption von Cs^+-Ionen aus 0.019 *N* NH_4Cl an mit 10.5 *N* HNO_3 bei 68°C behandelten Proben verschiedener Chelonaustauscher. ○, MC 50/1379; ⊡, Chelex 100; ●, Dowex A-1.

TABELLE II

Cs-SORPTION AUS 0.019 *N* NH_4Cl AN MIT HNO_3 BEHANDELTEN WOF MC 50-PROBEN

Nr.	C_{HNO_3} *(N)*	*Temp. (°C)*	*Reaktions-zeit (h)*	*COOH-Kapazität (mäquiv./g)*	K_d *(ml/g)*	*K'*
1	Original	—	—	4.42	73.7	0.317
2	6.7	68	1	4.37	61.5	0.267
			2	4.07	58.4	0.273
			3	3.67	53.4	0.276
			4	2.34	34.9	0.283
			4.8	2.09	25.4	0.231
			6	2.14	25.7	0.228
			7	2.29	43.2	0.358
			8	2.51	56.7	0.429
			15.75	3.58	86.8	0.461
			25	4.07	95.3	0.445
			48	4.42	104	0.449
3	10.5	68	0.5	3.80	64.7	0.323
			0.75	3.52	45.5	0.246
			1	1.95	27.9	0.272
			1.5	1.93	28.4	0.280
			2	3.5	51.9	0.282
			3	3.0	59.5	0.377
			4	3.5	85.9	0.466
			6	3.98	98.0	0.468
			16	4.74	97.1	0.389
			25	4.80	104	0.410

deten kernständigen COOH-Gruppen eine höhere Selektivität für die Cs-Sorption besitzen. Diese entspricht der für die schwachsauren Kationenaustauscher Wof CP und W of CA20 gefundenen Selektivität, für die unter analogen Bedingungen *K'*-Werte von 0.52 bestimmt wurden.

Die für die Cs-Sorption erhaltenen Ergebnisse zeigen, dass die Behandlung mit HNO_3 die Kationenaustauscheigenschaften nur vorübergehend verschlechtert und dass nach Wiederanstieg der COOH-Kapazität sogar eine Verbesserung der Kationenaustauschsorption beobachtet wird.

Eine Erklärung dafür ist, dass die Kationenaustauscheigenschaften der Originalharze im Neutralbereich im wesentlichen durch die freien, d.h. nicht durch Zwitterionenbildung gebundenen, COOH-Gruppen bestimmt werden. Die Abspaltung der tertiären Aminodiessigsäuregruppen führt zum Verlust dieser freien COOH-Gruppen und damit zu einer Verschlechterung der Kationenaustauscheigenschaften. Infolge Neubildung von kernständigen, freien COOH-Gruppen nach längeren Reaktionszeiten steigt die Kationensorption dann erneut an.

Sorption von Anionen

In den Figuren 3 und 4 sind die Ergebnisse der $PdCl_4^{2-}$-Sorption aus 1 *N* HCl und in den Figuren 5 und 6 die der TcO_4^--Sorption aus 1 *N* HNO_3 dargestellt. Die erhaltenen Ergebnisse machen deutlich, dass die Sorption von Anionen und anionischen Komplexen mit zunehmender Reaktionszeit abnimmt, d.h., dass die Reaktion mit HNO_3 auch zu einer Verschlechterung der Anionenaustauscheigenschaften führt. Die Geschwindigkeit der Abnahme der Anionensorption nimmt mit der HNO_3-Konzentration zu, bei einer Arbeitstemperatur von 68°C ist auch bei einer HNO_3-Konzentration von 3 *N* eine Langzeitstabilität nicht gegeben (siehe auch Tabelle I). Die Abnahme der K_d-Werte für die Sorption von Anionen beruht auf der vollständigen Abspaltung der Aminodiessigsäuregruppen durch die ein wesentlicher Teil der N-Atome, an die die Anionenaustauscheigenschaften gebunden sind, verloren gehen.

Bei allen Harzen beobachtet man aber eine deutlich Restsorption mit K_d-Wer-

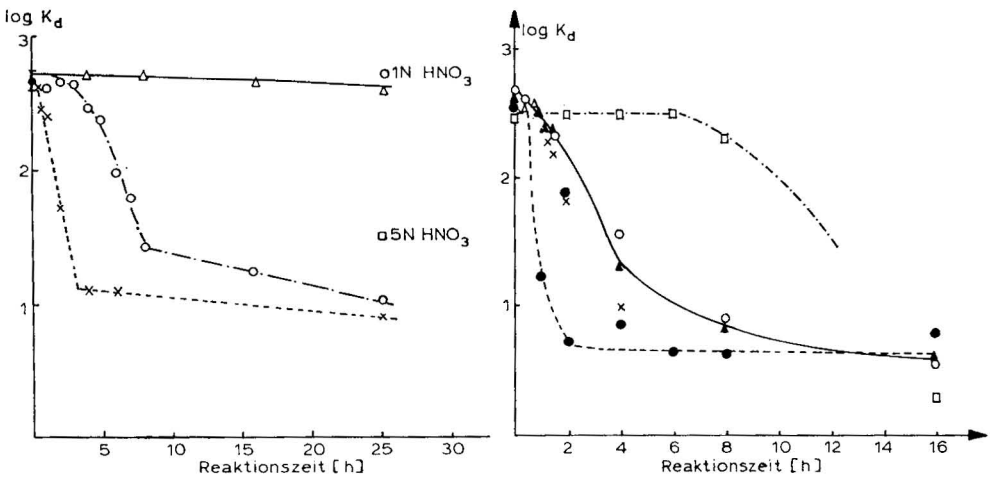

Fig. 3. Sorption von $PdCl_4^{2-}$-Ionen aus 1 *N* HCl an mit HNO_3 bei 68°C behandelten Wof MC 50-Proben. △, 3.2 *N* HNO_3; ○, 6.7 *N* HNO_3; ×, 10.5 *N* HNO_3.

Fig. 4. Sorption von $PdCl_4^{2-}$-Ionen aus 1 *N* HCl an mit 10.5 *N* HNO_3 bei 68°C behandelten Proben verschiedener Chelonaustauscher. ○, MC 50/1379; ×, MC 50/1429; △, MC 50/1431; ⊡, Chelex 100; ●, Dowex A-1.

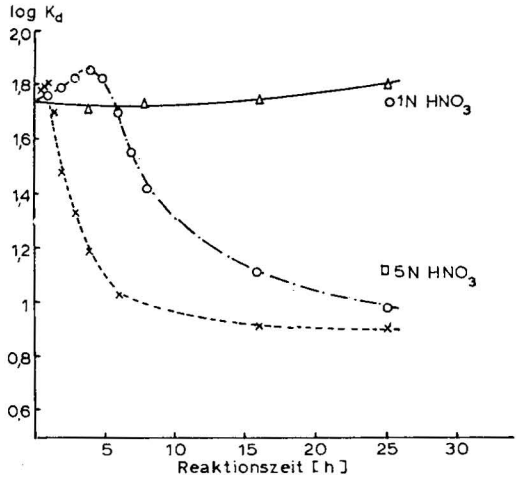

Fig. 5. Sorption von TcO_4^--Ionen aus 1 *N* HNO_3 an mit HNO_3 bei 68°C behandelten Wof MC 50-Proben. △, 3.2 *N* HNO_3; ○, 6.7 *N* HNO_3; ×, 10.5 *N* HNO_3.

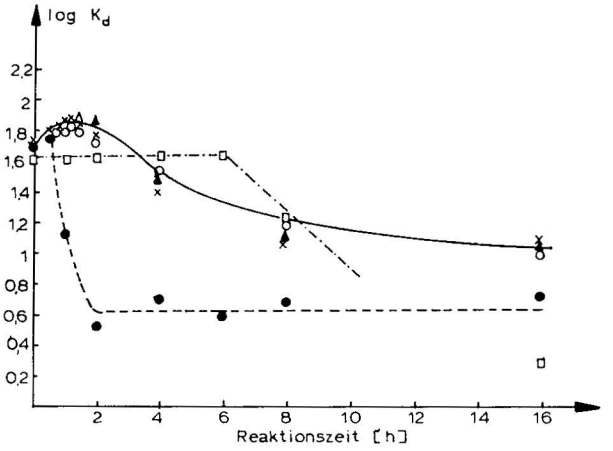

Fig. 6. Sorption von TcO_4^--Ionen aus 1 *N* HNO_3 an mit 10.5 *N* HNO_3 bei 68°C behandelten Proben verschiedener Chelonaustauscher. ○, MC 50/1379; ×, MC 50/1429; ▲, MC 50/1431; ⊡, Chelex 100; ●, Dowex A-1.

ten von 4–12 ml/g, die an die im Harz verbleibende Aminoessigsäuregruppe bzw. deren N-Nitrosoverbindung gebunden ist. Die unterschiedliche Restsorption der verschiedenen Austauschertypen hängt deshalb in erster Linie vom Anteil der Aminoessigsäuregruppen an der Gesamtkapazität des Originalharzes ab.

Sorption von Pd(II) aus 3 N HNO_3

Über die Komplexbildung von Pd(II) mit NO_3^--Ionen liegen in der Literatur bisher keine Ergebnisse vor, deshalb ist auch über die Natur der Pd-Spezies in 3 *N* HNO_3 nichts bekannt.

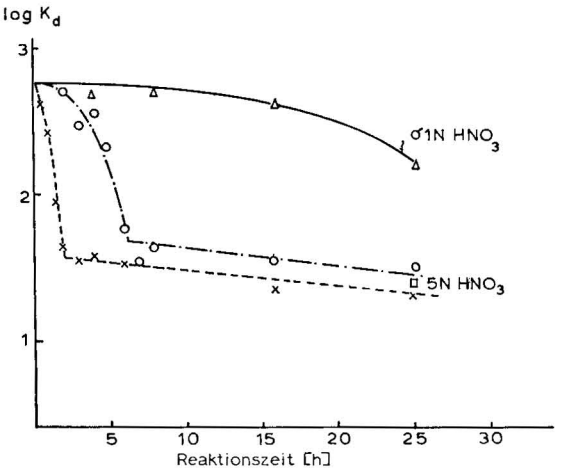

Fig. 7. Sorption von Pd(II) aus 3 *N* HNO_3 an mit HNO_3 bei 68°C behandelten Wof MC 50-Proben. △, 3.2 *N* HNO_3; ○, 6.7 *N* HNO_3; ×, 10.5 *N* HNO_3.

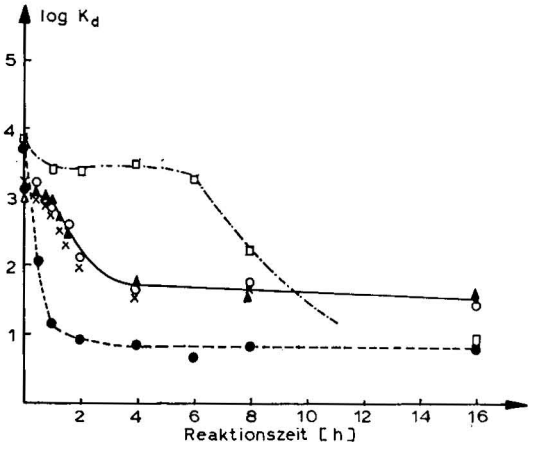

Fig. 8. Sorption von Pd(II) aus 3 *N* HNO_3 an mit 10.5 *N* HNO_3 bei 68°C behandelten Proben verschiedener Chelonaustauscher. ○, MC 50/1379; ×, MC 50/1429; ▲, MC 50/1431; ⊡, Chelex 100; ●, Dowex A-1.

Aus Sorptionsuntersuchungen von Pd(II) an verschiedenen Harztypen müssen wir schliessen, dass im Fall von Austauschern mit einer Aminocarbonsäure als funktionelle Gruppe bei der Pd-Sorption aus 3 *N* HNO_3 die Chelatbildung des Pd(II) mit der funktionellen Gruppe eine Rolle spielt. Das zeigen die hohen Verteilungskoeffizienten von 10^2–10^4 ml/g für verschiedene Chelonaustauscher im Gegensatz zu K_d-Werten von 20–50 ml/g im Fall von Anionenaustauschern mit quaternären Trimethylammoniumgruppen[4].

Man kann deshalb annehmen, dass das System Pd(II)–3 *N* HNO_3 zur Charakterisierung der chelatbildenden Eigenschaften der mit HNO_3 behandelten Austauscher geeignet ist. Die Ergebnisse sind in den Figuren 7 und 8 dargestellt.

Aus der schnellen Abnahme der Pd-Sorption folgt, dass bei Einwirkung von HNO_3 mit der Abspaltung der Aminodiessigsäuregruppen auch die Fähigkeit des Harzes zur Chelatbildung weitgehend verlorengeht, d.h. andererseits, dass im Fall des Pd(II) die chelatbildenden Eigenschaften im wesentlichen an die Aminodiessigsäuregruppen gebunden sind. Die Restsorption mit K_d-Werten von 5–30 ml/g muss auch in diesem Fall den im Harz verbleibenden Aminoessigsäuregruppen zugeordnet werden. Die höchste Restsorption beobachtet man mit K_d-Werten von 30 ml/g bei den Wof MC 50-Proben.

Weiterhin ergibt sich, dass die neugebildeten kernständigen COOH-Gruppen keinen Beitrag zur Chelatbildung des Harzes leisten.

Zusammenfassung

Die dargelegten Ergebnisse zeigen, dass die Einwirkung von HNO_3 auf Chelonaustauscher, wie Dowex A-1, Chelex 100 und Wof MC 50, und die damit verbundene Abspaltung der Aminodiessigsäuregruppe zu wesentlichen Veränderungen der Sorptionseigenschaften dieser Harze führen.

Die Kationenaustauschsorption verschlechtert sich zunächst infolge Abnahme der COOH-Kapazität, steigt aber dann durch die Neubildung von COOH-Gruppen über den Ausgangswert an.

Die Anionenaustauschsorption und die chelatbildenden Eigenschaften nehmen durch die Abspaltung der Aminodiessigsäuregruppen und die damit verbundene Verringerung des N-Gehaltes wesentlich ab. Die im Harz verbleibende Aminoessigsäuregruppe bedingt eine gewisse Restsorption, die je nach dem Anteil dieser Gruppen an der Gesamtkapazität des Originalharzes bis zu 20% des Ausgangswertes betragen kann. Bei Harzen mit einem hohen Anteil an Aminodiessigsäuregruppen (z.B. Chelex 100) beträgt die Restsorption $<1\%$. Für die Praxis ergibt sich daraus die Schlussfolgerung, dass Chelonaustauscher dieses Typs für den Einsatz in salpetersauren Lösungen, insbesondere bei HNO_3-Konzentrationen von $\geqslant 3\ N$ und erhöhten Temperaturen wenig geeignet sind, da sich die Sorptionseigenschaften bereits nach geringen Kontaktzeiten verändern.

DANK

Der Autor ist Frau G. Hanke für die zuverlässige experimentelle Mitarbeit und Frau C. I. Rommel für die Durchführung einer Vielzahl von N-Bestimmungen zu besonderem Dank verpflichtet.

ZUSAMMENFASSUNG

Die Änderung der Sorptionseigenschaften von Chelonaustauschern mit Aminoessigsäure- und Aminodiessigsäuregruppen vom Typ Wof MC 50, Chelex 100 und Dowex A-1 nach Behandlung mit HNO_3 wird untersucht.

Die HNO_3-Konzentration wurde im Bereich von 1–10.5 N und die Reaktionszeit von 0.5–24 h variiert. Die Reaktionstemperatur betrug 68°C. Durch die Abspaltung der Aminodiessigsäuregruppen werden die Sorptionseigenschaften verändert.

Die Sorption von Kationen (Cs^+–0.019 N NH_4Cl) nimmt ab, geht durch ein Minimum und steigt infolge Zunahme der COOH-Kapazität erneut an. Die Sorption

von Anionen (TcO_4^- – 1 *N* HNO_3) und anionischen Komplexen ($PdCl_4^{2-}$ – 1 *N* HCl) nimmt in Korrelation mit dem N-Gehalt ab, das gleiche gilt für die Sorption infolge Chelatbildung (Pd II– 3 *N* HNO_3).

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CHROMATOGRAPHIC BEHAVIOUR OF AROMATIC COMPOUNDS ON ANION-EXCHANGE RESINS IN VARIOUS CARBOXYLATE FORMS

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SUMMARY

Phenols, benzyl alcohols and aromatic carboxylate ions were chromatographed on an anion-exchange resin in aqueous acetate, glycolate, butyrate and tartrate solutions. Non-polar interactions of alkyl groups and hydrogen bonding between phenolic hydroxyl protons and the resin led to increased distribution coefficients of both ions and non-electrolytes, while internal hydrogen bonding between phenolic and vicinal methoxyl groups suppressed the hydrogen bonding with the resin. The distribution coefficients of most aromatic carboxylate ions were higher in 0.5 *M* than in 0.3 *M* sodium tartrate solution. A large increase in the activity coefficients of the aromatic ions in the eluent with increasing tartrate concentration explains this abnormal effect, which parallels the high salting out of aromatic non-electrolytes in tartrate compared to the other eluents.

INTRODUCTION

In anion exchange of strongly hydrophilic ions such as aldonate ions and oligomers built up from sugars and carboxylate anions the distribution coefficients decrease with increasing molecular volume[1,2]. Calculations based on the Gibbs–Donnan equation show that the pressure–volume term has a predominant influence on the equilibrium in systems of this type. Reversed affinity orders, *e.g.*, for the series $CH_3(CH_2)_nCOO^-$ and $^-OOC(CH_2)_nCOO^-$, are explained by non-polar interactions which exert a larger effect than the pressure–volume term[3,4]. Little work has been devoted to studies of the ion-exchange affinity of aromatic carboxylate anions in aqueous solution, while the sorption of the undissociated acids has been studied by several investigators[5]. The main purpose of this investigation is to compare the effect of various substituents in aromatic carboxylate ions, and in other aromatic compounds, on the sorption onto anion-exchange resins in sodium salt solutions of aliphatic carboxylic acids.

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EXPERIMENTAL

Four batches containing equal amounts (by weight) of undried Dowex 1-X8 (10–17 μm) in the acetate form were slurried in water and transferred to four columns with an inner diameter of 2.4 mm. The exchange capacity was 0.99 mmol. This was determined in separate batches which were converted into the chloride form and washed with water. The chloride ions were eluted with sodium nitrate and determined potentiometrically.

One of the columns was conditioned by pumping 0.5 *M* sodium acetate solution through it for 1 h. The bed volume of the conditioned column was 0.75 ml. The other columns were converted into the glycolate, butyrate and tartrate forms by treatment with sodium salt solutions and conditioned in 0.5 *M* solution. The bed volume for the glycolate and butyrate columns was virtually the same as that obtained for the acetate resin, but was 5% lower for the tartrate form. This shrinkage was disregarded when calculating the distribution coefficients D_v from the relationship

$$D_v = \bar{v}/X - \varepsilon_I$$

where $\bar{v}$ is the peak elution volume, X the column volume and ε_I (= 0.4) the relative interstitial volume. The difference between ln D_v for a parent compound, *e.g.*, phenol, and a substituted compound, *e.g.*, 4-methylphenol, in the same medium is denoted by Δ.

The nominal linear (empty tube) flow-rate was 14.0 cm min^{-1}. The aromatic compounds were dissolved in boiled distilled water. Sparingly soluble acids were added as their sodium salts. Single compounds were applied to the column and chromatographed to check the purity and determine the retention volume and response in the UV detector at 254 and 280 nm. The reported retention data were obtained with well separated mixtures of three to five compounds (2.0 μmol per ml of each). In all the experiments the injected volume was 25 μl.

The influence of the loaded amount of aromatic compounds was studied in 0.5 *M* sodium acetate at pH 7.0. For 2-methoxyphenol, which gave symmetric peaks, the elution volume was unaffected when the loaded amount was varied between 0.02 and 0.4 μmol. Addition of benzyl alcohol, benzoic acid and 4-methoxybenzoic acid (0.1 μmol of each) had no influence on the position of 2-methoxyphenol. For 4-hydroxy-3-methoxybenzaldehyde, which exhibited some tailing, an increased loading from 0.02 μmol to 0.1 μmol led to a decrease in the retention volume of 3%.

RESULTS

Influence of the eluent composition

The eluents contained sodium salts of acetic, glycolic, *n*-butyric and L-tartaric acids and free acids so that the pH was 7.0. At this pH, variations of $\pm$ 0.1 pH unit had no detectable effect on the retention volumes for the aromatic carboxylate anions and for weakly acid solutes such as phenols and benzaldehyde, while the positions of 4-hydroxybenzaldehyde and hydroxymethoxybenzaldehydes, which are partially ionized under the applied conditions, were affected. At 60°C the D_v of 4-hydroxybenzaldehyde in 0.5 *M* sodium acetate was 56 at pH 6.0, 97 at pH 7.0 and 140 at

pH 8.0. Far-reaching conclusions cannot therefore be drawn from small differences in retention data of these compounds.

Table I shows that an increased sodium acetate concentration led to a large decrease in D_v for the hydroxybenzaldehydes, thus illustrating that ion exchange contributed markedly to the sorption of these solutes. Conversely, an increased eluent concentration resulted in an increased D_v value for unsubstituted benzaldehyde which is non-ionized at pH 7.

TABLE I

DISTRIBUTION COEFFICIENTS FOR BENZALDEHYDE AND DERIVATIVES AT pH 7.0 AND 60°C IN SOLUTIONS OF SODIUM SALTS

Substituents	*Acetate*		*Glycolate*	*Butyrate*	*Tartrate*
	0.5 M	*1.0 M*	*0.5 M*	*0.5 M*	*0.5 M*
None	7.1	7.9	7.1	6.4	12.6
4-Hydroxy	97	75	68	57	79
4-Hydroxy-3-methoxy	93	63	67	41	77
4-Hydroxy-3,5-dimethoxy	76	48	62	27	71

Similarly, salting out was observed for the phenols and benzyl alcohols. The increase in D_v was between 11 and 31% when 1 *M* instead of 0.5 *M* sodium acetate was used (Table II). For sodium tartrate the D_v increased by 17–41% when the tartrate concentration was raised from 0.3 to 0.5 mol l^{-1}. In both acetate and tartrate the largest salting out effects were observed for the phenols with alkyl substituents while the lowest effects were found for 4-hydroxyphenol and 4-hydroxybenzyl alcohol.

To study further the salting out of non-ionized aromatic compounds the D_v was determined for benzyl alcohol, phenol and 4-methylphenol in acetate, glycolate, butyrate and tartrate solutions over a wider concentration range. In Fig. 1 ln D_v is plotted *versus* the molar eluent concentration (C). It is seen that straight line relationships were obtained. Hence, $\ln D_v - \ln D_v^0 = k_s C$ where the superscript 0 refers to the extrapolated value for pure water. The salting out parameter, k_s, differed somewhat for different compounds but no cross-over occurred. For all compounds the salting out parameters depended strongly on the medium. The slopes observed in sodium tartrate were much larger than those obtained in the other media. Even if the salting out parameters in tartrate are calculated from plots of ln D_v *versus* moles of carboxylate per litre instead of molarity, the values are significantly higher than those in the other media. The smallest salting out parameters were obtained in sodium butyrate. Since butyrate anions are more hydrophobic than any of the other anions this result is consistent with the observation, discussed below, that hydrophobic interactions contribute markedly to the D_v values. As expected, larger salting out parameters were obtained in glycolate than in acetate.

According to the Gibbs–Donnan theory the molality in the resin phase (m_r) divided by that in the external solution (m) is determined by the equation

$$\ln m_r/m = -\pi \bar{v}/RT - \ln f_r/f$$

TABLE II

RETENTION DATA FOR PHENOLS, BENZYL ALCOHOLS, BENZOATES AND PHENYLACETATES IN ACETATE, GLYCOLATE, BUTYRATE AND TARTRATE SOLUTIONS AT 60°C

Parent compounds and substituents	*Acetate 0.5 M*		*Acetate 1.0 M*		*Glycolate 0.5 M*		*Butyrate 0.5 M*		*Tartrate 0.3 M*		*Tartrate 0.5 M*		*Activity coefficient ratio*	
	D_v	Δ	D_v	Δ	D_v	Δ	D_v	Δ	D_v	Δ	D_v	Δ	*Acetate* $f_{1.0}/f_{0.5}$	*Tartrate* $f_{0.5}/f_{0.3}$
Phenol	29.2		35.5		21.3		29.4		30.9		39.3		1.22	1.27
4-Methyl	59	0.71	73	0.72	43.9	0.72	56	0.64	64	0.73	86	0.78	1.24	1.34
4-Ethyl	121	1.42	153	1.46	88	1.42	113	1.34	138	1.49	195	1.60	1.26	1.41
4-*n*-Propyl	260	2.2	340	2.3	210	2.3	240	2.1	320	2.3	450	2.4	1.31	1.41
4-Hydroxy	37.8	0.26	42.0	0.17	22.6	0.06	33.1	0.12	31.5	0.02	37.4	−0.05	1.11	1.19
4-Methoxy	34.7	0.17	40.5	0.13	25.6	0.18	28.1	−0.05	39.8	0.25	50.0	0.24	1.17	1.26
2-Methoxy	20.1	−0.37	23.9	−0.39	17.3	−0.21	16.5	−0.58	25.6	−0.19	32.0	−0.21	1.19	1.25
Benzyl alcohol	6.0		7.3		5.4		5.5		6.9		9.0		1.22	1.30
4-Hydroxy	12.5	0.73	14.6	0.69	9.3	0.53	10.3	0.63	12.1	0.56	14.1	0.45	1.17	1.17
4-Methoxy	8.1	0.30	10.1	0.32	7.7	0.35	6.4	0.15	10.9	0.46	14.1	0.45	1.25	1.29
4-Hydroxy-3-methoxy	8.3	0.32	10.0	0.31	7.2	0.29	6.7	0.20	9.8	0.35	11.8	0.26	1.20	1.20
Benzoate	64		36.7		70		31.3		31.2		32.7		1.15	1.35
4-Methyl	131	0.71	75	0.72	147	0.74	56	0.58	68	0.77	73	0.81	1.15	1.38
4-Ethyl	260	1.4	153	1.43	310	1.48	109	1.25	152	1.58	171	1.65	1.16	1.45
4-*n*-Propyl	590	2.2	350	2.3	700	2.30	230	2.0	380	2.5	430	2.6	1.19	1.46
4-Hydroxy	156	0.88	83	0.82	121	0.54	83	0.97	49.6	0.46	47.9	0.38	1.06	1.25
4-Methoxy	100	0.44	58	0.47	110	0.44	42.2	0.30	53	0.53	55	0.52	1.16	1.34
4-Hydroxy-3-methoxy	100	0.44	54	0.39	91	0.26	44.0	0.34	37.8	0.19	37.9	0.15	1.08	1.29
4-Hydroxy-3,5-dimethoxy	63	−0.02	36.3	−0.01	63	−0.12	23.2	−0.30	29.8	−0.05	31.9	−0.02	1.15	1.38
Phenylacetate	40.0		21.7		44.8		19.4		20.6		21.5		1.09	1.35
4-Hydroxy	82	0.72	44.5	0.72	70	0.44	43.0	0.80	30.6	0.39	31.0	0.37	1.09	1.31
4-Methoxy	56	0.33	34.0	0.45	62	0.32	23.3	0.18	30.8	0.40	32.1	0.40	1.21	1.34
4-Hydroxy-3-methoxy	51	0.23	29.7	0.31	49.5	0.10	22.4	0.14	22.8	0.10	23.2	0.08	1.16	1.31
4-Hydroxy-3,5-dimethoxy	31.0	−0.25	18.7	−0.15	35.6	−0.23	11.4	−0.53	16.0	−0.25	17.5	−0.20	1.21	1.41

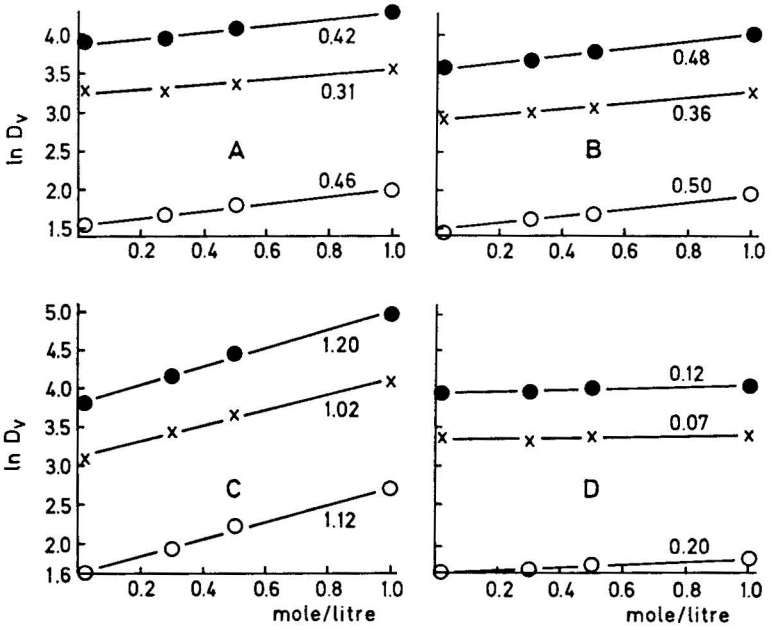

Fig. 1. Relationship between ln D_v and eluent concentration in sodium acetate (A), sodium glycolate (B), sodium tartrate (C) and sodium butyrate (D) at 60°C. O, Benzyl alcohol; X, phenol; ●, 4-methylphenol. Values given on the lines refer to the salting out parameter k_s (slope of the straight lines calculated by the least-squares method).

where π is the swelling pressure, $\bar{v}$ the partial molal volume of the solute and f_r/f the activity coefficient ratio[6]. A small increase in the eluent concentration will have a negligible influence on the swelling pressure and on the activity coefficients in the resin phase. As a good approximation, D_v is proportional to the molality ratio. This means that $\ln D_v - \ln D_v^0 = \ln f/f^0$. The ratio between the activity coefficients for the aromatic non-electrolyte at two concentrations of the eluent is therefore equal to the ratio between the distribution coefficients. The ratios $f_{1.0}/f_{0.5}$ determined in sodium acetate and $f_{0.5}/f_{0.3}$ determined in sodium tartrate are listed in Table II. Since the concentration of the non-electrolyte is low, f^0 can be taken as unity. Hence, $\ln f = k_s C$.

In agreement with the Gibbs–Donnan theory the D_v values for the carboxylate anions decreased markedly when the acetate concentration (pH 7.0) was increased from 0.5 to 1.0 mol l^{-1} (Table II). The decrease varied between 39% (4-methoxyphenylacetate) and 47% (4-hydroxybenzoate). Hence, it was significantly less than the value (50%) calculated for the exchange of ions of equal charge under the idealized conditions that the activity coefficient term, the swelling pressure and the concentration of eluting anions (in this case acetate) in the resin phase are unaffected by an increase in eluent concentration by a factor of 2. The individual differences for different aromatic ions show that specific interactions reflected in the activity coefficients are affected by the eluent concentration.

Under the same idealized conditions the calculated D_v values for the carboxylate anions in 0.5 *M* tartrate should be 23% lower than those in 0.3 *M* tartrate

solution. As shown in Table II a decrease of 3% was observed for 4-hydroxybenzoate ions while increased D_v values were obtained for the other ionic species investigated. The largest increase was obtained for 4-ethylbenzoate and 4-*n*-propylbenzoate (13%). Evidently, large interactions reflected in the activity coefficients must be responsible for the anomalous elution behaviour of the aromatic carboxylate anions in tartrate solution.

To elucidate further the abnormal elution behaviour in tartrate solutions, the D_v values for chloride ions were determined at various tartrate concentrations. When the concentration was changed from 0.3 to 0.5 mol l^{-1} the D_v decreased from 5.77 to 4.82 which corresponds to 16% while, as already mentioned, the decrease calculated for idealized conditions is 23%. In 0.1 *M* tartrate solution the D_v was 8.33 compared to 5.77 $(0.3/0.1)^{0.5} = 9.99$ calculated under idealized conditions from the value recorded in 0.3 *M* tartrate solution. The results show that the activity coefficient term cannot be disregarded even for the chloride–tartrate system, but that the deviations are much smaller than those observed for the aromatic carboxylate anions.

The aromatic carboxylate ions (A) were held so strongly in 0.02–0.10 *M* sodium acetate solutions, which are suitable eluents for strongly hydrophilic aliphatic carboxylate ions[2], that the D_v values could not be accurately determined. Accordingly, the calculated pressure–volume term in the Gibbs–Donnan equation was smaller than the activity coefficient term indicating that specific interactions were of great importance for the aromatic anions.

$$\ln\frac{[A]_r}{[A]} \cdot \frac{[B]}{[B_r]} = \pi(\bar{v}_B - \bar{v}_A)/RT + \ln f_{B_r} \cdot f_A/f_{A_r} \cdot f_B \qquad (= \ln k)$$

D_v is proportional to $[A]_r/[A]$. A is present in trace amounts. The activity coefficients in the resin phase are therefore determined by the concentration of the eluent anion, $[B]_r$. If the effect of the eluent concentration, [NaB], on the swelling pressure and the electrolyte invasion can be disregarded, we obtain

$$\frac{D_v'}{D_v''} \cdot \frac{[B]'}{[B]''} = \frac{f_A' \cdot f_B''}{f_B' \cdot f_A''}$$

where the superscripts refer to two different concentrations of the eluent.

For sodium acetate[7] the activity coefficient in 1 *M* solution is virtually the same as in 0.5 *M* solution. This means that the ratio between the activity coefficients of the aromatic anion in 1 *M* sodium acetate ($f_{1.0}$) to that in 0.5 *M* sodium acetate ($f_{0.5}$) is equal to the ratio between the distribution coefficients multiplied by 2. This ratio has been calculated for all aromatic anions studied. With the same approximations the ratio between the activity coefficient of the aromatic anions in 0.5 *M* sodium tartrate ($f_{0.5}$) to that in 0.3 *M* sodium tartrate is obtained from the ratio between the D_v values multiplied by 1.29. The results are given in Table II.

It is noteworthy that for all non-electrolytes the ratio $f_{1.0}/f_{0.5}$ in sodium acetate was lower than or equal to the ratio $f_{0.5}/f_{0.3}$ in sodium tartrate. This result shows that for all non-electrolytes the salting out was much more effective in sodium tartrate than in sodium acetate. In both media the lowest ratio was observed for the most hydrophilic species (4-hydroxyphenol and 4-hydroxybenzyl alcohol) while alkylphenols exhibited larger values.

For all benzoates and phenylacetates the ratio $f_{1.0}/f_{0.5}$ in sodium acetate was much lower than the ratio $f_{0.5}/f_{0.3}$ in sodium tartrate. In both media 4-hydroxybenzoate exhibited the lowest ratio among the benzoate anions, while the less hydrophilic species (alkylbenzoates and 4-hydroxy-3,5-dimethoxybenzoate) exhibited high values. Similarly, 4-hydroxyphenylacetate exhibited a lower ratio than 4-hydroxy-3,5-dimethoxyphenylacetate both in acetate and in tartrate.

The results show that the effect of an increase in eluent concentrations on the activity coefficients of the aromatic carboxylate anions in the eluent parallels the effect of an increased concentration on the activity coefficients of non-electrolytes of similar structure. In addition, the type of anion in the eluent has a similar influence on the activity coefficients in the external solution of both aromatic anions and uncharged aromatic compounds. Hence, interactions in the external solution in which the eluting anions are involved and which greatly affect the activity coefficients of the aromatic anions are of the same kind as the interactions which determine the salting out of the uncharged aromatic solutes. It should therefore be possible to predict the elution behaviour of an eluent during the elution of aromatic anions from salting out experiments with related non-electrolytes. As shown above (Fig. 1) sodium butyrate gave much smaller salting out effects for non-electrolytes than sodium acetate and sodium tartrate. Hence, it can be predicted that in butyrate solution the activity coefficient ratios for aromatic anions should be lower, and more close to unity, than the corresponding values determined for acetate and tartrate. This was confirmed by the results given in Table III. It is noteworthy that in butyrate, as in acetate and tartrate, the anions with 4-hydroxy-3-methoxy substituents exhibited lower activity coefficient ratios than the more hydrophobic anions with 4-hydroxy-3,5-dimethoxy substituents.

TABLE III

VOLUME DISTRIBUTION COEFFICIENT (D_v) FOR AROMATIC CARBOXYLATE ANIONS IN 0.3 *M*, 0.5 *M* AND 1.0 *M* SODIUM BUTYRATE AND THE RATIO BETWEEN THE ACTIVITY COEFFICIENTS (f) OF THE AROMATIC ANIONS IN THESE MEDIA AT 60°C

The activity coefficients for sodium butyrate at 25°C reported by Harned and Owen[7] were used in these calculations. If these activity coefficients are disregarded, $f_{0.5}/f_{0.3}$ decrease by 1% and $f_{1.0}/f_{0.5}$ by *ca.* 10%

	Distribution coefficients			$f_{0.5}/f_{0.3}$	$f_{1.0}/f_{0.5}$
Anion	*0.3 M*	*0.5 M*	*1.0 M*		
4-Methylbenzoate	88	56	26.8	1.08	1.06
4-Hydroxy-3-methoxybenzoate	79	44	19.1	0.94	0.96
4-Hydroxy-3,5-dimethoxybenzoate	37.1	23.2	10.6	1.06	1.01
4-Hydroxy-3-methoxyphenylacetate	40.1	22.4	10.4	0.94	1.03
3-Hydroxy-3,5-dimethoxyphenylacetate	17.4	11.4	5.5	1.11	1.07

Influence of substituents on the retention data

The sorption of aromatic non-electrolytes from aqueous solution onto different adsorbents, including anion-exchange resins, is markedly higher for solutes containing non-polar substituents than for the parent compound[5,8]. The elution order phenol < 4-methylphenol < 4-ethylphenol < 4-*n*-propylphenol (Table II) obtained in-

dependent of the ionic form of the anion exchanger shows that non-polar interactions including hydrophobic interactions (sorption enforced by water-structure[3]) contribute markedly to the observed distribution coefficients.

Enhanced non-polar interactions must also be responsible for the higher D_v of 4-alkylbenzoates compared to benzoate. In all media the Δ values for all alkylbenzoates were virtually the same as obtained for the corresponding 4-alkylphenols in the same medium. It should be recalled that under the applied conditions the phenols were non-dissociated while the aromatic carboxylic acids were almost 100% dissociated. Despite this difference the incremental change (decrease) in free energy for the transfer from the eluent to resin phase, resulting from the introduction of an alkyl group, was virtually the same for the non-electrolyte as for the ion. The results confirm that hydrophobic substituents have a marked influence on the ion-exchange equilibrium and show that under favourable conditions this effect can be predicted from independent data. For both phenols and benzoates the highest Δ values resulting from the introduction of the alkyl groups were found for 0.5 *M* tartrate solution, while the lowest values were observed in butyrate solution. An increase in Δ with increasing eluent concentration can be traced in sodium acetate solution for both phenols and benzoates. This concentration effect was larger in tartrate medium. The results are consistent with the observation that hydrophobic species are salted out more effectively by tartrate than by the other eluents.

Methoxyl groups are less hydrophobic than methyl groups. Accordingly, methoxyl groups in *para* positions in phenol, benzoate and phenylacetate led to a smaller increase in ln D_v than that observed for methyl groups. Again, the largest effect in 0.5 *M* solutions was obtained for the tartrate form and the smallest effect for the butyrate form. In butyrate medium the D_v for 4-methoxyphenol was slightly lower than that of phenol. The observation that Δ depends strongly on the parent compound indicates that the methoxyl group affects other factors of importance for the sorption than the non-polar interactions. Hence, 4-methoxyphenol is a weaker acid than phenol which means that the methoxyl group will decrease the hydrogen bonding between the phenolic hydroxyl group and the resin. Similarly, the substituent weakens the strength of benzoic acid which may affect the ion-exchange affinity of the 4-methoxybenzoate ion[3].

Hydrophilic substituents suppress the non-polar interactions and, provided that other effects are small, their introduction will lead to decreasing D_v values. Hence, the introduction of phenolic hydroxyl groups in uncharged aromatic compounds leads to a decreased sorption from aqueous solution[8] onto non-ionic styrene–divinylbenzene resins. For anion-exchange resins in the acetate form[5], and for other adsorbents[9] containing groups with a strong ability to participate in hydrogen bonding by serving as proton acceptors, hydrogen bonding with the adsorbent gives a positive contribution to ln D_v which can be larger than the decrease due to the lower hydrophobic interactions.

Table II shows that a phenolic hydroxyl group in the *para* position in benzoate and phenylacetate resulted in markedly increased distribution coefficients, indicating that hydrogen bonding between the phenolic proton and the resin contributes markedly to the ion-exchange affinity. The Δ values were lower in tartrate and higher in butyrate than in the other media. These values differed somewhat for the two types of aromatic anions. We conclude that the suppression of the non-polar interactions

had less effect on D_v than the hydrogen bonding with the resin and that the non-polar interactions have a greater influence on the equilibrium in tartrate medium than in butyrate.

In all media the effect of a second hydroxyl group in the *para* position in phenol was much less, and in 0.5 *M* tartrate Δ was slightly negative. The results indicate that hydrogen bonding between phenols serving as hydrogen donors and the resin contributes to the sorption not only on acetate resins but also on the other ionic forms, and that the medium has a great influence on the effect of a second hydroxyl group. The acid strength of 1,4-dihydroxybenzene is lower than that of phenol. Hence, hydrogen bonding between the first hydroxyl group and the resin is less favoured than for phenol. This can explain the comparatively low Δ values.

In agreement with previous investigations[5] under other conditions, 2-methoxyphenol was retained less strongly than phenol by the acetate resin. This is ascribed to intramolecular hydrogen bonding which weakens the hydrogen bonding to the resin. As shown in Table II, negative Δ values were also observed with the other resin forms. Similarly, 4-hydroxy-3-methoxybenzaldehyde and 4-hydroxy-3-methoxybenzyl alcohol were, in all media, held less strongly than the corresponding compounds lacking the methoxyl group. If hydrogen bonding between the phenolic hydroxyl group and a vicinal methoxyl group is also important for the retention of carboxylate anions, it can be predicted that 4-hydroxy-3-methoxybenzoate and 4-hydroxy-3-methoxyphenylacetate should exhibit lower D_v values than the corresponding anions without the methoxyl group. Table II shows that this holds true. Both for phenol and for the carboxylate anions the smallest effect of the methoxyl group on ln D_v was found in tartrate medium, while the largest effect was obtained in butyrate. A rough estimate of ln D_v for the substituted anions can be obtained by adding Δ for 2-methoxyphenol to ln D_v of the 4-hydroxy substituted anions. In all media the calculated values were slightly higher than the observed values. The results permit the conclusion that internal hydrogen bonding between phenolic hydroxyl groups and a vicinal methoxyl group leads to lowered D_v values of both non-ionized and ionized aromatic species.

In agreement with this conclusion, 4-hydroxy-3,5-dimethoxybenzoate and 4-hydroxy-3,5-dimethoxyphenylacetate exhibited lower D_v values than the corresponding anions with one methoxyl group. Again, the smallest effect of the second methoxyl group was obtained with the tartrate resin while the largest effect was found with the butyrate resin. The large decrease in ln D_v resulting from the second methoxyl group indicates that, in addition to a strengthened internal hydrogen bonding, the second methoxyl group leads to a steric shielding which will further suppress hydrogen bonding between the phenolic hydrogen in the aromatic anions and the resin. Effective internal hydrogen bonding and steric shielding can also explain the lower D_v of 4-hydroxy-3,5-dimethoxybenzaldehyde compared to 4-hydroxy-3-methoxybenzaldehyde.

Influence of the ionic form

The larger activity coefficients of the non-electrolytes in glycolate than in butyrate solutions (Fig. 1) shows that glycolate anions in the external solution have a greater ability than butyrate anions to push the aromatic solutes into the resin phase. It is therefore reasonable to assume that, at zero concentration in the external solution, glycolate ions in the resin would tend to push the solutes into the external

solution more effectively than the butyrate counter ions. This offers an explanation for the higher D_v^0 values observed for butyrate than for glycolate (Fig. 1). As expected, acetate takes an intermediate position. The results lend additional support to the conclusion that hydrophobic interactions contribute to the distribution coefficients for the non-electrolytes. It is noteworthy that the structure of the carboxylate counter ions has a modest influence on D_v^0. The observation that higher values were obtained for the tartrate resin than for the glycolate resin can be related to the lower swelling pressure for the resin containing divalent anions.

The selectivity coefficient $k_{B/Cl}$ for the ion-exchange equilibrium between butyrate and chloride ions on Dowex 1-X10 was found to be approximately 0.24 for resins with the exchange groups 50–95 % occupied by chloride ions[3]. For the exchange between acetate and chloride the corresponding value was approximately 0.12. The higher value for butyrate is explained by larger non-polar interactions. If the selectivity coefficients were independent of the relative amounts of ions in the resins the ratio between the D_v of the aromatic carboxylate anions in 0.5 *M* butyrate and their D_v in 0.5 *M* acetate should be approximately 0.5. The observed values varied between 0.37 and 0.53. The highest values were obtained for 4-hydroxybenzoate and 4-hydroxyphenylacetate, while the lowest values were recorded for benzoate and phenylacetate with 4-hydroxy-3,5-methoxy substituents. It is noteworthy that both for the unsubstituted anions and for the derivatives virtually the same ratio was found for benzoates and phenylacetates.

No relevant data which permit a prediction of the ratio between D_v in glycolate to that in acetate seem to be available. The observed ratio varied between 0.78 and 1.19. The lowest ratio was observed for the carboxylate ions with a phenolic hydroxyl group in the *para* position. In contrast to the ratio between D_v in butyrate and acetate, this ratio depended on the parent compound. For all investigated species a higher ratio was found for phenylacetates than for benzoates. The results indicate that interactions in which the hydroxyl group in the glycolate ions are involved exert a great influence on the ion-exchange equilibrium.

Influence of the temperature

The elution of benzaldehyde, 4-hydroxy-3-methoxybenzaldehyde and the three aromatic anions listed in Table IV was studied in 0.5 *M* solutions of sodium acetate, glycolate, butyrate and tartrate at 15, 30, 45 and 60°C. The distribution coefficients decreased markedly with increasing temperature. Plots of ln D_v *versus* $1/T$ gave straight lines for all investigated systems. Hence, the enthalpy change was independent of the temperature. The slope showed that the sorption was strongly exothermic. For reasons already mentioned, the results with the substituted benzaldehyde will not be discussed further. The results obtained with the other solutes are given in Fig. 2 and Table IV. The lowest numerical enthalpy values were, in all media, obtained for benzaldehyde and the highest values for 4-hydroxy-3-methoxybenzoate which was also held more strongly by all ionic forms of the resin than the other aromatic carboxylate ions investigated.

As already mentioned, the activity coefficients for the aromatic anions in the external solution depended on the eluent concentration. Any generally valid comparisons between the changes in free energy and entropy cannot therefore be based upon available data. The calculations of these thermodynamic functions have there-

TABLE IV

THERMODYNAMIC FUNCTIONS OF ANION-EXCHANGE EQUILIBRIA AT 30°C

Aromatic anions and eluents	$-\Delta\bar{H}$ *(kJ mol^{-1})*	$-\Delta\bar{G}$ *(kJ mol^{-1})*	$-\Delta\bar{S}$ *(J mol^{-1}/K^{-1})*
4-Hydroxy-3,5-dimethoxyphenylacetate			
0.5 *M* Sodium acetate	11.9	7.28	15.3
0.5 *M* Sodium glycolate	12.1	7.68	14.4
0.5 *M* Sodium butyrate	12.4	4.76	25.3
4-Hydroxy-3-methoxyphenylacetate			
0.5 *M* Sodium acetate	12.7	8.54	13.6
0.5 *M* Sodium glycolate	12.3	8.54	12.3
0.5 *M* Sodium butyrate	13.4	6.70	22.0
4-Hydroxy-3-methoxybenzoate			
0.5 *M* Sodium acetate	14.6	10.43	13.7
0.5 *M* Sodium glycolate	14.5	10.23	13.9
0.5 *M* Sodium butyrate	15.8	8.44	24.1

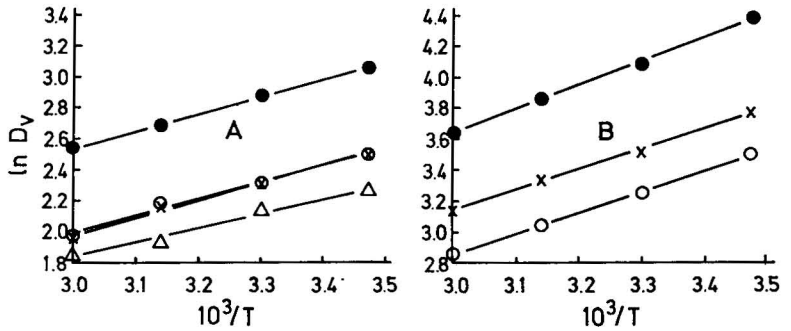

Fig. 2. Dependence of ln D_v on $1/T$. A, Benzaldehyde in 0.5 *M* solutions of: tartrate (●), $\Delta H = -9.48$: acetate (X), $\Delta H = -9.34$; glycolate (○), $\Delta H = -9.08$; butyrate (△), $\Delta H = -7.64$. B, Various anions in 0.5 *M* sodium tartrate: 4-hydroxy-3-methoxybenzoate (●), $\Delta H = -12.8$; 4-hydroxy-3-methoxyphenylacetate (X), $\Delta H = -11.0$; 4-hydroxy-3,5-dimethoxyphenylacetate (○), $\Delta H = -11.3$. The change in enthalpy, ΔH, is in kJ mol^{-1}.

fore been restricted to the ion-exchange equilibria in 0.5 *M* solutions of the singly charged eluents. The apparent enthalpy change, $\Delta\bar{H}$, was calculated by the least-squares method from the straight line relationship between ln D_v (or ln k) and $1/T$. The $\Delta\bar{H}$ value refers to the transfer of the ions from, and into, the applied eluents. The corresponding changes in free energy ($\Delta\bar{G}$) and entropy ($\Delta\bar{S}$) were calculated from the equations:

$$\Delta\bar{G} = -RT \ln k$$
$$\Delta\bar{S} = (\Delta\bar{H} - \Delta\bar{G})/T$$

The results showed that, as for $\Delta\bar{H}$, the $\Delta\bar{S}$ values were virtually independent of the temperature, and therefore only the values obtained at 30°C are given in Table IV.

Table IV shows that the influence of the eluent anions on $\Delta\bar{H}$ was rather small for the studied aromatic anions. This may indicate that exothermic interactions (*e.g.*, hydrogen bonding with the resin) and endothermic interactions (*e.g.*, breakage of internal hydrogen bonds) were affected to a modest extent by the structural differences in the eluting anions. The change in entropy was negative in all systems. For acetate and glycolate solutions $-\Delta\bar{H}$ had a much larger influence on the change in free energy than the entropy term. The entropy change calculated for the acetate and glycolate systems differed only slightly, while $\Delta\bar{S}$ was more negative for the exchanges in butyrate. A possible explanation is that hydrophobic (water-structure enforced) effects which contribute to an increased $\Delta\bar{S}$ are more important in the less hydrophobic acetate and glycolate systems than in the butyrate systems. The results are consistent with the observation, mentioned above, that the contribution to ln D_v resulting from the introduction of an alkyl or methoxyl group in phenols and carboxylate anions is less or more negative in butyrate medium than in any of the other media under investigation.

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EFFECT OF SOLVENTS ON THE RESOLUTION OF NEUTRAL LIPIDS ON CHROMARODS*

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SUMMARY

The chromatographic behavior of neutral lipids on chromarods, used for the Iatroscan TH-10 analyzer, was evaluated and compared to that observed in adsorption thin-layer chromatography on silica gel. Various proportions of hexane, diethyl ether and formic acid were used in the developing solvent to determine changes in R_F values of the neutral lipid classes. In addition, acetic and propionic acids were investigated as substitutes for formic acid in the developing solvent. A knowledge of the chromatographic behavior of the neutral lipid classes, with systematic changes in the concentration of individual solvents in the developing solvent mixture, allows maximum resolution to be obtained. The R_F values of some lipid components change with extensive use of the chromarods (25 to 30 uses); however, the life of the chromarods can be extended by changing the proportion of the solvent components in accordance with the chromatographic characteristics determined in this study.

INTRODUCTION

The Iatroscan[1] is an instrument that combines the resolution capabilities of thin-layer chromatography (TLC) with the possibility of quantitation by using a flame-ionization detector. The application of this method is still in the experimental stages, and new developing solvents are continually reported.

The apparent similarity between the use of chromarods and adsorption TLC on plates coated with silica gel has tempted many workers to apply solvent systems successfully developed for TLC on silica gel directly to chromarods. However, it is evident from recently published data on the resolution of neutral lipids with chromarods, that the effects of solvent systems are quite different[2–6] from those applicable in adsorption TLC. The solvent systems containing hexane, diethyl ether and organic acid are either low in acid[2,3] or have no acid at all[4], giving the same separation sequence as with TLC, or are low in diethyl ether[5], which results in an inversion of the

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triglyceride (TG) and free fatty acid (FFA) sequence obtained in adsorption TLC. A separation of neutral lipids has also been reported in which a chlorinated solvent system is used[6].

In this communication, the effects of various solvents and solvent mixtures on the resolution of the neutral lipid classes will be examined.

EXPERIMENTAL

Instrument and operating conditions

The Iatroscan TH-10 TLC Analyzer, Mark II (Iatron Labs., Tokyo, Japan; Canadian distributor: Technical Marketing Associates, Mississauga, Canada) used, was equipped with a flame-ionization detector and an electronic stepping integrator. The flame-ionization detector was operated with a hydrogen flow-rate of 160 ml/min and an air flow-rate of 2 l/min. The scanning speed was 0.42 cm/sec. A two-pen linear recorder (Fisher Recordall, Model 5000) was used at 10 mV full-scale deflection and a chart speed of 0.47 cm/sec.

Procedure

The chromarods (type S; mean thickness of sintered coating of active silica gel adsorbent 75 μm) were cleaned and placed overnight in a 9 N H_2SO_4 solution, rinsed with distilled water, then dried at 110°C for 10 min and scanned twice in the Iatroscan before use. A mixture containing equal amounts (by weight) of cholesterol ester (CE), methyl ester (ME), triglyceride (TG), free fatty acid (FFA) and cholesterol (C) was purchased from Nu Check Prep (Elysian, MN, U.S.A.); oleic acid was the fatty acid used in this standard mixture. A heptane solution was prepared containing 30 μg/μl of the total mixture (or 6 μg/μl of each of the five compounds). It was later necessary to spike a portion of the standard solution with approximately double the amount of TG, and to prepare simpler mixtures, to differentiate between the TG, ME and FFA peaks in many instances. The chromarods, held in an appropriate frame, were spotted with the standard solution (1 μl) and placed in glass tanks lined with filter paper. A variety of solvent mixtures was used, and development was for *ca.* 11 cm on the chromarods. The rods were then dried at 110°C for 5 min, transferred into the Iatroscan and scanned. All R_F values quoted are the means of 10 rods developed simultaneously.

Developing solvents

All solvent mixtures used for development were prepared by mixing X volumes of diethyl ether with 100 — X volumes of hexane. The organic acids (formic, acetic or propionic) were added to the 100 ml of solution containing hexane and diethyl ether in amounts of 0.04 to 1 ml. All solvents were of reagent grade and were distilled in glass before use.

Thin-layer chromatography

High-performance Whatman TLC plates (Type HP-K; 10 × 10 cm) coated with silica gel were used. These plates are coated with a special silica gel of particle size 5 μm. The developing solvents were the same as used for the chromarods. To visualize the compounds, the TLC plates were sprayed with H_2SO_4–methanol (1:1) and heated.

RESULTS AND DISCUSSION

Results using thin-layer chromatography

In order to understand the chromatographic behavior of neutral lipids on chromarods, a study of the behavior of these lipid classes in adsorption TLC was useful. The well-known sequence of resolution in adsorption TLC on silica gel with mobile phases containing various proportions of hexane, diethyl ether and formic acid is shown in Table I. The mobility of CE, ME, TG and C was increased by increasing the proportion of the polar solvents (diethyl ether and formic acid). The effect of formic acid was not evident when the ratio of hexane to diethyl ether was 85:15, presumably because of the small change in the total volume of polar solvents. On the other hand, the migration of FFA in adsorption TLC was dependent on the organic acid, provided that more than 5% of diethyl ether was present in the developing solvent. The FFA did not migrate from the origin when the developing solvent was hexane–diethyl ether (95:5), even with the addition of 1 ml of formic acid (*i.e.*, hexane–diethyl ether–formic acid, 95:5:1). Similarly, a developing solvent containing hexane–diethyl ether, (85:15) did not move the FFA from the origin (not shown). However, the presence of 0.1 or 1 ml of formic acid in this hexane–diethyl ether mixture significantly increased the mobility of the FFA (Table I).

TABLE I

THE R_F VALUES OF NEUTRAL LIPIDS ON HIGH-PERFORMANCE TLC PLATES (TYPE HP-K)

Component	*R_F value*			
	Hexane–diethyl ether–formic acid			
	95:5:0.1	*85:15:0.1*	*95:5:1*	*85:15:1*
Cholesterol ester	0.44	0.73	0.58	0.71
Methyl ester	0.26	0.53	0.36	0.51
Triglyceride	0.05	0.32	0.09	0.31
Free fatty acid	0	0.09	0	0.17
Cholesterol	0.01	0.04	0.03	0.05

Results using chromarods: comparison with thin-layer chromatography conditions

It was evident from the results with chromarods (Fig. 1) that the developing solvent ideal for adsorption TLC (hexane–diethyl ether–formic acid, 85:15:1) gave no resolution of ME, TG and FFA (Fig. 1–5). It was therefore necessary to prepare several simpler lipid mixtures and to "spike" the five-component standard mixture with TG in order to resolve these complex chromatograms. In order to achieve a separation sequence similar to that observed in adsorption TLC, the formic acid content had to be lowered to 0.04 ml (Fig. 1–6). Another resolution of these lipid classes was reported by Sipos and Ackman[5], who used a developing solvent containing small amounts of diethyl ether (hexane–diethyl ether–formic acid, 97:3:1). As shown in Fig. 1-1, such a developing solvent results in inversion of the FFA and TG peaks as compared with adsorption TLC on silica gel.

A systematic approach was therefore undertaken to elucidate the chromatographic behavior of these lipid classes with change of diethyl ether content, keeping the

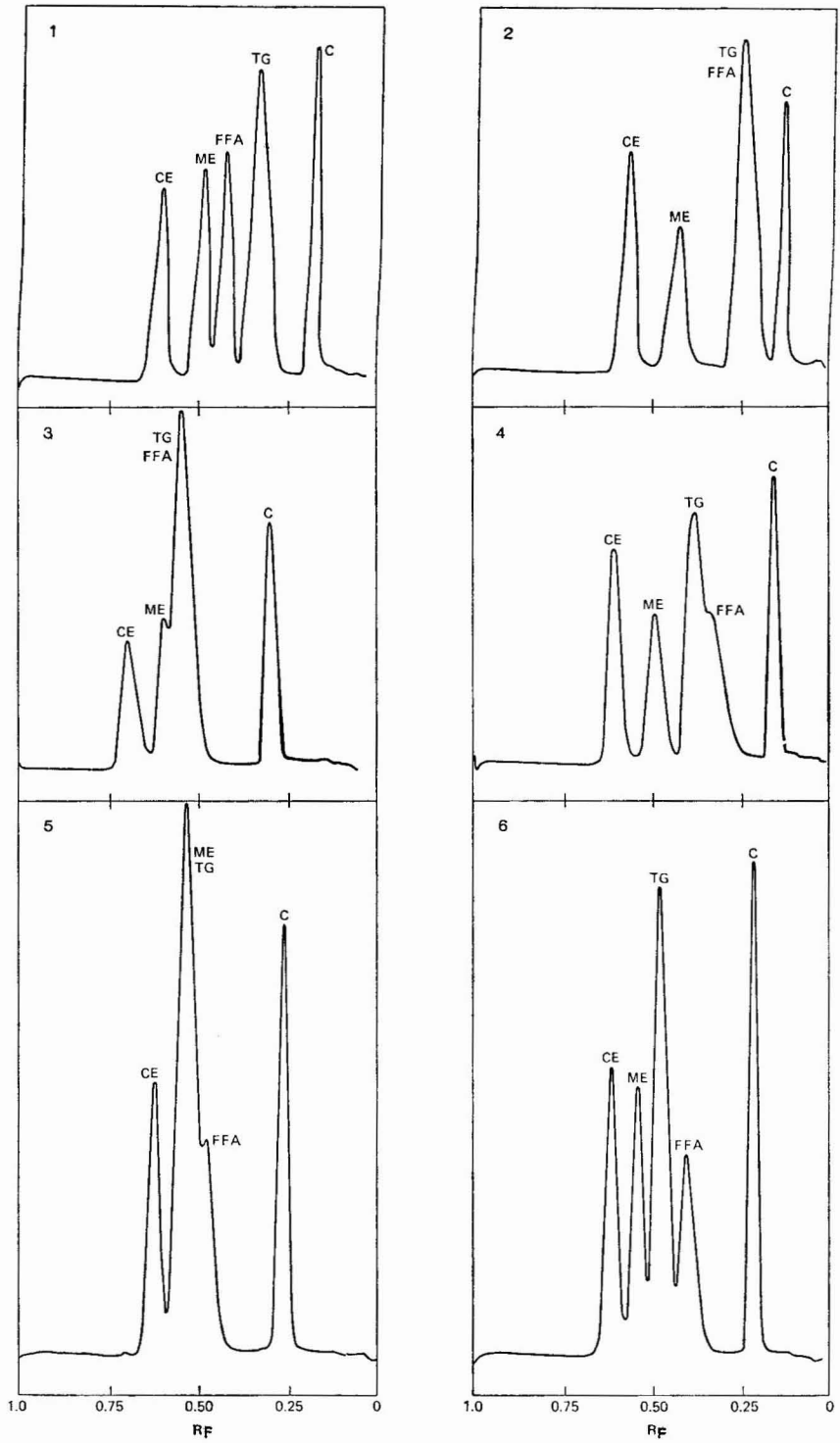

Fig. 1. Chromatograms showing the separation of neutral lipid classes on chromarods with solvents containing hexane–diethyl ether–formic acid in the proportions 95:5:1 (1); 95:5:0.04 (2); 90:10:1 (3); 90:10:0.04 (4); 85:15:1 (5); 85:15:0.04 (6); R_F values are indicated. CE = cholesterol ester; ME = methyl ester; TG = triglyceride; FFA = free fatty acid; C = cholesterol.

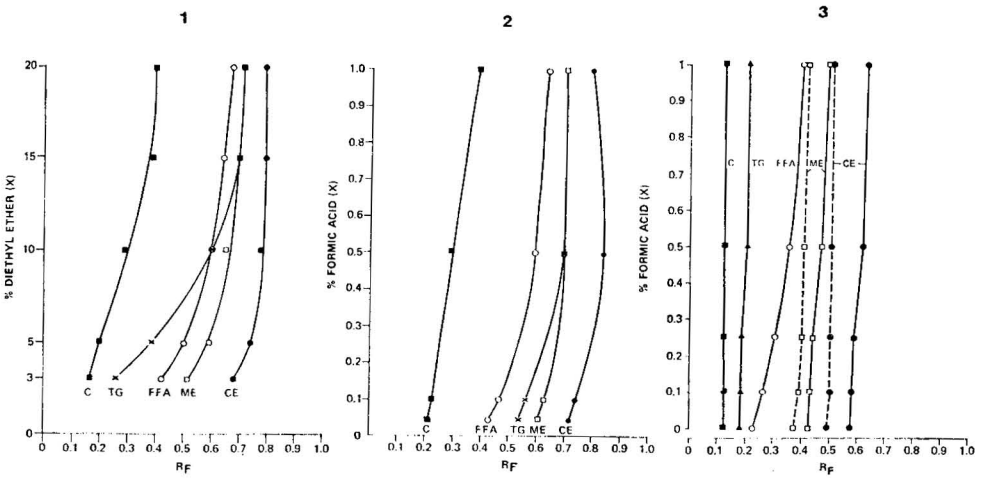

Fig. 2. R_F values of cholesterol (C), free fatty acid (FFA), triglyceride (TG), methyl ester (ME) and cholesterol ester (CE) plotted at various concentrations of hexane, diethyl ether and formic acid in the developing solvent. In Fig. 2-1, the concentration of diethyl ether is decreased, keeping the level of formic acid constant (hexane–diethyl ether–formic acid, 100−X:X:1). In Figs. 2-2 and 2-3, the concentration of formic acid is decreased, keeping the proportions of hexane and diethyl ether constant (85:15 in Fig. 2-2, and 97:3 in Fig. 2-3). The broken lines in Fig. 2-3 are the R_F values of ME and CE on chromarods used at least 25 to 30 times; the R_F values of C, TG and FFA on the used chromarods were the same as on new chromarods.

concentration of formic acid constant (Fig. 2-1). Conversely, the hexane–diethyl ether ratio was kept constant at 85:15 (Fig. 2-2) or 97:3 (Fig. 2-3), and the concentration of formic acid was changed.

Effect of diethyl ether

It is evident from Fig. 2-1 that a reduction of the diethyl ether content in the developing solvent retarded the relative mobility of TG more so than that of C, ME and CE; this effect was also noted for adsorption TLC. However, the relative mobility of FFA on chromarods paralleled that observed for C, ME and CE, which was totally different from the behavior of FFA in adsorption TLC. This difference in the relative mobility of FFA and TG therefore permitted separation of all five components at low concentrations of diethyl ether. The effect was an inversion of the TG and FFA resolution sequence on chromarods at hexane–diethyl ether–formic acid ratios of 95:5:1 and 97:3:1 compared with that observed in adsorption TLC. From Fig. 1-3, it can be seen that the developing solvent hexane–diethyl ether–formic acid (90:10:1) does not separate the TG and FFA peaks, which is contrary to the results of Tanaka *et al.*[7,8].

Effect of formic acid

As stated before, the developing solvent hexane–diethyl ether–formic acid (85:15:1) did not separate TG and ME on chromarods, and FFA was poorly resolved (Fig. 1-5). A reduction in the formic acid content lowered the relative mobility of both TG and FFA more so than that of ME, thus permitting separation of all five components (Fig. 2-2). A developing solvent of hexane–diethyl ether (85:15), with no formic

acid, gave the same separation sequence as did hexane–diethyl ether–formic acid (85:15:0.04) (see Fig. 1-6), but the FFA peak was slightly broader when the former developing solvent was used (results not shown). A broad FFA peak is evident from the results of Vandamme *et al.*[4]. The addition of small amounts of formic acid is therefore recommended in order to obtain a sharper FFA peak and a greater separation between FFA and C. As demonstrated by van Tornout *et al.*[2], the presence of a small amount of formic acid (light petroleum (b.p. 60–80°C)–diethyl ether–formic acid, 85:15:0.1) allowed the use of a long-chain alcohol as internal standard, which migrated between FFA and C.

In Fig. 2-3, the effect is shown of decreasing the amount of formic acid in the developing solvent hexane–diethyl ether (97:3) on the chromatographic behavior of the five lipid classes (solid lines only). The sequence of resolution of the lipid classes remained the same, but a significant decrease was observed in the relative migration of FFA.

Effect of extensive use of chromarods

The results in Fig. 1 and Fig. 2 (solid lines) were obtained from new chromarods. However, after about 25 to 30 developments on the chromarods, the TG and FFA peaks could no longer be resolved by hexane–diethyl ether–formic acid (85:15:0.04). A switch to the developing solvent hexane–diethyl ether–formic acid (97:3:1) to take advantage of the TG and FFA inversion proved unsuccessful, because the FFA and ME now migrated together (Fig. 2-3). A detailed study of these older chromarods was undertaken as shown in Fig. 2-3 (broken lines) to see whether or not the FFA and ME could again be separated by taking advantage of the relatively greater decrease of the R_F value of FFA compared with ME. The results are superimposed in Fig. 2-3; the R_F values of C, TG and FFA had not changed from new to old chromarods, but the R_F values of ME and CE were greatly reduced (broken lines). By reducing the formic acid content in the developing solvent containing hexane–diethyl ether (97:3), an effective resolution of these neutral lipid classes on the chromarods could be maintained beyond the 20–25 developments previously reported[5]. In fact, through knowledge of the chromatographic behavior of these lipid classes and the effects of various solvents, we have been able successfully to complete over 50 separations.

Effect of other organic acids

The effect of using organic acids other than formic acid was also investigated. Substitution of acetic or propionic acid for formic acid in the developing solvent hexane–diethyl ether–formic acid (85:15:0.1) did not improve the resolution of the five-component mixture (Table II). In fact, when acetic acid was used, the TG and FFA peaks were incompletely resolved. The R_F values of all components, except C, were increased with increasing chain-length of the organic acid.

Evaluation of the chromatographic behavior of the chromarods

With a knowledge of the chromatographic behavior of the common neutral lipid components and the effects of developing solvents, the investigator can select the most appropriate conditions for a given sample and extend the life expectancy of the chromarods. Further, conditions may be selected to provide separations when certain internal standards are used. For example, van Tornout *et al.*[2] recently recom-

TABLE II

THE R_F VALUES OF NEUTRAL LIPIDS ON CHROMARODS

The developing solvent was hexane–diethyl ether–organic acid (85:15:0.1).

Component	*R_F value*		
	Formic acid	*Acetic acid*	*Propionic acid*
Cholesterol ester	0.74	0.79	0.86
Methyl ester	0.63	0.67	0.74
Triglyceride	0.56	0.56	0.64
Free fatty acid	0.47	0.50	0.55
Cholesterol	0.23	0.20	0.23

mended the use of a long-chain alcohol as an internal standard. The use of the developing solvent that inverted the TG and FFA peaks (hexane–diethyl ether–formic acid, 97:3:1[5]) is not recommended, as it resulted in poor separation of TG and the long-chain alcohol. However, the use of small amounts of formic acid is recommended; this gives a sharper FFA peak and greater separation between FFA and C. The use of propionic acid instead of formic acid has the added advantage of giving still greater separation between C and FFA (Table II).

The chromatographic behavior of FFA on chromarods was markedly different from that in adsorption TLC. On chromarods, FFA migrated with an R_F value of 0.4 without an organic acid (Fig. 2-2) or a high ether content (Fig. 2-1) in the developing solvent; even with hexane–diethyl ether (97:3), the R_F value of FFA was still 0.25 (Fig. 2-3). This is totally unlike the chromatographic behavior of FFA in adsorption TLC. Further, the R_F values of the other lipid classes were also much greater on chromarods than on silica gel layers when identical solvents were used. This suggests that forces other than those operative in adsorption chromatography operate on chromarods. Presumably, capillary action (or other forces) could be involved, which acts independently of the developing solvent.

The present study clearly indicates that resolution of components and the effects and influences of developing solvents on chromarods are very different from those in adsorption TLC, despite the fact that silica gel is used in both techniques. A better understanding of the nature of separation on chromarods is therefore essential. Procedures involving separation on chromarods and flame-ionization detection have in the past 5 years been shown to have advantages and potential as a complementary technique in lipid research.

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GAS CHROMATOGRAPHIC AND GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC CHARACTERISATION OF METHANE THIOLSULPHONATES CARRYING FURTHER FUNCTIONAL GROUPS

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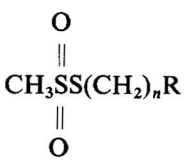
SUMMARY

A further group of methane thiolsulphonates have been characterised by gas chromatography (GC) and GC–mass spectrometry (MS). These compounds contain additional functional groups and have the general structure $CH_3SO_2S(CH_2)_nR$ (Type III) and $CH_3SO_2SCH_2CO(CH_2)_nR$ (Type IV). Although in a few cases some decomposition on GC to the respective sulphones was observed, all samples gave characteristic mass spectra and all but one could be characterised by combined GC–MS. Certain aspects of the GC and MS behaviour are briefly discussed.

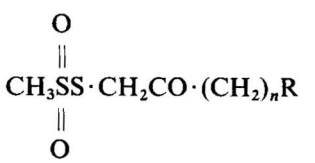
INTRODUCTION

The characterisation[1] and application in enzyme studies[2] of some thiolsulphonates and bisthiolsulphonates have recently been described. These and other studies[3,4] have shown that these compounds are generally amenable to characterisation by gas chromatography– mass spectrometry (GC–MS) and MS. A further group of thiolsulphonates has been synthesised in our laboratory. These new compounds are thiolsulphonates in which additional functional groups are separated from the methanethiolsulphonate moeity by methylene chains of varying lengths. Their characterisation by GC–MS is described in this report. These new compounds are broadly classified into two further structural types (for types I and II, see ref. 1):

Type III



Type IV



Individual structures are shown in Table I. It was found that during both GC and direct probe analyses, compounds 6, 7 and 8 showed a variable (pyrolytic?) loss of sulphur to form the corresponding sulphone. GC and MS data from these sul-

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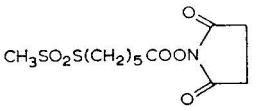
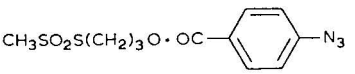
TABLE I

STRUCTURES AND GC RETENTION TIMES (ON OV-1) OF METHANE THIOLSULPHONATES AND SULPHONES

Number	*Compound structure*	*Oven temperature (°C)*	*Retention time (min) (min)*
1A	$CH_3SO_2S(CH_2)_3OH$	170	3.8
1B	$CH_3SO_2S(CH_2)_3$ OTMS*	170	4.1
2A	$CH_3SO_2S(CH_2)_5COOH$	220	3.9
2B	$CH_3SO_2S(CH_2)_5COO\ CH_3$	220	3.5
2C	$CH_3SO_2S(CH_2)_5COO$ TMS	220	5.0
	CH_3	140	4.5
3	$CH_3SO_2S(CH_2)_5CO\cdot NH\cdot CH\cdot COOCH_2CH_3$	140	4.5
4		—	—**
5		150	5.0
6A	$CH_3SO_2SCH_2CO\cdot(CH_2)_2COOCH_3$	—	—**
6B	$CH_3SO_2CH_2CO(CH_2)_2\ COOCH_3$	160	5.4
7A	$CH_3SO_2SCH_2CO(CH_2)_6COCH_2Cl$	270	4.2
7B	$CH_3SO_2CH_2CO(CH_2)_6COCH_2Cl$	270	5.8
8A	$CH_3SO_2S\ CH_2\ CO(CH_2)_{14}\ CH_3$	—	—**
8B	$CH_3SO_2CH_2\ CO(CH_2)_{14}\ CH_3$	250	3.0
8C	$HS.\ CH_2\ CO\ (CH_2)_{14}\ CH_3$	250	5.7
(8D)	$[-S\text{-}CH_2CO(CH_2)_{14}CH_3]_2$	250	16.5

* TMS = trimethylsilyl.

** Parent compounds did not elute.

phones are included for completeness because of their significance in characterisation. Compound 8 elutes from the GC only as the sulphone. Some aspects of the mass spectra of sulphones have been described.

EXPERIMENTAL

Specific synthetic details and enzyme studies will be reported elsewhere; however, in outline the synthetic routes are similar to that described for bismethanethiolsulphonates[2].

For analysis, the compounds were freshly dissolved in acetone or methylene chloride. GC and GC–MS conditions were as described previously[1] using an OV-1 column; the GC column temperatures are listed in Table I. Identities of GC decomposition products were initially obtained by GC–MS and this identification is used in Table I. All the compounds gave satisfactory analyses without the need for reduction to the thiol, but mass spectra for "intact" 4, 6A and 8A were obtained only by direct probe.

RESULTS

Gas chromatography

The retention times of compounds 1 to 5, 6B, 7A, 7B, 8B and 8C are summarised in Table I. Structures of compound 4, and the parent compounds 6A and 8A are also included; these did not elute unchanged from the GC. Structures 6B, 7B, 8B and 8C represent the major identified decomposition products, attributed to pyrolytic loss of sulphur on injection. Approximate proportions of the major peaks were as follows: 6B = 95%; 7A = 35%; 7B = 45%; 8B = 60%; 8C = 20%; 8D (see Discussion) = 10%. Other minor peaks (<10%) were observed; these were not analysed. Compounds 1 to 5 gave clean traces with little evidence of decomposition.

MS and combined GC–MS

Mass spectra were obtained for all compounds listed in Table I. Normalised partial spectra are given in tabular form (Table II) including the molecular ion (where present) and a 10-ion listing from *m/z* 50, inluding base peak. The list is biased towards diagnostic peaks at higher mass rather than high abundance, but less unique, ions at lower mass. Molecular ions are absent in some cases but these samples produced diagnostic peaks at high mass, due to loss of simple fragments.

Some features of the individual spectra are outlined below; M − 79 and M − 111 were observed except where noted.

Compound 1. A, small M^+, base peak *m/z* 91 (M − 79), small peak at *m/z* 137, showing an unexpected loss of SH. 1B no M^+ (M − 15 present), otherwise predictable fragmentation.

Compound 2. A, no M^+; base peak at *m/z* 64, probably SO_2, an unusual finding for these compounds. 2B and C, base peaks *m/z* 129 $[C_6H_9OS]^+$, probably cyclic.

Compound 3. M^+ present. Shows combination of predictable fragments with the base peak the well documented cyclic ion $[C_5H_9S^+]$ at *m/z* 101 and similar peaks at *m/z* 129, 131 $[C_6H_{11}OS]^+$ and 231 $[M - (79 + 15)]$.

Compound 4 (probe only). M^+ absent, base peak *m/z* 101. Prominent peaks from succinimide moiety (*m/z* 99 and 115) and also *m/z* 129 from the central portion of the molecule.

Compound 5. M^+ present, but not M − 79. Azide characterised by M − 28 at *m/z* 287 and peaks from phenyl azide[5] at *m/z* 90, 91, 118, 134. Other peaks at 73 (base, $C_3H_5S^+$) and 153 [M − OOC PhN_3].

Compound 6. A, M^+ absent, base peak *m/z* 115 formed by cleavage at the ketone, as is *m/z* 129 in this compound $[CH_2CO(CH_2)_2CO_2CH_3]^+$; 6B, M^+ present, other fragments corresponding to those from 6A with the addition of *m/z* 149 $[M - COOCH_3]^+$.

Compound 7. The 2,9-diketone structure gave complex spectra, the predominant fragmentations were due to McLafferty rearrangements giving peaks at *m/z* 191, 168, 136 and 137, together with simple α-cleavage with loss of $\cdot CH_2Cl$ or $CH_3SO_2(S)CH_2\cdot$ to give *m/z* 189, 233 and 264. The peak at *m/z* 111 is probably $[(CH_2)_5CHCO]^+$ (see Discussion). Also found were the ion series $[CH_3SO_2(CH_2)_n]^+$ at *m/z* 93, 107, 121 and 135 in 7B, and a weaker series in 7A ascribed to $[CH_3SO_2S(CH_2)_n]^+$ at *m/z* 125, 139 and 153. The values of the lower mass ions in this series thus imply that there has been an elision of CO from the chain. Some un usual fragments at high mass (*m/z* 266–264, 250–248) in 7A await further study.

TABLE II

TEN-ION LISTING OF MASS SPECTRAL PEAKS FROM METHANE THIOLSULPHONATES AND SULPHONES

M^+																															
1A		*1B*		*2A*		*2B*		*2C*		*3*		*4*		*5*		*6A*		*6B*		*7A*		*7B*		*8A*		*8B*		*8C*	
m/z	*I*	*m/z*	*I*	*m/z*	*I*	*m/z*	*I*	*m/z*	*I*	*m/z*	*I*	*m/z*	*I*	*m/z*	*I*	*m/z*	*I*	*m/z*	*I*	*m/z*	*I*	*m/z*	*I*	*m/z*	*I*	*m/z*	*I*	*m/z*	*I*		
170	0.69	242	—	226	—	240	—	298	—	325	1.9	323	—	315	4.9	240	—	208	1.1	314	—	282	5.7	364	0.50	332	1.2	286	7.9		
153	0.81	227	56	209	8.0	209	12	283	38	252	46	244	18	287	11	209	1.6	177	5.8	264	3.3	233	73	332	3.3	252	65	256	1.1		
139	3.6	169	80	161	20	161	23	219	28	246	28	209	55	224	12	194	3.0	176	2.2	248	3.8	191	22	285	4.8	239	13	239	100		
137	1.0	163	43	147	21	129	100	187	6.6	231	9.8	145	3.9	153	26	179	4.3	149	22	191	40	189	15	252	96	234	22	137	1.8		
125	3.9	147	7.7	129	90	127	33	171	70	209	12	131	16	146	23	161	6.6	129	3.2	189	37	149	26	239	82	194	19	123	3.5		
112	13	137	21	127	18	101	47	169	48	200	8.9	129	65	137	14	129	11	121	1.8	169	11	137	22	234	39	149	21	111	2.8		
106	2.1	135	14	113	33	97	7.2	137	74	159	16	116	16	134	28	123	5.3	115	100	149	20	125	58	194	30	136	85	109	7.9		
91	100	133	25	101	45	95	17	129	100	129	13	115	15	118	49	115	100	97	11	137	15	121	28	149	20	121	18	95	13		
90	44	115	9.1	95	20	87	53	117	24	118	96	101	100	106	28	88	45	88	45	121	16	111	48	137	75	111	22	85	26		
79	30	103	39	87	89	85	35	101	28	101	100	99	25	90	86	83	79	87	17	111	100	107	58	125	40	97	59	83	14		
64	47	73	100	64	100	67	40	75	64	67	70	87	30	73	100	65	14	79	45	69	71	69	100	57	100	57	100	71	43		

Compound 8 (8A probe only). All showed M^+, although of low intensity. Base peak of *m/z* 57 in 8A and 8B is a hydrocarbon fragment. The peak at M − 80 (*m/z* 252) in 8B is the only instance of hydrogen transfer, presumably influenced by the long hydrocarbon chain, to the eliminated methanesulphonyl group. This transfer is common in sulphonates[6]. Present also is *m/z* 111. The spectrum of 8C is dominated by α-cleavage to the keto group to give a base peak at *m/z* 239 through loss of $HSCH_2$. This is the only important peak at high mass.

DISCUSSION

Gas chromatography

All compounds except 4 produced peaks on the GC. Pyrolytic loss of sulphur (during injection) appears to be preferred to SO_2 loss[7,8] and also to the loss of $[CH_3SO_2]$ as observed previously[1] in SS'-polymethylenebis (methanethiolsulphonates). Thermal loss of $[CH_3SO_2]$ is observed only in the 8A–8C transition. A later eluting minor peak in the 8 group was tentatively identified by MS as the disulphide, 8D $[-SCH_2CO-(CH_2)_{14}CH_3]_2$ presumably from oxidation and cyclisation of 8C, but was not studied further.

Loss of sulphur from 7 was also observed on direct probe analyses (see below). Thermal loss of sulphur in type IV compounds may be attributable to the keto group in close proximity to the thiolsulphonate group; no sulphur loss was observed in 1–5. Loss of sulphur from 8A was so facile that no unchanged 8A eluted from the GC. The presence of a long hydrocarbon chain may also be influencial in these decompositions, *e.g.* 8A and 8C. The loss of $[CH_3SO_2]$ from polymethylenethiolsulphonates[1] similarly increases with increasing methylene chain length $[CH_2SO_2S\cdot(CH_2)_n\cdot S\cdot SO_2CH_3$, when $n > 8]$. Pyrolytic decomposition and disproportionation of thiolsulphonates, usually resulting in loss of SO_2, is known from other chemical studies[9] and has been used in synthetic sequences.

Mass spectrometry

The spectra in general showed major fragments similar to those found previously[1,3] or formed by predictable or established pathways. Hydrocarbon ions also were prominent in the long-chain compounds.

The tendency of type IV compounds to undergo thermal loss of sulphur on GC was mirrored by their behaviour on the heated direct probe. For example, the decomposition of 7A to 7B with parallel appearance of peaks due to free sulphur $(S8)$[10] was observed.

An alternative mode of sulphur loss, that occurring as a fragmentation process, is apparently preferred over the loss of SO_2 (except in 2A) observed in sulphonates[6] and certain thiolsulphonates[7,11,12]. That this loss is sulphur (and not O_2) is shown by subsequent loss of mass 79 $[CH_3SO_2]$, other fragments and S isotope peaks. Loss of mass 79 and 111 $[CH_3SO_2S]$ was observed from the appropriate compounds. However, a peak appearing at mass 111, in 7 and 8, not normally observed in thiolsulphonates so far examined, has an identity different from $[CH_3SO_2S]$ because the methane sulphonyl group is not normally charge-retaining[4]. The peak at *m/z* 111 is also present in those compounds where one sulphur has already been eliminated and is associated with those compounds which, in addition, contain a ketone group. A probable identity is $C_6H_{11}CO$.

The presence of a carbonyl group, especially in the longer-chain compounds, also suppresses the high intensity cyclic sulphide ions, but enhances ions formed via McLafferty rearrangements, and simple hydrocarbon ions. In addition to the predicted cyclic sulphur-containing ions at *m/z* 73, 87, 101 a further group of ions, containing the keto group, was found at *m/z* 129 $[C_6H_9OS]^+$, 131 $[C_6H_{11}OS]^+$ and a series of $[CH_3SO_2(S)(CH_2)_n]^+$ or $[CH_3SO_2SCH_2(CO)(CH_2)_n]^+$ were found for structures containing the keto grouping.

Smaller peaks from rearrangements and elisions are fairly numerous. The high propensity of sulphur compounds for rearrangements is well known, and where such a rearrangement ion is intense and diagnostically useful, it has been included in Table II. Complete assignments were not attempted in this study, some were ambiguous at low resolution and would require elucidation using higher resolution or isotopes. Certain similarities were seen with simple ethane and higher alkyl thiolsulphonates[1,3]; however other previously reported peaks[3,4,11] from thiolsulphonates were not observed, possibly because of the relatively complex nature of the acylthiol moieties and a predominance of peaks from these. Mass spectra of the sulphones (6B, 7B and 8B) showed general similarities to those of the thiolsulphonates from which they were derived and to published fragmentation behaviour[13] of sulphones. The major peaks are listed in Table II.

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PAIRED-ION REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HUMAN AND RAT CALCITONIN

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SUMMARY

Improved methods for isolation, characterization, and quantitation of immunochemically heterogeneous forms of calcitonin (CT) in tissue and plasma must be developed before the biological origins and clinical importance of CT moieties can be elucidated. We are now proposing reversed-phase high-performance liquid chromatography (RP-HPLC) as one possible means of achieving high recovery, high resolution of CT moieties. In this paper we report RP-HPLC analyses of trace amounts of radiolabeled and unlabeled synthetic human and rat CT. We have systematically evaluated our application of RP-HPLC by employing several elution modes, including isocratic and gradient elution, as well as several elution reagents. We determined that high recovery and high resolution were best achieved with alkyl ion-pairing reagents, such as tetrabutylammonium phosphate, pH 7.5, or sodium sulfonyl hexane, pH 3.5. The most sensitive UV detection of trace amounts of CT was achieved with tetrabutylammonium phosphate buffer (TBAP). We recommend for RP-HPLC of CT a C_{18}-bonded silica column and elution with a 20-min linear gradient of methanol–water (20:80 to 80:20, v/v) containing 0.005 *M* TBAP. Combined with appropriate extraction procedures, such as silica adsorption or immunoadsorbant chromatography, this paired-ion RP-HPLC method can be an important aid in achieving more accurate and extensive information about CT moieties in biological samples. This method will also allow the rapid, optical detection and quantitation of CT moieties recovered from tissues, and perhaps from plasmas.

INTRODUCTION

Improved immunoassay methods have demonstrated elevations of plasma calcitonin (CT) in many physiological and pathophysiological states, notably cancer, renal failure, and hypercalcemia[1–7]. Despite some discrepancies in apparent size and number of immunochemical forms of CT detected in hypercalcitoninemic states, gel chromatography analyses do confirm the existence of multiple forms[1–6]. The supposition is that the specific forms comprising hypercalcitoninemia differ in various pathophysiological states, and that these differences among the CT moieties reflect the

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pathogenesis of these states[1,2]. Despite the potential importance of this concept, its proof remains elusive. Sensitive immunoassays in concert with new methods for extracting and concentrating CT moieties could support extensive studies of CT heterogeneity[7–9]. But the labor involved in gel chromatography analysis and the limited resolution it now offers do not make this procedure a likely choice for accurately characterizing and elucidating the complex mixture of CT moieties expected to be recovered from plasma and CT-producing tissues.

We previously reported reversed-phase high-performance liquid chromatography (RP-HPLC) on octadecylsilyl-bonded silica for purification of certain vitamin D metabolites[10,11]. Recently, RP-HPLC methods have evolved for high resolution and efficient recovery of peptides[12–14]. These methods employ a stationary phase of octadecyl-bonded silica[12–14] and a mobile phase containing hydrophobic ion-pairing reagents[12,13]. The RP-HPLC method for CT we are reporting has developed systematically from our evaluation of ion-pairing reagents, solvent-buffer composition, pH, elution mode (isocratic *vs.* gradient), and flow rate. These components were studied not only in respect to their effects on the resolution and recovery of CT, but also in relation to their effects on sensitive detection of CT by UV absorbance and by radioimmunoassay.

MATERIAL AND METHODS

Spectroanalytic grade methanol (Burdick & Jackson Lab., Muskegon, MI, U.S.A.) and distilled–deionized water were filtered through fluoropore filters with Z-μm pore size (Millipore, Bedford, MA, U.S.A.) and thoroughly degassed prior to use. The ion-pairing reagents (Water Assoc., Milford, MA, U.S.A.) were tetrabutylammonium phosphate (TBAP) and sodium sulfonyl *n*-hexane (SSH); the final concentration of the ion-pairing reagents in methanol and in water was 0.005 *M*. In addition to paired-ion chromatography buffers, we also studied the following buffer systems: 0.01 *M* ammonium acetate, pH 4.5–6.0; 0.01 *M* Tris, pH 6.5–7.5; and 0.05 *M* sodium phosphate, pH 6.0–7.5.

CT preparations, radioimmunoassay and immunoextraction

Synthetic human CT (hCT) was obtained from Ciba-Geigy (Basel, Switzerland); synthetic rat CT (rCT) was analyzed for amino acids and obtained from Drs. D. Ontjes and C. Cooper at the University of North Carolina, Chapel Hill, NC, U.S.A.[7]. The synthetic CTs were radioiodinated by chloramine-T oxidation[15]. ^{125}I-CT monomer was purified by gel chromatography (30 × 0.7 cm column of Sephadex G-50, fine) in 0.2 *M* ammonium acetate, pH 5.8, containing 0.03% Brij; preparations of purified CT monomer (K_d = 0.44) had specific radioactivities ranging between 125 and 350 μCi/μg. Purified CT was lyophilized, stored for several days, and solubilized by overnight incubation in solvent-buffers used for RP-HPLC. Radioimmunoassays and immunoextractions of HPLC fractions employed rabbit antibodies to hCT with affinities for intact hCT and rCT and for carboxyterminal fragments of CT[1,7,16].

High-performance liquid chromatography

The HPLC apparatus consisted of the following components (Waters Assoc.):

U6K injector, 2 6000A pumps; 660 programmer; and 450 variable wavelength UV detector. Full-scale UV absorbance was expressed as a.u.f.s. A Hewlett-Packard 3380A recording integrator was used to register retention times and to obtain integrated UV-absorbance peak areas. A reversed-phase μBondapak C_{18} column (10 μm) (Waters Assoc.) was used in tandem with a CO:PELL PAC guard column (Whatman, Clifton, NJ, U.S.A.).

Both isocratic and gradient elution modes were examined for their effects on resolution and quantitation of hCT and rCT. The isocratic solvent systems we considered ranged from methanol–aqueous buffer (35:65 to 100% methanol with a pH range of 3.5 to 7.5. Constant flow-rates studied were between 1 and 2 ml/min. Both linear and asymptotic gradients of methanol–aqueous buffer (20:80 to 80:20) were run over a 20-min period (after 20 min the methanol:buffer ratio was maintained at 80:20) at a selected constant flow-rate of 1–2 ml/min. The radioactive and non-radioactive CT samples were applied to RP-HPLC in 20–50-μl volumes.

Polyacrylamide gel electrophoresis (PAGE)

Samples of ^{125}I-hCT dissolved in 50 μl sodium dodecyl sulfate (SDS)–urea–mercaptoethanol were electrophoresed by previously described methods[17]. Acrylamide concentration was 10%; bisacrylamide concentration was 1%; gels contained 8 *M* urea and 0.1% SDS.

RESULTS

Fig. 1 shows the elution profile of trace amounts of hCT and ^{125}I-hCT, previously purified by gel chromatography (Sephadex G-50)[28], following their application to an isocratic RP-HPLC system of methanol–TBAP (70:30), pH 7.5. The first radioactive peak (retention time of 3 min) had the same retention time as ^{125}I, was not immunoreactive, and could be eliminated by prior treatment of samples with an anion exchange resin, AG1-X8[15]. Compared to isocratic elution with other buffer systems (0.01 *M* ammonium acetate, pH 4.5–6.0; 0.01 *M* Tris, pH 6.5–7.5; and

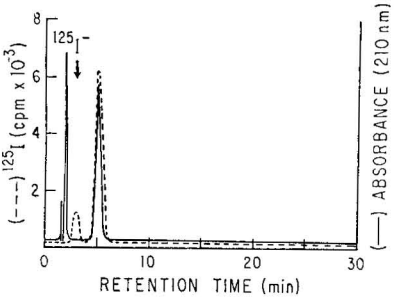

Fig. 1. Isocratic paired-ion RP-HPLC elution profiles of trace amounts of ^{125}I-hCT and hCT. Samples (10,000 cpm of ^{125}I-hCT or 100 ng of hCT) were chromatographed using a μBondapak C_{18} column in tandem with a CO:PELL PAC guard column and as eluent methanol–water (70:30) which contained 0.005 *M* TBAP, pH 7.5. Elution rate was 1.5 ml/min at 100 bar. Radioactivity (– – –) and UV absorbance at 210 nm (——) are the mean ± S.D. of 9 RP-HPLC procedures, with retention times of 3.0 ± 0.1 and 5.0 ± 0.2 min.

0.05 *M* sodium phosphate, pH 6.0–7.5), this system gives better resolution (narrower peak width and relatively longer retention time for hCT). Radioactive and non-radioactive hCT coeluted close to the solvent front with a retention time of 5.0 ± 0.2 min (mean ± S.D.). We achieved better hCT recovery (71 ± 12%, $n = 9$) with ion-pairing reagents (TBAP or SSH) than we did with other buffer systems. Systems without phosphate gave low (11.7 ± 1.6%) recovery; sodium phosphate buffer gave 44.9 ± 3.5% recovery, but the retention time was less than that obtained with TBAP or SSH (3.5 ± 0.1 min for sodium phosphate *vs.* 5.0 ± 0.2 min for TBAP or SSF).

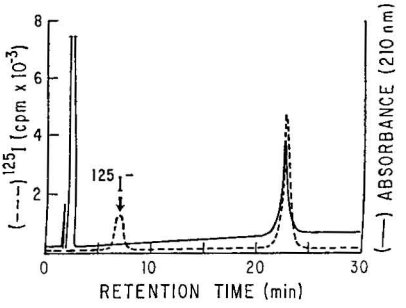

Fig. 2. Gradient paired-ion RP-HPLC elution profiles of trace amounts of ^{125}I-hCT and hCT. Samples (10,000 cpm of ^{125}I-hCT or 100 ng of hCT) were chromatographed on the columns described in Fig. 1 using a linear gradient of methanol–water (20:80 to 80:20) containing 0.005 *M* TBAP, pH 7.5, run over a 20-min period at an elution rate of 1.5 ml/min. Elution profiles were determined as in Fig. 1; results are the mean ± S.D. of 22 RP-HPLC procedures, with retention times of 6.5 ± 0.4 and 22.0 ± 0.3 min.

The elution profiles from 22 RP-HPLC analyses of hCT and ^{125}I-hCT using a 20-min linear gradient of methanol–water (20:80 to 80:20) in TBAP, pH 7.5, are summarized in Fig. 2. As in the isocratic system (Fig. 1), there was narrow peak width without double peaks or tailing[14]. With gradient elution, the retention time of hCT increased: hCT and ^{125}I-hCT eluted at 22.0 ± 0.3 min with good recovery (75.1 ± 9.9%, $n = 22$). We performed similar RP-HPLC using 0.005 *M* SSH, pH 3.5, instead of TBAP. In isocratic and in gradient elution modes the use of SSH gave hCT recovery comparable to TBAP and a retention time slightly longer, without peak widening. Acetic acid, used to adjust the pH of the SSH buffer, interfered with UV-absorbance monitoring below 230 nm[12,13,18]; we found TBAP much less UV-absorbing, even at 210 nm. A further advantage of TBAP over SSH emerged in radioimmunoassay studies. To detect possible interference of the ion-pairing reagents in CT radioimmunoassays, 10–100-μl aliquots of aqueous TBAP or SSH at various concentrations were tested for their effects on antibody–tracer binding[15,19]: 10 μl of 0.15 *M* SSH markedly decreased specific binding by 75%; 10 μl of 0.15 *M* TBAP caused 45% decrease in tracer–antibody binding. At lower concentrations (0.015 *M*), neither buffer interfered with radioimmunoassay performance. Subsequent studies were done only with TBAP.

Fig. 3 summarized 7 RP-HPLC elution profiles for purified rCT and ^{125}I-rCT applied to the same type of methanol–water gradient (0.005 *M* TBAP, pH 7.5) used to fractionate hCT (Fig. 2). The retention time (23 ± 0.2 min) for rCT was nearly identical to the retention time (22.2 ± 0.5 min) for hCT (Fig. 2).

The RP-HPLC elution profiles of purified hCT and ^{125}I-hCT after 2 months of storage in 0.01 *M* ammonium acetate buffer at −20°C are summarized in Fig. 4. Samples were lyophilized just before RP-HPLC. Radioiodide and hCT eluted with retention times similar to those noted for fresh samples (Fig. 2); recovery of radioactivity applied to HPLC was 71 ± 11%, $n = 6$. For both labeled and unlabeled hCT preparations there was good resolution of 3 additional peaks (9.0 ± 0.1, 11.0 ± 0.8, 16.3 ± 0.6 min), presumably fragments or aggregates of hCT. The radioactive peaks from RP-HPLC of fresh (Fig. 2) and stored (Fig. 4) ^{125}I-hCT were further characterized by pooling, lyophilizing, and immunoextracting specific peaks from several HPLC runs. The only immunoreactive peak was the latest eluting (22 min) and major peak, previously identified as hCT (Fig. 2). SDS–urea–polyacrylamide gel electrophoresis of the two-month-stored CT preparation showed fragments and higher molecular weight aggregates in addition to CT monomer (not shown). The resolution of these additional peaks was poor, and recovery of radioactivity was low, ranging between 24 and 40%.

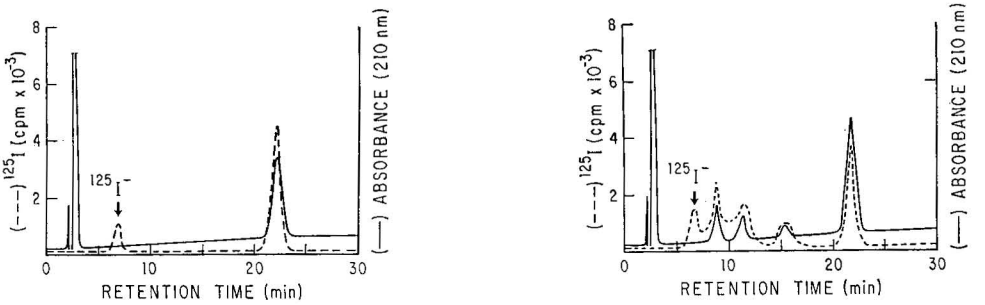

Fig. 3. Gradient paired-ion elution profiles of trace amounts of ^{125}I-rCT and rCT. Sample amounts (10,000 cpm or 100 ng), chromatography conditions, and radioactivity (- - -) or UV-absorbance (——) measurements were as described in Fig. 2. Results are the mean ± S.D. for 7 RP-HPLC procedures, with retention times of 7.0 ± 0.2 and 23.0 ± 0.2 min.

Fig. 4. Gradient paired-ion-elution profiles of trace amounts of ^{125}I-hCT and hCT stored frozen for 2 months in 0.01 *M* ammonium acetate. Samples (15,000 cpm of stored ^{125}I-hCT or 200 ng of stored hCT) were chromatographed and radioactivity (- - -) or UV absorbance (——) measured as described in Fig. 2. Results are the mean ± S.D. for 6 RP-HPLC procedures, with retention times of 6.5 ± 0.8, 9.0 ± 0.1, 11.0 ± 0.8, 16.3 ± 0.6, and 22.2 ± 0.5 min.

DISCUSSION

Gel chromatography, cation exchange chromatography and electrophoresis have been primary methods for partial separation of CT moieties[1–6,19–22]. To achieve resolution/purification of CT moieties it has been necessary to combine several of these time-consuming procedures, each associated with significant loss of immunoreactive CT moieties[20,22]. We initiated the present studies with the impression that RP-HPLC could well be an eventual means for efficient, high-resolution isolation, and perhaps even for direct quantitation, of CT moieties in biological samples. We have assessed recovery, peak resolution, and retention time of synthetic CTs (human and rat) with a variety of protein- and detergent-free solvent–buffer combinations and elution modes. CT recovery and CT resolution were decreased with standard chroma-

tography buffers: Resolution values* for the separation of ^{125}I and hCT were at best 3.7 for ammonium acetate, sodium phosphate, and other standard buffer systems examined; alkyl ion-pairing reagents, tetrabutylammonium phosphate (TBAP) or sodium sulfonyl hexane (SSH), characteristically gave resolution values of 4.0 or greater. In contrast to the poor recoveries noted with phosphate and other standard buffers (44.9 $\pm$ 3.5% or less), good recovery was achieved with alkyl ion-pairing reagents (75.1 $\pm$ 9.9%). The best resolution was obtained by gradient, as opposed to isocratic, elution mode (compare Figs. 1 and 2).

Comparisons of the two ion-pairing reagents, TBAP and SSH, revealed that each has certain advantages and limitations. SSH, pH 3.5, provided slightly longer retention times and better resolution. The improved resolutions with SSH could be due to alkyl-ion pairing with protonated amino groups of CT combined with partial ionic suppression of the free carboxyl ($pK = 3.65$) group of the aspartic acid residue[12,23,24]. It is very likely that lowering the pH, perhaps to 2, which decreases ionization of aspartate and further reduces hydrophilic interactions, would lengthen retention times, as a result of relatively increased hydrophobicity. Both effects, ion pairing- and ionic suppression, reduced hydrophilic interactions and made more dominant hydrophobic interactions between the CT–alkyl–ion complexes in the mobile phase and the stationary organic phase (octadecyl groups bonded to silica). At pH 7.5 there can be no ionic suppression of CT's major protonated sites[23], such as the epsilon-amino group in lysine and the alpha-amino group in the amino-terminal cysteine residue[23,24]. Both amino groups have pK values close to 10.5[23]. Still, TBAP at pH 7.5, nearly the upper pH limit for column-packing stability, gave nearly as good a resolution as did SSH at pH 3.5. This suggests that the increased retention time of CT during RP-HPLC with alkyl ion-pairing reagents is due mainly to the increased hydrophobicity of alkyl–ion–CT complexes and not to ionic suppression[12,13].

In comparison to SSH, TBAP is relatively UV-transparent at low wavelengths. This fact has permitted UV monitoring of peptide (amide) bonds at 210 nm, a wavelength close to the peak molar absorbance of peptide bonds[18]. Based on the height and resolution of UV-absorbance peaks with 100 ng of CT, there should be no problem detecting as little as 20 ng of CT by UV absorbance[18]. Appropriate derivatization of CT moieties could further decrease the amount of peptide needed for optical detection or make possible detection by sensitive fluorimetry[25]. This would make feasible rapid, direct detection of several ng of CT. Derivatization of free amino groups[25] should not affect immunoreactivity, which resides in the carboxyl-terminal region of CT[6,7,19,26]; this will allow immunological identification of trace amounts of peptides purified and quantitated by RP-HPLC of biological samples[18,27,29].

Further studies will be needed to assess paired-ion RP-HPLC for specific isolation and for quantitation of the multiple immunochemical forms of CT in tissue and plasma[1–6,18,27,28]. Based on our present results, such investigation should include the use of a microanalytical, octadecylsilyl-bonded silica column and methanol–water gradient elution employing TBAP, or some other UV-transparent quaternary ammonium cation, as a hydrophobic counter-ion. We are currently working out methods of

* Resolution values are derived from the equation $R_s = 2(V_r - V_r)/(W_1 + W_2)$ where V_r is the peak retention time and W is the peak width in min.

sample preparation and of peptide derivatization that will enable us to use this RP-HPLC method to study specific CT moieties in biological samples[7–9,25].

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GEL CHROMATOGRAPHIC COMPARISON OF THE MOLECULAR WEIGHT DISTRIBUTIONS OF AMPHOLINE, SERVALYTE AND PHARMALYTE CARRIER AMPHOLYTES USED IN ISOELECTRIC FOCUSING

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SUMMARY

The molecular weight distributions of Ampholines, Servalytes and Pharmalytes have been studied by gel chromatography on Bio-Gel P-4. The results corroborate our previous conclusions with respect to the dependence of the molecular weight on the isoelectric point and on the carrier ampholyte system. Evidence for association of carrier ampholyte molecules was also obtained. The unexpected highly heterogeneous molecular weight distribution of Ampholines permits a better understanding of their composition.

INTRODUCTION

In a recent paper[1] we presented a comparison of the buffer capacities and specific conductivities of focused Ampholines, Servalytes and Pharmalytes. It was shown that the ratio of these two properties is related to the molecular weight of the carrier ampholytes. We found that (i) for the three systems studied the molecular weight generally increases with decreasing isoelectric point and (ii) the mean molecular weight increases in the order Servalytes < Ampholines < Pharmalytes.

In this paper we present results of a comparative gel chromatographic study, undertaken with the aim of verifying the former results independently. For this study a polyacrylamide gel (Bio-Gel P-4) was used, which appeared to have a higher resolution for the carrier ampholytes than dextran gels (Sephadex G-15 and G-25).

In determinations of molecular weight by gel chromatography it is essential to use as calibration standards compounds that are structurally related to the investigated substances. Firstly, if the separation mechanism is genuine gel permeation, elution volumes are governed by molecular size, which, in turn, is related to molecular weight only for a series of structurally related compounds. Secondly, if in addition to gel permeation other mechanisms are operative (*e.g.*, ion exchange, adsorption) the use of such a series is the only way of correcting for the effect of these mechanisms.

In the gel chromatographic molecular weight determinations of carrier ampholytes published thus far[2,3], polyethylene glycol standards were used for calibration.

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However, aliphatic compounds carrying the same functional groups should be used. For Ampholines polyaminopolycarboxylic acids (of the EDTA type) are the most appropriate (some amino acids, considered as the lowest molecular weight analogue in this series, can also be used). For Servalytes analogous compounds, with sulphonic and phosphonic acid groups, are suitable. For Pharmalytes polyamines carrying hydroxyl and amino acid groups would be ideal standard compounds.

Unfortunately, very few and rather low-molecular-weight compounds of these types are available. As peptides are the only more or less related series of compounds of which members with molecular weights up to a few thousand are available, we used them also for comparison. Further, the behaviour of the aminocarboxylic acids was compared with that of some low-molecular-weight aliphatic carboxylic acids and miscellaneous compounds.

EXPERIMENTAL

Bio-Gel P-4, fine grade (Bio-Rad Labs., Richmond, CA, U.S.A.), Sephadex G-25, fine grade, and Sephadex G-15 (Pharmacia, Uppsala, Sweden) were swollen in aqueous 0.2 *M* sodium chloride.

Columns (K16/70, Pharmacia) were filled with these gels by the slurry technique and packed to a bed height of 64.5 cm (bed volume 129 ml) at an elution rate of about 15 $ml \cdot h^{-1}$. The columns were equipped with a sample valve (LV4, Pharmacia), a flow adaptor (A16, Pharmacia), a peristaltic pump (Minipuls 2, Gilson, Villiers-le-Bel, France) and a UV monitor (Uvicord-S, LKB, Stockholm, Sweden).

As a routine, 15-μl aliquots of commercially supplied Ampholines (LKB), Servalytes (Serva, Heidelberg, G.F.R.) and Pharmalytes (Pharmacia), diluted with aqueous 0.2 *M* sodium chloride to 1 ml, were applied to the columns and eluted with aqueous 0.2 *M* sodium chloride at a flow-rate of about 15 $ml \cdot h^{-1}$. The flow-rate was determined gravimetrically. The UV absorbance was measured at 206 nm.

The following compounds were used for calibration or comparison:

Amino acids: glycine (Merck, Darmstadt, G.F.R.), proline (Serva, Heidelberg, G.F.R.), arginine (Hoffmann-La Roche, Basel, Switzerland).

Peptides: GlyGly (Merck), GlyGlyGly, AlaPhe, GlyGlyLeu, LeuLeuLeu, GlyPhePhe, PhePhePhe (all from Sigma, St. Louis, MO, U.S.A.), ProGlyLysAlaArg, ValValValValValVal (both from Serva), PhePhePhePhePhe (Sigma), thymus hormone, bradykinin potentiator C, fibrinopeptide A, α-endorphin, apamin, insulin A-chain (all from Serva).

Proteins: cytochrome *C*, bovine serum albumin (both from Sigma).

EDTA analogues: ethylenediaminediacetic acid (Aldrich Europe, Beerse, Belgium), nitrilotriacetic acid (Merck), ethylenediaminetetraacetic acid (Serva), ethylene glycol bis-(2-aminoethyl) tetraacetic acid (Fluka, Buchs, Switzerland), diethylenetriaminetetraacetic acid (Aldrich Europe).

Aliphatic acids: formic acid (Baker, Phillipsburg, NJ, U.S.A.), acetic acid, oxalic acid, succinic acid, tartaric acid, citric acid (all from Merck).

Miscellaneous: sodium azide, acetone, ethylenediamine (all from Merck), N,N-bis-(2-hydroxyethyl)glycine, N-α-(acetamido)-2-aminoethanesulphonic acid, N-2′-hydroxyethylpiperazine-2-ethanesulphonic acid (all from Serva).

Aliquots of 2–3 mg of these compounds, dissolved in 1 ml of aqueous 0.2 *M*

sodium chloride, were applied to the column and monitored as described above. Ethylenediamine was labelled with the ^{14}C compound (Radiochemical Centre, Amersham, Great Britain) to a specific activity of 16 $nCi \cdot \mu mol^{-1}$. In that case 0.7-ml fractions of the eluate were collected and assayed for radioactivity by liquid scintillation counting.

RESULTS

In Fig. 1 the chromatograms of Ampholine, pH 6–8, on Sephadex G-15, Sephadex G-25 and Bio-Gel P-4 are given. As these results demonstrated that Bio-Gel P-4 gives the best separation of the Ampholine constituents, all further work was performed with this gel (see Discussion).

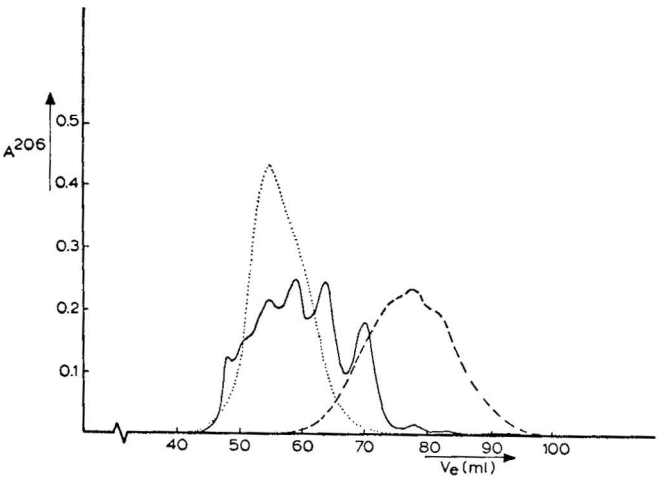

Fig. 1. Chromatograms of Ampholine, pH 6–8, on Sephadex G-15 (. . . . . .), Sephadex G-25 (---------) and Bio-Gel P-4 (———). Bed height: 64.5 cm. Sample: 15 μl.

In Fig. 2 the elution volume of the standard compounds is plotted against the logarithm of their molecular weights.

Fig. 3 shows the influence of the sample concentration for Ampholine, pH 3.5–10.

In Fig. 4 the chromatograms for the neutral pH range of Pharmalyte at two concentrations and of Pharmalyte, pH 3–10, are shown.

In Fig. 5 the elution pattern of a new lot (see Discussion) of Pharmalyte, pH 5–8, at two concentrations is given.

In Figs. 6, 7 and 8 the elution patterns of an acidic, a neutral and a basic pH range of Ampholine, Servalyte and Pharmalyte, respectively, are given.

In Figs. 9 and 10 the elution patterns of the basic Ampholine ranges, pH 9–11 and pH 8–9.5 and those of the acidic Ampholine intervals, pH 2.5–4 and pH 4–6, respectively, are compared.

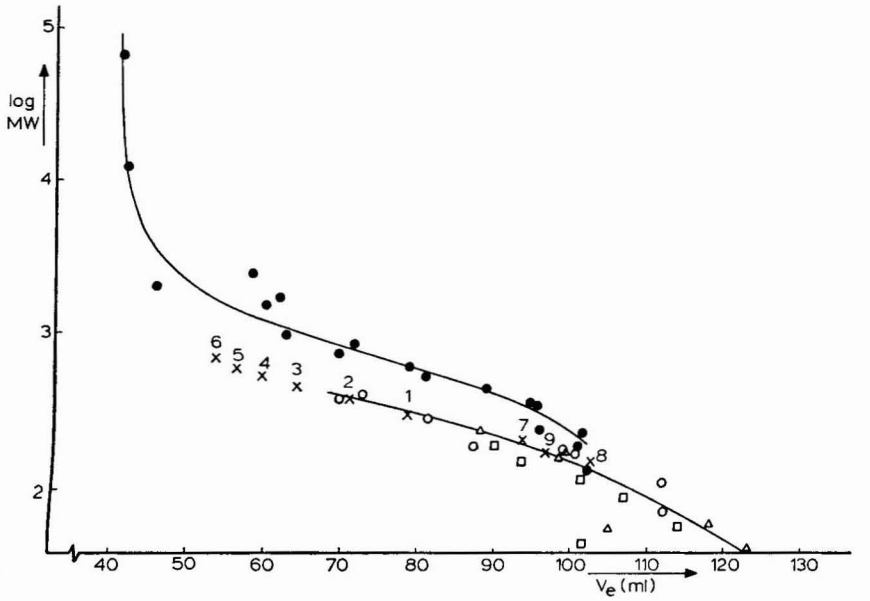

Fig. 2. Relationship between the logarithm of the molecular weights of standard compounds and their elution volumes, V_e, on Bio-Gel P-4. The standard compounds (for each category listed in order of increasing molecular weight) were as follows: *peptides and proteins* (●): GlyGly, GlyGlyGly, AlaPhe, GlyGlyLeu, LeuLeuLeu, GlyPhePhe, PhePhePhe, ProGlyLysAlaArg, ValValValValValVal, PhePhePhe PhePhe, thymus hormone, bradykinin potentiator C, fibrinopeptide A, α-endorphon, apamin, insulin A-chain, cytochrome C, bovine serum albumin; *EDTA analogues and amino acids* (○): glycine, proline, arginine, ethylenediaminediacetic acid, nitrilotriaceticacid, ethylenediaminetetraacetic acid, ethylene glycol bis-(2-aminoethyl)tetraacetic acid, diethylenetriamine pentaacetic acid; *aliphatic acids* (□): formic acid, acetic acid, oxalic acid, succinic acid, tartaric acid, citric acid; *miscellaneous* (△): sodium azide, acetone, ethylenediamine, N,N-bis-(2 hydroxyethyl)glycine, N-α-(acetamido)-2-aminoethane sulphonic acid, N-2′-hydroxyethylpiperazine-2-ethanesulphonic acid. The numbered points (×) refer to Ampholine constituents (see Discussion).

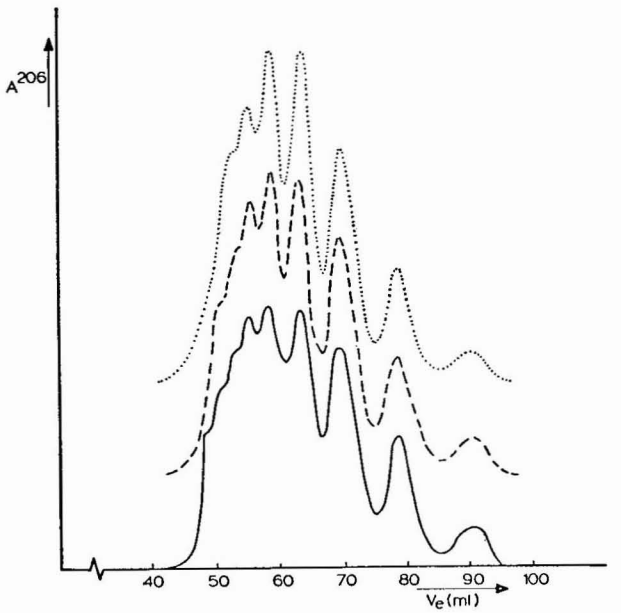

Fig. 3. Chromatograms of Ampholine, pH 3.5–10, at different concentrations, on Bio-Gel P-4. Samples: 15 μl (. . . . . .), 50 μl (---------) and 100 μl (———).

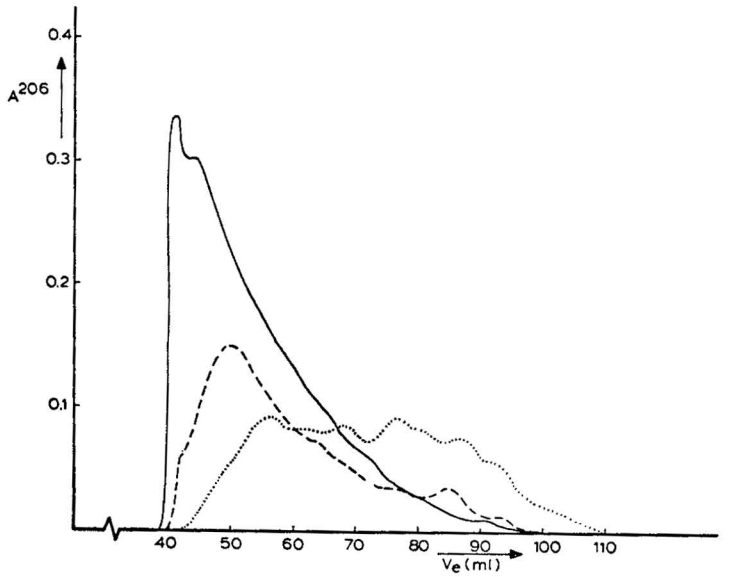

Fig. 4. Chromatograms of Pharmalytes on Bio-Gel P-4. Samples: 15 μl of Pharmalyte, pH 5–8 (———), 5 μl of Pharmalyte, pH 5–8 (------), and 10 μl of Pharmalyte, pH 3–10 (. . . . .).

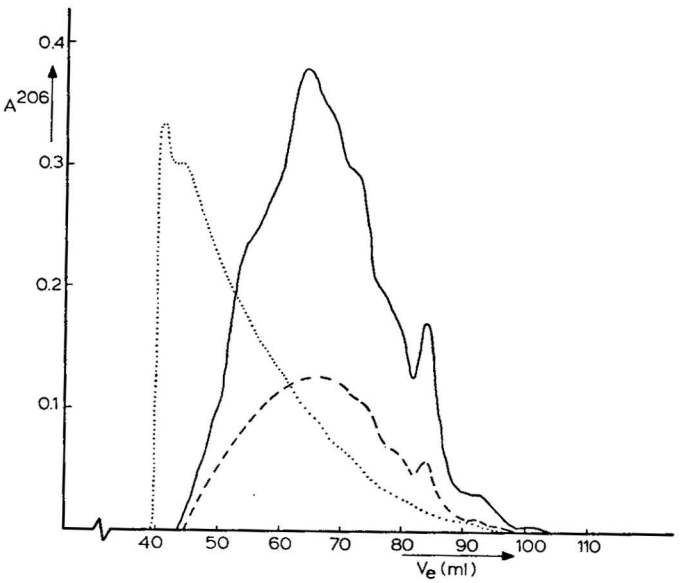

Fig. 5. Chromatograms of a new lot of Pharmalyte, pH 5–8 ,at different concentrations, on Bio-Gel P-4. Samples: 15 μl of Pharmalyte, pH 5–8 (———), and 5 μl of Pharmalyte, pH 5–8 (---------). For comparison, the chromatogram of an earlier lot of Pharmalyte, pH 5–8, from Fig. 4, is also given (. . . . .).

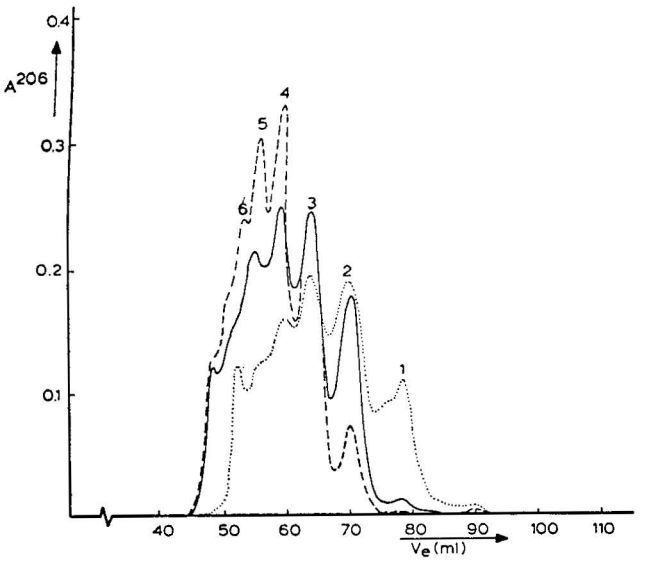

Fig. 6. Chromatograms of Ampholines on Bio-Gel P-4. Samples: 15 μl of Ampholine, pH 4–6 (--------), 15 μl of Ampholine, pH 6–8 (———), and 15 μl of Ampholine, pH 8–9.5 (. . . . .).

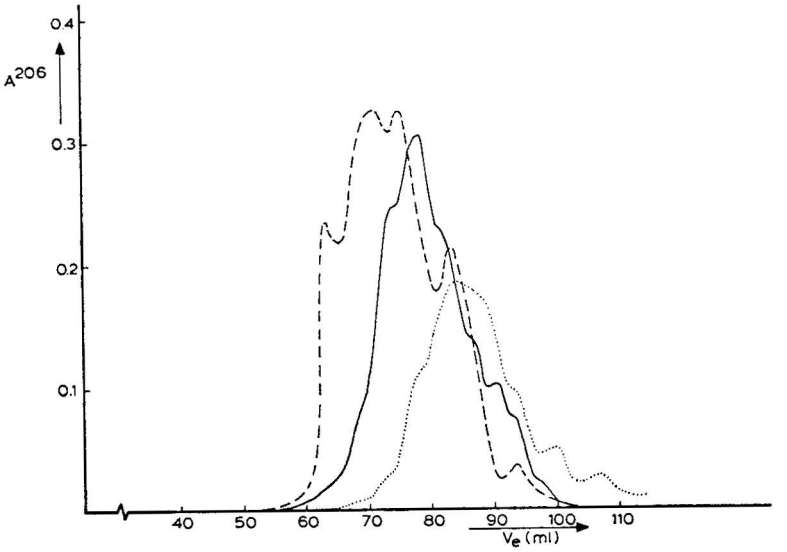

Fig. 7. Chromatograms of Servalytes on Bio-Gel P-4. Samples: 15 μl of Servalyte, pH 4–6 (---------), 15 μl of Servalyte, pH 6–8 (———), and 15 μl of Servalyte, pH 8–10 (. . . . .).

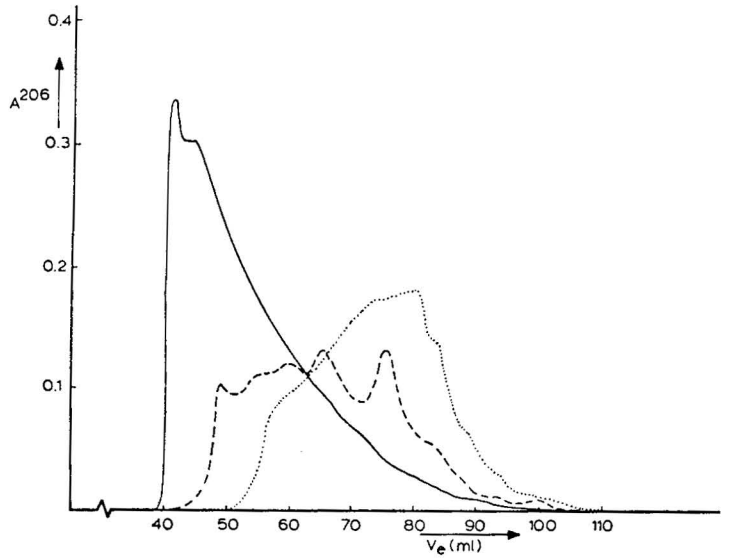

Fig. 8. Chromatograms of Pharmalytes on Bio-Gel P-4. Samples: 15 μl of Pharmalyte, pH 2.5–5 (---------), 15 μl of Pharmalyte, pH 5–8 (———), and 15 μl of Pharmalyte, pH 8–9.5 (. . . . .).

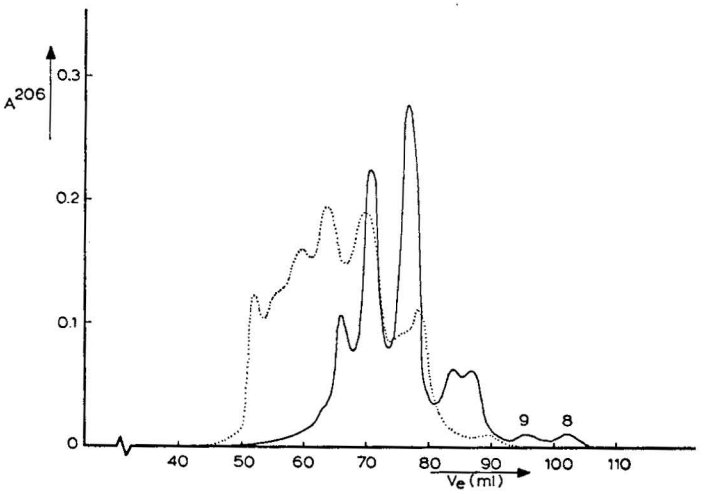

Fig. 9. Chromatograms of basic Ampholines on Bio-Gel P-4. Samples: 15 μl of Ampholine, pH 8–9.5 (. . . . .), and 15 μl of Ampholine, pH 9–11 (———).

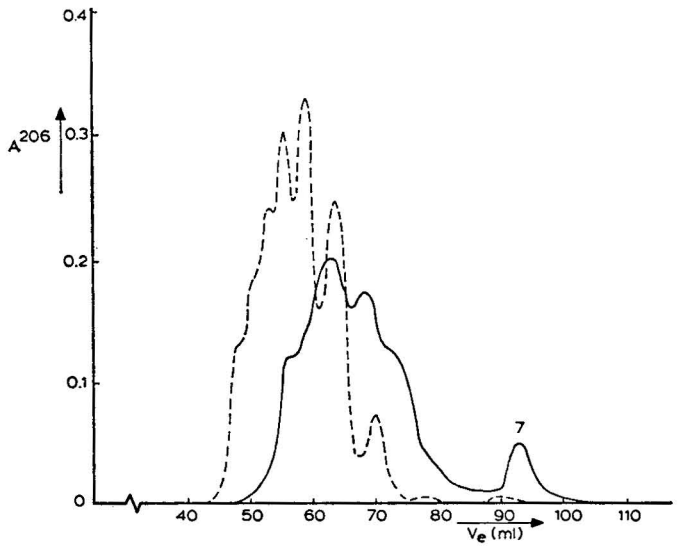

Fig. 10. Chromatograms of acidic Ampholines on Bio-Gel P-4. Samples: 15 μl of Ampholine, pH 4–6 (---------), and 15 μl of Ampholine, pH 2.5–4 (———).

DISCUSSION

Comparison of the gels

It can be seen in Fig. 1 that the resolution of Ampholine constituents by the gels increases in the order Sephadex G-15 < Sephadex G-25 < Bio-Gel P-4. For Sephadex G-25 and Bio-Gel P-4 this is partly due to a higher selectivity, *i.e.*, a smaller slope ($|d(\log M)/d(V_e)|$, where M = molecular weight and V_e = elution volume) of the calibration line, but mostly to a higher efficiency, *i.e.*, a smaller theoretical plate height with Bio-Gel P-4. The latter, determined from the elution peak of sodium azide (elution volume 123.0 ml), was 0.14 mm. In spite of several trials, we did not succeed in packing a Sephadex G-25 column with a better theoretical plate height than about 1 mm for the sodium azide elution peak*. A relatively smooth elution pattern on Sephadex G-25 was also obtained by other investigators[2,4] for ^{14}C-labelled Ampholine, pH 3.5–10. From the results in Fig. 1 we decided to perform all further work on Bio-Gel P-4.

Standardization of the gel

Fig. 2 clearly shows that peptides are retarded on Bio-Gel P-4 relative to com-

* The superior efficiency of Bio-Gel P-4 is due to its smaller maximum bead diameter and its narrower bead diameter distribution. For the fine grade of Bio-Gel P-4 used a hydrated bead diameter range of 37–75 μm is given by the manufacturer. The grades of Sephadex G-15 and G-25 used have, according to the manufacturer, dry bead diameter ranges of 40–120 and 20–80 μm, respectively. On the basis of the water regain[5] on swelling (1.5 and 2.5 ml·g^{-1}, respectively), the density[5] of the swollen gel (1.19 and 1.13 g·ml^{-1}, respectively) and a supposed density of the dry gels equal to that of sucrose (1.6 g·ml^{-1}), this corresponds to hydrated bead diameter ranges of 64–192 and 34–136 μm, respectively.

pounds of the same molecular weight without a peptide bond. The content of phenylalanine residues appears not to be a significant factor in the retention of the peptides. We ascribe this specific retardation to interaction of the peptide bond with the carbamyl groups of the polyacrylamide, presumably by hydrogen bonding.

With the exception of the points for acetone and formic acid, those of all the other standard compounds fall approximately on the same smooth line. However, this line does not represent the true gel permeation behaviour. As the bed volume of our column is 129 ml and the specific packed bed volume of Bio-Gel P-4 is about 6 ml per gram of xerogel[5], the total volume of stationary and mobile phase must be about $5/6 \times 129 = 107.5$ ml, if the gel matrix is assumed to have a density of 1 $ml \cdot g^{-1}$. This volume is very close to the elution volume of acetone (105 ml), which therefore can be assumed to exhibit no interaction with the gel. This means, however, that all the other low-molecular-weight standard compounds (with the exception of formic acid) are retarded by some kind of interaction with the gel.

This agrees with the work of Streuli[6] on Bio-Gel P-2 and P-6 in 0.01 *M* sodium chloride solution. In that study acetone (and tetrahydrofuran, having the same elution volume) is also assumed to indicate the total volume. On this basis, Streuli found $K > 1$ on Bio-Gel P-2 for all of the carboxylic acids used in our study and also for citric acid on Bio-Gel P-6. Whereas such a strong retardation is not manifest in our work, as a result of the higher ionic strength used, some interaction must be assumed to explain our results.

The fact that peptides and EDTA analogues exhibit a significantly different behaviour on this gel precludes the determination of the molecular weight of material eluting in peaks in the elution patterns of Pharmalytes, which are known to contain at least one peptide bond[7]. Moreover, a general consequence of practical importance is that the separation of peptides and carrier ampholytes subsequent to isoelectric focusing is, at least on this gel at 0.2 *M* ionic strength, more difficult than could be supposed from the difference in molecular weight. On the other hand, the determination of the molecular weight of material eluting in peaks in the elution patterns of Ampholines (and Servalytes), using EDTA analogues and amino acids as standards, is possible. However, it is hampered by the considerable scatter of points around the calibration line (Fig. 2), and by the lack of EDTA analogues with molecular weights exceeding 400.

Association of ampholytes

In Fig. 3 the influence of the Ampholine concentration is shown. It can be seen that with increasing concentration definite shoulders gradually appear in the elution pattern at elution volumes of 51 and 48 ml. We ascribe this effect to increasing association of Ampholine constituents with increasing concentration. The Ampholine sample concentrations in these experiments were 0.6, 2 and 4% (w/v). In the chromatograms of the acidic and neutral Ampholine pH ranges (Fig. 6), where the Ampholine sample concentration was 0.6% (w/v), shoulders and even a small peak appear at the same elution volumes (51 and 48 ml). These can also be ascribed to Ampholine associates as the relative concentration of a particular Ampholine species is almost three times higher in these narrow pH ranges than in the broad pH range used in Fig. 3. As the usual ampholyte concentration in isoelectric focusing is, on average, 2% (w/v), it is possible that association of Ampholines is manifest in isoelectric focusing, as postulated by

Gianazza *et al.*[8]. However, it should be realized that the conditions in this gel chromatographic work and in isoelectric focusing are different: the ionic strength in our work is 0.2 *M* whereas in isoelectric focusing it is exceedingly small[9]; on the other hand, the high electric field strength prevailing in isoelectric focusing is absent, of course, in gel chromatography.

Neutral Pharmalytes also show considerable material eluting at small elution volumes, as can be seen from the chromatograms for the pH range 5–8 at concentrations of about 0.2 and 0.6% (w/v) in Fig. 4. Whereas this could be partly due to association also [in the chromatogram for the broad pH range 3–10 (Fig. 4), where the relative concentration of all species is small, no material is found at small elution volumes], Pharmacia pointed out to us[10] that Pharmalyte of pH 5–8 contains many ampholyte species of high molecular weight. As the company was changing the production procedure for this pH range material at the time this paper was being prepared and intends to sell the new product by the time it is published, we also chromatographed an aliquot of a new lot of Pharmalyte, pH 5–8. In Fig. 5 the elution pattern of this new lot, at concentrations of about 0.2 and 0.6% (w/v), is shown. As can be seen, the content of high-molecular-weight material is significantly reduced in this new product.

Relationship between molecular weight and isolectric point

The results in Figs. 6–8 substantiate one of our previous conclusions[1], *viz.*, that the molecular weight of carrier ampholytes generally increases with decreasing isoelectric point. This effect is most convincingly demonstrated for the Servalytes (Fig. 7), but also for the Ampholines (Fig. 6) there is a gradual shift towards smaller elution volumes with decreasing isoelectric point. The neutral Pharmalytes (Fig. 8) present an exception to this rule as their main fraction has a much smaller retention volume than the acidic and basic Pharmalytes. As argued in the preceding section, this could be partly due to association of neutral Pharmalytes.

Fig. 9 contains, for two basic Ampholine pH ranges, another confirmation of the general rule. In Fig. 10 the acidic Ampholine ranges, pH 4–6 and pH 2.5–4, are compared. It can be seen that, contrary to the general rule, the most acidic interval contains relatively more low-molecular-weight material. This confirms our earlier finding (*cf.*, Fig. 3 in ref. 1).

Comparison of the molecular weight distribution of Ampholines, Servalytes and Pharmalytes

Figs. 6–8 substantiate our earlier findings (*cf.*, Fig. 3 in ref. 1). The high molecular-weight boundaries of the molecular weight distributions increase in the order Servalytes < Ampholines ≈ Pharmalytes for the acidic range, Servalytes < Ampholines < Pharmalytes for the neutral range and Servalytes < Pharmalytes < Ampholines for the basic range.

It is impossible to calculate mean molecular weights from the chromatograms. The ordinate in Figs. 3–10, absorbance at 206 nm, is proportional to the concentration of functional groups absorbing at 206 nm, *i.e.*, $\diagdown\!\!\diagup C{=}O$ for Ampholines and Pharmalytes and $\diagdown\!\!\diagup C{=}O$, $\diagdown\!\!\diagup P{=}O$ and $\diagdown\!\!\diagup S(=O)(=O)$ for Servalytes. Hence the sensitivity

of the measurement of the concentration of ampholyte species increases with increasing content of these functional groups, *i.e.*, in general, with decreasing elution volume. With Servalytes, however, nothing is known about the relative amounts of the absorbing groups in the ampholyte species. For the Pharmalytes we know neither the number nor the identity of the amino acids introduced. Even with Ampholines there remains some ambiguity as to the carboxylic acid content of ampholytes eluting in a particular peak (see the following section).

Assignment of the Ampholine peaks

The elution patterns of Ampholines (Figs. 3 and 6) show such well defined peaks that their identification can be attempted.

Of course, such an attempt is rendered difficult by the scattering of points around the calibration line (Fig. 2) and by the fact that we have no EDTA-like calibration compounds with molecular weights above 400, whereas most of the Ampholine peaks are eluted at smaller elution volumes than the highest EDTA analogue used. Therefore, the assignment proposed in this section must be regarded as highly speculative.

In a previous paper[1], the shift towards higher molecular weight with decreasing isoelectric point was explained as the result of a gradually increasing degree of substitution of amine hydrogens in a given polyamine with carboxylic acid residues. Hence, if the peaks in Fig. 6 are due to ampholyte molecules and if these molecules are separated on Bio-Gel P-4 according to their molecular weights, one might expect a shift of their elution volumes towards smaller values with decreasing isoelectric point. The peculiarity in Fig. 6 is, however, that almost all of the peaks appear at invariable elution volumes in the chromatograms for the acidic, neutral and basic pH range materials.

Therefore, we firstly ascertained that the peaks are due to genuine ampholyte species: although improbable in view of the high relative amount of material eluting in the peaks, they might be due to some contaminating non-ionizable compound, which, evidently, would be present in the three pH ranges and would elute at invariable elution volumes. For this purpose we chromatographed Ampholine, pH 6–8, on the cation exchanger SP-Sephadex at pH 4.9 and Ampholine, pH 4–6, on the anion exchanger DEAE-Sephadex at pH 8.2. Only on SP-Sephadex some UV-absorbing material eluted from the column in the break through volume, which upon re-chromatography on Bio-Gel P-4, however, did not reveal any peaks. In contrast, material eluting from the ion-exchange columns by the application of a salt gradient (0–1 *M* sodium chloride) did show the characteristic peaks upon re-chromatography on Bio-Gel P-4.

Secondly, we considered the possibility that the peaks in Fig. 6 are due to some specific interaction of Ampholines with the polyacrylamide gel, depending on the number of nitrogen atoms in the Ampholine constituents*, rather than on the

* Ampholines are prepared[11] by the reaction of acrylic acid with a mixture of polyamines, the exact composition of which is unknown. The relevant patent[12] mentions polyamines with more than four nitrogen atoms; in a theoretical calculation of the maximum number of different ampholyte species obtainable in this synthesis, Vesterberg[13] assumes the presence of polyamines having between two and nine nitrogen atoms; in the more recent literature[14,15] pentaethylenehexamine (PEHA) is assumed to be one of the principal constituents of the mixture.

molecular weight. In that case, a particular peak would represent ampholytes derived from a particular polyamine, independent of the degree of substitution with propionic acid residues, *i.e.*, independent of the isoelectric point. In fact, Streuli[6] demonstrated that the amides acetamide, urea and biuret are adsorbed on Bio-Gel P-2 to an extent increasing with the number of nitrogen atoms when 0.01 *M* sodium chloride solution is used as the eluent.

Such a specific interaction with Bio-Gel P-4 was ruled out in our work, however. Firstly, the elution volumes of the calibration compounds are correlated with their molecular weights and not with the number of nitrogen atoms. Secondly, we re-chromatographed material eluting from Bio-Gel P-4 in the peaks marked 3, 4 and 5 in Fig. 6 on Sephadex G-25 and found that the elution order remained unchanged.

Thus, it must be concluded that the peaks in Fig. 6 are due to ampholyte molecules which are separated by molecular sieving. Assuming that the pricipal constituents of Ampholines are derived from PEHA by the introduction of propionic acid (PA) residues*, we assign the peaks n in Fig. 6 to PEHA·nPA ($1 \leqslant n \leqslant 6$). In Fig. 2 the molecular weights of these compounds are also plotted as a function of the elution volumes; it strongly suggests the correctness of this peak assignment.

However, an alternative assignment should be considered. In Fig. 11 the

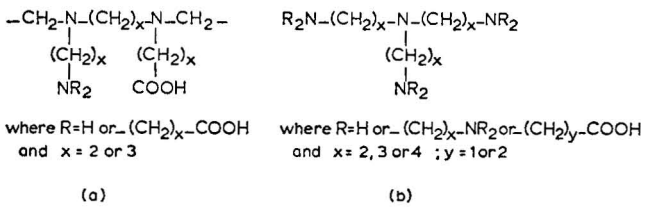

Fig. 11. General formula of Ampholines, according to the manufacturer[11] (a) and to Vesterberg[17] (b). The uncertainty with regard to the length of the carboxyl-connecting alkyl chain has been discussed by Righetti *et al.*[16]. As $y \neq 2$ is in contradiction with the stated[11] use of acrylic acid in the synthesis, we assume with these authors that only propionic acid residues ($y = 2$) are present in the normal-range Ampholines (p*I* 3.5–10).

general formula of Ampholines, as given by the manufacturer[11], is depicted. This formula allows the presence of polypropylenepolyamine, *e.g.*, pentapropylenehexamine-(PPHA) containing constituents, if $x = 3$ for *all* the nitrogen-connecting alkyl chains. Similarly, according to the general formula given by Vesterberg[17] (see also Fig. 11), even ampholytes derived from polybutylenepolyamines, *e.g.*, pentabutylenehexamine (PBHA), could possibly be present. The molecular weights of PEHA·nPA and PPHA·(n − 1)PA and of PPHA·(n − 1)PA and PBHA·(n − 2)PA differ by only 2, corresponding to a difference in elution volumes of about 0.2 ml. Such a small difference could easily have remained undetected in our experiments. In fact, as PEHA·nPA, PPHA·(n − 1)PA and PBHA·(n − 2)PA are expected to have widely different p*I* values, this would provide an explanation for the presence of peaks (with $n \geqslant 3$) at virtually the same elution volume in the elution patterns for different pH ranges. In spite of this, we do not believe in the presence of appreciable amounts of

* See footnote on p. 311.

these compounds. Firstly, we presume that the statement $x = 2$, 3 or 4 in conjunction with the above-mentioned formula is meant to cover the presence of ampholytes in which some, but not all, x values are different from 2. Secondly, if substantial amounts of ampholytes with $x = 3$ or 4 throughout were present, one would expect Ampholines to show adsorption on hydrophobic materials. We did not find, however, any adsorption in hydrophobic interaction chromatography on octyl-Sepharose under conditions (1 *M* sodium chloride) where the tripeptides GlyGlyVal, GlyGlyLeu and LeuLeuLeu, containing isopropyl and isobutyl chains, are significantly retarded[18].

Therefore, another explanation must be found for the coincidence of most elution peaks in the chromatograms for different pH ranges. Inasmuch as relatively small peaks or shoulders are concerned (*e.g.*, peaks 1 for the neutral and acidic Ampholines and shoulder 5 for the basic Ampholines) they could be due to PEHA ampholytes with an isoelectric point outside the stated pH range. As Ampholines are fractionated, subsequent to their synthesis, by isoelectric focusing at high concentration, some species could remain in the "wrong" pH range as a result of association with the main constituents. Upon dilution, as in our experiments, such complexes dissociate and the constituents would appear individually in the elution pattern. This explanation cannot apply, of course, to the prominent peaks in Fig. 6, as substantial amounts of such "wrong" ampholytes would adversely affect the pH gradient obtained in isoelectric focusing.

We think that for these prominent peaks the explanation is provided by the presence of ampholytes derived from the different PEHA isomers. It has been shown by Vesterberg[19] that upon the substitution of amine hydrogens of a polyamine with alkyl carboxylic acid groups the pK values of the amine groups are virtually unchanged. Thus, the pK values of the amine groups in the original polyamine can be used in a rough estimation of the pI values of ampholytes derived therefrom provided that the degree of substitution is low enough that the pK value of the carboxylic acid plays no role in determining the isoelectric point. In Table I the four steric isomers of PEHA are schematically depicted; also indicated are the number of primary, secondary and tertiary amine groups.

It can be seen that with increasing branching the number of primary amine

TABLE I

THE FOUR STERIC ISOMERS OF PENTAETHYLENEHEXAMINE (PEHA)

PEHA isomer	*Number of amine groups*		
	Primary	*Secondary*	*Tertiary*
α: o—o—o—o—o—o	2	4	0
β: o—o—o—o—o | o	3	2	1
γ: o—o—o—o—o | o	3	2	1
σ: o—o—o—o | | o o	4	0	2

groups (having relatively high pK values) increases, whereas the number of secondary amine groups (with relatively low pK values) sharply decreases. Hence, the introduction of, say, two PA groups into α-PEHA results in an ampholyte, the pI of which is determined by the pK values of one primary and one secondary amine group, whereas the same substitution into β-, γ- and δ-PEHA gives ampholytes with higher pI values, as they are governed by the pK values of two primary amine groups. Analogously, upon the introduction of three PA groups into PEHA, ampholytes will result with pI values, governed by two secondary (α-PEHA), one secondary and one primary (β- and δ-PEHA) and two primary (δ-PEHA) amine groups and therefore the pI value of PEHA·3PA isomers increases in the order $\alpha < \beta, \gamma < \delta$. Unfortunately, the magnitude of the pI difference between isomeric PEHA ampholytes cannot be given with certainty, as only the pK values of δ-PEHA are known[20]. We may assume, however, that the pK values of α-PEHA are equal to those of the linear tetraethylenepentamine isomer[20]. In Table II these values are given, together with the calculated pI values.

On the basis of these data, α-PEHA·3PA is expected to be present in Ampholine, pH 6–8, and δ-PEHA·3PA in Ampholine, pH 8–9.5, while both PEHA·3PA ampholytes of which, of course, many isomers exist, depending on the position of the PA residues, would appear at the same elution volume (*viz.*, that of peak 3 in Fig. 6). According to the above reasoning, peak 2 of the acidic Ampholine pH range cannot be PEHA·2PA. Nor can this peak be due to an ampholyted erived from a cyclic polyamine with only one primary amine group (Bergstedt and Widmark[21] demonstrated the presence of such piperazine derivatives in commercial PEHA). One would then expect a slight displacement (of about 2 ml) of the corresponding elution peaks, as these piperazine derivatives are not isomeric with PEHA, but differ by 26 molecular weight units from it. Such a displacement has not been observed, however. [Retardation on the gel of such piperazine derivatives relative to non-heterocyclic compounds, so as to give identical elution volumes, is improbable, as the piperazine derivative HEPES behaves as expected on the basis of its molecular weight (see Fig. 2)].

The well defined peaks in the elution patterns in Figs. 3 and 6 indicate that some constituents (*viz.*, if the above assignment is correct, PEHA isomers) of the polyamine mixture used in the synthesis of Ampholines either are predominant or react more efficiently with acrylic acid. This means that less different ampholyte species are present in normal-range Ampholines (pH 3.5–10) than could be assumed hitherto from the given general formulae (see Fig. 11)*.

In relation to the discussion of Vesterberg[17] on the extension of the pH range of Ampholines at both ends of the pH scale, *i.e.*, below pH 3.5 and above pH 10, it is of interest to reconsider the elution patterns in Figs. 9 and 10. As already argued previously[1], these comments can be taken as indicative of the hypothesis that the most acidic Ampholines are produced in different runs, *i.e.*, that they do not belong to the PEHA·xPA family. The same argument holds for the most basic Ampholines.

* The chromatograms of Servalytes (Fig. 7) show less distinct peaks than those of Ampholines. Thus, Servalytes have in general a more homogeneous molecular weight distribution. This is expected as Servalytes are prepared by introducing, in addition to carboxylic acid, also phosphonic acid and sulphonic acid groups into polyamines. Pharmalytes (Fig. 8) apparently have the most homogeneous molecular weight distribution of the three systems compared, which reflects the use of many different compounds in the synthesis.

TABLE II

DISSOCIATION CONSTANTS OF TWO PEHA ISOMERS AND ISOELECTRIC POINTS OF AMPHOLYTES DERIVED THEREFROM

PEHA isomer	*Temperature (°C)*	*Dissociation constant of amine groups*				*Isoelectric point of ampholyte with n propionic acid groups*		
		pK_1	pK_2	pK_3	pK_4	$n=1$	$n=2$	$n=3$
α	25	9.9	9.1	7.9	4.3	9.5	8.5	6.1
σ	20	10.2	9.7	9.1	8.6	10.0	9.4	8.9

Figs. 9 and 10 confirm the correctness of this hypothesis. In fact, almost all of the peaks appearing in the chromatograms of the most extreme pH ranges in these figures have elution volumes different from those of the PEHA·xPA system, present in the patterns of Ampholines with pI values between 3.5 and 10 (Figs. 3 and 6). Hence, these "new" peaks represent "new" ampholytes, not belonging to the "normal" family. Some of these "new" peaks can be identified. Thus, the prominent peak marked 7 in Fig. 10 may be assigned to methylaminopropanetricarboxylic acid (molecular weight 205). The eight steric isomers of this ampholyte, together with the seventeen isomeric amino-*n*-butanetricarboxylic acids and the eight aminoisobutanetricarboxylic acids, all obey the condition[17] that the carboxylic acid groups are close together, thereby providing the ampholytes with a low isoelectric point. The two peaks marked 8 and 9 in Fig. 9 are probably due to diaminopentanecarboxylic acid (molecular weight 146) and diaminoheptanecarboxylic acid (molecular weight 174), respectively. Many of the isomers of these ampholytes obey the condition[17] that the primary amine groups are far apart, thereby providing the ampholytes with a high isoelectric point. On the basis of these assignments, the points for peaks 7, 8 and 9 are tentatively included in Fig. 2.

CONCLUSIONS

(1) The resolution of Ampholine constituents by Bio-Gel P-4 is higher than that by the Sephadex G-15 and G-25 gels.

(2) The constituents of acidic and neutral Ampholine pH ranges associate at concentrations of about 2% (w/v). The neutral Pharmalyte pH range contains high-molecular-weight associates at even smaller concentrations.

(3) The molecular weight of carrier ampholytes generally increases with decreasing isoelectric point and, at constant isoelectric point, in the order Servalytes < Ampholines < Pharmalytes.

(4) The homogeneity of the molecular weight distribution increases in the order Ampholines < Servalytes < Pharmalytes. Presumably this is also the order of increasing number of different ampholyte species per pH unit.

(5) Normal-range Ampholines (3.5 < pI < 10) contain a large proportion of ampholytes derived from only a few polyamines, probably the isomers of pentaethylenehexamine.

(6) Extremely acidic and basic Ampholines (3.5 > pI > 10) contain large amounts of ampholytes derived from polyamines different from those of which the normal-range Ampholines are derived.

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EXAMINATION OF THE CONVERSION PRODUCTS OF PYRETHRINS AND ALLETHRIN FORMULATIONS EXPOSED TO SUNLIGHT BY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY*

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SUMMARY

Natural and synthetic pyrethrins in kerosene solution similar to commercial formulations were stored in clear soft-glass bottles and exposed to sunlight through clear window glass. Transition photoproducts were observed by gas chromatography (GC) for cinerin I and II, and jasmolin I and II, which revealed slightly shorter retention times than the pyrethrins. No photoproducts for pyrethrin I and II were observed, which may have polymerized or been converted into products of higher polarity. Critical studies on the photoproduct from jasmolin I indicated the isomerization of (*Z*)-pent-2-enyl side-chain of the rethrolone moiety to the (*E*)-isomer. Other circumstantial evidence indicated that a similar change occurred with jasmolin II and cinerin I and II. Allethrin, under similar conditions, had a photoproduct with a longer retention time, which was identified as an isomer with a cyclopropyl side-chain, the *d,l*-cyclopropylrethronyl *d-trans*-crysanthemate.

INTRODUCTION

The presence of "false" pyrethrins in pyrethrin extracts was reported by Brown and co-workers[1–4]. Brown *et al.*[4], utilizing the modified sulfur color test[5], examined the heated extracts and identified two isomers, isopyrethrin I and isopyrethrin II. The conversion products were noted when pyrethrin extracts were examined by gas chromatography with flame-ionization detection by Head *et al.*[6]; however, the products were not identified. The acid moiety of the photodegradation products of pyrethrins from the exposure of extracts to UV light was identified by thin-layer chromatography (TLC)[7]. Abe *et al.*[8] observed an unknown component of a pyrethrin film, irradiated with a 500-W incandescent lamp, by gas–liquid chromatography.

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Bullivant and Pattenden[9] applied direct photolysis to the major constituents of pyrethrins by exposing the material to a 100-W medium-pressure mercury-arc quartz-filtered light and monitored the reaction by gas chromatography (GC). Elliott[10] noted that new isomers were formed when *cis*-pyrethrolone and related compounds were heated, and he confirmed the tentative findings of Brown *et al.*[4] that isopyrethrin I and isopyrethrin II were found in heat-treated pyrethrin extracts. Recently, the formation of isopyrethrin I and isopyrethrin II were confirmed by gas chromatography–mass spectrometry (GC–MS) of the pyrethrin extracts in heat-treated sealed tubes[11].

In this study, extracts of pyrethrins and allethrin exposed to sunlight through clear window glass were examined by GC and GC–MS. Supporting data was obtained by GC, NMR and solid probe mass spectrometric analyses of isolated photoproducts of jasmolin I and allethrin which had been exposed to a 100-W mercury lamp.

EXPERIMENTAL

Chemicals

Refined natural pyrethrin extracts from Kenya, Tanzania and Ecuador were supplied by Dr. Dean Kassera of McLaughlin Gormley King Co., U.S.A.; the sample of Fumakilla Brand pyrethrin extract was received from T. Takano, Japan Pyrethrin Institute, Kyoto, Japan; and the World Standard pyrethrin extracts were supplied by V. M. Shah, Pyrethrum Marketing Board, Nakura, Kenya.

Two grams of each refined extract (20% pyrethrins) were weighed into a 50-ml volumetric flask and diluted to 50 ml with kerosene. Equal parts of the solution were transferred into an 8-oz Owens clear glass bottle and a 50-ml brown bottle.

Jasmolin I was isolated from a pyrethrum extract (66.5%, Dainippon Jotyugiku, Osaka, Japan).

Allethrin, 0.1 g (purity 90%, supplied by Sumitomo Chemical, Osaka, Japan), was weighed into a 50-ml volumetric flask and diluted to 50 ml with kerosene. The solution was divided in the same manner as described above.

Bioallethrin, *d-trans*-chrysanthemate of *d,l*-allethrolone (>93%), was supplied by Maruwaka Kagaku, Osaka, Japan.

Chromatography and analytical procedure

TLC of the mercury lamp-exposed jasmolin and allethrin was performed on 0.25-mm pre-coated silica gel $60F_{254}$ glass plates (Merck, Darmstadt, G.F.R.). The plates were developed with *n*-hexane–ethyl acetate (4:1). Detection was accomplished by spraying the plates with saturated ethanolic molybdophosphoric acid solution and heating at 110°C for 3–4 min. Dark blue spots were observed under 253.6-nm UV light.

Pure jasmolin I was isolated by column chromatography through two columns. The first column, 50 cm × 43 mm I.D., was slurry packed with 36.5 cm of 100–200-mesh silica gel (Kanto Chemical, Japan) in *n*-hexane. The Dainippon extract (6 g) was then added and eluted with 800 ml of 95:5 and 1200 ml of 90:10 *n*-hexane–ethyl acetate. Fractions of 7.8 g were collected and monitored by TLC for jasmolin I. Approximately 430 mg of crude jasmolin I were collected in fractions 22–28. The crude jasmolin I was purified on a second column, 100 cm × 20 mm I.D.,

prepared in the same manner as the first column except that 61.5 cm of silica gel was added. The same solvent mixtures were used with reduced volumes of 300 and 400 ml, respectively. The purified jasmolin I (100 mg) was collected in fractions 12–15. Isolation of the unknown allethrin photoproduct from 1 g of the Maruwaka allethrin formulation exposed to the mercury lamp was accomplished through a single column chromatographic step. The same column chromatographic procedure as used for the initial isolation of the crude jasmolin I isolated 290 mg of pure allethrin photoproduct in fractions 13–17.

Infrared (IR) spectra of carbon disulfide solutions of allethrin and its photoproduct were determined with a Shimadzu 27S grating infrared spectrophotometer.

Proton nuclear magnetic resonance (PMR) spectra of jasmolin I (J_I), allethrin and their corresponding photoproducts were obtained in deuterochloroform solution on a JEOL JNM-PMX60 NMR spectrometer.

Electron-impact mass spectra (MS) were obtained with a JEOL JMS-D400 mass spectrometer. The operating conditions were as follows: accelerating voltage, 3 kV; scan speed, 10 sec; multiplier, 1.3 kV; ionizing voltage, 75 eV; ionizing current, 300 μA; solid probe sample temperature settings, for J_I 70°C and for the photoproduct of allethrin 45°C.

The Hitachi 063 gas chromatograph was equipped with a 1 m × 3 mm I.D. stainless-steel column packed with 10% SE-30 on Chromosorb W (100–120 mesh), with an oven temperature of 210°C. The flow-rate of the carrier gas (nitrogen) was 40 ml/min.

The Shimadzu GC-6A gas chromatograph was equipped with a 2 m × 3 mm I.D. glass column packed with 10% SE-30 on Chromosorb W (100–120 mesh), with an oven temperature of 245°C.

The F & M Model 810 gas chromatograph was equipped with a flame-ionization detector (FID). A 60 cm × 3.2 mm I.D. glass column was packed with 2.5% XE-60 on Chromosorb W, acid washed, DMCS treated (60–100 mesh) [a mixture of equal parts of 3% XE-60 on Chromosorb W (80–100 mesh) and 2% XE-60 on Chromosorb W (60–80 mesh)] (see details in ref. 12). The operating conditions were as follows: injection port temperature, 190°C; column temperature, matrix temperature programming from 150 to 205°C; detector temperature, 225°C. The matrix temperature programming for the pyrethrin study was as follows. The initial temperature was set at 150°C and held there for 5 min starting from the initial rise of the solvent peak. The temperature was then programmed at 20°C/min to 175°C and held there until 2.25 min past the apex of the Py_1 peak. The oven temperature was then further increased at 20°C/min to 205°C and held there until the elution of the Py_2 group.

A matrix temperature program was used for the allethrin study. The initial temperature was set at 150°C and held there for 5 min after the initial rise of the solvent peak. The oven temperature was programmed at 20°C/min to 170°C and held there until the elution of allethrin and its photoproduct.

A Finnigan Model 3000 mass spectrometer was used with a sensitivity setting of 10^{-6} A/V, electron multiplier high voltage −2.00 kV and electron energy 69.5 V. The mass spectrum was taken at the apex of each peak. A glass column, 45 cm × 2 mm I.D., was packed with 2.5% XE-60 on Chromosorb W (60–100 mesh). The helium flow-rate was 20 ml/min and the system was matrix programmed as described above for the F & M 810.

Exposure to sunlight under mild conditions

The control solutions in the 50-ml brown bottles were stored in the dark. The solutions in the clear glass bottles were placed on a platform 3 in. away from a clear glass window for the exposure study. The entire bottle was exposed to sunlight for about 3 h each morning through a clear glass window facing the east side of the building. During the remainder of each day, no direct sunlight struck the bottles. The samples were exposed to sunlight for 121 days (period between June and September). The temperature of the liquid in the bottles ranged from 30 to 58°C on exposure to direct sunlight and decreased to 23 to 27°C during the shaded period of the day.

Photochemical reaction

The irradiations were performed with a 100-W high-pressure mercury vapor lamp made by Riko Kagaku Sanyo (Chiba, Japan). Before irradiation, the reaction solutions were purged with dry nitrogen for 1 h. The photochemical reactions were monitored by removing samples at intervals followed by GC analysis with an FID. Photolysis was continued until photoequilibrium was established. After the removal of the solvent by distillation *in vacuo*, the photoproducts were isolated and purified by chromatography.

RESULTS AND DISCUSSION

Six insecticidally active esters were identified in pyrethrin extracts: cinerin I (C_I), jasmolin I (J_I), pyrethrin I (Py_I), cinerin II (C_{II}), jasmolin II (J_{II}) and pyrethrin II (Py_{II}). The first three esters are grouped together as Py_1; the other three are grouped together as Py_2 (see Fig. 1).

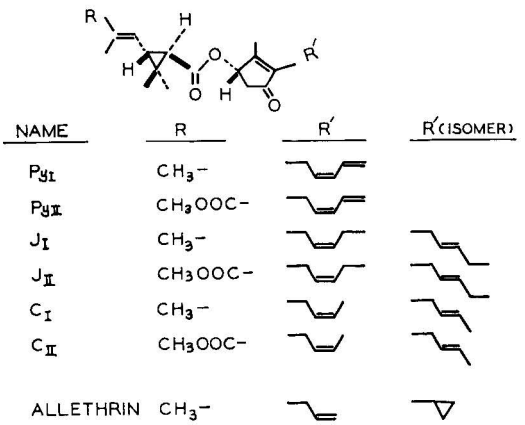

Fig. 1. Structures of the six insecticidally active esters of pyrethrins, the synthetic pyrethroid allethrin and their sunlight products (J_I', J_{II}', C_I', C_{II}' and allethrin).

The chromatogram of the unexposed extract from Kenya (Fig. 2A) revealed the six major constituents C_I, J_I, Py_I, C_{II}, J_{II} and Py_{II}. The chromatogram of the extract from Kenya exposed to sunlight (Fig. 2) revealed decreasing peak areas of C_I, J_I, Py_I, C_{II}, J_{II} and Py_{II}. Simultaneously, unknown peaks labeled C_I', J_I', C_{II}'

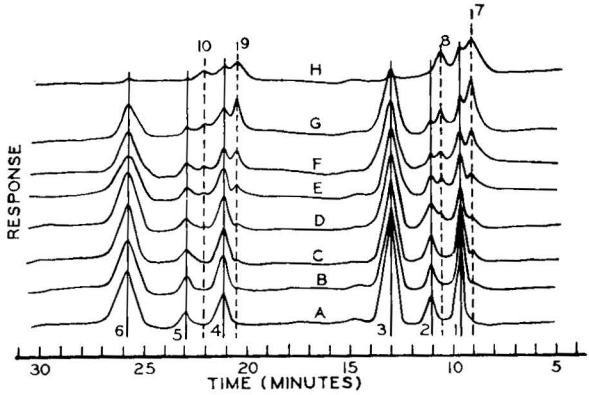

Fig. 2. Comparison of gas chromatograms of a pyrethrin formulation made with an extract from Kenya exposed to sunlight 0 (A), 8 (B), 29 (C), 39 (D), 52 (E), 56 (F), 63 (G) and 121 (H) days. Peaks: 1 = C_I; 2 = J_I; 3 = Py_I; 4 = C_{II}; 5 = J_{II}; 6 = Py_{II}; 7 = unknown C_I'; 8 = unknown J_I'; 9 = unknown C_{II}'; 10 = unknown J_{II}'.

and J_{II}' increased in area with increasing exposure to sunlight. Similar chromatograms were observed in sunlight-exposed extracts of Ecuador (Fig. 3), Fumakilla (Fig. 4) and Tanzania (Fig. 5).

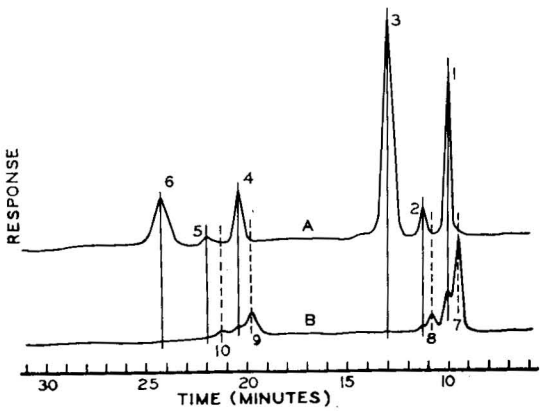

Fig. 3. Comparison of gas chromatograms of a pyrethrin formulation made with an Ecuador extract exposed to sunlight for 0 (A) and 100 days (B). Peaks as in Fig. 2.

Apparently, the sum of the areas of the reduced C_I, C_{II}, J_I and J_{II} and their conversion products are approximately equal to that of the original pyrethrin esters. When the sunlight exposure time was increased, the sum of the area calculation did not hold as it did initially. The area calculation was attempted on chromatograms of Tanzania extract used for the sunlight exposure study (see Fig. 6). Peak areas of C_I, C_I', C_{II} and C_{II}' were measured using a planimeter. The areas of J_I, J_I', J_{II} and J_{II}' were not taken as the resolutions of the peaks were poor.

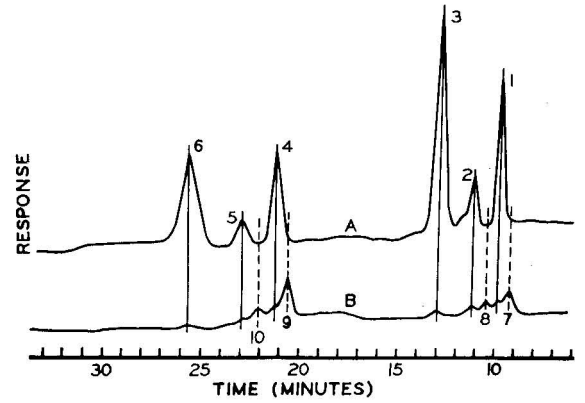

Fig. 4. Comparison of gas chromatograms of a pyrethrin formulation made with a Fumakilla Brand extract from Japan exposed to sunlight for 0 (A) and 100 days (B). Peaks as in Fig. 2.

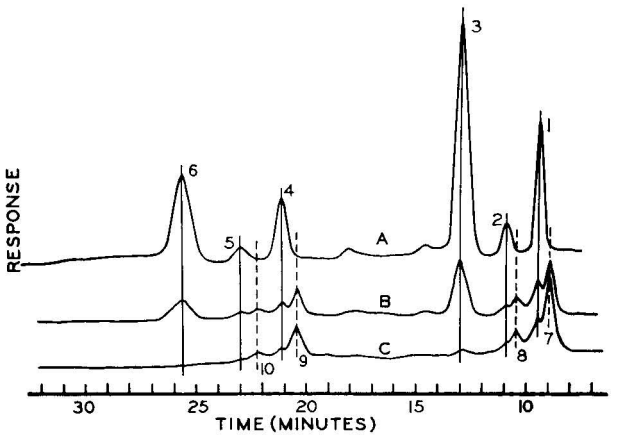

Fig. 5. Comparison of gas chromatograms of a pyrethrin formulation made with an extract from Tanzania exposed to sunlight for 0 (A), 28 (B) and 45 (C) days. Peaks as in Fig. 2.

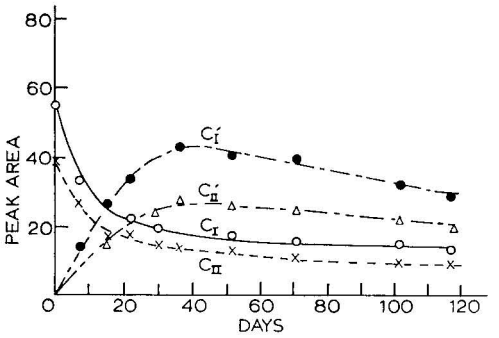

Fig. 6. Changes in concentration of C_I, C_I', C_{II} and C_{II}' with increasing sunlight exposure in a Tanzanian extract formulation. Arbitrary area units are used to express concentration.

The unknown peak areas reached their maximum between 35 and 50 days and thereafter decreased, and it may be assumed that sunlight products C_I' and C_{II}' were further converted into other products not detected by the GC parameters.

Miskus and Andrews[13] reported that window glass (usually soft glass) transmitted radiation above 300 nm, whereas borosilicate glass (Pyrex) was effective at 290 nm. They noted that pyrethrins were destroyed within the UV range 290–320 nm. The more effective range of radiation, 290–300 nm, was excluded by soft glass; thus any changes in the pyrethrin or allethrin properties would be expected to be milder. If the unknown compounds C_I' C_{II}', J_I' and J_{II}' were oxidation products, they would be polar in nature and would not be chromatographed at the indicated retention time positions.

The gas chromatogram of the pyrethrin extract from Tanzania (Fig. 5A) showed peaks 1–6, identified as C_I, J_I, Py_I, C_{II}, J_{II} and Py_{II}, respectively. When exposed to sunlight, the extract showed a different chromatogram (Fig. 5B and C). GC–MS revealed spectra (see Table I) very similar to those of compounds C_I, C_{II}, J_I and J_{II} for peaks corresponding to compounds C_I', C_{II}', J_I' and J_{II}'. Molecular ions were observed for C_I', J_I', C_{II}' but not for J_{II}'. The molecular ion for J_{II}' was not observed owing to its relatively lower concentration. With the GC–MS system used, it was very difficult to observe the molecular ions of the Py_2 group because of their low intensities of less than 0.1 % relative. However, Py_I and Py_{II} were not observed; the former almost disappeared and the latter completely disappeared from the chromatogram after exposure for 45 days. The major peaks 7 and 9 had shorter retention times relative to C_I and C_{II}, respectively.

The GC–MS spectra indicate that the molecular weights of unknown compounds C_I', J_I', C_{II}' and J_{II}' were identical with those of the starting materials. Similarly, the acid moiety and ester bond of the unknown compounds showed no change compared with those of the starting materials. Therefore, it is presumed that the unknown compounds were the isomers of the starting material which was modified in the alcohol moiety. However, Py_I and Py_{II} photoproducts were not chromatographed. They may be converted into substances of higher polarity or polymerized and not detected by GC.

For example, 150 mg of jasmolin I in 200 ml of *n*-hexane were irradiated with a high-pressure mercury vapor lamp for 1 h to obtain a 2:1 mixture of the photoproduct and the starting material. The GC elution order of jasmolin I and its mercury lamp-induced photoproduct coincided with its sunlight-exposure product. Isolation of the photoproduct was accomplished by collection of the product as it eluted from the Hitachi 063 gas chromatograph. The IR and mass spectra of the photoproduct almost agreed with those of jasmolin I (See Table I, J'*). However, the PMR spectrum of the photoproduct differed slightly from that of jasmolin I in several shift positions. The PMR spectrum of the photoproduct in $C^2H_2Cl_3$ indicated $\delta = 0.94$ ppm (3H, t, $J = 7.3$ Hz, CH_2CH_3), $\delta = 1.14$ ppm (3H, s, CH_3), $\delta = 1.26$ ppm (3H, s, CH_3), $\delta = 1.41$ ppm (1H, d, $J = 5.4$ Hz, CHCO), $\delta = 1.72$ ppm (6H, s, $(CH_3)_2C=$), $\delta = 2.03$ ppm (3H, s, $CH_3C=$), $\delta = ca.$ 2.0 ppm (obscured, 2H, CH_2, CH_3), $\delta = ca.$ 2.0 ppm (obscured, 1H, $(CH_3)_2C=CH-CH$), $\delta = ca.$ 2.2 ppm (obscured, 1H, dd, CHHCO), $\delta = 2.89$ ppm (1H, dd, $J = 6.0$ and 18.4 Hz, CHHCO), $\delta = 2.91$ ppm (1H, d, $J = 4.3$ Hz, $=C-CH_2-C=$), $\delta = 4.90$ ppm (1H, dm, $J = ca.$ 8 Hz, $Me_2C=CH$), $\delta = 5.26$–5.54 ppm (2H, CH = CH), $\delta = 5.66$ ppm (1H, dm, CH–O).

TABLE I

INTENSITIES OF FRAGMENT IONS IN THE MASS SPECTRA OF A SUNLIGHT-EXPOSED FORMULATION MADE WITH TANZANIA EXTRACTS

Values are expressed as a percentage of the base peak.

m/e	C_I'	C_I	J_I'	$J_I'^*$	J_I	C_{II}'	C_{II}	J_{II}'	J_{II}
360						0.07	0.08		
344								0.5	0.3
343								0.5	0.7
331			0.2	1.7	0.1	0.1			
330			0.4	3.6	0.2	0.2	0.1		
329						0.4	0.4		
318	0.04								
317	0.1	0.1							
316	0.35	0.33							
212						1.2	1.3	1.6	1.4
211						1.4	1.2	1.7	1.3
169	0.6	0.8	0.5		0.4	0.7	0.8	3.2	1.2
168	2.8	2.9	3.1	5.3	2.9	3.3	3.4	5.0	4.7
167	0.8	0.8	1.0		0.6	25.0	25.5	27.8	29.6
166								2.3	1.3
165			1.9	2.9	2.1			3.6	2.7
164			15.7	19.8	15.4			9.6	14.6
163			8.9	9.6	3.0	1.2		27.3	34.3
162			4.5	5.3	2.5	1.5		5.9	8.2
161			4.1			1.1		2.3	2.6
160								0.4	0.3
153	2.5	2.2	3.4	4.6	2.4	1.6	1.4	4.5	1.9
152	0.4	0.3					0.8	1.8	0.9
151	2.9	3.6				2.6	2.3	3.6	1.4
150	21.4	19.2				17.2	15.2		
149	10.5	5.8		9.6		53.8	28.8		
148	5.5	4.2				12.6	6.9	6.4	1.4
147	1.2	0.7				2.5	1.5	4.1	3.7
146								1.4	1.5
145								4.1	5.9
136				4.6					
135	1.5	1.0	12.0	17.8	9.8	8.8	7.4	34.0	40.1
134			1.6	3.1	1.0		2.8	5.5	4.4
133	5.2	4.9	11.0	4.8	6.0	13.7	8.2	13.7	14.6
132			0.5					2.3	1.1
131			1.1			4.4	2.3	4.5	1.6
125	3.0	2.4	4.6		2.6	3.0	4.7	7.3	6.1
124	10.3	10.0	10.8	11.1	10.9	1.0		2.7	1.6
123	100.0	100.0	100.0	100.0	100.0	4.2	3.4	18.3	6.1
122	3.9	3.9	3.6	3.1	2.3	8.0	6.2	6.4	6.1
121	25.7	23.5	22.3	12.0	13.4	72.0	57.6	56.9	41.8
120							1.4	4.1	3.9
119		1.8		2.4		4.6	3.4	6.9	5.7
109	4.7	2.8	9.4	7.0	7.7	6.0	6.1	9.1	12.6
108	7.0	7.5	6.4	4.6	5.9	13.7	12.5	12.3	15.0
107	15.2	16.6	16.4	10.8	14.3	100.0	100.0	100.0	100.0
106	2.5	2.3	1.8		1.5	4.7	3.9	6.8	3.6
105	11.3	11.0	8.9	6.7	7.0	22.0	17.0	22.8	17.5

TABLE I *(continued)*

m/e	C_I'	C_I	J_I'	J_I'*	J_I	C_{II}'	C_{II}	J_{II}'	J_{II}
104					0.7			3.6	1.3
103	1.9	2.2			1.5		2.7	5.0	3.0
97	1.6	0.8		2.4	2.7			4.5	3.6
96	1.8	0.8		2.2	2.4			3.6	2.4
95	8.7	14.2	8.9	5.8	7.4		3.2	6.8	6.0
94	4.0	3.6	3.6	3.9	3.9		6.4	4.7	7.4
93	36.6	37.5	30.8	17.8	28.8	81.0	67.8	70.5	68.9
92	3.4	3.4	4.1	3.9	3.1	7.2	6.0	9.3	6.9
91	12.4	23.7	23.1	14.2	19.8	50.0	57.3	45.5	47.1
85	2.0	1.1	11.3	2.4	2.9		1.0	15.6	2.2
84	0.6	1.3	4.8	2.4	1.2		0.2	7.3	1.2
83	4.4	4.1	18.9	4.1	6.0	5.0	4.1	14.0	8.2
82	1.3	3.9	5.1	2.4	4.2	2.0	1.8	7.0	3.5
81	40.6	42.0	41.2	19.3	41.4	8.5	6.7	16.2	16.5
80	4.0	4.1	4.8	3.1	5.1	5.6	4.6	11.6	8.2
79	24.0	25.4	26.0	11.1	24.0	40.3	33.4	49.0	42.2
78	4.4	4.8	4.8	2.9	4.1	8.4	5.9	7.0	6.4
77	21.0	22.4	22.0	11.3	17.4	36.6	30.2	37.2	32.4
73	1.8	1.1	38.4		8.1			3.7	2.2
72			1.5		0.6			3.3	0.5
71	2.2	2.8	16.6	2.4	5.4				
70		1.1	4.9	2.4	2.3				
69	16.8	13.3	30.2	9.6	19.4	18.5	8.9	28.0	21.4
68	1.8	1.9	2.9	2.4	2.9	1.5	1.5	1.4	4.0
67	14.8	16.0	21.6	8.7	16.7	13.5	10.9	21.0	19.4
66	2.7	3.2		2.4	3.0	4.7	4.1	3.7	5.5
65	7.6	8.8	8.5	5.1	7.8	13.5	11.4	16.3	14.8
59					1.7	14.8	11.9	16.3	15.5
57	9.3	13.8	44.3	7.2	17.2	1.2	0.5	37.2	23.3
56	2.1	2.5	9.0	4.3	4.2	2.3	1.6	4.7	4.4
55	30.0	38.0	64.0	17.3	47.9	43.0	33.6	60.5	79.6
54				2.4					
53	5.5	17.0	14.8	7.0	13.7	18.5	15.3	25.6	19.1
52		2.9		2.4	1.9	2.5	2.8		
51	3.6	5.1		3.6	3.3	6.5	4.6		
45				2.4					
44				2.7					
43	44.5	63.5	87.0	16.6	58.5	34.6	29.9	91.0	70.9
42				2.2					
41	37.0	44.0	75.0	22.9	54.3	32.0	27.8	70.0	65.0
40				2.4					
39	14.0	22.0	18.0	6.0	13.4	19.0	16.5	28.0	21.4
29	18.0	24.8	39.4		18.3	31.0	27.9	46.5	26.2
27	8.9	17.4	13.1		11.0	12.4	9.5	18.6	15.0

* Photoproduct of jasmolin I exposed to a 100-W mercury lamp. Electron impact spectra were obtained from a solid probe analysis of the isolated photoproduct using a JOEL JMS-D100 mass spectrometer.

That is, the shift positions of methyl, 1-methylene and 4-methylene protons on the pent-2-enyl group in the photoproduct were downfield 0.05–0.08 ppm from those of the corresponding protons in jasmolin I. Pent-2-enyl group CH=CH protons

in the photoproduct also absorbed from $\delta = 5.26$ to 5.54 ppm; these protons in jasmolin I absorbed from $\delta = 5.08$ to 5.53 ppm.

The photoproduct was the (*E*)-isomer of jasmolin I. Bullivant and Pattenden[14] also reported the formation of an (*E*)-olefin of jasmolin I on irradiation with a high-pressure mercury vapor lamp.

A similar type of sunlight exposure study was conducted with the synthetic pyrethroid allethrin. Sunlight-exposed and unexposed samples were examined by GC. The chromatograms are shown in Fig. 7. The retention time of the unknown peak in exposed allethrin was longer than that of the starting material, in contrast to the conversion products C_I', J_I', C_{II}' and J_{II}'.

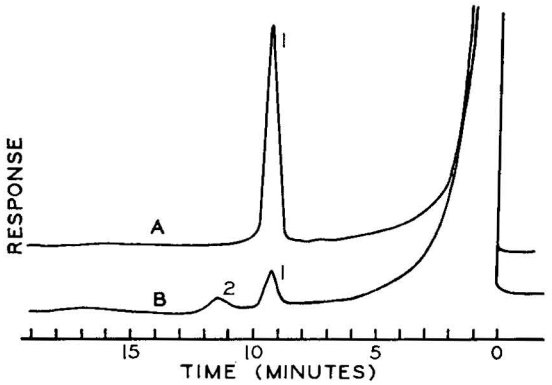

Fig. 7. Comparison of gas chromatograms of a *d-trans*-allethrin formulation exposed to sunlight for 0 (A) and 30 days (B). 1 = *d-trans*-allethrin; 2 = unknown product of *d-trans*-allethrin.

The peak area of the unknown of allethrin increased with time until a plateau was observed after 3 weeks with a corresponding decrease in the allethrin conversion products thereafter (see Fig. 8). The GC–MS study of the unknown material isolated from sunlight-exposed allethrin revealed (see Table II) that it had a fragmentation pattern, including the same apparent molecular ion (*m/e* 302), similar to that of the

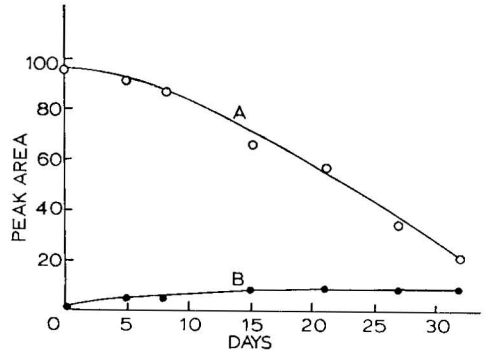

Fig. 8. Comparison of the relative concentration changes of allethrin (A) and photoproduct (B) with increasing exposure to sunlight.

TABLE II

INTENSITIES OF FRAGMENT IONS IN THE MASS SPECTRA OF SUNLIGHT-EXPOSED (A) AND UNEXPOSED (B) ALLETHRIN

Values are expressed as a percentage of the base peak.

(A) Exposed						*(B) Unexposed*					
m/e	%	*m/e*	%	*m/e*	%	*m/e*	%	*m/e*	%	*m/e*	%
303	0.3	115	1.4	69	30.1	303	0.12	106	1.3	57	7.1
302	1.2	111	3.2	68	3.1	302	0.6	105	5.9	56	1.3
285	0.04	110	1.2	67	22.0	169	0.4	104	0.3	55	18.5
284	0.36	109	6.9	66	4.1	168	3.3	103	1.0	54	1.6
169	1.1	108	9.6	65	15.3	167	1.0	97	1.0	53	13.3
168	5.7	107	56.6	64	1.0	154	0.2	96	1.0	52	2.3
167	1.0	106	2.0	63	1.9	153	2.5	95	7.1	51	3.7
153	2.2	105	9.3	59	1.6	152	0.2	94	2.6	50	0.9
152	0.74	104	0.7	58	1.6	151	0.5	93	22.0	45	0.44
151	2.2	103	2.0	57	22.0	150	0.2	92	4.7	44	2.1
150	3.1	99	0.9	56	3.3	149	0.6	91	28.7	43	40.6
149	1.1	98	0.9	55	37.7	139	0.7	85	1.0	42	2.8
145	1.1	97	3.0	54	3.0	138	0.2	84	0.3	41	46.0
139	1.1	96	2.0	53	21.1	137	2.3	83	3.5	40	3.1
138	0.5	95	13.3	52	4.7	136	20.0	82	3.4	39	17.1
137	4.0	94	5.0	51	7.4	135	2.8	81	40.6	29	29.0
136	30.6	93	44.8	50	1.9	134	1.1	80	4.7	28	5.3
135	30.0	92	8.4	45	1.4	133	1.0	79	45.6	27	13.9
134	11.6	91	50.0	44	5.3	125	2.5	78	3.5	15	0.7
133	3.2	85	2.7	43	68.1	124	10.5	77	14.0		
131	1.1	84	1.1	42	4.3	123	100.0	71	1.5		
125	2.7	83	7.5	41	69.0	122	2.0	70	0.7		
124	11.2	82	4.9	40	5.7	121	4.4	69	10.0		
123	100.0	81	56.1	39	25.4	119	1.0	68	1.5		
122	4.9	80	7.4	29	44.3	111	0.8	67	13.6		
121	6.4	79	74.6	28	8.2	110	0.6	66	2.7		
119	3.1	78	6.7	27	23.2	109	3.1	65	10.0		
118	0.4	77	25.3	15	3.1	108	5.5	63	1.1		
117	2.2	71	3.9			107	43.3	59	0.6		

unexposed allethrin standard. The relative intensities of the various peaks differed with a general increase in intensity of the fragment ions below *m/e* 123, compared with the allethrin standard. In both instances *m/e* 123 is the base peak, and there appears to be no change in the acid moiety and the ester bond. Only the relatively low intensity fragment ions *m/e* 284 and 285 of the photoproduct were not observed in the allethrin spectrum.

One gram of bioallethrin in 200 ml of *n*-hexane was irradiated with a high-pressure mercury vapor lamp for 24 h to obtain a 15:2 mixture of the photoproduct and the starting material. The photoproduct was isolated from the allethrin formulation by elution through a silica gel column. GC performed on the Hitachi GC-063 instrument using a 10% SE-30 column revealed the same elution order of the photoproduct generated by the mercury lamp.

The PMR spectrum of the photoproduct of allethrin in $CDCl_3$ indicated $\delta = 0.55$–0.93 ppm (2H, m, cyclopropyl group), $\delta = 1.14$ ppm (3H, s, CH_3), $\delta =$

0.93–1.54 ppm (3H, m, cyclopropyl group), δ = 1.25, 1.28 ppm (3H, s, CH_3), δ = 1.39 ppm (1H, d, J = 1.39 Hz, CHCO), δ = 1.71 ppm (6H, s, $(CH_3)_2C=$), δ = 2.07 ppm (3H, s, $CH_3C=$), δ = *ca.* 2.1 ppm (obscured, 1H, $(CH_3)_2C=CH$–C*H*), δ = *ca.* 2.2 ppm (obscured, 1H, dd, C*H*HCO), δ = 2.81 ppm (1H, dd, J = 6.0 and 18.2 Hz, CH*H*CO), δ = 4.90 ppm (1H, dm, J = 7.2 Hz, $(CH_3)_2C=CH$), δ = 5.63 ppm (1H, broad, CHO).

The mass spectrum of the mercury lamp-induced photoproduct had the same fragmentation pattern as that of the sunlight photoproduct of allethrin including the fragment ions *m/e* 284 and 285. The *m/e* 284 and 285 ions were not observed in the mass spectrum of the original standard allethrin. The photoproduct was identified as *d,l*-cyclopropylrethronyl *d-trans*-chrysanthemate, which has been previously observed in photodecomposition studies of allethrin in petroleum solutions[9,14–16]. A di-π-methane rearrangement involving hydrogen migration of the central $-CH_2-$ grouping of the prop-2-enyl side-chain has been proposed as the mechanism in the rearrangement of the alcohol moiety's prop-2-enyl side-chain to a cyclopropyl ring[9,16,17].

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GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC DETERMINATION OF DOPAMINE IN SUBREGIONS OF RAT BRAIN

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SUMMARY

A quantitative gas chromatographic–mass spectrometric assay was developed for the determination of dopamine in subregions of rat brain. Five tissue punches weighing approximately 1.7 mg each were taken from the nucleus accumbens and four neostriatal regions differing in anterior–posterior level. The dopamine extracted from the tissue was treated with pentafluoropropionic anhydride (PFP) and quantitatively formed dopamine-$(PFP)_3$. The derivatizing procedure took 15 min and the retention time for dopamine-$(PFP)_3$ and its deuterated analogue [1,1,2,2-$^2H_4$]dopamine-$(PFP)_3$) was 120 sec. Selective ion monitoring was utilized to monitor the gas chromatographic effluent. Ions were generated by electron impact ionization. The assay was able to measure concentrations of 1 nanogram dopamine per milligram protein. An anterior–posterior gradient of dopamine was observed in the striatum. This assay should be useful in studies examining the effects of experimental manipulations on dopamine content in relatively small areas of brain tissue.

INTRODUCTION

Much experimental effort has been expended to evaluate the physiological and behavioral functions of brain neuronal systems utilizing dopamine (DA) as a neurotransmitter. Studies using fluorescence histochemistry (Ungerstedt[1], Lindvall and Björklund[2]) have shown the major brain DA systems to originate from cell bodies in midbrain which project their axons to terminate in several forebrain areas, particularly neostriatum (caudate and putamen and nucleus accumbens/olfactory tubercle. This projection is topographically organized, with different cell groups in the midbrain synapsing upon different structures and portions of structures in forebrain (Fallon and Moore[3]).

In order to evaluate the effects of experimental manipulations (lesions, stimulation, drugs, etc.) on subcomponents of the DA projection, it is necessary to be able to

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determine DA levels in small regions of the forebrain terminal areas. Several analytical methods have been developed for the determination of dopamine in small (less then 5 mg) samples of rat brain tissue. Enzymatic isotopic methods of Brownstein *et al.*[4] have reported dopamine levels of 96.6 $\pm$ 6.5 ng/mg protein in nucleus caudatus and 88.4 $\pm$ 10.3 ng/mg protein in caudate-putamen obtained from stainless steel punches. Tassin *et al.*[5] measured a progressively decreasing content of endogeneous dopamine (100–40 ng/mg protein) in microdiscs from the anterior to posterior neostriatum.

Recently, mass spectrographic methods have become available for dopamine assays. Koslow *et al.*[6] reported a gas chromatographic–mass spectrometric (GC–MS) method for dopamine determination using alpha-methyldopamine as internal standard and measured an anterior–posterior gradient in punched samples of the nucleus caudate-putamen. Freed *et al.*[7] have used a direct inlet probe chemical ionization method with [$^2H_2$]dopamine as internal standard and determined an average value for whole rat corpus striatum of 9.49 $\pm$ 0.46 ng/mg tissue. Other GC–MS methods, with [$^2H_3$]dopamine as internal standard, have reported 8.11 $\pm$ 0.15 ng/mg tissue in each pair of striata (Wiesel[8]), and 12.73 $\pm$ 1.50 ng/mg tissue using one whole rat caudate nucleus tissue for each sample (Kilts *et al.*[9]).

This paper describes a GC–MS assay for dopamine in subregions of rat neostriatum. In this study [1,1,2,2-$^2H_4$]dopamine was used as the internal standard with pentafluoropropionic anhydride (PFP) as the derivatizing agent.

EXPERIMENTAL

Materials

Analytical grade [1,1,2,2-$^2H_4$]dopamine obtained from Merck & Co., Rahway, NJ, U.S.A. was used as the internal stanard. This solution was made by dissolving the isotope (final concentration of 91.9 ng [$^2H_4$]dopamine per 175 μl of solution) in 100 ml of deionized water with 10% trichloroacetic acid (Sigma, St. Louis, MO, U.S.A.) and 0.1% sodium metabisulfite (Fisher Scientific, Pittsburgh, PA, U.S.A.). Calibration plots were made with dopamine obtained from Sigma Chemical Company. The derivatizing agent used was PFP (Pierce, Rockford, IL, U.S.A.); however, other derivatizing agents such as trifluoroacetic anhydride (TFA) and heptafluoropropionic anhydride (both from Pirece) could be used, although the TFA derivative is somewhat less stable.

Instrumentation

MS was accomplished using a Finnigan 4000 GC/MS quadrupole mass analyzer spectrometer with a Model 6000 automated data system. GC was performed on a 1.8 m $\times$ 2 mm I.D. glass column with 3% OV-17 on Gas-Chrom Q, 100–120 mesh. The column temperature was maintained at 160°C, injection port at 175°C, jet separator at 190°C and the ion source at 250°C. The voltage of the electron multiplier was 1800 volts with an ionization potential of 70 eV. The ions were measured at *m/e* 428, 428.1 and 431, 431.1 for dopamine and [$^2H_4$]dopamine, respectively. A Varian Cary 118 ultra-violet–visible spectrophotometer was used for the protein assay with a fixed wavelength of 725 nm.

Sample Collection

Adult male CFE albino rats (Charles River, MA, U.S.A.) were used in all experiments. Animals were killed with a guillotine and the brains quickly removed from the skull and frozen in powdered dry ice. The brains were kept frozen on the stage of a Super Histofreeze (Scientific Products) and five contiguous 1 mm-thick sections were cut using a sliding microtome. The landmark for the first section was the nucleus accumbens at AP 10.0 (brain atlas of Pellegrino and Cushman[10]). As soon as they were cut, the brain slices were transferred to a cold plate and two tissue punches were taken from each slice. These punches were from symmetrical locations in the left and right hemispheres and were combined for further analysis. Punches were made with stainless steel tubing of 1.35 mm I.D. The punched samples were transferred to plastic test tubes containing 175 μl of the standard solution, immediately homogenized by sonication (Heat Systems Model W-220F) for 5–10 sec and the tubes placed in ice. These tubes were then centrifuged for 10 min at 500 g to precipitate the protein after which 125 μl of the supernatant was removed, placed in 1.5 ml micro test tubes and shaken for 10 min with 150 μl of isooctane. The micro tubes were centrifuged at 15,000 g (Eppendorf micro centrifuge) for 4 min and the organic layer discarded. The samples were placed in a desiccator and nitrogen was passed into the desiccator for 10 min. The samples were then vacuum-dried and derivatized with 20 μl of derivatizing agent (50% ethyl acetate and 50% PFP). All samples were run immediately using 1 μl injection. Retention time of dopamine and [$^2H_4$]dopamine was 120 sec. The tissue precipitate was analyzed for protein as described by Lowry *et al.*[11] and the tissue concentration of dopamine is reported on the basis of milligrams protein content.

To determine the relative intensity of the ions to be monitored, microgram samples of the hydrochloride salts of dopamine and $^2H_4$-dopamine were weighed (Kahn electrobalance) in various ratios and the mixtures analyzed by measuring the intensity of ions 428 and 431. A graph of intensity ratio (as measured by the area of each selected ion monitoring (SIM) signal) *versus* the ratio of amounts of dopamine and [$^2H_4$]dopamine was constructed (Fig. 1). A least-squares fit ($R = 0.997$) of the data was performed, resulting in the following equation:

$$\text{ng DA} = 0.749 \frac{\text{Area DA}}{\text{Area D4DA}} + 0.041 \text{ ng D4DA} \quad (1)$$

Eqn. 1 was used to calculate the dopamine content of the tissue samples.

RESULTS AND DISCUSSION

Ion intensities

A graph of the ion intensity ratio of dopamine and [$^2H_4$]dopamine *versus* the molar ratio of the two compounds should result in a slope of unity if each structure generates ions of equal intensity. Even on a weight basis the slope should be within 2.1% of unity. As the graph indicates, however, the deuterated dopamine generates an ion of significantly lower intensity at *m/e* 431 than did the dopamine at *m/e* 428. This can be understood by examining Fig. 2 where the fragmentation leading to these ions is depicted for a general catecholamine structure. It can be seen that the ions are

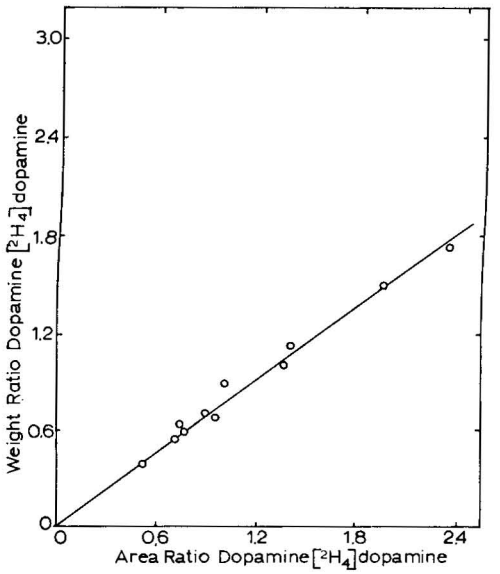

Fig. 1. Graph of intensity ratio *versus* area ratio for dopamine and [$^2H_4$]dopamine.

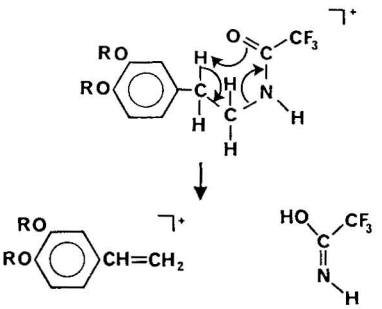

Fig. 2. McLafferty rearrangement of general catecholamine structure leading to ions monitored in SIM of catecholamines.

formed through a McLafferty rearrangement[12] in which a hydrogen or deuterium atom migrates from the beta carbon to the carbonyl oxygen through a six membered transition state. Thus the difference in ion intensities is due to a deuterium isotope effect. The deuterium isotope effect arises from several contributing factors. The most important of these are (1) the difference in zero-point energy between a bond to hydrogen and the corresponding bond to deuterium, which is on the order of 1.2–1.5 kcal/mole, and (2) the reduced velocity of passage over the potential energy barrier of a reaction involving displacement or loss of deuterium in place of hydrogen[13]. The first effect, that of lower zero-point energy, is less important in a reaction of the type in Fig. 2 because a new bond with the leaving atom is being formed as the old one is broken, cancelling the zero point effect to some extent.

When energy is readily available, such as at high temperature or after ionization with 70 eV electrons, the isotope effect should approach the square root of the

mass ratio, in this case 1.4[13]. When the data of Fig. 1 are calculated on a molar ratio basis, the isotope effect is found to be 1.37, which is in good agreement with the square root of the mass ratio.

Dopamine assay

Fragmentation patterns of dopamine-$(PFP)_3$ have been previously described by Koslow *et al.*[14] and Gelpi *et al.*[15]. As stated earlier an additional mass fragmentogram at one tenth of an a.m.u. higher mass was monitored for the dopamine and the internal standard to increase the signal to noise ratio. When the areas for each pair were calculated, errors in the data were easily checked and precision was improved over measuring 428 and 431 only. In Fig. 3, a mass fragmentogram from one of the samples obtained from rat striatum is shown. Assurance of specificity was obtained by the fact that the chromatographic retention time of the endogenous compound was identical to that of its deuterated analog.

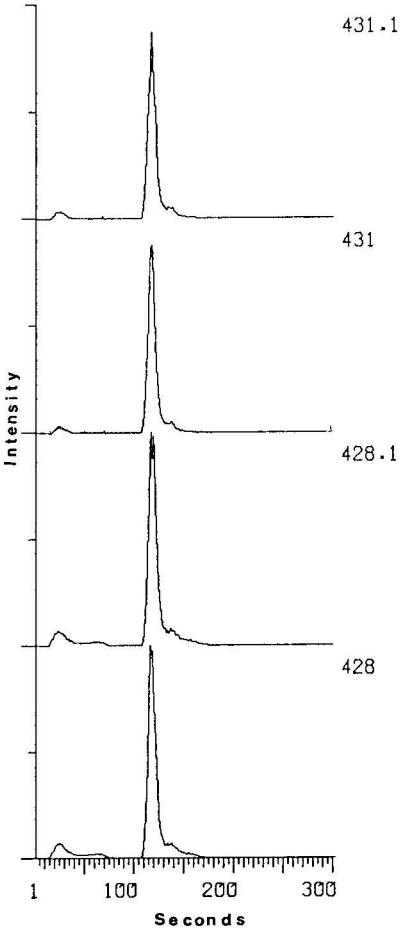

Fig. 3. Selected ion monitoring of ions 428 from dopamine and 431 from deuterated dopamine. The signal was also monitored at 428.1 and 431.1 to improve the signal to noise ratios.

In Fig. 4, the tissue punch placement can be seen. The first punch was a 1 mm-thick tissue slice containing the nucleus accumbens and a portion of olfactory tubercle; the next four tissue punches were taken from the four successive 1 mm-thick tissue slices in the striatum. Fig. 5 is a plot of ng dopamine/mg protein from the five contiguous sections of tissue; the anterior–posterior striatal gradient observed in this study agrees with Koslow *et al.*[6] and Tassin *et al.*[5].

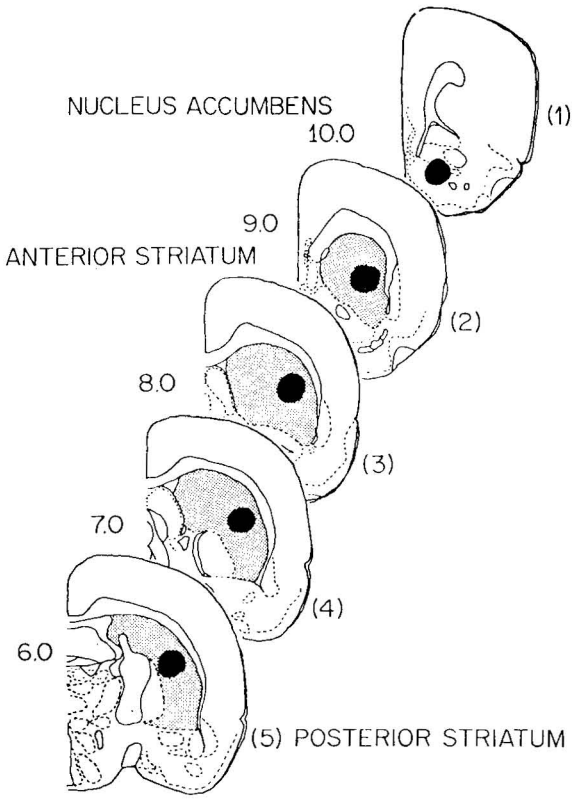

Fig. 4. Tissue punch placement. The first punch was from a 1-mm thick tissue slice containing portions of nucleus accumbens and olfactory tubercle; the next four punches were taken from successive 1-mm thick tissue slices in the striatum (shaded structure). The numbers on the left refer to the anterior–posterior axis coordinates in the brain atlas of Pellegrino and Cushman[10]. Punch diameter, 1.35 mm.

Dopamine loss in the interval between sacrifice and sonication does not seem to be a major problem. Moleman *et al.*[16] found very little change in dopamine levels in dissected and undissected rat striata a few minutes after killing the rat and removing the brain. A 15% decrease in dopamine level was observed in dissected tissue 20 min after removal of the brain although this effect was not statistically significant. In our samples the tissue was sonicated within 5 min after sacrifice.

Protein assay

The average weight of representative tissue samples in the combined punches

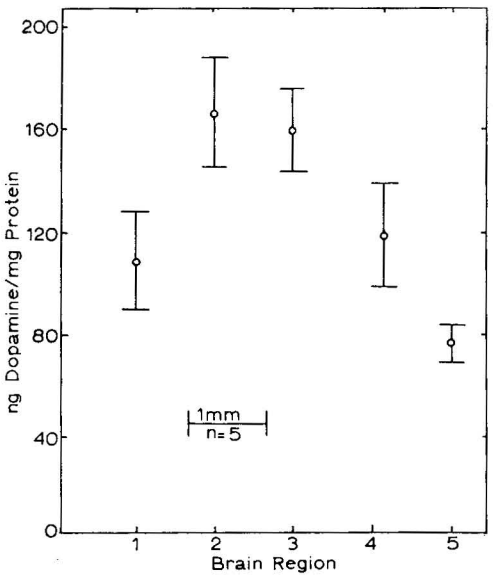

Fig. 5. Average (± S.D.) values of ng dopamine/mg protein in each brain region. Region 1 is the nucleus accumbens, regions 2–5 are the striatum with regions 2 and 5 the anterior and posterior striatal regions, respectively.

(one punch from the right hemisphere and one punch from the left hemisphere) from each brain section was 1.72 mg tissue (wet weight). An average of 0.224 mg protein/mg tissue was obtained in this study.

CONCLUSION

This method is useful for determination of dopamine in different DA terminal areas of rat brain. As found by others[5,6] an anterior–posterior gradient of DA content was observed in the neostriata of the rat brains in this study. A large number of samples can be run on a daily basis (*e.g.*, 35–50 samples from 7–10 rats). The GC–MS method is sensitive and specific. The limit of detection was 1 ng dopamine per mg protein. The assay described in this paper has been found useful in testing the hypothesis that the dopaminergic innervations of subregions of the striatum are functionally different[17].

The results of this assay demonstrate that levels of dopamine observed depend on the region of tissue sampled. This observation has important implications in functional studies of brain dopamine systems. For example, we have found that the degree of correlation between levels of dopamine in striatum and various behaviors is enhanced if dopamine in subregions of striatum is considered rather than total striatal dopamine[17].

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DETERMINATION OF RESIDUAL VINYL CHLORIDE IN POLY(VINYL CHLORIDE) RESINS

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SUMMARY

The determination of residual vinyl chloride (VC) in poly(vinyl chloride) (PVC) powders based on thermal desorbtion in a carrier gas stream and trapping of the VC is described. A trap is installed in the heated injection port of a gas chromatograph and desorbed volatiles are chromatographed by a two-stage chromatographic system. While VC is purged through an analytical column, the first column is cleaned by back-flushing. The method has been tested on various types of PVC powders and has no limit of sensitivity.

INTRODUCTION

Owing to the carcinogenic properties of vinyl chloride (VC)[1,2], it must be analysed both in poly(vinyl chloride) (PVC) and in PVC materials. Modern requirements for the purity of PVC range from a few parts per million downwards[2,3]. These severe requirements for the purity of PVC are conditioned by wide fluctuations in VC content in PVC resins and products depending on the technology of PVC production and processing.

Purity is especially important for foodstuffs packing materials, as foodstuffs should not contain more than 50 ppb of VC[4,5], and there is a linear relationship between the VC concentration in packing materials and that subsequently found in packed foodstuffs[6] and cosmetics[7]. Different foodstuffs extract VC to different extents[8].

The determination of VC in foodstuffs is difficult; the direct injection method is unsatisfactory[9], and the headspace method is generally used[6,8,9], frequently combined with mass spectrometry for greater reliability[10,11]. Conversion of VC into dibromochloroethane[12] and combustion of VC combined with electrochemical detection of hydrogen chloride[13] are selective methods used in VC trace analysis.

Various methods have been described for the determination of residual VC in PVC. In one method[14,15] the polymer is dissolved in a suitable solvent and a portion of the polymer solution is chromatographed. However, possible clogging of the

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chromatograph evaporator by the polymer is a serious inconvenience in this method. The sensitivity is *ca.* 10 ppm.

Gaseous contaminants in PVC may be trapped on heating the polymer in a vacuum, measuring the liberated gas and analysing it by gas chromatography or IR or mass spectrometry[16,17].

The solution mode of the headspace method that is generally used is inconvenient as it requires time for dissolving the sample and equilibration, and the purity of the solvent must be high with only very low contents of impurities that may be chromatographed simultaneously with VC[6,18,19]. Analysis of the gas phase above the solid PVC is a labourious and slow procedure, demanding precautions against possible loss of VC[20].

Owing to these difficulties we preferred a dynamic method to static methods for removal of VC from PVC powder.

The principle and possibilities of the mode have been described briefly[20,21], but no routine analysis has been suggested so far.

EXPERIMENTAL

Auxiliary equipment

The system for the separation and trapping of volatiles from PVC is installed in the cover of the detector oven of a Tsvett-104 chromatograph (OCBA, U.S.S.R.). The system is shown schematically in Fig. 1. The system for feeding the gas into the device includes a pressure-reducing valve, a pressure gauge and a needle valve to adjust the carrier gas flow-rate in the tube containing PVC and in the trap. For cooling the trap should be dipped half its height into a Dewar vacuum flask containing liquid nitrogen.

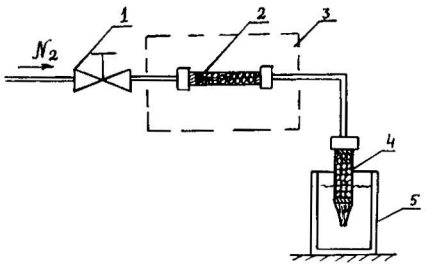

Fig. 1. System for trapping volatiles from PVC powder. 1 = Needle valve; 2 = tube with PVC; 3 = oven; 4 = trap containing sorbent; 5 = Dewar flask containing liquid nitrogen.

Preliminary procedures

The system should be assembled as shown in Fig. 2. The system consists of two columns (pre-column and analytical column) connected to the instrument through a six-port switching valve. Depending on the position of the switching valve, the columns may work under conditions of direct purging (solid line) or back flushing (dotted line). For observing the back-flushing the pre-column may be connected to the second flame-ionization detector (FID).

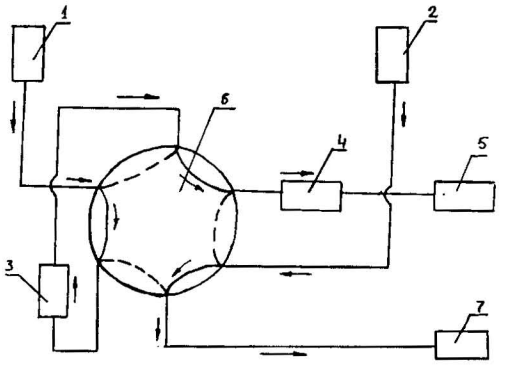

Fig. 2. Schematic flow diagram with six-port switching valve. 1,2 = First and second channels of injection port; 3 = pre-column; 4 = analytical column; 5,7 = detectors; 6 = six-port switching valve. Arrows show carrier gas flow in direct purging.

Fig. 3 shows the installation of the trap in the injection port. As there is a ground-glass joint at the end of the trap, the trap enters into the corresponding connection with the ground-glass joint, connected to the evaporator in the same manner as the glass column.

The traps are removed from the evaporator with tweezers that have narrow tips with cuts.

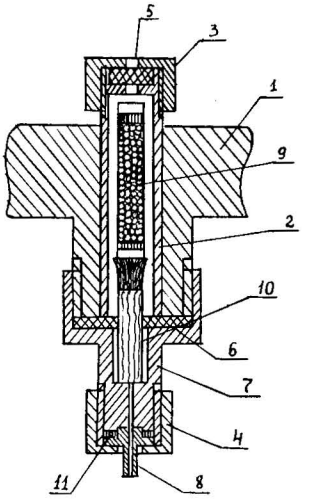

Fig. 3. Installation of trap in evaporator. 1 = Oven body; 2 = evaporator body; 3,4 = nuts; 5,6 = silicone-rubber seals; 7 = union; 8 = stainless-steel capillary tube; 9 = trap filled with sorbent; 10 = ground-glass joint coupling, filled with glass-wool; 11 = aluminium gasket.

Column preparation

The pre-column is filled with a sorbent consisting of 15% Apiezon L on Chezasorb AW, with particle dimensions of 0.210–0.360 mm (Chemapol, Prague,

Czechoslovakia). The sorbent for the analytical column is Polysorb 1 (0.125–0.250 mm fraction) or Chromosorb 104. The pre-column and analytical column are made of glass, with dimensions 100 × 0.4 cm I.D.

The column system is assembled and conditioned for 24 h at 150°C in the back-flushing mode.

Chromatographic conditions

The carrier gas is nitrogen at flow-rates, measured at ambient temperature in the analytical column exit, of 8.5 ml/min during direct purging, 50 ml/min during back flushing and 10 ml/min at the trap exit during desorption of volatiles from PVC. The desorption time is 15 min. The following temperatures are used: column oven, 80°C; evaporator, 230°C; and detector oven, 110°C. The flow-rates of hydrogen and air are 40 and 400 ml/min, respectively. The scale is from $5 \cdot 10^{-7}$ to $5 \cdot 10^{-11}$ a.u.f.s., depending on the VC concentration. The amount of polymer taken is 20–200 mg.

Determination of efficiency of analytical column

It is necessary to establish the efficiency of the analytical column by using a model mixture of methyl chloride, VC and ethyl chloride and determining the selectivity coefficient (K_S), separation factor (K) and number of theoretical plates (N):

$$K_S = \frac{t_{R_2} - t_{R_1}}{b_2 + b_1}$$

$$K = \frac{t_{R_2} - t_{R_1}}{t_{R_2} + t_{R_1}}$$

$$N = 5.54 \cdot \left(\frac{t_{R_2}}{b_2}\right)^2$$

where t_{R_1} is the retention time of VC (sec), t_{R_2} is the retention time of ethyl chloride (sec) and b_1 and b_2 are the peak widths of VC and ethyl chloride, respectively, measured at half the peak height.

The determination is carried out in the back-flushing mode, introducing the mixture through the injection port.

The analytical column should satisfy the following requirements: $K_{\text{methyl chloride–VC}} > 0.8$, $K_{\text{VC–ethyl chloride}} > 2.0$, $K_{S\ \text{VC–ethyl chloride}} > 0.30$ and $N_{\text{ethyl chloride}} > 500$.

Determination of vinyl chloride zone cut-off time

To determine the time required for the VC zone to pass through the pre-column, it is necessary to install a T-joint before the analytical column so that the greater portion of the flow enters the analytical column and lesser portion arrives at the detector. The resulting VC peak in the chromatogram is used for a rough estimation of the time required for the PVC zone to pass through the pre-column and enter the analytical column. The final VC zone cut-off time is determined from the VC and ethyl chloride peak areas, depending on the valve switching time. To accom-

plish this the system is assembled as shown in Fig. 2 (without a T-joint). A mixture of VC and ethyl chloride is introduced through the sample loop. By varying the cut-off time of the VC zone it is feasible to make the final determination. If the exclusion time is properly selected (*i.e.*, if the time of switching the valve from direct purging to back-flushing is determined correctly), the VC peak areas show good reproducibility when replicate measurements are carried out. The results demonstrate that the VC zone entered the analytical column completely.

Calibration regression equation

The relationship between peak area and VC content is interpreted by the least-squares method. Coefficients (a, B) for the regression equation are found:

$$y = a + Bx$$

where y is the VC peak area in mm^2 (S) and x is the absolute VC content in mg (P); or

$$S = a + BP$$

Hence,

$$P = \frac{S - a}{B} \tag{1}$$

The VC concentration in the sample being analyzed in parts per million (Q) would be given by

$$Q = \frac{P \cdot 10^6}{\text{weighed amount of sample (mg)}}$$

Substituting from eqn. 1 instead of P we obtain

$$Q = \frac{(Sf - a) \cdot 10^6}{B \cdot \text{weighed amount of sample (mg)}} \tag{2}$$

where f is a scale conversion coefficient, *i.e.*, a coefficient that equals in current units (A) the ratio of the scale used for analysis to the scale used for calibration.

The sensitivity by the method of Freed and Mujsce[22], A_1 (= B in eqn. 1), correlates well with the sensitivity, A_2, taken from the method of absolute calibration, *i.e.*,

$$A_2 = \frac{\sum_{n=1}^{10} S/10 \text{ (mm}^2\text{)}}{\text{VC in sample (mg)}}$$

where $\sum_{n=1}^{10} S$ is the sum of the areas of ten VC peaks obtained on its introduction into the evaporator through the sample loop.

Good agreement between the sensitivities A_1 and A_2 (to within 5%) permits absolute calibration to be employed for the calculation of the residual VC levels in the range from hundredths to thousands parts per million.

Sequence of analysis

A weighed portion of PVC from 20 to 200 mg is held in the glass tube between two glass-wool plugs and is located in the detector oven with the help of nuts, after which the trap is adjusted. The oven cover is closed and the oven is heated.

The trap is immersed in the Dewar flask containing liquid nitrogen, then the flow of carrier gas is started. After the trapping, the trap is disconnected and fitted into the chromatograph evaporator, and simultaneously a stop-watch is started. The switching valve is in the direct purging position but, after the cut-off time, the valve is switched into the back-flushing position.

During the time interval from purging to back-flushing the next tube, filled with a weighed portion of polymer for desorption, is installed in the detector oven. Fig. 4 shows a chromatogram of a VC zone obtained under the above conditions. The analysis time is about 20 min.

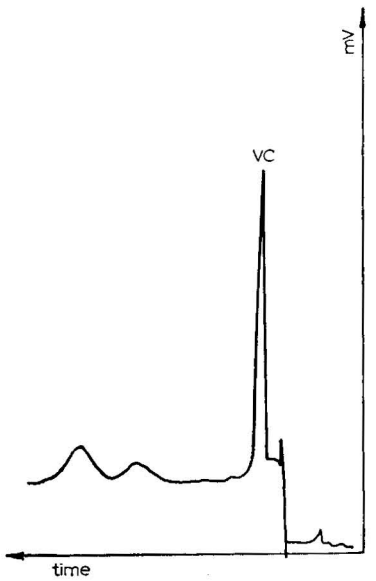

Fig. 4. Chromatogram obtained during separation of VC band on analytical column.

RESULTS AND DISCUSSION

According to the literature, the specific surface area of PVC powder is less than 10 m^2/g, and it is doubtful whether PVC powder can retain VC by adsorption[23]. Hence the major proportion of VC is absorbed in the polymer particles. Owing to the small diffusion coefficients, it is difficult to carry out the removal of VC quantitatively. An abrupt increase in the diffusion coefficient occurs above the glass transition point, so VC can be removed quantitatively from small polymer particles in the chromatographic regime, *i.e.*, by continuous purging a stream of carrier gas through the polymer layer. The only limitation is the narrow temperature range between the glass transition point at 80°C and decomposition at 130°C, so it is necessary to maintain the operating temperature carefully.

The polymer was placed in a small glass tube and heated to 110°C, with simultaneously the stream of nitrogen purging through it. Such a technique, combined with chromatography and on-line mass spectrometry, facilitates the investigation of polymer volatiles[24].

To concentrate stripped volatiles, they were transferred into a trap cooled by liquid nitrogen, after which the trap was placed in the injection port of the chromatograph for further analysis[25,26].

However, it must be noted that there are no data on PVC impurities, except for residual VC. The first analysis of volatiles removed from PVC shows that they have a complex composition, containing many components at different concentrations, complete analysis of which was possible only with a high-efficiency column and mass spectrometry.

Together with VC, methyl and ethyl chlorides were found among PVC contaminants with a relatively high vapour pressure[27]. This mixture was used as a model for the examination of the analytical column. The behaviour of these compounds when chromatographed on polymer sorbents is well known[28].

The complex composition of the volatiles demands a very careful chromatographic separation, as constant control by mass spectrometry is difficult. That is why a double column system for chromatographic separation was suggested. A narrow fraction containing VC was transferred from the first column (pre-column) to the second column (analytical), then the pre-column was purged with carrier gas in the reverse direction (back-flushing) to clean the column from other components and to prepare it for further analysis[29]. The choice of the analytical column and the separation conditions was made very carefully as a complete VC separation was needed.

Both of the columns should be operated in one oven, otherwise the instrumentation required for analysis would be too complex.

For maximal sensitivity it was preferred to use the device at a high sensitivity ($2 \cdot 10^{-11}$–$5 \cdot 10^{-11}$ a.u.f.s.), so the background signal should be minimal. Columns with polymeric sorbents are preferable. Chromosorb 104 (one of the most polar polymeric sorbents) or Polysorb 1 are the best.

The time of the valve switching for transferring the VC band from the pre-column to the analytical column was selected so that the VC passed through the pre-column completely, as indicated by an alteration of the ethyl chloride peak area eluted after VC.

Determination of vinyl chloride in PVC of different types

Berens[23] showed that VC stripping depends on sample morphology, and this gave rise to doubts during the investigation. The diffusion time for spherical particles depends on the square of the diameter of a particle, so this method can be applied to polymer powders consisting of loose particles, which in turn consist of smaller subparticles with diameters of a few microns. In this case the diffusion time is several minutes at temperatures near 100°C. Glassy particles of larger diameter in the polymer retard the removal of VC. In order to study this aspect, some types of polymers with different glassy particles contents were investigated (Table I).

Samples of different types were analysed by our method and the solution method; the results of the latter do not depend on the type of polymer used. Table II shows the good agreement obtained.

TABLE I
CHARACTERISTICS OF DIFFERENT TYPES OF PVC

PVC type	*Specific surface area (m^2/g)*	*Homogeneity* (glassy particle content)*
C-58	0.34	Very low
C-70	1.20	$\frac{1.2}{6}$; $\frac{1.66}{8.8}$
C-63M	0.66	Many fiine particles
PVC filler	0.35	$\frac{3}{15}$; $\frac{3}{15}$
Microsuspension	3.7	High

* PVC homogeneity was measured according to the U.S.S.R. State Standard. The numerator is the average number of glassy particles in a square 10 × 10 cm; the denominator is the number of glassy particles in the film of the same square and volume of 0.1 cm^3.

TABLE II
COMPARISON OF RESULTS FOR THE ANALYSIS OF PVC SAMPLES

PVC	*Sample No.**	*VC content (ppm)***	
		Solution method	*Proposed method*
C-70	1	330	315
	2	120	127
C-63M	1	130	148
	2	87	97
C-58	1	88	93
	2	347	356
C-66	1	416	450
	2	65	49

* Different VC removal conditions used in polymerization process.
** Average of two results.

Control experiments

Control experiments showed the complete desorption of VC from the polymer and its quantitative transfer into the chromatographic column by the cooled trap. To verify the complete removal of VC, the VC liberated was trapped for 15 min, as described under Experimental. For trapping VC the trap was packed with sorbent (15% Apiezon L on Chezasorb AW, particle size 0.210–0.360 mm) and cooled by liquid nitrogen. The first trap was replaced with a new one for a further 15 min. The traps were analysed for their VC contents and the second trap was tested on the most sensitive scale ($5 \cdot 10^{-12}$ a.u.f.s.). There was no VC from any type of PVC in the second trap.

The completeness of VC trapping and desorption from the trap in the injection port was tested in the following way. The VC sample was injected either in the cooled trap by a sample valve or without a trap directly on to the column. The VC peak areas (average of 10 measurements) were 863.1 and 859.7 mm^2, respectively.

It is known that a considerable amount of water is adsorbed on the PVC powder surface. According to the literature[30], the sensitivity of the FID is decreased by water which is eluted together with other components; consequently, it was necessary to check whether the bands of VC and water overlap. A glass tube con-

taining calcium carbide was placed before the FID and a water sample was injected into the evaporator. The detector recorded the signal of acetylene. The retention time for water is 5 min 56 sec., whereas that for VC under the same conditions is 1 min 43 sec. Hence it is evident that water elutes later and is removed by back-flushing.

Calibration

For VC calibration we used the method proposed by Freed and Mujsce[22]. This consists in the dehydrochlorination of 1,2-dichloroethane to VC in a pre-column packed with anhydrous potassium carbonate at 300°C, and gives a linear calibration graph of amount of VC *versus* peak area in the range 1–125 ng. We investigated amounts of VC in the range 10–400 ng and have obtained a linear calibration graph in the range 10–200 ng. In attempts to prepare higher VC levels, we observed the non-linear character of the dependence, probably because of the tube dimensions and the amount of potassium carbonate used the large amount of sample has insufficient time to react.

The sensitivity of the method obtained from the calibration graph interpreted by the least-squares method was compared with absolute calibration of detector by injection of VC through the inlet sample valve. The results were in good agreement, the discrepancy being about 5%.

Statistics

It was interesting to find how the relative standard deviation changes in relation to VC content. Table III shows the results for the analysis of five PVC samples with different VC contents. Obviously the relative standard deviation increases with decreasing VC content.

TABLE III

VARIATION OF RELATIVE STANDARD DEVIATION WITH VC CONTENT*

Sample No.	$\bar{x} = \frac{\Sigma x_i}{n}$ *(ppm)*	n	$S = \sqrt{\frac{\sum_{i=1}^{n}(x_i - x)^2}{n-1}}$ *(ppm)*	$S_r = \frac{S}{\bar{x}} \cdot 100$ *(%)*
1	1118.0	4	53.4	4.8
2	710.6	4	51.9	7.3
3	72.32	4	5.46	7.5
4	2.46	4	0.25	10.2
5	0.24	4	0.034	14.1

* x_i = VC content; $\bar{x}$ = arithmetic mean; n = number of samples; S = root-mean square; S_r = relative standard deviation.

It should be noted that PVC resin loses noticeable amounts of VC on storage. Thus in one sample the VC content decreased from 2230 to 70 ppm after storage for 1 week. Owing to this the root-mean-square deviation and the relative standard deviation were measured not by special experiments, involving multiple analysis of one sample (not less than 20 measurements), but by current data[31]. The VC content varied from 70 to 350 ppm and all measurements were duplicated. S and S_r were measured according to the equations

$$S = \sqrt{\frac{1}{2m} \sum_{i=1}^{m} d_i^2}$$

$$S_r = \frac{1}{2m} \sqrt{\sum_{1=1}^{m} \frac{(d_i)^2}{\bar{x}}}$$

where d_i is the difference between duplicate measurements and m is the number of samples. It was found that S_r was 5%.

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GAS CHROMATOGRAPHIC COLUMN FOR THE RAPID DETERMINATION OF CONGENERS IN POTABLE SPIRITS

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SUMMARY

The determination of minor components, including fatty acids, in potable spirits has been accomplished by using acid-washed (AW) Carbopack B, an example of graphitized carbon black, modified with PEG 20M. Quantitative data for selected compounds show that good precision can be obtained even at the level of a few parts per million. The determination of minor components in a commercial sample of Scotch whisky was carried out by using AW Carbopack B modified with 6.6% of PEG 20M.

Components present in concentrations of about 0.5 ppm can be determined with a precision of about 30%. An additional column packing (AW Carbopack B + 3.35% of PEG 20M) was used for determining those few components of minor interest which cannot be separated by the former column packing.

INTRODUCTION

The determination of individual congeners or secondary constituents present in alcoholic beverages is of importance not only to those engaged in the production of spirits, but also to Food and Drug Administration and Customs and Excise workers and toxicologists. This type of analysis is complicated by the large number of congeners present in small proportions.

The direct gas chromatographic method is undoubtedly the best method for determining individual minor components in potable spirits as it offers maximal simplicity, rapidity, sensitivity and accuracy[1–4]. There is a need for a chromatographic column able to separate microamounts of alcohols, acids, aldehydes and esters present in a water–ethanol mixture in a few minutes. Although there has been considerable work in this direction, no chromatographic system described in the literature is completely satisfactory. Whenever direct chromatography of aqueous alcohol solutions has been attempted, various difficulties have been encountered. Firstly, stationary phases are generally intolerant to water injections. Consequently, the chromatographic column deteriorates after some period of continuous use. Secondly, so far as we know the columns used for the analysis of higher alcohols and esters in alcoholic beverages are not able to elute acids. The official method for the determina-

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tion of acids in potable spirits is a titration which determines total acid and the results are calculated as acetic acid. Thirdly, the chromatographic determination of some minor components in distillates is hindered by the presence of excess amounts of ethanol. Last, in almost all instances the separation of 2-methylbutan-1-ol from 3-methylbutan-1-ol cannot be achieved by using conventional chromatographic columns. The quantitative determination of these two components is of importance in establishing the quality of an alcoholic beverage.

Recently, using graphitized carbon black (Carbopack B), suitably modified with 3% of polyethylene glycol (PEG) 20M and 2.4% of 1,3,5-tricarboxybenzene (trimesic acid), we were able to determine individual congeners present in an Italian potable spirit[5]. However, this column packing has two limitations: firstly, its relatively low thermal stability does not permit the column to be operated at temperatures higher than 160°C; secondly, the percentage of trimesic acid is a very critical parameter. At slightly higher percentages of trimesic acid, alcoholic compounds are eluted as tailed peaks owing to their tendency to form esters with the acidic modifier. If, on the other hand, the percentage of the acidic deactivating agent is decreased, acids are not eluted linearly.

Very recently, it has been shown that washing the surface of graphitized carbon black is effective in removing the surface sites that are responsible for chemisorption of acids[6]. The result is that the acidic deactivating agent is no longer necessary in order for symmetrical peaks for trace amounts of acidic eluates to be obtained.

The object of this paper is to show that the individual contents of minor components present in potable spirits, even at sub-parts per million levels, can be determined in less than 30 min by using acid-washed Carbopack B modified with PEG 20M. This column packing has been shown to be very stable for long periods of continuous use. Quantitation data reported for some congeners of interest show that good precision can be achieved at the level of a few parts per million. The chromatographic profile and the quantitative determination of minor components present in an actual sample of whisky are also presented.

EXPERIMENTAL

Carbopack B, which is an example of graphitized carbon black, was kindly supplied by Supelco (Bellefonte, PA, U.S.A.).

The procedure for washing Carbopack with an acidic solution has been recently reported[6]. An aqueous solution of phosphoric acid (0.1 *M*) was used to wash the carbon surface as a first attempt. Very good results were obtained in terms of peak symmetry for both acids and alcohols if acid-washed (AW) Carbopack B was coated with more than 4% of PEG 20M. On the other hand, slightly tailed peaks for alcohols were noted at surface coverages of PEG 20M lower than 4%. This fact can be explained by assuming that, after the chemical treatment, traces of phosphoric acid still remain on the carbon surface, even after washing it with large amounts of distilled water. Then, at low surface coverages of PEG 20M, the terminal hydroxyl groups of the stationary phase are not sufficient to deactivate chemisorbed phosphoric acid, which gives strong interactions with alcoholic eluates.

This problem was eliminated by washing Carbopack B with an aqueous solution of a low-boiling acid, such as acetic acid, at a concentration of 0.3 *M*. By

changing the nature of the acid, no differences were noted in the gas chromatographic characteristics of the PEG 20M + AW Carbopack system, provided that the percentage of PEG 20M was higher than 4%. On the other hand, untailed peaks for alcohols were observed even on coating the acetic acid-washed Carbopack surface with relatively small amounts of modifying liquid.

The procedure for coating Carbopack B was similar to that reported previously[7].

Glass columns (2 m × 2 mm I.D.) were filled with the packing material by following a procedure described elsewhere[8]. The packed columns were conditioned under a flow of nitrogen at 240°C for about 15 h. Nitrogen was used as the carrier gas.

Standards were supplied by Fluka (Buchs, Switzerland). Ethanol used as the solvent was supplied by Carlo Erba (Milan, Italy). This compound contained 24.3 ppm (w/w) of methanol, 1.3 ppm of acetaldehyde and 1.1 ppm of acetic acid, as determined in our laboratory.

A Carlo Erba Model GI gas chromatograph equipped with a flame-ionization detector was used.

For measurements of peak areas a Hewlett-Packard 3385-A integrator was used.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatogram obtained after injection of an artificial ethanol–water (1:1) mixture containing 40–60 ppm of each individual compound that may be present in natural alcoholic beverages. The elution was obtained by using AW Carbopack B modified with 6.6% of PEG 20M.

It can be seen that small amounts of acids, alcohols and aldehydes are eluted as symmetrical peaks. Also, under the conditions used only the peak for isobutanal, which is a congener of minor interest, is obscured by excess amounts of ethanol.

Using the column described above, accurate determinations of congeners of practical interest in potable spirits can be performed. It also appears that overlapping of some peaks of compounds of minor interest could be a partial limitation to the use of the system.

It has been reported that useful modifications to the fractionating power of liquid-modified graphitized carbon black can be achieved not only by changing the modifying liquid but also by varying its relative amount[9]. In order to fractionate all of the components of the complex mixture considered, many attempts were made by changing both the modifying liquid and its percentage, but no system was found to be completely suitable for the required purpose.

Therefore, we resorted to using an additional column packing capable of fractionating isobutanol, pentanal and pentan-2-ol from the other components of the artificial mixture. AW Carbopack B modified with 3.35% of PEG 20M was used with the purpose of determining the three compounds mentioned above; this column packing is more effective than the former in separating the two isoamyl alcohols of interest. This allows the accurate determination of these two compounds without the use of an electronic integrator.

Chemisorption of acids does not occur after acid washing of the surface of the carbon black. To substantiate this result and to determine the minimal amounts of

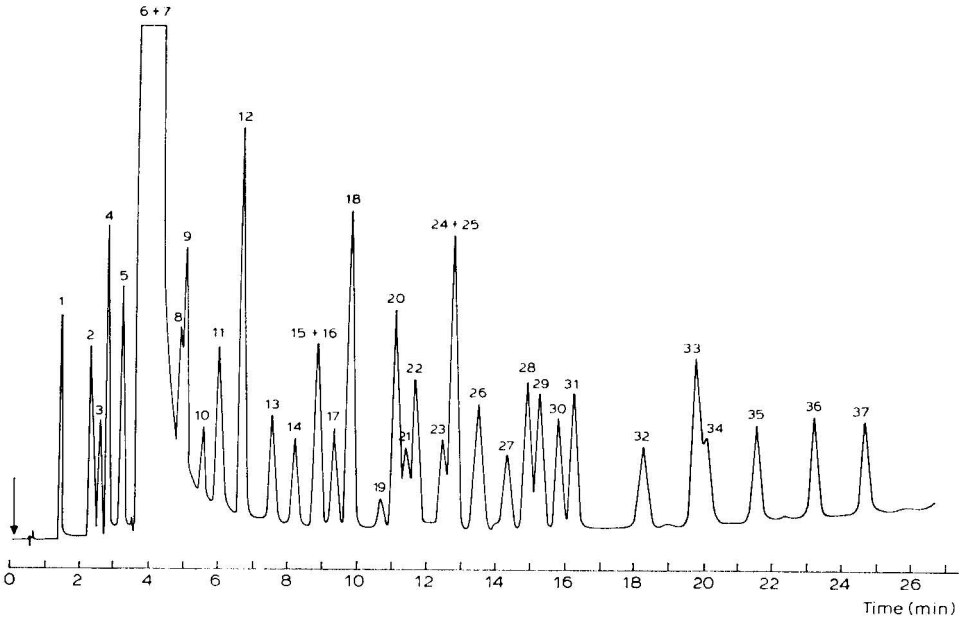

Fig. 1. Chromatograms of an artificial water–ethanol mixture eluted on AW Carbopack B modified with PEG 20M. Column, 2.0 m × 2 mm I.D; packing, AW Carbopack B (100–120 mesh) + 6.6% of PEG 20M; carrier gas, nitrogen; dead time, 32 sec; temperature, programmed from 80°C to 200°C at 4°C/min; sample size, 1 μl. Peaks: 1 = acetaldehyde; 2 = methanol; 3 = propanal; 4 = acetone; 5 = methyl acetate; 6 = ethanol; 7 = isobutanal; 8 = butanal; 9 = isopropanol; 10 = ethyl acetate; 11 = diacetyl; 12 = propanol; 13 = isopentanal; 14 = *sec.*-butanol; 15 = pentanal; 16 = ethyl propionate; 17 = propyl acetate; 18 = isobutanol; 19 = acetal; 20 = butanol; 21 = ethyl isobutyrate; 22 = 3-methylbutan-2-ol; 23 = 3-pentanol; 24 = 2-pentanol; 25 = isobutyl acetate; 26 = ethyl butyrate; 27 = butyl acetate; 28 = 2-methylpentan-1-ol; 29 = 3-methylpentan-1-ol; 30 = acetic acid; 31 = pentanol; 32 = isoamyl acetate; 33 = furfural; 34 = propionic acid; 35 = hexanol; 36 = isobutyric acid; 37 = butyric acid.

congeners in distillates that can still be measured with good precision, quantitative measurements of some compounds were made by using the above two column packings. Measurements were carried out by using the same experimental conditions as reported in the caption to Fig. 1. Methyl amyl ketone was chosen as the internal standard. Standard solutions at concentrations lower than 100 ppm were prepared by diluting the starting solution containing the reference compound with the ethanol–water solvent mixture. The results are reported in Table I. It appears that quantitative determinations at a level of 2 ppm can be made with an error of about 5% for compounds that are eluted well after ethanol. At the same level of concentration, the uncertainty in the measurement is higher than 5% for butanal and isopropanol, because these two compounds are eluted just after the broadened peak for excess amounts of ethanol. The anomalous behavior of the response factor of methanol can be explained by considering that the high-purity ethanol used for calibrated test solutions contains 24.3 ppm of methanol, plus 1.1 ppm of acetic acid and 1.3 ppm of acetaldehyde.

TABLE I

QUANTITATIVE DATA FOR SELECTED COMPOUNDS ELUTED ON AW CARBOPACK B + 6.6% OF PEG 20M

Results are concentrations (ppm, w/w) ± standard deviations for 6 determinations.

Compound	*Concentration (ppm, w/w)*			
	100	*50*	*10*	*2*
Acetaldehyde	0.920 ± 0.013	0.930 ± 0.012	1.05 ± 0.06	1.35 ± 0.09
Methanol	1.22 ± 0.013	1.85 ± 0.05	4.27 ± 0.27	16.0 ± 1.5
Isobutanal*	0.937 ± 0.014	0.910 ± 0.013	0.900 ± 0.051	0.87 ± 0.09
Butanal	0.941 ± 0.016	0.931 ± 0.033	0.842 ± 0.092	0.702 ± 0.18$_a$
Isopropanol	1.101 ± 0.013	1.110 ± 0.022	1.171 ± 0.052	1.504 ± 0.123
Ethyl acetate	0.943 ± 0.011	0.942 ± 0.018	0.937 ± 0.037	0.929 ± 0.056
Propanol	1.031 ± 0.013	1.033 ± 0.015	1.028 ± 0.043	1.029 ± 0.071
2-Methyl-pentan-1-ol	1.007 ± 0.014	1.007 ± 0.016	1.010 ± 0.032	1.013 ± 0.052
Acetic acid	0.551 ± 0.008	0.580 ± 0.015	0.572 ± 0.026	0.684 ± 0.039
Propionic acid	0.864 ± 0.011	0.871 ± 0.020	0.878 ± 0.039	0.912 ± 0.054
Hexanol	1.091 ± 0.015	1.093 ± 0.026	1.073 ± 0.046	1.068 ± 0.054
Butyric acid	1.011 ± 0.013	1.016 ± 0.024	1.100 ± 0.054	1.016 ± 0.056

* Data obtained by using AW Carbopack B + 3.35% of PEG 20M.

The column packing used for quantitation has a relatively high thermal stability. Thus, the increase in the baseline during temperature programming is minimal. This is made evident by the fact that 2 ppm of well retained compounds, such as hexanol and butyric acid, can be determined with good precision.

Fig. 2 shows the chromatogram obtained after direct injection of 1.5 μl of

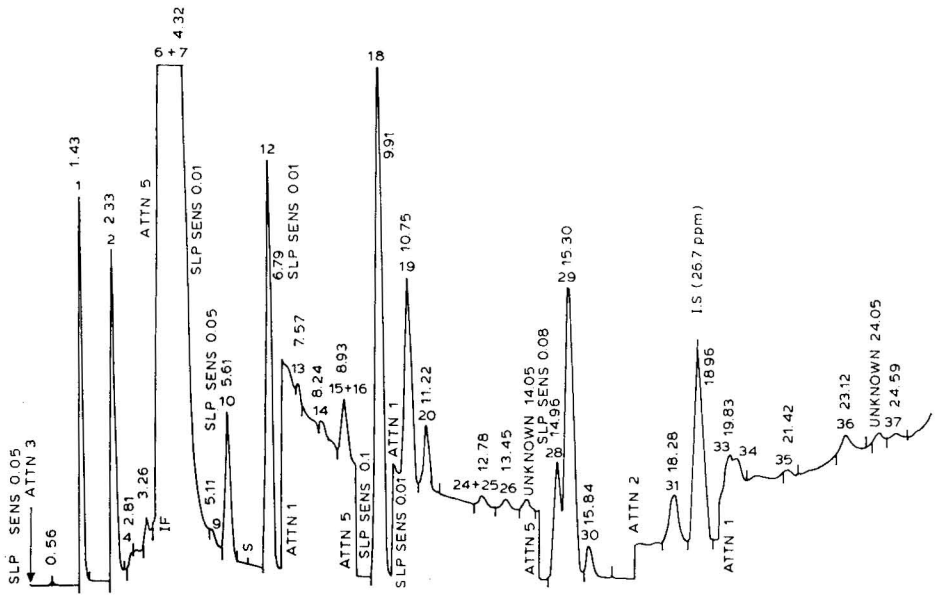

Fig. 2. Chromatogram of a commercial sample of Scotch whisky (Johnny Walker Red Label). Experimental conditions and peak numbering as in Fig. 1A.

an actual sample of Scotch whisky (Johnny Walker Red Label), using AW Carbopack B + 6.6% of PEG 20M.

An additional column packing of AW Carbopack B + 3.35% of PEG 20M was used for the determination of those few components which are not separated by the former column packing. Methyl ethyl ketone was used as the internal standard in this instance as the peak for methyl amyl ketone overlaps that for isoamyl acetate.

The results obtained are given in Table II. The mean standard deviations obtained over a set of ten determinations for components present at concentration under 1 ppm were about 25%. As can be seen, the agreement between the data for isoamyl alcohols obtained with the two column packings is good. The increase in the baseline just before the appearance of the ethanol peak is due to water. This disturbance by water does not affect significantly the determination of traces of acetone and methyl

TABLE II

DETERMINATIONS OF CONGENERS IN A COMMERCIAL SAMPLE OF SCOTCH WHISKY

Results are concentrations (ppm, w/w) ± standard deviations for 6 determinations.

Congener	*AW Carbopack B + 6.6% of PEG 20 M*	*AW Carbopack B + 3.35% of PEG 20M*
Acetaldehyde	24 ± 2	
Methanol	34 ± 2	
Acetone	0.41 ± 0.05	
Methyl acetate	2.4 ± 0.5	
Isobutanal	— —	1.6 ± 0.3
Isopropanol	1.8 ± 0.6	1.7 ± 0.2
Ethyl acetate	82 ± 1	
Propanol	260 ± 2	
Isopentanal	0.54 ± 0.07	
sec.-Butanol	0.42 ± 0.06	
Pentanal	2.85 ± 0.09	0.2 ± 0.1
Ethyl propionate		2.8 ± 0.3
Isobutanol	415 ± 3	
Acetal	11.5 ± 0.5	
Butanol	1.4 ± 0.2	
Pentan-2-ol	0.56 ± 0.09	Traces
Isobutyl acetate		0.4 ± 0.1
Ethyl butyrate	0.61 ± 0.03	
Unknown (t_R: 14.05 min)	0.68 ± 0.07	
2-Methylbutan-1-ol	101.2 ± 0.9	106.5 ± 0.6
3-Methylbutan-1-ol	282 ± 2	297 ± 1
Acetic acid	54 ± 1	
Isoamyl acetate	7.4 ± 0.4	
Furfural	3.1 ± 0.4	
Propionic acid*		1.2 ± 0.1
Hexanol	0.3 ± 0.1	
Isobutyric acid	0.6 ± 0.2	
Unknown (t_R: 24.05 min)	0.6 ± 0.2	
Butyric acid	0.5 ± 0.2	

* Data for propionic acid were obtained from the second column as the electronic integrator added the areas for furfural and propionic acid.

acetate. By using AW Carbopack B modified with 3.35% of PEG 20M the isopropanol peak appears on the terminal part of the tail of the peak for ethanol, thus making the determination of traces of isopropanol more accurate than by using AW Carbopack B modified with 6.6% of PEG 20M.

As far as column stability is concerned, no significant variation of column performance was noted during continuous use for 2 months. During this period, about 200 samples of aqueous alcoholic solutions were injected directly into the column packings. The only precaution used was to keep the columns at 220°C overnight after a few days of use, in order to eliminate from the column high-boiling compounds contained in the caramel that is used as an additive for whisky.

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Note

Error incurred in gel permeation chromatography by using the elution peak volume in lieu of the elution mean volumes in the calculation of K_{av}

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The model of chromatography suggested by Lapidus and Amundson[1] says that the concentration profile in a packed bed follows the equation

$$D\frac{\delta^2 c}{\delta Z^2} = U\frac{\delta c}{\delta Z} + \frac{\delta c}{\delta t} + \frac{I\delta n}{\alpha\delta t} \tag{1}$$

where D = axial dispersion coefficient
c = concentration in moving phase
Z = axial position, $0 \leqslant Z \leqslant L$
t = time, $t \geqslant 0$
U = fluid velocity through bed interstices
α = fractional bed void volume

and

$$n = Kc \tag{2}$$

which is the condition of equilibrium between mobile phase concentration, c, and immobile phase concentration, n (K = partition coefficient). Substituting eqn. 2 into eqn. 1 yields

$$\bar{D}\frac{\delta^2 c}{\delta Z^2} = \bar{U}\frac{\delta c}{\delta Z} + \frac{\delta c}{\delta t} \tag{3}$$

where

$$\bar{D} = D/\left(1 + \frac{K}{\alpha}\right) \tag{4}$$

$$\bar{U} = U/\left(1 + \frac{K}{\alpha}\right) \tag{5}$$

If a moving frame of reference is adopted wherein

$$t = Z - \bar{U}t \tag{6}$$

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then eqn. 3 becomes

$$\frac{\delta c}{\delta t} = \bar{D}\frac{\delta^2 c}{\delta x^2}$$

where x is a coordinate which moves with the center of mass of the concentration, c. The conditions on this equation are

$$\text{at } t = 0,\ c = \mathrm{H}(x)c_0\frac{Q}{V} \tag{7}$$

where $c_0\mathrm{H}(x)$ is a pulse function of concentration c_0 and V is the column volume. The solution to this problem is then[2]

$$\frac{cV}{c_0 Q} = \frac{L}{(4\pi\bar{D}t)^{1/2}}\exp -(x^2/4\bar{D}t) \tag{8}$$

which may be rearranged to

$$\frac{cV}{c_0 Q} = \frac{\exp -\left\{\left(1 - \frac{v}{V}t\right)^2/4\left(\frac{v}{V}t\right)\left(\frac{\bar{D}}{\bar{U}L}\right)\right\}}{\left\{4\pi\left(\frac{v}{V}t\right)\left(\frac{\bar{D}}{\bar{U}L}\right)\right\}^{1/2}} \tag{9}$$

where v = the interstitial fluid volume flow-rate
Q = sample volume
L = the column length.

It is important to note that

$$\frac{\bar{D}}{\bar{U}L} = \frac{D}{UL} \tag{10}$$

so that this term is independent of K. In order to estimate the error caused by using peak concentrations to mark the eluent volume instead of the mean concentration, it is necessary to compute the difference between the time when the peak arrives and the mean passes. The peak concentration arrives at

$$\left(\frac{v}{V}t\right)_{\text{peak}} = \left\{\left(\frac{\bar{D}}{\bar{U}L}\right)^2 + 1\right\}^{1/2} - \frac{\bar{D}}{\bar{U}L} \tag{11}$$

while the mean concentration arrives at

$$\left(\frac{v}{V}t\right)_{\text{mean}} = 1.0 \tag{12}$$

so that the difference between the two is

$$\Delta\left(\frac{v}{V}t\right) = \left(\left(\frac{\bar{D}}{\bar{U}L}\right)^2 + 1\right)^{1/2} - \left(\frac{\bar{D}}{\bar{U}L} + 1\right) = ERR \tag{13}$$

which ensures that the peak and the mean volumes are always different. This term is a measure of the fractional error incurred by using the peak elution volume and, according to eqn. 10, it has no dependence on K. Defining

$$K_{\text{av}} = \frac{V_e - V_0}{V_\tau - V_0} \tag{14}$$

where V_e is the mean concentration *elution* volume, V_τ is the total column volume accessible to small molecules and V_0 is the column void volume.

The corresponding peak elution volumes are then

$$\left.\begin{aligned} V_e^P &= (1 - ERR)V_e \\ V_\tau^P &= (1 - ERR)V_\tau \\ V_0^P &= (1 - ERR)V_0 \end{aligned}\right\} \tag{15}$$

where ERR is a function only of the column parameters and not of the partition coefficient so that

$$K_{av}^P = \frac{V_e^P - V_0^P}{V_\tau^P - V_0^P} = \frac{(1 - ERR)V_e - (1 - ERR)V_0}{(1 - ERR)V_\tau - (1 - ERR)V_0} = K_{av} \tag{16}$$

so that there is no net error incurred by using peak concentration volumes to measure K_{av} so long as all the volumes are measured according to the peak value.

It is important to consider the error that is routinely made in gel permeation chromatography. Both Bio-Rad[3] and Pharmacia[4] define V_τ as the volume occupied by the entire gel phase plus the interstitial bed volume. Using this definition of V_τ automatically introduces an error into the calculation of K_{av} because no solute may occupy the solid part of the gel phase. Also, because experimental measurement of V_e and V_0 is usually done using peak volumes the total error present in much of the reported data is

$$\frac{\Delta K_{av}{}^*}{K_{av}} \approx \frac{V_{gel}}{V_\tau - V_0} + \frac{ERR\ V_\tau}{V_\tau - V_0} \tag{17}$$

where the first term on the right is approximate and is roughly the percent of solids in the gel phase, usually between one and ten percent and the second term represents the error incurred by using the mean total volume with peak elution and void volumes. This term may often be on the order of a few percent and these two terms are *never* offsetting. Also, this total error is large enough and of the correct sign to account for the difference between the theoretical and experimental measurement of K_{av} (ref. 5).

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* $\Delta K_{av} = \dfrac{V_e^P - V_0^P}{V_\tau - V_0^P} - \dfrac{V_e^P - V_0^P}{V_\tau^P - V_0^P}$, where $V_\tau \approx V_{gel} + V_\tau$.

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Note

Zur Korrektur von R_F-Werten nach Galanos und Kapoulas

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Trotz wesentlicher Fortschritte auf dem Gebiet der Experimentiertechnik und der Herstellung hochwertiger Sorbentien und Fertigschichten sind die retentionswirksamen Einflussfaktoren in der Dünnschichtchromatographie nicht vollständig zu beherrschen. Die Bewertung der Trennresultate anhand der R_F-Werte ist daher nur mit Einschränkung möglich. Galanos und Kapoulas[1] haben 1964 ein empirisches Korrekturverfahren vorgeschlagen, mit dem nichtkontrollierbare Einflüsse auf den R_F-Wert eliminiert werden können. Die Brauchbarkeit dieser R_F-Korrektur ist vor allem durch einen Ringversuch unterstrichen worden[2]. Eine kritische Wertung hat Geiss vorgenommen[3].

Es war Zweck der vorliegenden Arbeit festzustellen, in welchem Umfang das chromatographische Milieu geändert werden kann, ohne die Grenzen praktisch vertretbarer Schwankungen des korrigierten R_F-Wertes zu überschreiten. Mit geringfügigen Abänderungen ist der erwähnte Ringversuch weitergeführt worden.

THEORIE

Der korrigierte R_F-Wert (R_F^c) einer Substanz i wird nach der folgenden Gleichung erhalten:

$$R_{F_i}^c = a \cdot R_{F_i} + b$$

Die Konstanten dieser Geradengleichung sind hier Übertragungsfaktoren, mit denen der R_F-Wert aus einem, in gewissen Grenzen beliebigen, System j* rechnerisch in ein willkürlich gewähltes Bezugssystem übertragen werden kann. Zur Bestimmung der Konstanten chromatographiert man zwei Bezugssubstanzen, I und II, in beiden Systemen. Dann gilt

$$a = \frac{R_{F_{II}}^c - R_{F_I}^c}{R_{F_{IIJ}} - R_{F_{IJ}}} \quad (R_{F_{II}} > R_{F_I})$$

und

$$b = R_{F_{II}}^c - a \cdot R_{F_{IIJ}}$$

* Unter "System" wird die Gesamtheit aller auf den R_F-Wert einwirkenden Faktoren verstanden.

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Die Gültigkeitsgrenzen dieses Korrekturverfahrens sind theoretisch nicht ableitbar. Formal sind sie an die Bedingung geknüpft, dass sowohl die Substanz *i* wie auch die Bezugssubstanzen der Geradengleichung genügen. Die Erfüllung dieser Bedingung kann von vornherein für chemisch verwandte Substanzen unter isokratischen Trennbedingungen erwartet werden.

EXPERIMENTELLES

Für alle Untersuchungen wurden Trägerplatten 8.5 × 10 cm (Photodiagläser) verwendet. Die Distanz Eintauchlinie–Start betrug 10 mm, die Trennstrecke 75 mm. Folgende Trennkammertypen kamen zum Einsatz:

(1) Normalkammer für aufsteigende Entwicklung, gesättigt (modifizierter Hohlglasziegel, Fig. 1a);

(2) Sandwichkammer für aufsteigende Entwicklung (Eigenbau, Fig. 1b);

(3) Spezialkammer für horizontale Entwicklung und Milieukontrolle (Eigenbau, Fig. 1c).

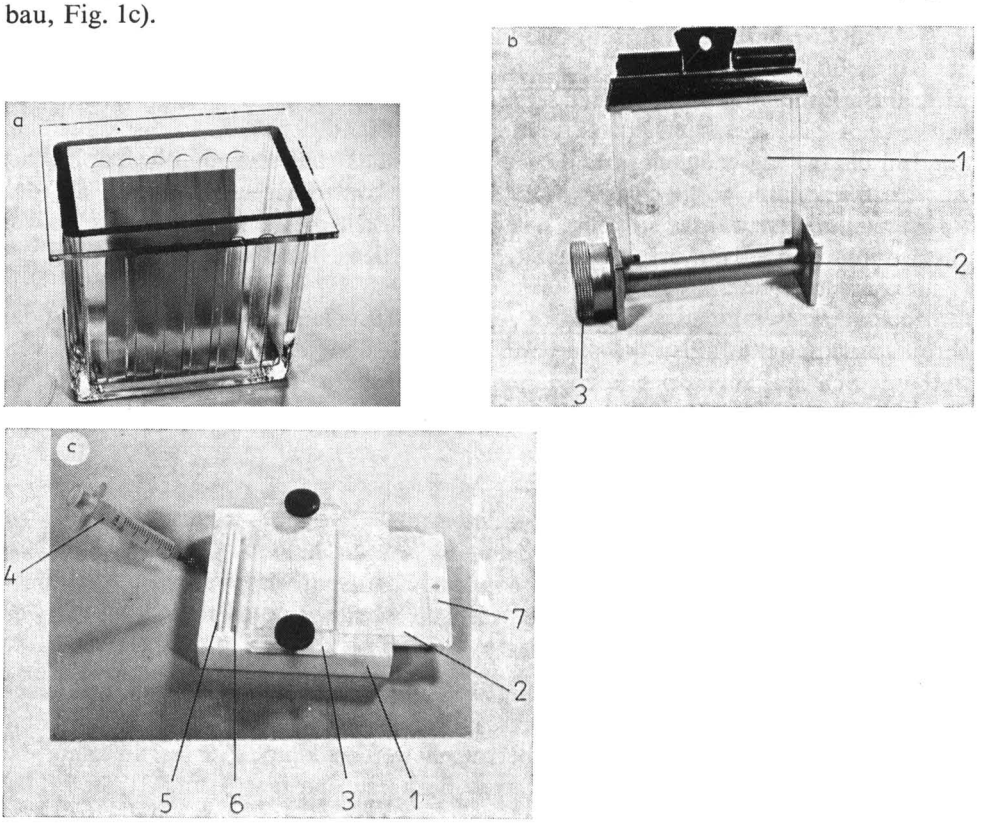

Fig. 1. Entwicklungskammern für das Plattenformat 8.5 × 10 cm. (a) Normalkammer (modifizierter Hohlglasziegel). (b) Sandwichkammer (Eigenbau); 1 = Sandwichkammer mit DC-Platte, 2 = Fliessmittelbehälter, 3 = Verschraubung von 2. (c) Konditionierkammer (Eigenbau); 1 = Trennkammer (PTFE), 2 = DC-Platte, 3 = Verschlussbügel, 4 = Injektionsspritze für Fliessmittelzufuhr, 5 = Docht zur Fliessmittelübertragung, 6 = Konditionierraum, 7 = Trennschieber zum Verschliessen von 6.

Als Sorbentien wurden Kieselgel und Aluminiumoxid verschiedenster Qualität und Herkunft verwendet (Tabellen I und II). Testsubstanzen waren Sudan G für Kieselgel und Sudan III für Aluminiumoxid. Als Bezugssubstanzen dienten Indophenol und Buttergelb bzw. *p*-Aminoazobenzol und *p*-Methoxyazobenzol. Die Fliessmittel waren Benzol, Toluol und Chloroform, alle chromatographisch rein.

TABELLE I

VERSUCHSBEDINGUNGEN DER IN TABELLE III AUSGEWERTETEN 16 SYSTEME

M = Glühprodukt von Bayerit; N = Glühprodukt von Böhmit; O = Glühprodukt von Hydrargillit (600°C, 30 h); P = Al_2O_3 G (Merck, Darmstadt, B.R.D.); R = Al_2O_3 T (Merck); S = Al_2O_3 basisch (Woelm, Eschwege, B.R.D.); T = Al_2O_3 Sauer (Woelm); NK = Normalkammer; SK = Sandwichkammer; + = Sättigung durch Kammerauskleidung bzw. fliessmittelgetränkte Gegenschicht; − = ohne Sättigung; ? = nicht kontrolliert; B = Benzol; T = Toluol.

Lfd. Nr.	*Sorbens*	*Kammertyp*	*Sättigung*	*rel. Feuchte in %*	*Fliessmittel*
1	M	NK	+	16.1	B
2	M	NK	+	45.6	B
3	N	NK	+	16.1	T
4	N	NK	+	35.2	B
5	N	NK	−	?	B
6	O	SK	+	45.6	B
7	O	NK	+	16.1	B
8	O	NK	+	?	T
9	P	SK	−	?	T
10	R	SK	−	45.6	B
11	R	NK	+	45.6	B
12	R	NK	−	?	T
13	S	SK	−	35.2	B
14	S	NK	+	16.1	B
15	T	NK	+	35.2	B
16	M	SK	−	45.6	T

TABELLE II

VERSUCHSBEDINGUNGEN DER IN TABELLE IV AUSGEWERTETEN 21 SYSTEME

A = Kieselgel G (Merck); B = Kieselgel H (Merck); C = Kieselgel DC (Woelm); D = Kieselgel S (Macherey, Nagel & Co., Düren, B.R.D.); E = Kieselgel G (Macherey, Nagel & Co.); F = Kieselgel G Fertigplatte (Merck); NK = Normalkammer; SK = Sandwichkammer; KK = Konditionierkammer; + = DC Platte 30 mm bei 120°C im Trockenschrank erhitzt; − = kleine Aktivierung; T = Toluol; Bz = Benzol; Chl = Chloroform.

Lfd. Nr.	*Sorbens*	*Kammertyp*	*Aktivierung*	*Fliessmittel*
1	A	NK	+	T
2	B	NK	−	T
3	C	NK	−	Bz
4	C	NK	−	T
5	E	NK	+	Bz
6	E	SK	+	Bz
7	A	SK	−	T
8	D	SK	−	Chl
9	A	SK	+	Chl
10	A	KK	+	T
11	B	KK	+	T
12	E	KK	−	Bz
13	F	KK	−	Chl
14	D	NK	+	T
15	A	NK	+	Chl
16	B	SK	−	T
17	E	SK	+	T
18	F	NK	−	T
19	F	KK	+	Bz
20	F	SK	+	T

In zwei zusammenfassenden Übersichtsexperimenten wurden die Parameter Schichtqualität, Trennkammertyp, Schichtaktivität und Fliessmittel willkürlich zu 16 Systemen für Aluminiumoxidschichten (Tabelle I) bzw. Systemen für Kieselgelschichten (Tabelle II) kombiniert. Um dabei der noch immer verbreiteten Praxis zu entsprechen, wurde bei der Kieselgelserie die Änderung der Schichtaktivität durch halbstündiges Erhitzen der Schichten bei 120°C im Trockenschrank und anschliessendes Abkühlen im Exsikkator vorgenommen.

ERGEBNISSE UND DISKUSSION

In Fig. 2a sind die $R_F{}^c$-Werte von Sudan G (3) den R_F-Werten auf Kieselgelen verschiedener Herkunft (A–F) gegenübergestellt. Die Entwicklung erfolgte in der Sandwichkammer mit Chloroform als Fliessmittel. Die Schichtaktivität hat sich frei eingestellt. Die Bezugswerte wurden dem oben genannten Ringversuch entnommen (zitiert in Lit. 3): R_F (Indophenol) = 0.132; R_F (Buttergelb) = 0.467. Alle Werte sind aus fünf Einzelresultaten gemittelt. Während sich die R_F-Werte zwischen 0.24 und 0.57 ändern, liegen die $R_F{}^c$-Werte im Bereich 0.21–0.23; sie sind befriedigend konstant.

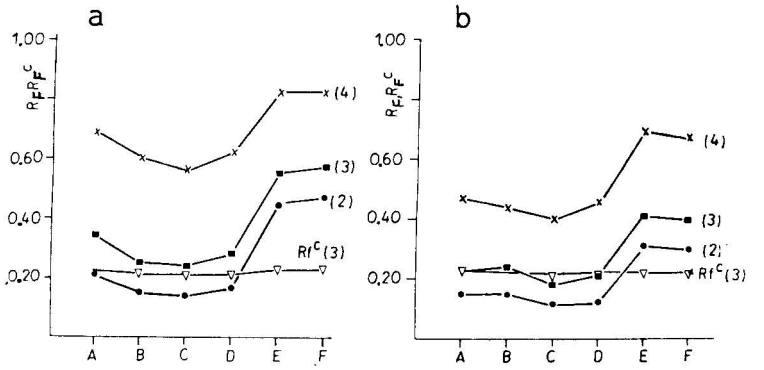

Fig. 2. R_F- und $R_F{}^c$-Werte auf Kieselgelschichten. (a) Sandwichkammer; (b) gesättigte Normalkammer (Auskleidung mit fliessmittelgetränktem Filterpapier). Testsubstanz: Sudan G (Kurve 3); Bezugssubstanzen: Buttergelb (4) und Indophenol (2); R_F-Werte der Bezugssubstanzen: R_F (4) = 0.47; R_F (2) = 0.13 (entnommen aus Lit. 3); Fliessmittel: Benzol; relative Feuchte (Schichtaktivität): nicht kontrolliert; Sorbentien: A–F (vgl. Tabelle II).

Die Fig. 2b zeigt die entsprechenden Ergebnisse in einer Normalkammer. Die R_F-Werte sind erwartungsgemäss erniedrigt, der Unterschied in den $R_F{}^c$-Werten ist dagegen statistisch nicht signifikant. Die Veränderung des R_F-Wertes mit der relativen Feuchte in der Trennkammer kann ebenfalls mit dem Korrekturverfahren kompensiert werden, wie Fig. 3a für Aluminiumoxidschichten zeigt. An diesem Beispiel wird ausserdem demonstriert, dass die Wahl der Bezugssubstanzen für das Funktionieren des Korrekturverfahrens von entscheidender Bedeutung sein kann. Die $R_F{}^c$-Werte in der Fig. 3a wurden mit den Bezugssubstanzen (2) und (4) berechnet. Diese wandern auf dem Chromatogramm zu beiden Seiten der Testsubstanz. Mit den Bezugssubstanzen (4) und (5) bzw. (1) und (5), die einseitig bzw. in grösserer Entfernung zur Testsubstanz laufen, werden stark fallende bzw. schwankende $R_F{}^c$-Werte gemessen. Diese Resultate zeigen, dass die dem Korrekturverfahren zugrunde liegende Voraussetzung der proportionalen Veränderung aller R_F-Werte beim Wechsel

TABELLE III

R_F- UND R_F^c-WERTE, GEMESSEN IN DEN 16 SYSTEMEN DER TABELLE I

I = *p*-Aminoazobenzol; II = *p*-Methoxyazobenzol; s = Sudan III. Mittelwert: $R_{Fs}^c = 0.644 \pm 1.8\%$; Bezugssystem: Nr. 13.

Lfd. Nr.	R_{FI}	R_{FII}	R_{Fs}	R_{Fs}^c
1	0.31	0.62	0.49	0.64
2	0.28	0.62	0.47	0.63
3	0.36	0.70	0.57	0.65
4	0.36	0.71	0.57	0.65
5	0.43	0.84	0.66	0.62
6	0.58	0.91	0.78	0.64
7	0.33	0.67	0.53	0.64
8	0.29	0.69	0.52	0.64
9	0.36	0.85	0.65	0.64
10	0.62	0.95	0.81	0.64
11	0.46	0.68	0.59	0.64
12	0.33	0.69	0.53	0.63
13	0.37	0.83	0.63	0.63
14	0.34	0.65	0.51	0.62
15	0.21	0.60	0.40	0.62
16	0.37	0.84	0.60	0.62

TABELLE IV

R_F- UND R_F^c-WERTE, GEMESSEN IN DEN 20 SYSTEMEN DER TABELLE II

I = Indophenol; II = Buttergelb; s = Sudan G. Bezugsgrössen: $R_{FI} = 0.132$; $R_{FII} = 0.467$ (entnommen aus Lit. 3).

Lfd. Nr.	R_{FI}	R_{FII}	R_{Fs}	R_{Fs}^c
1	0.06	0.41	0.12	0.19
2	0.13	0.58	0.21	0.21
3	0.08	0.43	0.15	0.20
4	0.10	0.58	0.21	0.21
5	0.08	0.44	0.16	0.20
6	0.06	0.41	0.17	0.23
7	0.11	0.60	0.22	0.20
8	0.47	0.85	0.69	0.33*
9	0.21	0.63	0.38	0.27*
10	0.06	0.41	0.14	0.20
11	0.04	0.39	0.14	0.23
12	0.13	0.56	0.20	0.19
13	0.21	0.60	0.37	0.27*
14	0.00	0.24	0.05	0.20
15	0.11	0.50	0.25	0.25
16	0.10	0.53	0.21	0.21
17	0.10	0.55	0.22	0.22
18	0.11	0.56	0.22	0.22
19	0.09	0.47	0.18	0.21
20	0.04	0.40	0.10	0.19

* Statistische Ausreisser; Fliessmittel = Chloroform; Mittelwert: $R_{Fs}^c = 0.206 \pm 8.3\%$.

des chromatographischen Systems nur in begrenzten Chromatogrammbereichen erfüllt ist. Grundsätzlich werden proportionale R_F-Änderungen umso eher zu erwarten sein, je geringer die Differenzen der R_F-Werte sind. Dass darüber hinaus die Grenzbereiche des Chromatogramms wegen des dort stark geänderten Fliessmittelprofils zu meiden sind, hat Geiss[3] bereits unterstrichen (vgl. Fig. 3a, Kurve 5).

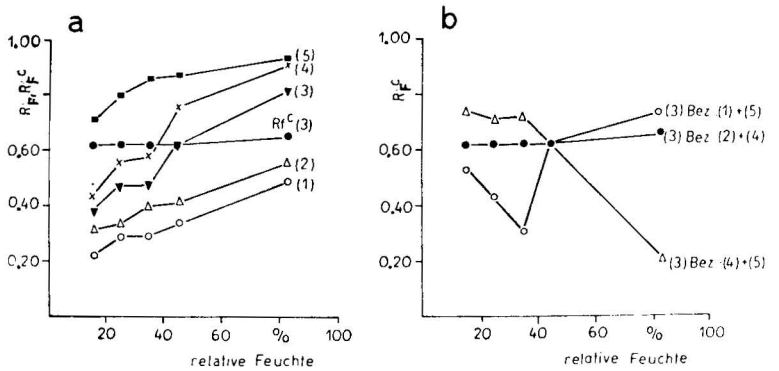

Fig. 3. Abhängigkeit des R_F- une R_F^c-Wertes von der relativen Feuchte in der Trennkammer. (a) R_F-Werte und R_F^c-Wert von Sudan III; (b) R_F^c-Werte von Sudan III für verschiedene Bezugssubstanzen. Testsubstanz: Sudan III (Kurve 3); Bezugssubstanzen: *p*-Hydroxyazobenzol (1), *p*-Aminoazobenzol (2), *p*-Methoxyazobenzol (4), Azobenzol (5); Bezugssystem: 45.6% rel. F; Fliessmittel: Toluol; Sorbens: Aluminiumoxid G (Merck).

Die Resultate der beiden Übersichtsversuche sind in den Tabellen III und IV zusammengefasst. Wie zu erwarten, schwanken die R_F-Werte beträchtlich. Die R_F^c-Werte dagegen sind in Anbetracht der teilweise extrem geänderten Versuchsbedingungen erstaunlich konstant. Für Sudan G ergibt sich $R_F^c = 0.21 \pm 8.3\%$. Aus dem erwähnten Ringversuch folgt für den gleichen Farbstoff $R_F^c = 0.22 \pm 6.4\%$. Auf Aluminiumoxidschichten hat Sudan III einen R_F^c-Wert von 0.64 bei einem prozentualen Fehler von nur 1.8% (Tabelle III). Die in Klammer gesetzten Werte der Tabelle IV sind statistisch gesicherte Ausreisser, die mit Chloroform als Fliessmittel erhalten wurden. Zwar hat Chloroform in der Snyderschen[4] Fliessmittelskala mit $\varepsilon^0 = 0.26$ nahezu die gleiche Fliessmittelstärke wie Benzol ($\varepsilon^0 = 0.25$), bedingt aber eine andere Selektivität, so dass das Korrekturverfahren versagt. Andererseits erweist sich hier Toluol dem Benzol gegenüber als gleichwertig; ein Grund mehr, dieses wegen der wesentlich geringeren Toxizität grundsätzlich vorzuziehen.

Als völlig unbrauchbar hat sich das Korrekturverfahren erwiesen, wenn mehrkomponentige und zudem sehr heteropolare Fliessmittelgemische verwendet werden[5]. Dann waren die Einflüsse der Schichtaktivität und des Kammertyps (Sandwich- oder gesättigte Normalkammer) auf den R_F-Wert nicht mehr zu kompensieren. Die Ursache hierfür ist sicher in der Entmischung der Fliessmittel zu sehen. Dann ist die Trennschicht auch im engeren R_F-Bereich nicht mehr gradientfrei, so dass die R_F-Werte nicht mehr proportional, d.h. mit dem Steigungsmass *a* der Geradengleichung verändert werden.

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Note

High-performance liquid chromatography of *Solanum* and *Veratrum* alkaloids

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Since our first publication on high-performance liquid chromatography (HPLC) of steroidal alkaloids in 1976[1], only two papers have appeared on this subject. Bushway *et al.*[2] have described the separation of potato glycoalkaloids, and Crabbe and Fryer[3] have applied HPLC to the quantitative analysis of solasodine glycosides. While we have used adsorption chromatography on a silica column for the aglycones, reversed-phase partition chromatography was applied in both cases where the more polar glycoalkaloids were analyzed.

We have continued to rely on adsorption, but the substitution of Zorbax-Sil for the coarser Porasil A has greatly improved resolution and shortened the analysis time. Instead of increasing the polarity of the eluent, we now use an increase in flow-rate for accelerating the elution of more polar steroids. The use of a UV detector is an obvious improvement over the testing of fractions by thin-layer chromatography, practiced earlier.

EXPERIMENTAL*

The HPLC apparatus was assembled from commercially available components. A solvent reservoir was connected to the inlet of a single-piston reciprocating pump (Model 110; Altex, Berkeley, CA, U.S.A.). From the pump outlet stainless-steel tubing led to a sample injection valve (Model 7125; Rheodyne, Berkeley, CA, U.S.A.) with a loop volume of 100 μl and from there to the column inlet.

The column consisted of two stainless-steel tubes, 250 × 4.6 mm I.D., prepacked with Zorbax-Sil (particle size 6 μm, DuPont, Wilmington, DE, U.S.A.), connected in series. The column outlet was connected to the inlet of a variable-wavelength detector (Model 155, Altex) with a flow cell having a 10-mm pathlength and a 20-μl volume, which was set at 213 nm, 0.2 full scale. The effluent from the detector was returned to the reservoir.

The signal from the detector was fed into a single-channel recorder (Model 335; Linear, Irvine, CA., U.S.A.), which was set at 10 mV. Only *n*-hexane was HPLC

* Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

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grade ("Distilled-in-Glass" quality; Burdick & Jackson Labs., Muskegon, MI, U.S.A.); the other solvents were of reagent grade.

We have tested 14 *Solanum* and *Veratrum* alkaloids in two groups. The less polar compounds, mainly *Solanum* alkaloids, were separated by use of *n*-hexane–methanol–acetone (18:1:1) as the eluent (Fig. 1). After the elution of the first four steroids, the flow-rate was increased from 0.3 ml/min to 1.5 ml/min. The more polar *Veratrum* alkaloids were separated by use of *n*-hexane–ethanol–acetone (18:1:1) as the eluent (Fig. 2) at a flow-rate of 1.0 ml/min. After the elution of the first two steroids, the flow-rate was increased to 1.6 ml/min.

Rubijervine was included in both groups and served as a reference for the determination of the relative retention times. These were found to vary by no more than $\pm$ 5%. As expected, rubijervine, having 2 hydroxyl groups, was more polar than any of the monohydroxysteroids in the first group (Fig. 1). Tomatillidine, which

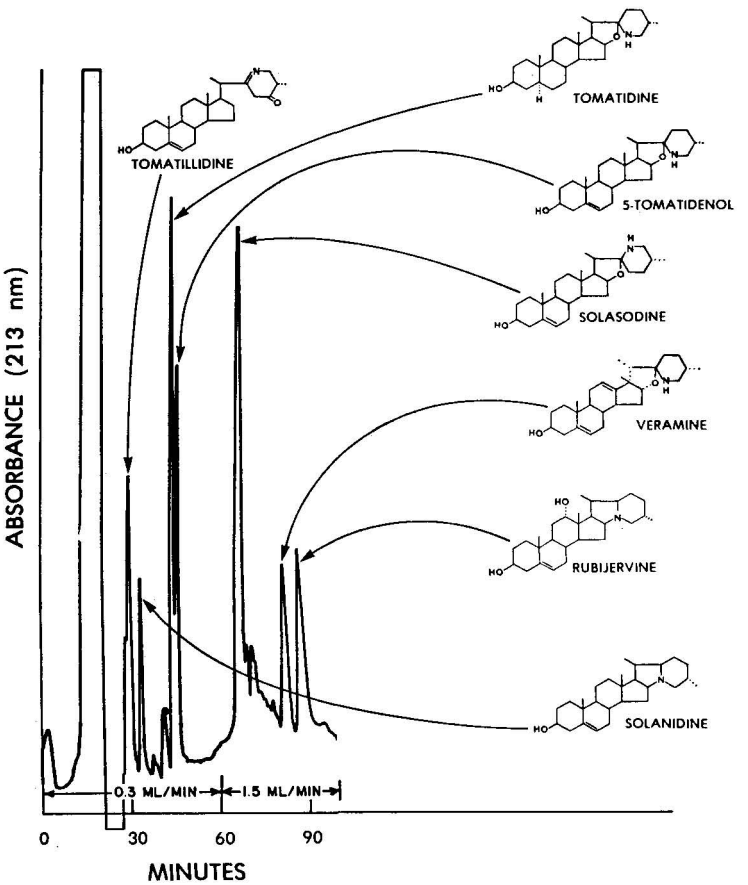

Fig. 1. Separation of the less polar steroidal alkaloids by HPLC. A mixture of 6.3 μg tomatillidine, 13 μg solanidine, 210 μg tomatidine, 13 μg 5-tomatidenol, 84 μg solasodine, 70 μg veramine, and 40 μg rubijervine in 20 μl dichloromethane-2-propanol (1:1) was applied to a Zorbax-Sil column, 500 $\times$ 4.6 mm I.D. Eluent, *n*-hexane–methanol–acetone (18:1:1); flow-rate, 0.3 ml/min; pressure, 800 p.s.i.; for 60 min. Afterwards, flow-rate, 1.5 ml/min; pressure 5000 p.s.i. Detector at 213 nm, range 0.2; recorder speed 6 cm/h, span 10 mV.

would be expected to show an intermediate polarity, being a monohydroxymonoketone, turned out to be the least polar compound, apparently because it has a tertiary nitrogen. This is followed by solanidine with its condensed ring system. Tomatidine was slightly less polar than its Δ^5-analog, 5-tomatidenol. However, solanidine was not separated from its 5α-analog, demissidine (not shown). The separation of the two 22-epimers, 5-tomatidenol and solasodine, is remarkable. In contrast to earlier results[1], the order of elution of solanidine and tomatidine was reversed.

Among the more polar alkaloids (Fig. 2), the condensed-ring isomers isorubijervine and rubijervine were eluted ahead of the C-*nor*-D-*homo*-steroids, as expected. However, isorubijervine with its primary hydroxyl at C-18 would have been expected to be more polar than rubijervine with its secondary hydroxyl at C-12. Certainly,

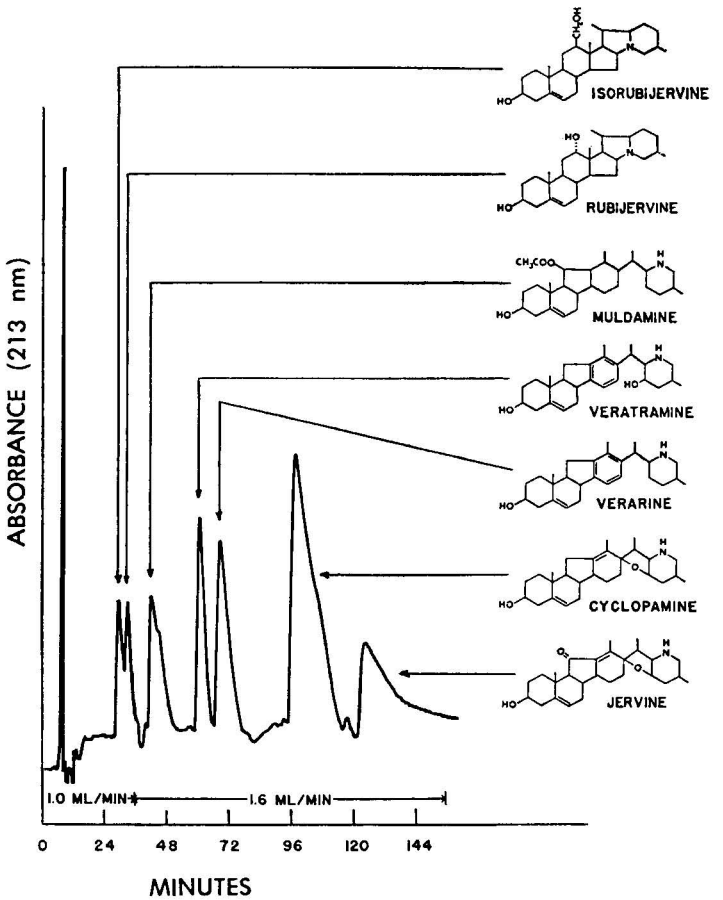

Fig. 2. Separation of the more polar steroidal alkaloids by HPLC. A mixture of 16 μg rubijervine, 56 μg isorubijervine, 210 μg muldamine, 55 μg veratramine, 30 μg verarine, 183 μg cyclopamine, and 540 μg jervine in 31 μl 2-propanol was applied to the same column as in Fig. 1. Eluent, *n*-hexane–ethanol–acetone (18:1:1); flow-rate, 1.0 ml/min; pressure, 2800 p.s.i.; for 36 min. Afterwards, flow-rate, 1.6 ml/min; pressure, 5000 p.s.i. For all other conditions, see Fig. 1.

steric phenomena are at play here, as is evident from the totally unexpected elution order of veratramine (2 hydroxyls) and verarine (1 hydroxyl). Otherwise, the sequence is as expected. The method obviously lends itself to the purification of alkaloids. Most of our reference compounds were found to contain impurities, which were difficult to remove by the methods available heretofore.

ACKNOWLEDGEMENTS

Gifts of reference compounds from D. F. Johnson (Laboratory of Chemistry, NIAMDD, National Institutes of Health, Bethesda, MD, U.S.A.) and from R. F. Keeler (Poisonous Plant Research Laboratory, ARS, SEA, U.S. Department of Agriculture, Logan, UT, U.S.A.) are gratefully acknowledged.

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Note

N-Chlormethyl-4-nitro-phthalimid als Derivatisierungsreagens für die Hochleistungs-Flüssigkeits-Chromatographie

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(Eingegangen am 22. April 1980; geänderte Fassung eingegangen am 13. Juni 1980)

Die, heute in der Hochleistungs-Flüssigkeits-Chromatographie (HPLC) üblich verwendeten, UV-Detektoren sind für Verbindungen mit schwachen Chromophoren nur ungenügend empfindlich. Diesem Umstand kann meist durch Derivatisierung etwaiger funktioneller Gruppen mit stark UV- bzw. fluoreszenzaktiven Reagenzien begegnet werden; eine Zusammenstellung der wichtigsten Reaktionstypen wird in den Übersichtswerken von Lawrence und Frei[1], sowie Blau und King[2] gegeben. Kürzlich stellten Lindner und Santi[3] eine neue Reagenziengruppe für die Derivatisierung von Karbonsäuren, Phenolen und Barbituraten (OH– und –NH acide Verbindungen), die N-Chlormethyl-phthalimide, vor. Weitere Untersuchungsergebnisse über N-Chlormethyl-4-nitro-phthalimid (ClMNPI), als stark UV-aktives Derivatisierungsreagens für Barbiturate und Karbonsäuren werden nun in vorliegender Arbeit beschrieben.

Ein, dem ClMNPI in seiner Reaktivität, ähnliches Derivatisierungsreagens stellt das 1-Chlormethylisatin (CMI) dar[4–6], welches hinsichtlich seiner Anwendbarkeit für die HPLC von Gübitz[7] untersucht wurde.

EXPERIMENTELLES

HPLC-System

Sämtliche Analysen wurden auf einem HPLC-System folgender Komponenten durchgeführt: Pumpe 110 A (Altex); UV-Detektor 1036 A (Hewlett-Packard); Integrator 3380 (Hewlett-Packard); Injektionssystem 7120 (Rheodyne). Als Trennsystem dienten Säulen (200 × 4.6 mm I.D.), gefüllt mit LiChrosorb RP-8 (7 μm; Merck, Darmstadt, B.R.D.).

Reagenzien

Die Lösungsmittel (Merck) hatten analytischen Reinheitsgrad und kamen ohne weitere Reinigung zur Anwendung. ClMNPI wurde analog der Methode von Böhme und Schwartz[12] synthetisiert. Das ClMNPI, sowie dessen Lösungen in trockenem Acetonitril sind bei Raumtemperatur und unter Lichtabschluss über mehrere Monate unzersetzt haltbar. Die Barbiturate hatten den für pharmazeutische Zwecke zugelassenen Reinheitsgrad.

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Derivatisierungsreaktionen

Methode A. Ein bis 100 μM Karbonsäuren bzw. Barbiturate werden in konische 1-ml Gefässe mit mindest 10-fachem molaren Überschuss ClMNPI und 3-fach molarem Überschuss TEA in 100 μl Acetonitril versetzt, verschlossen und 3 h bei 60°C gehalten. Nach Abkühlung und Zentrifugation können die Reaktionslösungen direkt auf die Säule injiziert werden.

Methode B. Wie Methode A, nur mit dem Unterschied, dass die Base TEA durch *ca.* 20-fachen Überschuss an fein gepulvertem $KHCO_3$, sowie 3-fach molarem Überschuss an Kronenäther (18-crown-6 der Firma Fluka, Buchs, Schweiz) ersetzt wird.

ERGEBNISSE UND DISKUSSION

Das Schema der ClMNPI Reaktion mit Barbituraten ist in Fig. 1 dargestellt. Die Struktur der Umsetzungsprodukte wurde durch Kernresonanz- und Massenspektroskopie gesichert, wobei besonders erwähnenswert ist, dass im Falle von $R_3 = H$ (z.B. Butalbital) beide NH-Gruppen derivatisiert werden und ein di-N-Methyl-4-nitro-phthalimid (-MNPI) Kondensationsprodukt des Barbiturates entsteht. In Barbituraten sind die pK_a Werte der beiden NH-Gruppen unterschiedlich ($pK_{a(1)} = ca.$ 7.3 bis 8.0 und $pK_{a(2)} = ca.$ 12.5) (Lit. 8); die zweite Dissoziationsstufe entspricht einer sehr schwachen Säure, deren Acidität aber trotzdem ausreicht um obige Reaktion einzugehen.

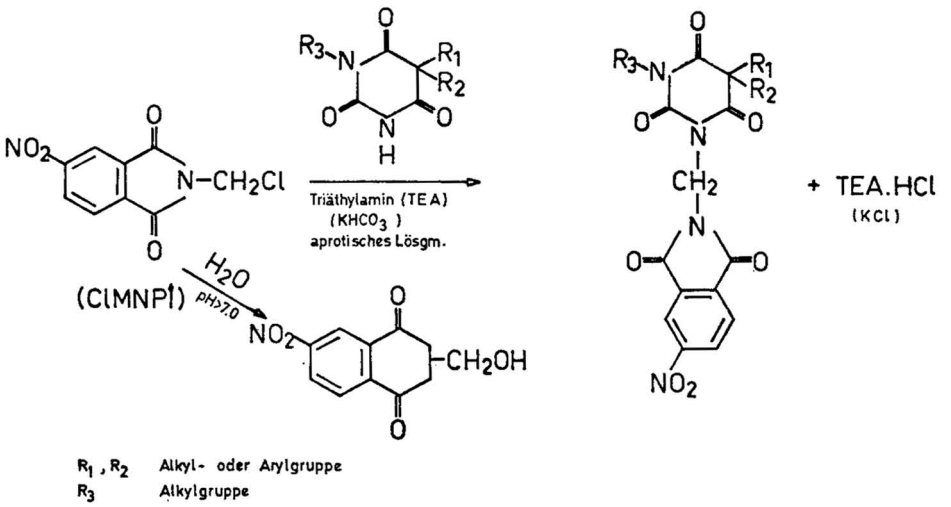

Fig. 1. Schema der ClMNPI-Reaktion mit Barbituraten.

Den reaktionskinetischen Verlauf der Butalbital-Derivatisierung gibt Fig. 2 graphisch wieder, wobei das Zwischenprodukt, das monosubstituierte MNPI–Butalbital, chromatographisch ebenfalls erfasst werden konnte (Fig. 3).

Die Interpretation der experimentellen Reaktionskurven der Hexobarbital–ClMNPI Reaktion (Fig. 4), wo nur eine freie NH-Gruppe zur Derivatisierung zur

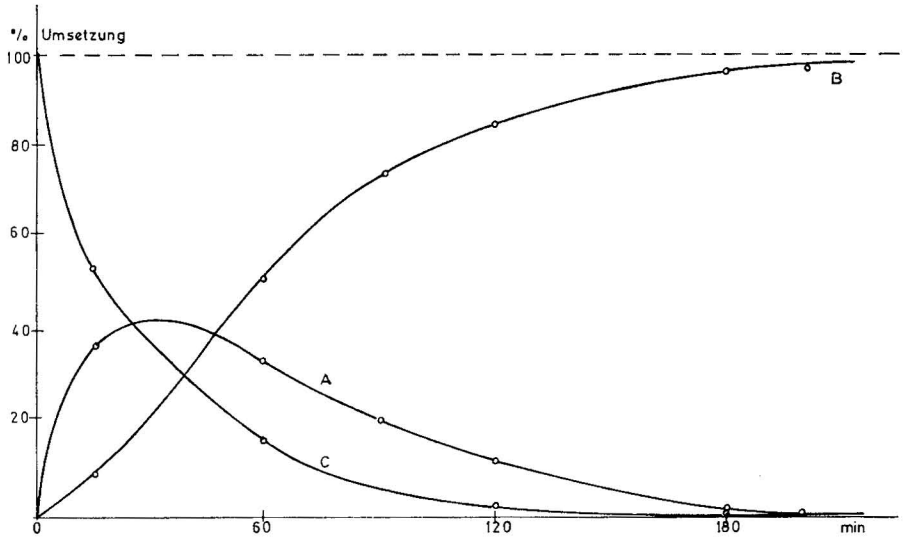

Fig. 2. Reaktionsumsatz von Butalbital mit ClMNPI in Acetonitril bei 60°C. Reaktionsbedingungen: 2 m*M* Butalbital + 6 m*M* TEA + 28 m*M* ClMNPI/ml Acetonitril. A = mono-MNPI-Butalbital; B = di-MNPI-Butalbital; C = Butalbital.

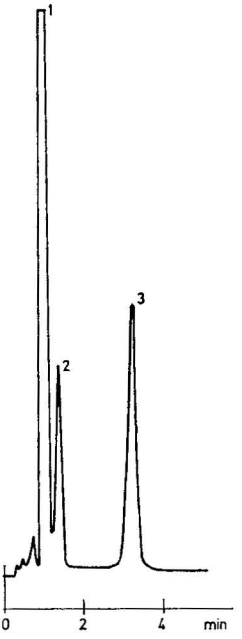

Fig. 3. Trennung von mono- und di-MNPI-substituiertem Butalbital. Säule 200 × 4.6 mm I.D., gepackt mit RP-8, 7 μm; mobile Phase Wasser–Acetonitril (55:45), Durchflussgeschwindigkeit 1 ml/min; UV bei 254 nm. Peakbezeichnung: 1 = ClMNPI; 2 = mono-MNPI-Butalbital; 3 = di-MNPI-Butalbital.

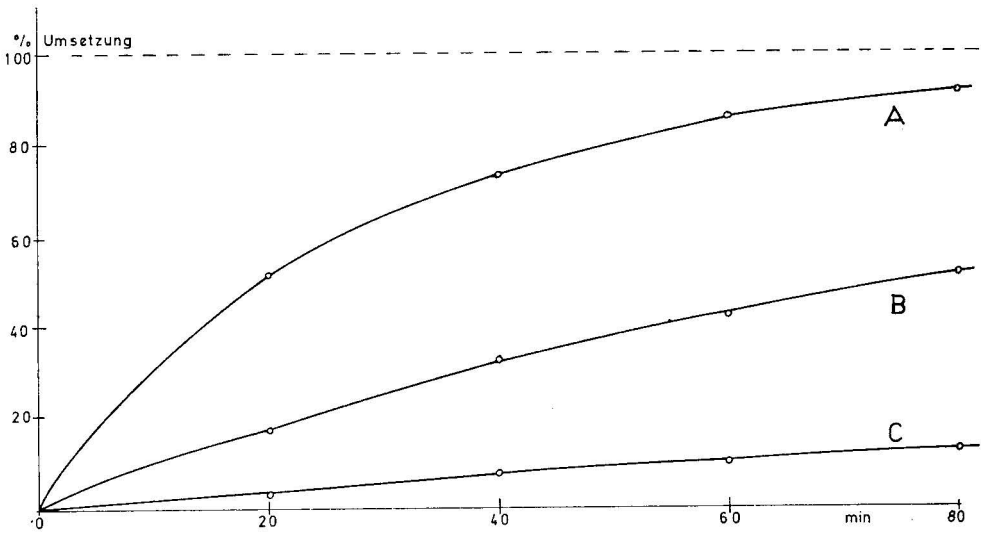

Fig. 4. Reaktionsumsatz von Hexobarbital mit ClMNPI in Acetonitril bei 60°C. Reaktionsbedingungen: A = wie Fig. 2; B = 2 m*M* Hexobarbital + 6 m*M* TEA + 4 m*M* ClMNPI/ml Acetonitril; C = zehnfache Verdünnung von B.

Verfügung steht, zeigt, dass diese Reaktion nach dem Gesetz zweiter Ordnung, wobei sie jeweils erster Ordnung in Bezug auf die beiden Reaktionspartner A (Barbiturat) und B (ClMNPI) ist, verläuft. Dies ergibt sich aus der graphischen Darstellung des log([B]/[A]) als Funktion der Reaktionszeit, wofür man annähernd eine Gerade erhält; es gilt somit die Beziehung[9]

$$v = -\frac{d[A]}{dt} = k[A]\,[B]$$

Vermutlich ist auch die Folgereaktion im Falle einer Zweifachderivatisierung, wie z.B. beim Butalbital, zweiter Ordnung, doch reichen die experimentellen Daten für eine derartige Festlegung nicht aus.

Karbonsäuren reagieren ebenfalls nach dem Gesetz zweiter Ordnung. Sofern keine sterischen Hinderungen die ClMNPI-Alkylierungsreaktion saurer Protonen beeinflussen, darf man annehmen, dass die Reaktionsgeschwindigkeit mit sinkendem pK_a Wert der aciden Gruppen steigt.

Faktoren, welche die Reaktionsgeschwindigkeit der ClMNPI-Umsetzung mit Barbituraten und Karbonsäuren (NH- und OH-acide Verbindungen) beeinflussen

(a) Wie in Fig. 1 dargestellt, läuft die Reaktion nur dann ab, wenn dem sehr aktiven Chlor im ClMNPI ein Salz einer aciden Verbindung angeboten wird. Demzufolge können die K-(Na-)Salze der Säuren direkt eingesetzt oder intermediär durch Zugabe von überschüssigem, festen, feingekörnten $KHCO_3$ zum Reaktionsmedium dargestellt werden. In beiden Fällen verwendet man vorteilhaft Kronenäther als Phasentransferkatalysator, um die ClMNPI Reaktion rasch ablaufen zu lassen. Durst *et al.*[10] setzten erstmals mit Erfolg Kronenäther als Katalysator für derartige Alkylierungsreaktionen ein.

Auch intermediär gebildete Ammoniumsalze der sauren Verbindungen (Triäthylammonium-Salze), welche durch Zugabe von überschüssigem Amin zur Säurekomponente entstehen, gehen in quantitativer Ausbeute die ClMNPI-Reaktion ein. Die Umsetzung der Ammoniumsalze verläuft allerdings langsamer als die der, durch Kronenäther katalysierten, Kaliumsalze[7,10].

(b) Nachdem die ClMNPI Reaktion zweiter Ordnung ist, erzielt man Reaktionszeitverkürzungen durch Erhöhung des Reagenzüberschusses, welcher für die Barbituratderivatisierung mindest zehnfach sein muss. Ebenso sollten die Reaktionspartner in möglichst hoher Konzentration im Reaktionsmedium vorliegen (Fig. 4).

(c) Selbstverständlich bringt auch eine Temperaturerhöhung Reaktionszeitverkürzung. Als praktikable Umsetzungstemperatur erweist sich 60°C (Trockenschrank).

(d) Die Art des Reaktionsmediums (aprotisches Lösungsmittel) bestimmt ebenfalls die Reaktionsgeschwindigkeit; sie nimmt in Richtung DMF > Acetonitril > Aceton ab. Kleinere Mengen von Wasser stören meist nicht, da die ClMNPI Umsetzung zum N-Hydroxymethyl-4-nitro-phthalimid (OHMNPI) nur langsam verläuft.

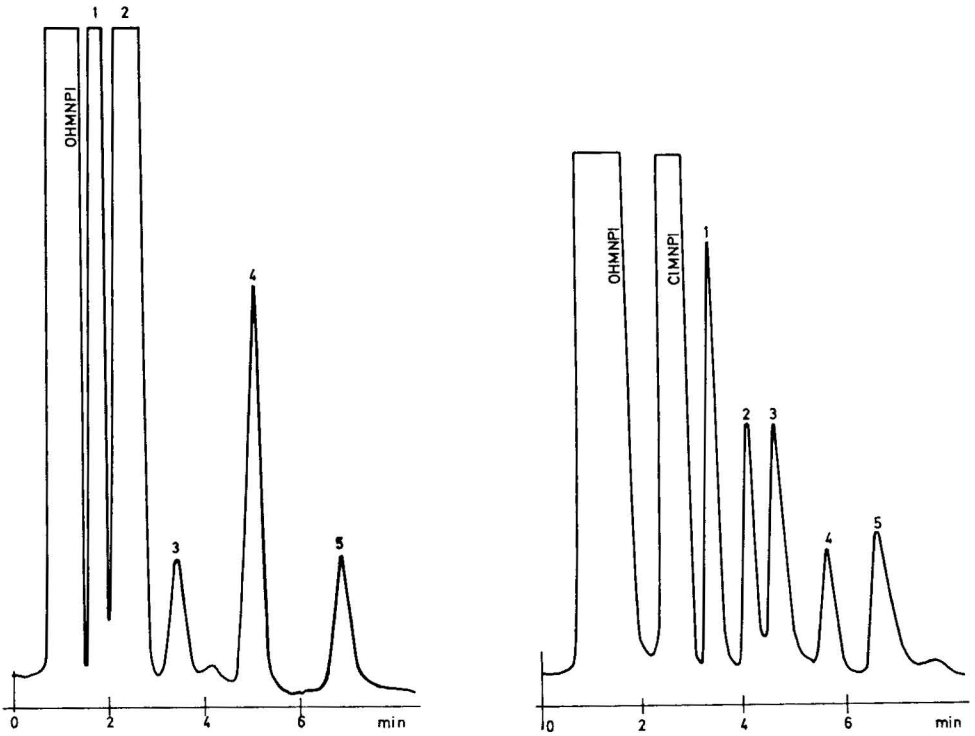

Fig. 5. Trennung von di-MNPI-substituierten Barbituraten. Säule wie Fig. 3; mobile Phase: Methanol-Wasser (11:9), Durchflussgeschwindigkeit 2 ml/min; UV bei 254 nm; Peakbezeichnung: 1 = Barbital; 2 = Phenobarbital; 3 = Aprobarbital; 4 = Butalbital; 5 = Cyclobarbital.

Fig. 6. Trennung von MNPI-derivatisierten Fettsäuren. Säule wie Fig. 3; mobile Phase: Acetonitril–Wasser (4:6), Durchflussgeschwindigkeit 2 ml/min; UV bei 254 nm; Peakbezeichnung: 1 = Essigsäure; 2 = Propionsäure; 3 = Buttersäure; 4 = Valeriansäure; 5 = 2-Äthylbuttersäure.

Abschliessend werden Anwendungsbeispiele obiger Reaktion gezeigt. Fig. 5 stellt die Trennung eines derivatisierten Barbituratgemisches dar, wobei die Substanzpeaks mit einer Absolut-Konzentration von *ca.* 30 ng pro Komponente vorliegen. Bei einem Signal zu Rausch-Verhältnis von 3:1 sind bei 254 nm etwa 3 ng derivatisiertes Phenobarbital, dem etwa 5 pmol freies Phenobarbital entsprechen, nachzuweisen und gilt in etwa für sämtliche Barbiturate. Auch für die niederen derivatisierten Fettsäuren erreicht man obige Nachweisempfindlichkeit; ihre Trennung wird in Fig. 6 demonstriert.

Zusammenfassend ersieht man, dass mit dem ClMNPI für stark und schwach saure Gruppen ein reaktionsfähiges Derivatisierungsreagenz mit hoher UV-Empfindlichkeit bei 254 nm zur Verfügung steht. Die chromatographischen Eigenschaften der Derivate gestatten den Einsatz einfacher Reversed-Phase Trennsysteme. Die ClMNPI Derivatisierung eignet sich auch gut für Barbiturat-Monitoring in Serum und Plasma, da die Nachweisempfindlichkeiten weit unterhalb der therapeutischen Blutspiegelkonzentrationen liegen.

DANK

Für die ausgezeichnete Mitwirkung an dieser Arbeit sei Herrn H. Ruckendorfer gedankt.

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Note

Trennung von Benzoesäurederivaten aus Pflanzenextrakten über Sephadex G-10-Säulen

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Zur Isolierung von Naturstoffen, Herbiziden und deren Metaboliten aus Pflanzen werden verschiedene Methoden genutzt. Zum Beispiel sind Benzoesäure und deren Derivate besonders durch Verteilungsverfahren aus Pflanzenextrakten abgetrennt worden[1,2]. Zur Fraktionierung von Stoffgemischen wird vielfach die Gelchromatographie an Sephadexsäulen eingesetzt. Hierbei wird vor allem das Prinzip der Filtration durch Dextrangele angewendet. Dextrangele haben eine hohe Affinität zu verschiedenen Verbindungen[3]. Janson[4] beschrieb die Adsorption von aromatischen Verbindungen an Sephadex. Die Adsorptionsstärke kann durch verschiedene Faktoren beeinflusst werden[5].

Ziel der vorliegenden Arbeit ist es, die Möglichkeit der Trennung von Benzoesäure und deren Derivaten aus Pflanzenextrakten mit Hilfe der Sephadex G-10-Säulenchromatographie zu untersuchen.

MATERIAL UND METHODEN

Zur Trennung von Extraktkomponenten verwendeten wir eine Sephadex G-10-Säule (30 × 1.5 cm). Auf das Sephadex G-10-Gel trugen wir eine 1 cm hohe Sephadex G-50-Schicht auf. Diese Schicht verhindert das Aufwirbeln der Oberfläche und dient gleichzeitig als Sammelschicht. Die Säule wurde an ein Uvicord II 8 300 mit dem Detektor 8303 A und einem Fallbügelschreiber 6520 (LKB, Uppsala, Schweden) angeschlossen. Das Eluat wurde bei 258 nm photometriert.

Als Versuchsobjekt diente die Zuckerrübensorte *Beta vulgaris cv. hymona*. Die Pflanzen wuchsen 4 Wochen im Gewächshaus. Je 5 g Blätter wurden gefriergetrocknet. Die Homogenisation des getrockneten Materials erfolgte im Mörser mit 10 ml 80%igem Ethanol. Nach 20stündiger Extraktion wurde 10 min bei 5000 *g* zentrifugiert. Dieser Überstand diente den weiteren Untersuchungen.

Zur Bestimmung des Verteilungskoeffizienten verwendeten wir die Verbindungen in einer Konzentration von 10^{-3} mol·l^{-1}. Als Testsubstanz wurde *o*-Chlorbenzoesäure eingesetzt. Die Berechnung des Verteilungskoeffizienten (K_D), der die Verteilung des zu untersuchenden Stoffes zwischen der mobilen Phase und dem inneren Volumen der stationären Phase ausdrückt, erfolgte nach folgender Gleichung[6]:

$$K_D = \frac{V_e - V_0}{V_i}$$

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V_e = Elutionsvolumen der Verbindung (ml), V_0 = Zwischenkornvolumen oder Volumen ausserhalb der kugelförmigen Partikel (ml); V_i = Inneres Volumen oder Volumen der Flüssigkeit innerhalb des Gels (ml).

Es wurde mit dem Elutionsvolumen einer Modellverbindung (Aceton) abzüglich dem Zwischenkornvolumen (V_o) bestimmt. Zur Bestimmung des Elutionsverhaltens setzten wir verschiedene Lösungen ein. Die Lösungen hatten folgende Zusammensetzung:

(1) pH 4.0: 61.5 ml, 0.1 mol·l^{-1} Citronensäure; 38.5 ml, 0.2 mol·l^{-1} Na_2HPO_4. (2) pH 4.4: 0.1 mol·l^{-1} KH_2PO_4. (3) pH 7.0: 29.1 ml, 0.1 mol·l^{-1} NaOH; 50 ml, 0.1 mol·l^{-1} KH_2PO_4 (mit Aqua dest. auf 100 ml auffüllen). (4) pH 9.0: 4.6 ml, 0.1mol·l^{-1} HCl; 50 ml, 0.025 mol·l^{-1} $Na_2B_4O_7 \cdot 10H_2O$ (mit Aqua dest. auf 100 ml auffüllen). (5) 0.05 mol·l^{-1} HCl. (6) 0.05 mol·l^{-1} NaOH.

ERGEBNISSE UND DISKUSSION

Bei der Trennung wurden jeweils 0.5 ml der Lösung auf die Sephadex-Säule aufgetragen. Die im ersten Teil angegebenen Lösungen benutzten wir zur Elution. Die Säule wurde vor der Verwendung mit dem jeweiligen Elutionsmittel gewaschen. Zur Bestimmung des Zwischenkornvolumens bzw. des inneren Volumens verwendeten wir 50 µl Dextran blau 2000 (10 mg/ml) bzw. 10 µl Aceton. Mit dem Elutionspuffer von pH 7.0 erhielten wir das in Fig. 1 dargestellte Profil.

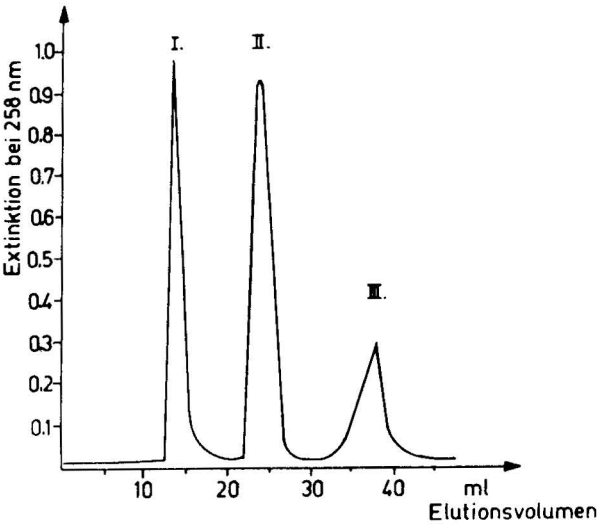

Fig. 1. Chromatogramm der *o*-Chlorbenzoesäure auf einer Sephadex G-10-Säule. I = Dextran blau 2000 (500 µg); II = Aceton (10 µl); III = *o*-Chlorbenzoesäure (30 µg). Elutionsmittel: Puffer pH 7; Durchflussgeschwindigkeit: 50 ml/h.

Bei den Trennungen mit den angegebenen Lösungen konnten wir die in Tabelle I dargestelleten K_D-Werte berechnen.

Aus dieser Tabelle ist eindeutig die Tendenz zu erkennen, dass die verwendete *o*-Chlorbenzoesäure im sauren Bereich einen höheren K_D-Wert besitzt als im alkali-

TABELLE I

EINFLUSS DES ELUTIONSMITTELS AUF DEN VERTEILUNGSKOEFFIZIENTEN (K_D) VON *o*-CHLORBENZOESÄURE BEI DER SÄULENCHROMATOGRAPHIE AUF SEPHADEX G-10

Elutionsmittel	K_D-*Wert*
Puffer pH 4.0	2.6
Puffer pH 4.4	3.6
Puffer pH 7.0	2.1
Puffer pH 9.0	0.4
0.05 mol/l NaOH	1.0
0.05 mol/l HCl	∞

schen Bereich. Die Zusammensetzung des Elutionsmittels spielt offensichtlich eine entscheidende Rolle, wie beim Vergleich der K_D-Werte der Puffer von pH 4.0 und 4.4 zu erkennen ist. Sie wurde aber bei weiteren Untersuchungen nicht berücksichtigt.

Zum weiteren Arbeiten setzten wir auf Grund der vorliegenden Ergebnisse als Elutionsmittel 0.05 mol·l^{-1} NaOH bzw. 0.05 mol·l^{-1} HCl ein. Bei der Verwendung von Natronlauge entspricht der Verteilungskoeffizient dem Wert des Acetons. Bei 0.05 mol·l^{-1} HCl als Elutionsmittel wird die Benzoesäure nicht von der Sephadex-Säule eluiert. Es entsteht eine reversible Bindung an das Gel der Säule. Sie kann durch nachfolgendes Eluieren mit einem alkalischen Elutionsmittel gelöst werden.

Diese reversible Bindung an das Sephadex-Gel wurde für folgende Verbindungen nachgewiesen: Benzoesäure, deren Monochlor-, Mononitro-[7], Monohydroxy-Derivate, Anthranilsäure, *o*-Methoxybenzoesäure, 2,5-Dihydroxybenzoesäure, 2,3-Dihydroxybenzoesäure, 2,3,5-Trijodbenzoesäure und 2,4-Dichlorphenoxyessigsäure.

Weitere Verbindungen wurden nicht zur Testung eingesetzt. Verantwortlich für die Bindung an das Gel der Säule ist die Struktur des Moleküls. Für alle getesteten Verbindungen sind der Benzolring und die Carboxylgruppe charakteristisch. Die Affinität für π-elektronenreiche Verbindungen und die hydrophobe Wechselwirkung von Ionenkomplexen an Dextrangele wurden bereits mehrfach als Ursache für die Veränderung des K_D-Wertes beschrieben[3-5,8-11].

Bei der Testung der Aminosäuren Tyrosin und Phenylalanin auf ihr chromatographisches Verhalten konnten wir ebenfalls eine Veränderung des K_D-Wertes nachweisen[4]. Sie zeigten unter den angegebenen Bedingungen aber keine Bindung an das Gel der Säule.

Die Struktur der Substanz, die Ionenzusammensetzung und der pH-Wert des Elutionsmittels spielen bei der Bindung eine wichtige Rolle.

Die Bindungsfähigkeit der Benzoesäure und der angeführten Derivate wurde zur Trennung aus Pflanzenextrakten genutzt (Fig. 2).

Zur Trennung mischten wir 0.5 ml Pflanzenextrakt mit 0.5 μCi ^{14}C-Benzoesäure-7 (= 1.04 μg). Das Gemisch wurde sofort auf eine Sephadex G-10-Säule aufgetragen, die vorher mit 0.05 mol l^{-1} HCl gewaschen wurde. Die Reihenfolge der eingesetzten Elutionsmittel ist aus der Fig. 2 ersichtlich.

Im Szintillationszähler wurde die Radioaktivität der erhaltenen Fraktionen gemessen. Die Ergebnisse sind in Tabelle II dargestellt. In der Fraktion 9 tritt die grösste Impulszahl auf. Die Aktivität in den anderen Fraktionen ist unbedeutend. Anhand

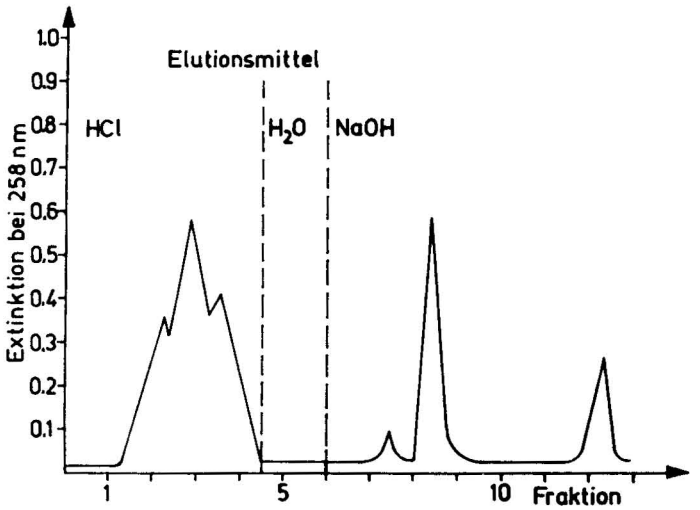

Fig. 2. Chromatogramm eines Pflanzenextraktes mit ^{14}C-Benzoesäure-7 auf einer Sephadex G-10-Säule. (Fraktion 9 Benzoesäure-Peak, Erläuterungen im Text.)

TABELLE II

MESSUNG DER RADIOAKTIVITÄT DER EINZELNEN ELUATFRAKTIONEN NACH DER IN FIG. 2 DARGESTELLTEN FRAKTIONIERUNG AUF EINER SEPHADEX G-10-SÄULE

	Fraktion					
	1–8	*9*	*10*	*11*	*12*	*13*
dpm	etwa 100	31,700	350	180	200	50

der auftretenden Radioaktivität kann die Fraktion 9 als Benzoesäurefraktion charakterisiert werden.

Die vorgestellte Methode ist somit geeignet, Benzoesäurederivate, die die freie Carboxylgruppe besitzen, aus Pflanzenextrakten abzutrennen.

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MEETINGS

SYMPOSIUM ON SEPARATION SCIENCE AND TECHNOLOGY FOR ENERGY APPLICATIONS

The annual symposium on "Separation Science and Technology for Energy Applications" will be held May 5–8, 1981, in Gatlinburg, TN, U.S.A. It is sponsored by the Department of Energy's Office of Basic Energy Sciences and Oak Ridge National Laboratory (ORNL). Anthony P. Malinauskas, ORNL Chemical Technology Division is general chairman. The symposium will include half-day sessions in computer modeling of separation processes, the recovery of valuable materials from dilute sources, the use of surfactants in separation processes, separation methods in fossil fuel utilization, and liquid–liquid extraction. Requests for additional information may be addressed to Anthony P. Malinauskas, Oak Ridge National Laboratory, P.O. Box X, Oak Ridge, TN 37830, U.S.A.

1ST INTERNATIONAL SYMPOSIUM ON CHROMATOGRAPHY IN BIOCHEMISTRY, MEDICINE AND ENVIRONMENTAL RESEARCH

This first International Symposium on Chromatography in Biochemistry, Medicine and Environmental Research will be held from June 16–17, 1981 in Venice, Italy. The symposium will discuss all the latest aspects of chromatography and its areas of applications, including biochemistry, medicine, toxicology, drug research, forensic science, clinical chemistry and pollution. The proceedings of the symposium will be published by Elsevier Scientific Publishing Company. Participants wishing to present a paper and/or poster communication should address themselves for detailed information to the organizing committee at the following address: Dr. A. Frigerio, Secretariat, Italian Group for Mass Spectrometry in Biochemistry and Medicine, c/o Istituto di Ricerche Farmacologiche – Mario Negri –, Via Eritrea 62, 20157 Milan, Italy. Papers should be submitted before January 31st, 1981, in English.

This symposium is being organized in conjunction with the 8th International Symposium on Mass Spectrometry in Biochemistry, Medicine and Environmental Research, which will be held on June 18–19, 1981 also in Venice, Italy.

CALENDAR OF FORTHCOMING MEETINGS

Oct. 19–23, 1980
Washington, D.C., U.S.A.

Annual Meeting of Assoc. of Official Analytical Chemists

Contact:
K.M. Eominaya, Box 540, Benjamin Franklin Station, Washington, D.C. 20044, U.S.A.

Oct. 29–31, 1980
Milan, Italy

New Trends in Antibiotics – Research and Therapy

Contact:
Fondazione Giovanni Lorenzini,
Via Monte Napoleone, 23, 20121 Milan, Italy.
Tel. (02) 702267 and 783868.

Nov. 11–15, 1980
Milan, Italy

1st African and Mediterranean Congress of Clinical Chemistry

Contact:
Secretariat, 1st African and Mediterranean Congress 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.

Feb. 17–20, 1981
Vienna, Austria

Euro Food Chem I

Contact:
Verein Osterreichischer Chemiker,
Dr. Werner Pfannhauser, FECS-WPFC-Secretary,
Eschenbachgasse 9, A-1010 Wien, Austria. Tel. 0-22-2/574249.

March 9–13, 1981
Atlantic City, N.J., U.S.A.

1981 Pittsburgh Conference

Contact:
John A. Queiser, Programme Chairman,
1981 Pittsburgh Conference,
2523 Greenboro Lane, Pittsburgh, PA 15220, U.S.A.
Tel. (412) 795-7110.

March 23–June 5, 1981
Uppsala, Sweden

Course: Biochemical Separation Methods

Contact:
Secretary, Eva Linder, Institute of Biochemistry,
University of Uppsala, Box 576, S-751 23 Uppsala, Sweden.

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 3–7, 1981
Hindelang, (Bavarian Alps), F.R.G.

4th International Symposium on Capillary Chromatography

Contact:
Dr. J. Rijks, Laboratory of Instrumental Analysis,
Eindhoven University of Technology, P.O. Box 513,
NL-5600 MB Eindhoven, The Netherlands.

May 5–8, 1981
Gatlinburg, TN, U.S.A.

Separation Science and Technology for Energy Applications

Contact:
A.P. Malinauskas, Oak Ridge National Laboratory,
P.O.Box X, Oak Ridge, TN 37830, U.S.A.

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. (Further details published, Vol. 194, No. 3).

May 20–22, 1981
Eger, Hungary

Symposium on the Analysis of Steroids

Contact:
Prof. S. Görög, c/o Hungarian Chemical Society,
1061 Budapest VI., Anker köz 1, Hungary.

June 16–17, 1981
Venice, Italy

1st International Symposium on Chromatography in Biochemistry, Medicine and Environmental Research

Contact:
Dr. A. Frigerio, Italian Group for Mass Spectrometry in Biochemistry and Medicine, c/o Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy. Tel. 35.54.546.

June 18–19, 1981
Venice, Italy

8th International Symposium on Mass Spectrometry in Biochemistry Medicine and Environmental Research

Contact:
Dr. A. Frigerio, Italian Group for Mass Spectrometry in Biochemistry and Medicine, c/o Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy. Tel. 35.54.546.

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. (Further details published in Vol. 195, No. 2).

Aug. 23–28, 1981
Espoo, Finland

Euroanalysis IV – Triennial 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) 421352.

Sept. 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).

NEW BOOKS

Advances in infrared and Raman spectroscopy, edited by R.J.H. Clark and R.E. Hester, Heyden & Son, London, 1980, **Vol. 6,** 372 pp., price £ 28.00, DM 128.00, ISBN 0-85501-186-6; **Vol. 7,** 416 pp., price £ 38.00, DM 174.00, ISBN 0-85501-187-4.

Reagents for organic synthesis, Vol. 8, by M. Fieser and L. Fieser, Wiley, Chichester, New York, 1980, *ca.* 896 pp., price *ca.* US$ 47.90, £ 21.95, ISBN 0-471-04834-8.

Chemical tables for laboratory and industry, by W. Helbing and A. Burkart, Wiley Eastern (Wiley), New Delhi, 1980, 272 pp., price US$ 9.75, £ 4.25, ISBN 0-85226-368-6.

Identification and analysis of plastics, by J. Haslam, H.A. Willis and D.C.M. Squirrel, Heyden & Son, London, 2nd ed., 1980, 756 pp., price £ 28.00, DM 128.50, ISBN 0-85501-193-9.

The use of microprocessors, by M. Aumiaux, Wiley, Chichester, New York, 1980, *ca.* 176 pp., price *ca.* US$ 24.00, £ 8.80, ISBN 0-471-27689-8.

Gas chromatography with glass capillary columns, by W. Jennings, Academic Press, New York, London, **2nd ed.,** 1980, XIII + 320 pp., price US$25.00, ISBN 0-12-384360-X.

Toxic metals and their analysis, by E. Berman, Heyden & Son, London, Philadelphia, Rheine, 1980, 304 pp., price £12.00, US$27.00, DM 56.00, ISBN 0-85501-468-7.

A dictionary of scientific units, by H.G. Jerrard and D.B. McNeill, Chapman & Hall, London, **4th ed.,** 1980, ca. 240 pp., price £10.00, ISBN 0-412-22360-0 (hardback) or £5.95, ISBN 0-412-22370-8 (paperback).

The infrared spectra of complex molecules, Vol. 2, Advances in infrared group frequencies, by L.J. Bellamy, Chapman & Hall, London, **2nd ed.,** 1980, *ca.* 328 pp., price *ca.* £12.00, ISBN 0-412-22350-3.

Acute toxicity in theory and practice: with special reference to the toxicology of pesticides, by V.K.H. Brown, Wiley, Chichester, New York, 1980, *ca.* 192 pp., price *ca.* US$ 35.75, £ 13.00, ISBN 0-471-26690-1.

Statistical theory and methodology of trace analysis, by C. Liteanu and I. Rîcă, Ellis Horwood (Wiley), Chichester, 1980, 446 pp., price £28.00, ISBN 0-85312-108-7.

Advanced inorganic chemistry: A comprehensive text, by F.A. Cotton and G. Wilkinson, Wiley, Chichester, New York, **4th ed.,** 1980, 1414 pp., price £13.75, US$31.60, ISBN 0-471-02775-8.

Progress in physical organic chemistry, Vol. 13, edited by R.W. Taft, Wiley, Chichester, New York, 1980 *ca.* 688 pp., price *ca.* US$60.00, £27.50, ISBN 0-471-06253-7.

Advances in chromatography, Vol. 18, edited by J.C. Giddings, E. Grushka, J. Cazes and P.R. Brown, Marcel Dekker, New York, Basel, 1980, XV + 292 pp., price SFr.88.00, ISBN 0-8247-6960-0.

Liquid chromatography of polymers and related materials II., edited by J. Cazes and X. Delamare, Marcel Dekker, New York, Basel, 1980, VIII + 262 pp., price SFr.80.00, ISBN 0-8247-6985-6.

Organic chemistry, by T.W.G. Solomons, Wiley, Chichester, New York, **2nd ed.,** 1980, *ca.* 1216 pp., price *ca.* US$29.95, £15.00, ISBN 0-471-04213-7.

Introduction to protein sequence analysis, by L.R. Croft, Wiley, Chichester, New York, 1980, *ca.* 256 pp., price *ca.* US$15.80, £5.75, ISBN 0-471-27710-X.

Water chemistry, by V.L. Snoeyink and D. Jenkins, Wiley, Chichester, New York, 1980, 480 pp., price £13.50, US$31.00, ISBN 0-471-05196-9.

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	197/1 197/2 198/1	198/2 198/3 198/4 199	200 201	202/1 202/2 202/3
Chromatographic Reviews			184/1	184/2					184/3			184/4	
Biomedical Applications		181/1	181/2	181/ 3-4	182/1	182/2	182/ 3-4	183/1	183/2	183/3	183/4		

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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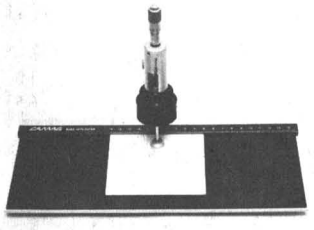

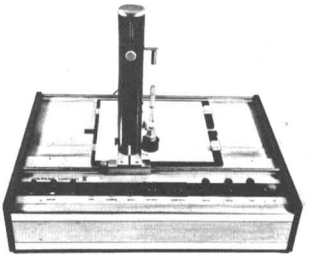

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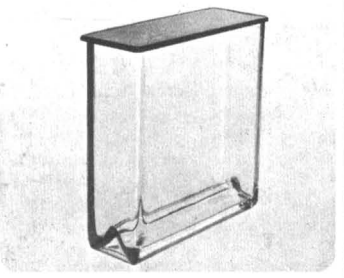

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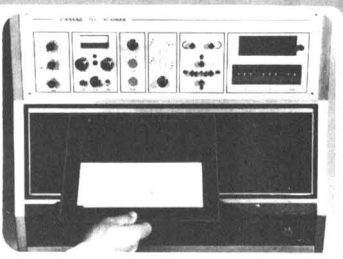

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